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Lipopolysaccharide suppresses IgE-mast cell mediated reactions

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Abstract

Background—Clinical and experimental analyses have identified a central role for IgE/FcεRI mast cells in promoting IgE-mediated anaphylaxis. Recent data from human studies suggest that bacterial infections can alter susceptibility to anaphylaxis.

Objective—We examined the effect of LPS exposure on the induction of IgE-mast cell-(MC) mediated reactions in mice.

Methods—C57BL/6 WT, TLR-4^{-/-} and IL10^{-/-} mice were exposed to LPS and serum cytokines (TNF and IL-10) were measured. Mice were subsequently treated with anti-IgE and the symptoms of passive IgE-mediated anaphylaxis, MC activation, Ca²⁺-mobilization and expression of FcεRI on peritoneal MCs were quantitated.

Results—We show that LPS exposure of C57BL/6 WT mice constrains IgE-MC mediated reactions. LPS-induced suppression of IgE-MC mediated responses was TLR-4-dependent and associated with increased systemic IL-10 levels, decreased surface expression of FcεRI on MCs and loss of sensitivity to IgE activation. Notably, LPS-induced desensitization of MCs was short-term with MC sensitivity to IgE reconstituted within 48 hours which was associated with recapitulation of FcεRI expression on the MCs. Mechanistic analyses revealed a requirement for IL-10 in LPS-mediated decrease in MC FcεRI surface expression.

Conclusions & Clinical Relevance—Collectively, these studies suggest that LPS-induced IL-10 promotes the down regulation of MC surface FcεRI expression and leads to desensitization

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of mice to IgE-mediated reactions. These studies indicate that targeting of the LPS-TLR-4-IL-10-pathway maybe used as a therapeutic approach to prevent adverse IgE-mediated reactions.

Keywords

IgE; mast cells; anaphylaxis; innate immunity and TLR-4 signaling

Introduction

Anaphylaxis is a severe, life-threatening allergic reaction that affects both children and adults in the United States (1). The most common inciting agents (33.2% of reactions) are foods, particularly peanuts and nuts, and food-induced anaphylaxis (FIA) hospitalization rates for children in the US have more than doubled from 2000 to 2009 (2). An anaphylactic reaction encompasses a variety of symptoms that may affect one or more target organs including gastrointestinal (GI), cutaneous, respiratory and cardiovascular systems (3, 4). Clinical and experimental analyses have identified a central role for IgE / FcεRI / mast cell (MC) s and MC-derived mediators, including histamine, platelet-activating factor (PAF), serotonin, proteases (tryptase and chymase) and lipid-derived mediators (prostaglandins [PGD₂] and leukotrienes [LTC₄, LTD₄ and LTE₄]), in promoting the clinical manifestations associated with food-triggered anaphylaxis (5–11).

Recent studies have revealed the presence of co- or augmentation factors including exercise, alcohol consumption, non-steroidal anti-inflammatory drugs (NSAID) and infection that can increase susceptibility to anaphylaxis (12). Epidemiological studies suggest that cofactors contribute to ~ 1.5 – 30% of all anaphylactic reactions in adults (12). One cofactor that seems to be important in anaphylaxis is infection where it is reported to be a relevant cofactor in 2.5–3% of anaphylactic reactions in children and in 1.3–11% in adults (12–14). Indeed, current guidelines for specific immunotherapy (SIT) with pollen or Hymenoptera venoms advise to discontinue SIT in case of infection (12, 15).

The underlying molecular basis of how infection can act as a cofactor and exacerbate anaphylaxis is not well understood. The primary means by which cofactors are thought to alter the likelihood of an anaphylactic reaction following allergen exposure through modulating allergen bioavailability and threshold for cellular activation (12). Infectious agents (e.g. viruses and bacteria) upon penetration of epithelial surfaces immediately encounter innate immune cells such as dendritic cells, macrophages and through recognition of distinct evolutionarily conserved structures on pathogens, termed pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs) e.g. Toll like receptors (TLRs) leads to the onset of an innate immune response (16–20). Activation of TLRs on mononuclear cells, including dendritic cells (DCs), monocytes, and macrophages (MΦs) (21) stimulates a NFκB-responsive pro-inflammatory cytokine (e.g. IL-1, IL-6, IL-10, Type I interferons and TNF) response (22–27).

MCs also express a number of different pattern-recognition receptors, including Toll-like receptors (TLRs) (28, 29). TLR-ligands can directly activate MCs by stimulating mitogen-activated protein kinases (MAPKs)- NFκB- and phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt signaling cascades causing release of MC mediators (30–33). The impact

of TLR activation on MC degranulation and effector function is controversial. Evidence suggests that TLR-ligands (e.g. TLR-2) can suppress MC activation and degranulation via modulation of FcεRI expression and cellular compartmentalization (34–36). Alternatively, prolonged TLR-ligand exposure promotes IgE-mediated MC activation (37, 38) stimulating cytokine release without affecting degranulation or synthesis of eicosanoids (33).

