The Journal of Immunology

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J Immunol 2013; 190:1614-1622; Prepublished online 16 January 2013;
doi: 10.4049/jimmunol.1202932
http://www.jimmunol.org/content/190/4/1614

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Leukotriene B_4 Enhances Innate Immune Defense against the Puerperal Sepsis Agent *Streptococcus pyogenes*

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Puerperal sepsis is a leading cause of maternal mortality worldwide. *Streptococcus pyogenes* [group A *Streptococcus*; (GAS)] is a major etiologic agent of severe postpartum sepsis, yet little is known regarding the pathogenesis of these infections. Tissue macrophages provide innate defense against GAS, and their actions are highly regulated. The intracellular second messenger cAMP can negatively regulate macrophage actions against GAS. Because leukotriene (LT) B_4 has been shown to suppress intracellular cAMP in macrophages, we hypothesized that it could enhance innate defenses against GAS. We assessed the capacity of LTB_4 to modulate antistreptococcal actions of human macrophages, including placental and decidual macrophages and used a novel intrauterine infection model of GAS in mice lacking the 5-lipoxygenase enzyme to determine the role of endogenous LTs in host defense against this pathogen. Animals lacking 5-lipoxygenase were significantly more vulnerable to intrauterine GAS infection than were wild-type mice and showed enhanced dissemination of bacteria out of the uterus and a more robust inflammatory response than did wild-type mice. In addition, LTB_4 reduced intracellular cAMP levels via the BLT1 receptor and was a potent stimulant of macrophage phagocytosis and NADPH oxidase–dependent intracellular killing of GAS. Importantly, interference was observed between the macrophage immunomodulatory actions of LTB_4 and the cAMP-inducing lipid PGE_2, suggesting that interplay between pro- and anti-inflammatory compounds may be important in vivo. This work underscores the potential for pharmacological targeting of lipid mediator signaling cascades in the treatment of invasive GAS infections. ***The Journal of Immunology*****, 2013, 190: 1614–1622.**

Worldwide, >500,000 women die per year as a complication of childbirth (1). A principal cause of maternal death is puerperal sepsis, which results in >75,000 maternal deaths per year globally (2). The Gram-positive bacterium *Streptococcus pyogenes*, also known as a group A *Streptococcus* (GAS), is the most common cause of severe postpartum sepsis (3). Puerperal GAS is an important cause of maternal death in resource-poor settings, and it is a reemerging problem in industrialized countries (3). For example, the rate of maternal deaths from sepsis increased in the U.K. between 2000 and 2008, primarily because of increases in postpartum GAS (4). Significant gaps exist in understanding fundamental mechanisms of GAS pathogenesis and host defense in the female reproductive tract that lead to intrauterine infection and sepsis.

Pregnancy is associated with major shifts in immune surveillance (5), as the maternal immune system must be "detuned" to accommodate the immunologically distinct fetus (6). Despite this, the mother's immune system must be able to detect and respond to potentially pathogenic organisms. However, some pathogens have evolved mechanisms to evade host defense, apparently taking advantage of the immunological shifts associated with pregnancy. For example, certain Gram-positive bacteria are adept at causing pregnancy-related infections, including *Listeria monocytogenes*, *S. pneumoniae*, GAS, group B *Streptococcus*, and the clostridia (3, 7, 8). The incidence of invasive GAS is >25 times higher for postpartum than for nonpregnant women, but the reasons for this are not defined (7). It is also unclear why only a subset of women exposed to GAS develops overt disease. Bacterial virulence factors are important determinants of GAS infection, but these alone do not predict the development and clinical severity of infection (9). Greater attention must be paid to the basic mechanisms of disease pathogenesis, with an emphasis on host–microbial interactions.

Tissue macrophages are critical cellular immune barriers against invasive GAS infection (10, 11), although this has not been examined in the reproductive tract. Macrophage phagocytosis and intracellular killing of tissue-invasive microbes are highly regulated processes that present an important first line of defense against potential pathogens. The host factors that regulate the

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Received for publication October 23, 2012. Accepted for publication December 6, 2012.