In this study, we used a reductionist approach to directly examine the impact of innate immune activation on IgE-FcεRI-mediated mast cell reaction in vivo and in vitro. We show that treatment of mice with the classical innate TLR-4 antigen LPS, inhibited IgE-MC-mediated anaphylactic reactions. We show that LPS-induced temporal suppression of IgE-MC-mediated response was associated with decreased surface expression of FcεRI on MCs and subsequent loss of sensitivity to IgE activation. Notably, LPS enhances systemic IL-10 levels and the LPS-mediated decrease of surface expression of FcεRI on MCs was IL-10-dependent. Collectively, our studies reveal that LPS can desensitize mice to IgE-mediated MC activation by downregulation of FcεRI via an IL-10-mediated process. These studies suggest that treatment of mice with TLR-4 agonists can induce MC desensitization to IgE activation and prevent IgE-mediated responses.

Methods and Materials

Animals

WT C57BL/6 and *Il10*^{-/-} (C57BL/6) mice were originally obtained from Jackson Laboratory and Charles River Laboratories but continual breeding was then performed in house. All mice were maintained in a barrier facility, and animals were handled under approved protocols of the Institutional Animal Care and Use Committee from Cincinnati Children's Hospital Medical Center. Experiments were performed on litter mate- and strain- and age-matched mice (6–12 weeks old).

Reagents

LPS derived from *Escherichia coli* (O55:B5) (Sigma, St. Louis, MO, USA), EM-95 (rat IgG2a anti-mouse IgE mAb (38), (kind gift of Professor Fred Finkelman at Cincinnati Children's Hospital Medical Center) were diluted in normal saline to a final volume of 200 μl per mouse. CD16/CD32, FcεRI-APC, ST2-PerCP-Cy5.5, and cKit-PECy7 were purchased from BD Biosciences (San Jose, CA, USA) and Biolegend (San Diego, CA, USA). RPMI complete media (Invitrogen, Carlsbad, CA, USA), IL-3 (Peprotech, Inc. Rocky Hill, NJ, USA), SCF (Peprotech, Inc. Rocky Hill, NJ, USA), anti-TNP-BSA /TNP-BSA (BD Biosciences San Jose, CA USA), Fluo-4, AM (Molecular Probes, Eugene OR, USA) and PNAD (4-Nitrophenyl N-acetyl-β-D-glucosaminide) and probenecid (Sigma-Aldrich, St. Louis, MO, USA) were used according to instructions.

Acute systemic inflammation and endotoxin shock

Six to twelve-week-old C57BL/6 WT and *Il10*^{-/-} mice were intraperitoneal i.p. injected with different doses of LPS (0.01, 0.1, 1 and 10 mg/kg). Rectal temperatures were monitored every 30 min for 2 – 4 hrs with a rectal probe following the LPS challenge (Physitemp Model BAT-12) to assess for evidence of hypothermia.

Passive systemic anaphylaxis

Six- to twelve-week-old C57BL/6 WT and *H10^{-/-}* mice were injected intravenously (i.v.) 20 µg anti-IgE (Clone: EM-95), as we have previously described (39–41). Systemic anaphylaxis was monitored by measuring changes in rectal temperature at 0, 15, 30, 45 and 60 minutes after anti-IgE injection using a rectal probe (Physitemp Model BAT-12) (39–41).

Hematocrit measurement

60 min following anti-IgE challenge blood was collected from the orbital sinus into heparinized capillary tubes and centrifuged for 5 min at 10,000 rpm. Hematocrit (percentage of packed red blood cell volume) was calculated as the length of packed RBCs divided by the total length of serum and red cells in the capillary tube and multiplied by 100% as previously described (11).

mMCPT-1 Enzyme Linked Immunosorbent Assay

Mouse serum was collected 1 h after EM-95 i.v. injection, and MCPT-1 in serum was measured by means of mouse MCPT-1 (mMCP-1) ELISA Ready-SET-Go!, according to the manufacturer's instructions (eBioscience, San Diego, CA, USA). In short, after the 96 well plate was coated with capture antibody and blocked with ELISA diluent, serial standard or serum sample dilutions, detection antibody, Avidin-HRP and TMB Solution were added to the wells, respectively and developed at room temperature. Afterwards, the stop solution was added to each well, and the optical density (O.D) was read with an ELISA plate reader at 450 and 570 nm. To determine concentration, the O.D value (570 nm) was subtracted from O.D value (450 nm) and the calculated concentration of mMCPT-1 was derived from O.D. of known concentrations of mMCPT-1 according to the standard curve.

Peritoneal mast cell analyses

Peritoneal wash, lymph node (LN) and MC analyses were performed as previously described (42). In brief, mice were euthanized and a hypodermic needle was inserted into the abdominal cavity (18 gauge). Sterile saline + 10% Fetal Bovine Serum (FBS) (10 ml) was injected into the peritoneal cavity, the abdominal region was gently massaged for 1 min and the fluid removed. The peritoneal lavage was centrifuged at 1200 rpm for 5 min at room temperature. The supernatant was poured off and Red Cell Lysis Buffer (RCLB) (Sigma-Aldrich, St.Louis, MO, USA) was added. After RCLB, cells were counted and re-suspended in FACS buffer (PBS/ 0.5% BSA or 1% FCS). The single-cell suspensions were washed with FACS buffer (PBS/ 1% BSA or FCS) and incubated with combinations of the following Abs: CD16/CD32 combinations of the mAb-fluorochrome conjugates (100 µl PBS/BSA, 1 µl FcεRI-APC, 1 µl ST2-PerCP-Cy5.5, 0.5 µl cKit-PE-Cy7, 1µl CD19-APC, 1µl B220-PerCP-Cy5.5 or CD5-PE) at 4°C for 30 min in the dark. The cells were measured on a BD LSR Fortessa I or FACS Canto following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA) and analyzed using the FlowJo 10 software (FlowJo LLC, Ashland, OR, USA). Data were expressed as mean fluorescence intensities (MFI) of labeled cells.

Calcium (Ca²⁺) mobilization assay by flow cytometry

C57BL/6 mice were i.p. injected with vehicle (saline) or 1mg/kg LPS, and 24 hrs later peritoneal cells were harvested by lavage as described above. Cells were treated with RBC lysis buffer, washed with Ca²⁺-free buffer (HBSS, 0.1% BSA, 2mM Probenecid), then stained for 30 min with FcεRI, CD117, ST2, and Fluo-4 in Ca²⁺-free buffer. Cells were washed and resuspended in Ca²⁺-containing buffer (HBSS, 0.1% BSA, 2mM probenecid, 1.8mM CaCl₂) prior to flow cytometry analyses. MCs were identified as FcεRI⁺, cKit⁺ and ST2⁺, and MFI of Fluo-4 was measured 5 mins following mock stimulant (Ca²⁺-containing buffer alone) or anti-IgE stimulation. The % change in Fluo-4 MFI was determined by Fluo-4 MFI of FcεRI⁺, cKit⁺ and ST2⁺ cells (mock stimulant) divided by Fluo-4 MFI of FcεRI⁺, cKit⁺ and ST2⁺ cells (anti-IgE stimulation) multiplied by 100.

Bone marrow (BM) mast cell culture

BM cells were isolated from 6- to 12-week-old C57BL/6 mice, which were cultured as previously described (43, 44). Briefly, C57BL/6 mice femurs and tibias were harvested, and marrow was collected and suspended in RPMI complete media consisting of RPMI supplemented with 10% fetal bovine serum (Atlanta Biologicals Lawrenceville, GA, USA), penicillin (100 U/ml), streptomycin (100 mg/ml), sodium pyruvate (1 mM), nonessential amino acids (0.1 mM), Hepes (25 mM), and 2-β-mercaptoethanol (50 μM). BM cells were cultured in the presence of IL-3 and SCF (20 ng/ml) for four weeks. Bone marrow-derived mast cells (BMMC) were examined for FcεRI, and c-Kit expressions by flow cytometry, when >95% of the cells were FcεRI and c-Kit double positive.

β-Hexosaminidase release assay

BMMC degranulation was measured by β-hexosaminidase release as described previously (42). BMMCs were incubated with 0.5 μg/ml anti-TNP-BSA at 37°C, 5% CO₂ for 18 h, and the sensitized BMMCs were challenged with TNP-BSA (10 ng/ml or 100 ng/ml) by incubation at 37°C, 5% CO₂ for 30 min. For measurement of β-hexosaminidase activity, supernatants were incubated with PNAG (4-Nitrophenyl N-acetyl-β-D-glucosaminide) in β-hexosaminidase buffer (0.1M Citric acid + 0.1M trisodium citrate) for 1 hr, followed by quenching of the enzymatic reaction by addition of 2 M NaOH and absorbance was measured at 405 nm using an ELISA plate reader. Cell lysates were used to estimate total β-hexosaminidase activity as a positive control. BMMCs incubated only with anti-TNP-BSA were used as negative control. Degranulation was expressed using the percentage of total β-hexosaminidase activity.

Peritoneal MΦs

Peritoneal wash and macrophage (MΦ) analyses were performed as previously described (45). Mice were intraperitoneally injected with 1 mL 3.85% thioglycolate as previously described (45). Three days after the i.p. injection, a peritoneal lavage was performed, and cells were centrifuged and suspended in 25 mL 10% FBS complete DMEM and plated for 4 h for adherence. Cells were then washed with PBS and detached with trypsin/EDTA (Life Technologies; Carlsbad, CA) at 37°C for 5 min and suspended in 10% FBS complete DMEM. Cells were then gently scraped off and suspended in 10% FBS complete DMEM

and LPS stimulation analyses and IL-10 analyses of supernatants was performed as described (45).

Statistical analysis

Data are expressed as mean $\pm$ SD, unless otherwise stated. In experiments comparing multiple experimental groups, statistical differences between groups were analysed using the one-way ANOVA parametric and a Tukey's multiple comparison post-test. In experiments comparing two experimental groups, statistical differences between groups were determined using a Student's t-test. $P < 0.05$ was considered significant. While repeated-measures two-way ANOVA was performed for rectal temperature data. All analyses were performed with Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA).