This work was supported by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior Grant 2652110 and Fundação de Amparo a Pesquisa do Estado de São Paulo, Brazil (to E.M.S., L.H.F.); an Investigators in the Pathogenesis of Infectious Disease grant from the Burroughs Wellcome Fund (to D.M.A.); a Scientific Exchange Fellowship from the International Society for Infectious Diseases (to E.M.S.); and National Institutes of Health Grants T32HL007749 (to K.L.M.) and HL103777-03 (to C.H.S.).

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Abbreviations used in this article: DM, decidual macrophage; 8E, 10E-trienoic acid; GAS, group A *Streptococcus*; 12-HHT, 12(S)-hydroxyheptadeca-5Z; HK, heat killed; 5LO, 5-lipoxygenase; LT, leukotriene; NADPHox, NADPH oxidase; PM, placental macrophage; PTX, pertussis toxin; RFU, relative fluorescence unit; ROI, reactive oxygen intermediate; $RPMI^{+/+}$, RPMI 1640 supplemented with 1% antibiotic–antimycotic and 10% charcoal/dextran-treated FBS; $RPMI^{+/-}$, RPMI 1640 supplemented with 1% antibiotic–antimycotic in the absence of serum; THY, Todd Hewitt Broth plus 5% of yeast; TSA–blood, tryptic soy agar plus 5% sheep blood.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1202932

capacity of macrophages to respond to GAS have not been well characterized. Defining the mechanisms governing antimicrobial functions of tissue macrophages provides an opportunity to develop innovative measures for preventing or treating invasive bacterial infections in susceptible hosts. Recently, host-derived lipid mediators have been found to regulate the capacity for macrophages to respond to GAS (12), but this has not been explored in the context of reproductive immunology.

Eicosanoids are immunoregulatory lipids that are oxygenated metabolites of the cell membrane constituent molecule arachidonic acid. They are potent modulators of innate immunity, including macrophage behaviors (13–17). For example, recent studies suggest that the cyclooxygenase-derived PG compound, PGE_2, suppresses macrophage phagocytosis and intracellular killing of GAS through acutely activating the synthesis of the intracellular second messenger molecule cAMP (18). In general, receptor-dependent increases in cAMP suppress macrophage immune responses, possibly an evolved mechanism for limiting the amplitude and duration of inflammatory responses (19). In contrast, the 5-lipoxygenase (5LO)–derived eicosanoid leukotriene (LT) B_4 has been reported to stimulate macrophage phagocytosis and intracellular killing of several pathogens in association with suppression of intracellular cAMP production (17, 20). The ability of LTB_4 to enhance host elimination of streptococci has been most clearly defined in a model of lung infection caused by *S. pneumoniae* (21–24). In addition, the administration of aerosolized LTB_4 to mice with preexisting pneumococcal pneumonia increased mononuclear phagocyte/macrophage accumulation in the lungs, NADPH oxidase (NADPHox) subunit expression in pulmonary macrophages, and pulmonary bacterial clearance (25).

Leukotriene B_4 is synthesized from arachidonic acid when an acute Ca^{2+} flux is induced in cells following activation by inflammatory mediators or pathogens (26, 27). Two G protein–coupled receptors for LTB_4 inhibit adenylate cyclase. The BLT1 receptor (high affinity) is expressed primarily in leukocytes, including granulocytes, eosinophils, macrophages, and differentiated T cells, whereas the BLT2 receptor (low affinity) is expressed in many cell types (28–32).

It is notable that both PGE_2 and LTB_4 are found in the gravid uterus (33–35), and both have immunoregulatory actions that might play a role in defining a postpartum woman's vulnerability to invasive bacterial infection. Because PGE_2 stimulates intracellular cAMP to suppress macrophage immune defenses against GAS (18), we hypothesized that LTB_4 would *stimulate* macrophage actions against this organism because its actions on cAMP are opposite to those of PGE_2. We further speculated that mice unable to generate LTB_4 because they lack the gene encoding 5LO would be susceptible to intrauterine GAS infection. Both in vivo and in vitro models of infection were used to assess the role of LTs as immunoregulators of innate defense against GAS.