RESULTS

LPS pretreatment abrogates IgE-MC mediated responses in C57BL/6 mice

To test the impact of LPS on IgE-MC-dependent reactions, C57BL/6 WT mice received an intraperitoneal (i.p.) injection of LPS (0.01, 0.1 and 1 mg/kg) and twenty-four hours later received i.v. injection of anti-IgE mAb (EM-95) and evidence of anaphylactic shock (hypothermia) was examined (Fig. 1A). Low dose (0.01 and 0.1 mg/kg) LPS exposure of C57BL/6 WT mice did not induce a significant endotoxic-induced shock response as evidenced by the lack of observed temperature loss within two hours of LPS exposure or rise in serum TNF levels (Fig. 1B and results not shown). In contrast, i.p. injection of 1 mg/kg LPS did promote endotoxic shock as evidenced by $>1^{\circ}\text{C}$ loss in body temperature and rapid rise in serum TNF levels (Figs. 1B and 1C). Murine-based evidence suggests that MCs are involved in the LPS-induced shock response (46). We examined evidence of MC degranulation and observed no significant increase in serum mMCPT-1 within two hours of LPS treatment (Figure 1D) suggesting no significant mucosal MC activation following LPS exposure.

To test whether LPS pretreatment modified IgE-mediated anaphylactic responses, twenty-four hours following LPS challenge, mice were i.v. injected with anti-IgE mAb and evidence of IgE-induced shock (hypothermia) and mast cell activation were examined. Anti-IgE treatment of WT C57BL/6 mice induced a rapid shock response characterized by significant temperature loss ($> -4^{\circ}\text{C}$) at thirty minutes following antibody treatment (Fig. 1E). The shock response was associated with MC activation as evidenced by increased mMCPT-1 (Fig. 1F). Pretreatment of mice with LPS (0.01, 0.1 and 1 mg/kg) suppressed the IgE-MC-induced hypothermia in a dose-dependent manner (Fig. 1E). Notably, the reduction in anaphylaxis symptoms was associated with decreased MC activation (Fig. 1F). Examination of Fc ϵ RI expression on peritoneal mast cells (pMCs) (c-Kit $^{+}$ Fc ϵ RI $^{+}$ ST2 $^{+}$) revealed a reduction in Fc ϵ RI on the surface of MCs in LPS-treated mice compared with vehicle-treated mice (Fig. 1G–I). Moreover, we show that the mean fluorescence intensity (MFI) of Fc ϵ RI on pMC's was reduced in LPS treated compared with the untreated group (Fig. 1G – I). Based upon these observations we concluded that LPS exposure suppresses IgE-MC-mediated anaphylaxis and that this was associated with decreased Fc ϵ RI expression on MCs.

LPS decreases surface FcεRI expression and abrogates peritoneal mast cell (pMC) sensitivity to IgE activation

In our previous studies, we have demonstrated that the IgE-MC-mediated shock response requires IgE, FcεRI on the surface of mast cells, mast cell activation and histamine-induced hypovolemic shock (6, 42). LPS pretreatment of WT mice had no impact on total serum IgE (Total serum IgE levels (ng/ml): 87.7 ± 28.6 vs. 121.1 ± 48.2 ; Vehicle- vs. LPS-treated + i.v. WT C57BL/6 challenged mice respectively, mean $\pm$ SEM; $n = 7$ mice per group). Further, LPS pretreatment did not impact histamine-induced hypovolemic shock in WT C57BL/6 mice (Maximum Temperature loss ($^{\circ}\text{C}$): -2.3 ± 0.5 vs. -1.9 ± 0.3 ; Hemacrit (%) 54.0 ± 0.8 vs. 52.8 ± 1.4 ; Vehicle- vs. LPS-treated + i.v. histamine (2 mg) challenged mice respectively, $n = 7$ and 10 mice per group). Examination of FcεRI expression on pMCs twenty-four hours following LPS exposure and prior to anti-IgE treatment revealed that that 0.01–1 mg/kg LPS exposure reduced the level of FcεRI mean fluorescence intensity (MFI) on pMCs (Fig. 2A and B). Notably the level of FcεRI expression on pMCs was reduced by ~40% compared with pMC's from vehicle-treated C57BL/6 mice (Fig. 2C). To determine whether the MC population in LPS-treated mice were responsive to IgE, we examined intracellular calcium [Ca^{2+}]_i release in LPS-exposed pMCs following anti-IgE activation. Anti-IgE stimulation of pMC's (c-Kit⁺, FcεRI⁺ ST2⁺) from vehicle-treated WT C57BL/6 mice led to increase intracellular Fluo-4 indicating IgE-induced Ca^{2+} -mobilization (Fig 3). In contrast, addition of anti-IgE to pMC's from LPS-treated mice did not change Fluo-4 MFI indicating that LPS-pretreatment prevented IgE-induced Ca^{2+} -mobilization.

LPS-induced suppression of IgE-mediated anaphylaxis is TLR-4-dependent

Previous studies have revealed a TLR2- and TLR4-dependent dysregulation of IgE-FcεRI mast cell-responses via synergistic production of cytokines (47). To determine the danger-associated signal that mediated down regulation of FcεRI and IgE-mediated responses, we examined the requirement of TLR-4 on LPS-mediated suppression of IgE-mediated reactions. The LPS used in our experiments was extracted from *Escherichia coli* (O55:B5) which predominantly consists of TLR-4-specific agonists. Notably, we show that the LPS-induced protection against IgE-mediated responses was lost in Tlr4^{-/-} mice (Fig. 4) as the level of shock (hypothermia) (Fig. 4A), hematocrit (Fig. 4B) and serum MCPT-1 (Fig. 4C) were comparable to that of vehicle-treated TLR4^{-/-} mice and LPS-treated WT mice challenged with anti-IgE (Fig. 4A–C). Based upon these observations we concluded that LPS-induced suppression of IgE-mediated passive anaphylaxis was TLR4-dependent.

LPS-attenuation of IgE-mediated anaphylaxis is short term

To determine the kinetics of LPS-induced protection from IgE-mediated reactions, C57BL/6 mice were treated with LPS (1 mg/kg) and challenged with anti-IgE between 24–72 hr following LPS treatment. We show that LPS-induced protection was diminishing by 48 hrs and was lost by 72 h following LPS challenge (Figure 5A and B). Notably, the loss in protection of IgE-mediated reactions was associated with the return of FcεRI expression on pMCs (Fig. 5E – G) and increased MC activation (Figure 5D). We concluded that LPS induced protection from IgE-mediated reactions is short term and associated with decreased FcεRI expression levels.

Direct effect of LPS on BMMC FcεRI expression and degranulation

Previous studies have revealed that BMMCs derived from WT C57BL/6 mice express TLR4/MD2 (48, 49). Furthermore, LPS stimulation of BMMCs promotes a pro-inflammatory cytokine response (IL-6 and TNF) (48, 49). To ascertain if LPS directly decreased FcεRI expression on MCs, we examined FcεRI expression on BMMCs following twenty-four hours LPS exposure. Four-week culture of BM cells from WT C57BL/6 mice in the presence of IL-3 and SCF led to the generation of BMMCs (c-Kit⁺ FcεRI⁺ ST2⁺) (Supplemental Figure S1A). Stimulation of BMMCs with LPS for twenty-four hours led to a dose-dependent decrease in FcεRI expression on BMMCs (Supplemental Figure S1A–C). Notably, the reduction in FcεRI MFI on BMMCs while statistically significant was reduced by ~10% and did not inhibit IgE-activation of BMMCs (Supplemental Figure S1D). Collectively, these studies suggest that LPS can directly decrease FcεRI expression on MC populations, however the level of reduction is small and insufficient to prevent IgE-mediated MC activation.

LPS-attenuation of pMC FcεRI expression is in part IL-10-dependent

Previous studies have reported that the cytokine IL-10 can reduce FcεRI expression on human and mouse MCs (50, 51). I.p. injection of LPS to WT C57BL/6 mice induced a 10-fold increase in systemic IL-10 levels (Figure 6A). Examination of FcεRI expression on pMCs (c-Kit⁺ FcεRI⁺ ST2⁺) revealed that the LPS-induced reduction of FcεRI on the surface of MCs was abrogated in LPS-treated *Il10*^{−/−} mice (Fig. 6B). Moreover, we show that the reduction in MFI of FcεRI on pMC's from LPS treated *Il10*^{−/−} mice was significantly less than that observed in WT mice (Fig. 6B). Based upon these observations we concluded that LPS-induced down regulation of FcεRI on MCs was in part dependent on IL-10.

Discussion

Herein, we demonstrate that LPS challenge of C57BL/6 WT mice inhibits IgE-MC mediated reactions via TLR-4-dependent mechanism and that this was associated with decreased mast cell activation and decreased surface expression of FcεRI on mast cells. Notably, we show that LPS-induced desensitization of mast cells to IgE was short-term with mast cell sensitivity reconstituted within 48 hours which was associated with recapitulation of FcεRI expression on the mast cells. Studies in *Il10*^{−/−} mice revealed that LPS-induced reduction FcεRI expression on the mast cells was in part dependent on IL-10. These studies indicate that LPS desensitizes mice to IgE-MC mediated reactions via IL-10.

MCs express TLR receptors, including TLR-4 and expression and function is thought to be influenced by maturation and mast cell type (30) (29) (52). Murine MCs particularly mature MCs such as peritoneal cell-derived MCs respond more potently to TLR receptor activation(52). Human MCs express similar pattern of TLR receptors, however in contrast to murine MCs, expression of TLR receptors is somewhat more variable and require cytokine priming(53, 54). Moreover, pretreatment of human MCs with IFNγ stimulates TLR-4 expression and leads to a more sustained LPS-induced MC-derived cytokine response(54).

Furthermore, IL-4 and serum sCD14 priming of cord blood MCs is required for LPS-induced but not proteoglycan (PGN)-induced TNF response (53).

In vivo studies revealed that LPS reduced FcεRI expression on MCs and decreased sensitivity to IgE activation and this was in part dependent on IL-10. Notably, we show that LPS treatment of WT C57BL6 mice increased systemic IL-10 levels. Previous studies have reported a biphasic increase in IL-10 following LPS treatment of mice (55). LPS through activation of TLR-4 on myeloid cell compartment (monocytes and macrophages) promotes an early increase in IL-10 within ninety minutes following challenge, followed by a second increase between 8 – 12 hrs (19, 55). IL-10 being a potent immunosuppressive cytokine is thought to down regulate proinflammatory cytokine production and promote the development of LPS tolerance (56, 57). MCs express TLR receptors, including TLR-4 and expression and function is thought to be influenced by maturation and mast cell type (29, 30, 52). Both murine MCs and human MCs express similar pattern of TLR receptors, however in contrast to murine MCs, human expression of TLR receptors is somewhat more variable and require cytokine priming (52–54). As TLR activation of MC promotes pro-inflammatory cytokine production (TNF, IL-1β, IL-6 and IL-13) (46, 58, 59), our observation that LPS-induced IL-10 decreased MC FcεRI expression is consistent with the immunosuppressive role of IL-10.