Materials and Methods

Reagents

RPMI 1640 culture medium and antibiotic solution (penicillin and streptomycin) including an antimycotic (amphotericin) were from Invitrogen (Carlsbad, CA). Charcoal-stripped and dextran-treated FBS was from HyClone Laboratories (Waltham, MA). Pertussis toxin (PTX), cytochalasin D from *Zygosporium mansonii*, and apocynin were obtained from EMD Chemicals–Calbiochem (Gibbstown, NJ). FITC, trypan blue, DNase, hyaluronidase, collagenase, Percoll, citrate buffer, fucoidan, saponin, PMA, nonenzymatic cell dissociation solution, $NaHCO_3$ buffer, and sodium bicarbonate were from Sigma-Aldrich (St. Louis, MO). LTB_4, PGE_2, 12(S)-hydroxyheptadeca-5Z, 8E, 10E-trienoic acid (12-HHT), U75302, and A23187 were from Cayman Chemicals (Ann Arbor, MI). Todd Hewitt Broth, tryptic soy agar, and yeast extract were from BD Biosciences (San Jose, CA). Sheep blood was from Remel (Lenexa, CA). Ficoll-Hypaque was obtained from GE Healthcare (Piscataway, NJ). Lysis buffer for erythrocytes was purchased from eBioscience (San Diego, CA). FLUO-4 NW used for intracellular calcium assays and 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (carboxy H2DCFDA) for reactive oxygen species measure were provided from Molecular Probes (Grand Island, NY). ELISA kits to measure intracellular cAMP were from Enzo Life Sciences (Farmingdale, NY). Magnetic MACS CD14 microbeads (human) were from Miltenyi Biotec (Auburn, CA). Required dilutions of all compounds were prepared immediately before use, and equivalent quantities of vehicle were added to the appropriate controls. Compounds requiring reconstitution were dissolved in DMSO (Sigma-Aldrich).

Animals

Age-matched, 6- to 8-wk-old, female, nonpregnant, C57BL/6 wild-type or 5LO-deficient mice ($5LO^{-/-}$) mice on a C57BL/6 background were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were treated according to National Institutes of Health guidelines for the use of experimental animals, with approval of the University of Michigan Committee for the Use and Care of Animals.

Bacteria

The GAS M1T1 strain 5448 was generously provided by Dr. Malak Kotb of the University of Cincinnati (Cincinnati, OH), and aliquots were grown in Todd Hewitt Broth plus 5% of yeast (THY). Estimates of bacterial concentrations in inocula were made by comparing the absorbance of light at 600 nm (OD_{600}) and using a standard curve of CFU per milliliter versus OD_{600}. The actual CFU per milliliter concentrations were confirmed for all bacterial suspensions used in these studies by culturing serial 10-fold dilutions on tryptic soy agar plus 5% sheep blood (TSA–blood) plates. For phagocytosis experiments, heat-killed (HK) FITC-labeled GAS were used. These were generated as previously described for *S. pneumoniae* (36).

Intrauterine infection

GAS cultures were grown 18 h, shaking at 200 rpm, at 37°C in 10 ml THY; the bacteria were then centrifuged 3000 rpm for 10 min, and the pellet was washed twice in sterile 1× PBS and resuspended in 1 ml total volume. Dilutions were prepared in 1× PBS to obtain the correct approximate inoculum of CFUs per mouse. The actual inoculum was determined by plating serial 10-fold dilutions of the PBS suspension onto TSA–blood plates. The infection was performed in anesthetized animals. A low 2.0-cm midline abdominal incision exposed the right uterine horn, the uterine horn was ligated at the cervical junction to prevent inoculum loss, and 20 μl of prepared culture was directly injected into the horn (37, 38). For survival experiments, mice were monitored every 2 h on weekdays and 2× per day on weekends for 1 wk after inoculation.