Our demonstration that the LPS-mediated decrease in MC FcεRI expression was predominantly mediated by IL-10 is also consistent with previous studies showing IL-10 alone is sufficient to directly reduce FcεRI expression on human and mouse MCs and IgE-mediated anaphylaxis (50, 51). Norton and colleagues have previously demonstrated that IL-10 stimulation of BMMCs leads to decreased FcεRI expression. Consistent with this observation, they reported that administration of recombinant IL-10 or genetic overexpression of IL-10 inhibited MC FcεRI expression and suppressed the production of IgE-mediated MC-derived cytokine and anaphylaxis (60). Notably, in their studies, rIL-10 suppressed pMC FcεRI expression by ~50% which is in line with our observed ~45% reduction of FcεRI expression on pMCs from LPS-treated mice supporting the argument of a major contribution for IL-10 in the LPS-induced response. Our demonstration that direct *in vitro* stimulation of BMMC's with LPS led to a modest decrease in FcεRI, a reduction that was significantly smaller than that observed *in vivo* (~10% vs 45% reduction [Supplemental Figure S1 and Figure 2E]) and insufficient to abrogate IgE-mediated activation is also consistent with the requirement of an indirect effect of LPS on MCs likely through activation of the myeloid cell-derived IL-10 compartment.

The cellular source of IL-10 that suppresses FcεRI on MCs is not yet delineated. A recent series of elegant *in vitro* and *in vivo* studies by Kim and colleagues identified IL-10 producing CD5⁺ B cells as a potent suppressor of IgE-MC responses (61, 62). Notably, CD5⁺ B cell-derived IL-10 dependent reduction in IgE-MC responses was through reduction of the abundance of the Src family kinases (Syk, Fyn and Fgr) and subsequent FcεRI-mediated signaling (62). Examination of the presence of peritoneal CD5⁺ B cells following LPS challenge revealed a dramatic reduction in the frequency of peritoneal CD5⁺ CD19⁺ B220⁺ cells (> 75% reduction) in the LPS-treated vs Vehicle-treated group (Supplemental Figure S2A). Notably, this was specific to the peritoneal cavity as the levels of CD5⁺ CD19⁺

B220⁺ cells in the mesenteric LN were comparable between groups (Supplemental Figure S2B). These data would be consistent with Xu et al., showing that LPS downregulates CD5 expression on B cells and suggest that CD5⁺ B cells are not the likely source of IL-10(63). We have recently demonstrated that i.p. LPS administration to WT mice stimulates an increase in inflammatory cell populations including resident macrophages (F4/80⁺ CD11b⁺ Ly6G⁻, Ly6C^{low}) and inflammatory macrophages (F4/80⁺ CD11b⁺ Ly6G⁻, Ly6C^{high})(45). Examination of the thioglycolate-elicited peritoneal macrophages from naïve WT mice revealed a significant increase in IL-10 production following LPS stimulation (Supplemental Figure S2C). These data suggest that LPS stimulation of peritoneal macrophages leads to IL-10 production and suggests that in this model system, peritoneal macrophages are a likely source of IL-10.

In contrast to our observations, recent reports indicate that IL-10 can enhance IgE-mediated mast cell responses in vivo (64). However, the requirement of IL-10 in the induction of IgE-MC-dependent food allergic response appeared to be related to the capability of IL-10 to alter MC proliferation and numbers (64). Previous studies by us and others have reported a requirement for small intestine (SI) MC expansion in IgE-mediated MC-dependent food allergic responses in mice. While IL-10 alone is not sufficient to promote MC expansion, in combination with SCF and IL-3, IL-10 amplifies IL-3 and SC-mediated MC development (65, 66). Deletion of IL-10 in mice diminishes the level of mast cell expansion and thus ablates IgE-dependent food allergic response (67). Our studies have primarily focused on the acute effect of LPS-induced IL-10 responses (< 48 hours) on IgE-MC function independent of the effect on total mast cell numbers. Consistent with this we did not observe any effect of LPS on peritoneal MC numbers (results not shown).

A number of studies have previously examined the effect of LPS on antigen-induced anaphylaxis in mice, however these studies have primarily focused on IgG-mediated reactions (68, 69). Systemic antigen challenge in mice can induce anaphylaxis via IgE- and IgG-dependent pathways (70). Furthermore, at high systemic antigen dose, anaphylaxis is predominantly mediated by the IgG-pathway (6). Thus, the previous studies describing LPS-mediated suppression of systemic antigen-induced anaphylactic responses in mice is likely inhibiting IgG-dependent responses, not IgE-reactions. In our studies, we utilize anti-IgE monoclonal antibody to specifically examine the effect of LPS on IgE-MC mediated anaphylactic response.