Quantitation of bacterial loads

After 24 h of infection, mice were euthanized, and bacterial load was determined in the blood, spleen, and uterus. The blood was serially diluted in sterile 1× PBS and cultured on TSA–blood at 37°C for 18 h. The spleen and uterus were homogenized with a handheld tissue tearer in 1 ml sterile 1× PBS, serially diluted in PBS, and then cultured on TSA–blood at 37°C for 18 h. Colonies were counted as previously described (37).

Human subjects

Following appropriate informed consent, human decidual tissue was obtained from healthy adult women aged 18–44 y who were undergoing elective surgical termination of pregnancy during the first trimester. Human placental tissue was obtained from healthy women undergoing cesarean section at the term of pregnancy. Human peripheral blood was obtained from healthy adults who were not smokers, not taking nonsteroidal anti-inflammatory drugs, not taking oral or inhaled steroids, and not pregnant. Blood was collected by venous puncture into heparinized collection tubes. These studies were reviewed and approved by the University of Michigan Institutional Review Board.

Cell culture

THP-1 monocytic cells were obtained from the American Type Culture Collection (ATCC TIB-202; Manassas, VA) and cultured in RPMI 1640 supplemented with 1% antibiotic–antimycotic and 10% charcoal/dextran-treated FBS ($RPMI^{+/+}$). Cells were passaged every 2–4 d and were used only through the 10th passage, at which time a new culture was started. THP-1 cells were differentiated into macrophage-like cells by culturing with 100 nM of PMA in $RPMI^{+/+}$ for 18 h at 37°C with 5% CO_2. Cells

were detached from the flask with nonenzymatic cell dissociation solution, scraped after 5 min of incubation at 37°C with 5% CO_2. The protocol was adapted as described before (39, 40). PBMCs, placental macrophages (PMs), decidual macrophages (DMs), and peritoneal macrophages were cultured in $RPMI^{+/+}$ for 18 h at 37°C with 5% CO_2 at a density of 2×10^5 cells per well in 6-, 96-, or 384-well plates (Costar, Corning, NY), depending on the experiment.

Isolation and culture of PMs and DMs

The procedure for DM and PM culture was similar to that used for THP-1 cells above. Isolation was adapted from a previously described protocol (38, 41). Briefly, the tissue was collected in 50-ml conical tubes containing 15 ml sterile PBS and washed three times with 50 ml PBS at 1500 rpm for 10 min. The tissue was weighed and minced into small pieces with autoclaved scissors and weighed again to determine final grams collected. After this, the fragments were placed into 50-ml conical tubes with digestion solution containing DNase, collagenase, and hyaluronidase at 10 ml per gram of tissue. Cells were filtered through a 280-μm autoclaved metal sieve, followed by 180- and 80-μm autoclaved nylon screens. Cells were centrifuged again and resuspended in 25% Percoll diluted in cold $RPMI^{+/+}$ and overlaid onto 50% Percoll plus 2 ml PBS on top of the density gradient. $CD14^+$ cells were performed using magnetic MACS large-cell separation column system (Miltenyi Biotec) according to the manufacturer's instructions and adapted as previously reported (38).

Human PBMC isolation

Human PBMCs were isolated from heparinized blood by Ficoll-Hypaque gradient density centrifugation by 20 min at 1700 rpm, 22°C, without active breaking. The PBMCs were collected, transferred to new tubes, and washed with PBS at 1400 rpm × 10 min at room temperature. The pellets were broken and pooled together in one tube and washed again. The erythrocytes were lysed after the addition of 10 ml of lysis buffer at 22°C × 10 min. The cells were washed again; the final solution was resuspended in 50 ml PBS, and total cells were counted. $CD14^+$ cells were negatively selected using magnetic MACS small-cell separation column system (Miltenyi Biotec) according to the manufacturer's instructions.

Isolation and culture of peritoneal macrophages

Resident PMs were collected from uninfected mice. Mice were euthanized by CO_2 inhalation, and peritoneal cavities were washed twice with 5 ml cold RPMI 1640 supplemented with 1% antibiotic–antimycotic in the absence of serum ($RPMI^{+/-}$). Collected fluid was placed in conical tubes on ice and centrifuged at 1500 rpm for 10 min. Cell counts were performed immediately, and 2×10^5 cells per well were added to culture in 384-well plates (Costar), followed by incubation at 37°C, 5% CO_2, for 2 h. After this period, the media were changed to $RPMI^{+/+}$, and adherent cells were cultivated by 18 h incubation at 37°C, 5% CO_2, as published before (42, 43).

Fluorometric phagocytosis assay with FITC-labeled GAS

The phagocytosis of unopsonized FITC-labeled HK GAS was assessed using PMA-treated THP-1 cells, PBMCs, DMs, PMs, and resident PMs. All cell types were plated onto 384-well tissue culture–treated plates with 2×10^5 cells per well, and the phagocytosis was performed as previously reported (20, 38, 44). After an overnight rest, media were removed, and $RPMI^{+/-}$ containing vehicle or different stimuli were added as indicated in the figure legends. Specific treatments included LTB_4 (15-min pretreatment, 1–1000 nM), PGE_2 (15-min pretreatment, 1 μM), 12-HHT (15-min pretreatment, 0.001–1 μM), U75302 (15-min pretreatment, 10 μM), and PTX (18-h pretreatment, 3 μg/ml). After appropriate incubations, GAS were added at multiplicity of infection of 150:1 and incubated at 37°C with 5% CO_2 for 3 h. Extracellular bacterial fluorescence was quenched with trypan blue (500 μg/ml in 0.09 M citrate buffer) for 15 min in the dark before the fluorescence was determined on a microplate fluorometer ($485_{ex}/535_{em}$), SPECTRAMax GEMINI EM (Molecular Devices, Sunnyvale, CA). A minimum of eight replicates were done for each condition in each experiment. Fluorometric data were expressed in arbitrary relative fluorescence emitted from intracellular phagocytosed bacteria (relative fluorescence unit $[RFU]_i$) and was determined by subtracting the fluorescence from unquenched, extracellular bacteria (RFU_{ex}) from the total fluorescence of the experimental well (RFU_{total}). The RFU_{ex} was determined using the fluorescence of cells exposed for 30 min to the phagocytosis inhibitor cytochalasin D (20 μg/ml). Thus, the PI = RFU_i = $RFU_{total} - RFU_{ex}$ (44, 45).

Bacterial killing assay

PMA-treated THP-1 cells were adjusted to a suspension of 2×10^5 cells per well in 100 μl $RPMI^{-/-}$ in replicates of 8 per condition in a 96-well plate and was centrifuged at 500 rpm for 30 s to settle all cells into a monolayer. Cells adhered for 1 h before starting with the treatments. One set of replicate conditions within each plate was treated with cytochalasin D for 30 min. Cells were pretreated with the NADPHox inhibitor apocynin 100 μM and/or LTB_4 10 nM for 30 and 15 min respectively before infecting cells with a 10:1 multiplicity of infection of live GAS. The plates were centrifuged again to synchronize bacterial contact with the monolayer and incubated for 30 min. After this time, all media were removed and plates washed gently 3 times with $RPMI^{+/-}$ to remove nonphagocytosed bacteria. Then 100 μl of 0.5% saponin in PBS was added and incubated for 10 min. Serial dilutions were made and the lysates were plated onto TSA–blood plates; CFUs were counted 24 h later (adapted from Refs. 14 and 22).

Measurement of intracellular reactive oxygen intermediates

Reactive oxygen intermediate (ROI) generation was performed with 1.2×10^5 PMA-treated THP-1 cells per well, using 384-well plates, according to our previously reported protocol (14, 22, 46). Medium was replaced the following morning, and cells were rested for 2 d. The cells were incubated with the ROI-sensitive fluorophore carboxy-H2DCFDA at 10 μM for 30 min, followed by two washes with HBSS. Then LTB_4 (10 nM), live unopsonized GAS 10:1, or both were added and incubated for 30 min. The fluorescence intensity was measured at an excitation wavelength of 493 nm and emission wavelength of 522 nm, using the fluorescent plate reader (above).