There are several limitations with respect to our studies employing *Il10*^{-/-} mice related to LPS responsiveness and immune dysfunction. We were unable to challenge LPS-treated *Il10*^{-/-} mice with anti-IgE to show that the LPS-mediated decrease in MC FcεRI expression was IL-10-dependent owing to the exaggerated LPS-induced shock response in *Il10*^{-/-} mice. However, we did show that LPS-induced reduction of FcεRI on the surface of MCs was abrogated in LPS-treated *Il10*^{-/-} mice compared with WT mice. Furthermore, anti-IgE treatment of *Il10*^{-/-} mice induced a similar anaphylactic response to what is observed in WT mice (Maximum Temperature loss (°C): WT vs *Il10*^{-/-} mice; - 5.8 ± 2.0 vs. - 5.4 ± 1.4; Anti-IgE (EM-95) (20μg/200μl i.v.) challenged WT vs *Il10*^{-/-} mice respectively, n = 10 and 4 mice per group) indicating that the absence of IL-10 does not impact IgE-MC responses. However, given the considerable immune dysfunction in the *Il10*^{-/-} mice it is possible that

these mice possess altered hematopoietic and non-hematopoietic responsiveness to IgE-MC responses. Despite these limitations our data suggests that LPS-induced down regulation of FcεRI on mast cells was in part dependent on IL-10.

Our *in vivo* studies reveal that LPS was sufficient to decrease MC FcεRI expression and decrease sensitivity to IgE activation. We currently do not know whether the unresponsiveness of MCs to IgE activation is exclusively due to the down regulated FcεRI on MC populations and/or induction of MC desensitization. TLR-2 and TLR-4 activation of MCs is not sufficient to promote a Ca²⁺ signal (33) and our *in vitro* BMMC analyses revealing decreased FcεRI expression in the absence of any impact on MC degranulation suggest that the LPS predominantly acts through down regulation of FcεRI. However, the effect of LPS on FcεRI expression on BMMCs was greatly diminished compared with *in vitro* pMC populations and the level of reduction maybe insufficient to decrease sensitivity to IgE activation. Previous *in vitro* BMMC studies have revealed that IL-10 while decreasing surface FcεRI expression, can also down-regulate expression of critical IgE-FcεRI downstream signaling molecules Syk, Fyn, Akt and STAT-5 (60, 62). It is possible that a combinatory effect of decreased surface expression of FcεRI and reduced IgE-FcεRI signaling machinery maybe required to drive the lack of responsiveness to IgE.

The potential clinical significance of LPS-induced suppression of MC responsiveness to IgE activation relates to the possible utilization of this pathway to prevent the onset of IgE-mediated reactions during allergen exposure. Allergen immunotherapy is a therapeutic approach to desensitize individuals to environmental and food antigens by manipulation of the immune system by administering increasing doses of allergen over time (71). The administration of increasing doses of specific allergens through a subcutaneous, oral, rectal or sublingual route over a period of weeks to months has been shown to be efficacious and prevent reactions upon accidental exposure (71–74). Unfortunately, subjects undergoing allergen immunotherapy such as oral Immunotherapy (OIT) to foods experience allergic reactions during desensitization, with approximately 20% having severe reactions requiring injection of epinephrine (75, 76). Recently, studies have revealed that usage of adjunctive therapy with Omalizumab (Xolair) decreased the number of adverse reactions during desensitization protocols (77–79). We speculate that usage of TLR4 agonists maybe an additional safe adjunctive therapeutic approach to prevent adverse reactions during desensitization protocols. Indeed, there is the recent development of TLR-4 agonists clinically either alone or in combination with antigens to enhance immune response and allergen immunotherapy approaches efficacy (80, 81). Moreover, clinical trials employing TLR4 agonists (Pollinex Quattro) were shown to be safe and in combination with allergen immunotherapy capable of decreasing reaction rates (80). Notably, experimental-based studies have previously demonstrated that TLR4 agonists can suppress allergic disease; however, this was through alteration of the adaptive T-cell response and generation of inhibitory IgG (82–84). We identify an additional pathway by which LPS can suppress allergic inflammation suppression through down regulation of FcεRI on MCs.

In summary, we show that LPS exposure of C57BL/6 WT mice transiently suppressed the development of IgE-MC mediated anaphylactic reactions and that LPS-induced suppression was associated with decreased mast cell activation and decreased surface expression of

FcεRI. These studies suggest that TLR-4 agonists maybe used as an adjunctive therapeutic approach to prevent adverse IgE-mediated reactions alone or in combination with allergen immunotherapy approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. LPS suppresses IgE-mediated reactions in C57BL/6 mice

(A) The experimental protocol is shown. Mice were i.p. injected with 0, 0.01, 0.1 or 1 mg/kg LPS, and 24 h later, were i.v. injected with 20 µg EM-95, and every mouse was subjected to peritoneal wash 1 h after EM-95 injection. The peritoneal cells were measured and analyzed by flow cytometry. MC were identified as c-Kit⁺, FcεRI⁺ and ST2⁺. (B) Rectal temperature change from 0 to 2 h after LPS i.p. injection. Serum TNF (C) and MCPT1 (D) concentration at 1 or 2 h after i.p. injection. Rectal temperature change from 0 to 60 min (F) and serum MCPT-1 levels in Vehicle- and LPS-treated WT C57BL6 mice following after EM-95 i.v. injection. (G). Representative dot plot and histogram of FcεRI expression on FSC^{low}

SSC^{high} C-Kit⁺ ST2⁺ peritoneal mast cells from Vehicle- (Upper panel) and LPS-treated (Lower panel) C57BL6 mice. Representative overlay histogram (H). and quantification of FcεRI MFI on FSC^{low} SSC^{high} C-Kit⁺ ST2⁺ peritoneal mast cells from Vehicle- and LPS-treated C57BL6 mice. Data represent mean ± SEM. n=4–10 per group; Data were analyzed by one-way or two-way ANOVA and Tukey's multiple comparison post-test. *P<0.05, **P<0.01, ***P<0.001.