Measurement of intracellular cAMP

For intracellular cAMP measurement, 3×10^6 PMA-treated THP-1 per well were used. The medium was changed to $RPMI^{+/-}$, and cells were incubated for 0, 15, 60, 300, 600, and 900 s with 10 nM LTB_4 for some experiments or 60 s with 10 nM LTB_4 plus 900 s, followed by 1 μM of PGE_2, for others. Culture supernatants were aspirated, and the cells were lysed by incubation for 10 min with 0.1 M HCl at room temperature, followed by disruption using a cell scraper (20). Intracellular cAMP levels were determined by ELISA according to the manufacturer (Enzo Life Sciences).

Measurement of LTB_4 and cytokines

Uteri were removed 24 h postinfection to measure LTB_4 and cytokines. Briefly, tissue was homogenized (Tissuemiser; Fisher Scientific, Suwanee, GA) in 1 ml PBS, centrifuged, and stored at −80°C until assayed. The OD of samples was determined at 405 nm on a microplate reader (VERSAMAX; Molecular Devices, Sunnyvale, CA), and concentrations of eicosanoids were calculated based on a standard curve. The sensitivity for LTB_4 was 5.63 pg/ml. A specific enzyme immunoassay was used to quantify LTB_4 (Enzo Life Sciences) according to the manufacturer's instructions. The tissue was recovered and stored at −80°C until assays were performed to determine the levels of IL-17A, IL-6, IL-1β, TNF-α, IL-10, IFN-γ, and MCP-1 by ELISA (R&D Duoset; R&D Systems). ELISAs were performed by the University of Michigan Cancer Center Cellular Immunology Core (14).

Analysis of calcium mobilization

PMA-treated THP-1 cells were used at a density of 4×10^5 cells per well, using 96-well, white-bottom plates (BD Biosciences). The cells were incubated at 37°C plus 5% CO_2 for 1 h to rest, and then the media were removed from the adherent cell culture, to eliminate sources of baseline fluorescence, particularly esterase activity. A volume of 100 μl of the dye-loading solution was added to each well immediately. The plate was incubated for 30–45 min at 37°C, and after this, LTB_4 (10 nM) and our positive control A23187 (1 μM) were added in the specific wells at the moment of the reading. Fluorescence was measured with excitation at 494 nm and emission at 516 nm. The assay was performed using a Fluo4-NM Calcium Kit (Invitrogen) according to the manufacturer's instructions and adapted according to previous works (47, 48).

Statistical analysis

Data are presented as the mean ± SEM and were analyzed with the Prism 5.0 statistical program (GraphPad Software). Comparisons among three or more experimental groups were performed with one-way ANOVA, followed by a Bonferroni correction, and between two experimental groups a Student *t* test was used, unless otherwise stated. Differences were con-

sidered significant for $p < 0.05$. Animal survival experiments were evaluated for differences using a Mantel–Cox log-rank test.

Results

5LO$^{-/-}$ mice have enhanced susceptibility to intrauterine GAS infection

Leukotrienes, including LTB_4, are important endogenous regulators of immune defense against bacterial pathogens (21, 25, 49, 50), suggesting that they might be involved in supporting host defense against GAS. To test this, wild-type C57BL/6 mice or 5LO$^{-/-}$ mice were subjected to intrauterine GAS infections and monitored for survival, uterine bacterial clearance, and bacterial dissemination. As illustrated in Fig. 1A, 5LO$^{-/-}$ mice were significantly more susceptible to overwhelming infection caused by GAS than were wild-type mice. It was found that 80% of the wild-type mice survived after 7 d, whereas only 30% of 5LO$^{-/-}$ mice survived ($p < 0.0001$). The enhanced susceptibility of 5LO$^{-/-}$ mice to intrauterine GAS infection was correlated with an impaired ability of these genetically deficient mice to contain the infection within the uterus (Fig. 1B). At 24 h postinfection, the number of bacteria that disseminated to the spleen in 5LO$^{-/-}$ mice was 1.48 times greater than that observed for wild-type mice. Levels of LTB_4 were measured in uterine homogenates from wild-type and 5LO$^{-/-}$ mice following GAS infection. As shown in Fig. 1C, intrauterine infection with GAS significantly increased LTB_4 production in the wild-type uterus. However, uterine tissues obtained from infected 5LO$^{-/-}$ mice did not reflect an increase in LTB_4 generation, compared with uninfected tissues. Interestingly, as evidenced in Fig. 1D, inflammatory mediator generation, including IL-1β, IL-6, IL-17, and MCP-1, was markedly increased in the uterine tissues of infected 5LO$^{-/-}$ mice compared with wild-type animals.