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Figure 2. LPS decreased surface expression of FcεRI on peritoneal mast cells
 (A) Overlay histogram and (B) quantification of MFI of FcεRI on FSC^{low} SSC^{high} c-Kit⁺ ST2⁺ peritoneal MC cells from Vehicle- and LPS-treated C57BL6 mice. (C). Quantification of MFI of FcεRI on FSC^{low} SSC^{high} c-Kit⁺ ST2⁺ peritoneal MC cells from Vehicle- and LPS-treated C57BL6 mice before and after anti-IgE treatment. Data represent mean ± SEM. n=4 mice per group; Data were analyzed by one-way or two-way ANOVA and Tukey's multiple comparison post-test *P<0.05, **P<0.01.

Figure 3. pMCs are unresponsive to IgE activation following LPS exposure

Quantification of percentage (%) induction of Fluo-4 MFI in FSC^{low} SSC^{high} c-Kit⁺ FcεRI⁺ ST2⁺ peritoneal MC cells following anti-IgE activation from Vehicle- and LPS-treated C57BL6 mice. Peritoneal cells from Vehicle- and LPS-treated C57BL6 mice were loaded with Fluo-4 and stained for MC markers (c-Kit⁺ FcεRI⁺ ST2⁺) and analyzed for Fluo-4 MFI prior to and following anti-IgE treatment. % Increase MFI Fluo-4 was calculated by Fluo-4 MFI of FSC^{low} SSC^{high} c-Kit⁺ FcεRI⁺ ST2⁺ peritoneal MC cells (mock stimulant) divided by Fluo-4 MFI of FSC^{low} SSC^{high} c-Kit⁺ FcεRI⁺ ST2⁺ peritoneal MC cells (following anti-IgE stimulation) multiplied by 100. Data represent mean ± SEM. n= 6 and 9 mice per group; Data were analyzed by Student's T-test *P<0.05.

Figure 4. LPS-mediated suppression of IgE-mediated reactions in C57BL/6 mice is TLR4-dependent

(A) Rectal temperature change from 0 to 60 min, (B) hemacrit and (C) serum MCPT-1 levels 1 h following anti-IgE stimulation in from Vehicle- and LPS-treated WT and *Tlr4*-deficient (*Tlr4*^{-/-}) C57BL6 mice. Data represent mean $\pm$ SEM. N = 5 and 7 per group; Data were analyzed by Student's T-test.

Figure 5. Temporal suppression of IgE-mediated reactions in C57BL/6 mice by LPS

(A) experimental regime (B) Rectal temperature change from 0 to 60 min, (C) Hematocrit and (D) serum MCPT-1 levels at 60 min in LPS-treated WT C57BL6 mice following anti-IgE challenge. C57BL6 mice were i.p. injected with vehicle (+) or 1 mg/kg LPS and 24, 48 or 72 hours later received an i.v. injection of anti-IgE (20 µg) and evidence of shock response was assessed. Data shown for Vehicle (+) is of the 24-hour time point. Data represent mean ± SEM. N = 4–8 per group; Data were analyzed by one-way ANOVA and Tukey's multiple comparison post-test *P<0.05, **P<0.01, ***P<0.001. (E) Representative dot plot and histogram of FcεRI expression of FSC^{low} SSC^{high} c-Kit⁺ ST2⁺ peritoneal MC

cells from C57BL6 mice following 24 (Upper panels), 48 (middle panels) and 72h (lower panels) LPS-treatment. (F) Overlay histogram and (G) quantification of MFI of FcεRI on FSC^{low} SSC^{high} C-Kit⁺ ST2⁺ peritoneal MC cells from 24–72 h LPS-treated C57BL6 mice. Data represent mean ± SEM. N = 7 – 10 mice per group; Data were analyzed by one-way or two-way ANOVA and Tukey's multiple comparison post-test *P<0.05, **P<0.01.

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Figure 6. LPS-induced down regulation of pMC FcεRI expression is IL-10-dependent

(A). Peripheral blood IL-10 levels in Vehicle and LPS-treated C57BL/6 mice. Mice were i.p. injected with 0 or 1 mg/kg LPS, and 24 h later sera were taken and IL-10 levels measured by ELISA. (B). Quantification of % reduction of FcεRI MFI of FSC^{low} SSC^{high} c-Kit⁺ FcεRI⁺ ST2⁺ peritoneal MC cells was calculated by dividing the mean MFI of FcεRI of FSC^{low} SSC^{high} c-Kit⁺ FcεRI⁺ ST2⁺ peritoneal MC cells from LPS-treated mice by mean MFI of FcεRI of FSC^{low} SSC^{high} c-Kit⁺ FcεRI⁺ ST2⁺ peritoneal MC cells from Vehicle-treated mice multiplied by 100. Data represent mean ± SEM. n = 7 and 3 mice per group; Data were analyzed by Student's T-test *P < 0.05.