LTB_4 enhances the phagocytosis of unopsonized GAS

The importance of LTs in supporting clearance of GAS in vivo and the role of macrophages in host defense against GAS (10, 11) suggested that LTB_4 might regulate macrophage interactions with GAS in vitro. Thus, primary human macrophages, mouse macrophages, or THP-1 cells were pretreated with various concentrations of LTB_4 (1–100 nM) that were found to enhance macrophage phagocytosis in other model systems (20, 50, 51). As outlined in Fig. 2A–E, LTB_4 significantly increased the phagocytosis of unopsonized GAS by THP-1 cells, human PBMCs, human DMs, human PMs, and mouse PMs. Because the actions of LTB_4 on THP-1 cells were similar to primary cells, we used this human macrophage–like cell line for mechanistic studies to build a model of how LTB_4 regulates phagocyte function during GAS infection.

The high-affinity, Gα_i-coupled BLT1 receptor for LTB_4 modulates phagocytosis in THP-1 cells

LTB_4 has two major G protein–coupled receptors: the high-affinity BLT1 receptor and the low-affinity BLT2 receptor (28). The BLT1 receptor has been implicated in macrophage immunoregulation by LTB_4 (52) through a PTX-sensitive suppression of intracellular cAMP (20). We confirmed that THP-1 cells express the BLT1 receptor (39) by Western immunoblot analysis (not shown) and demonstrated that the LTB_4-mediated enhancement of GAS phagocytosis by THP-1 cells was completely blocked by the BLT1 receptor antagonist U75302 (Fig. 3A). Further, 12-HHT, an agonist for the BLT2 receptor that is more potent than LTB_4 (28), had no significant effect on THP-1 internalization of unopsonized bacteria (Fig. 3B).

The capacity of LTB_4–BLT1 signaling to enhance GAS phagocytosis suggested that LTB_4 reduced cAMP in these cells. We measured intracellular cAMP in macrophages after exposure to

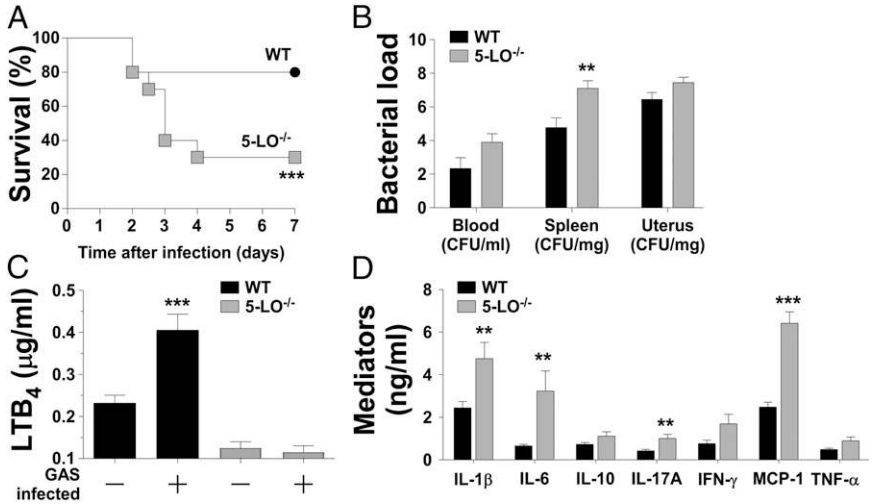

FIGURE 1. Leukotrienes are important determinants of survival and containment of intrauterine GAS infection in mice. (**A**) Wild-type C57BL/6 or 5LO$^{-/-}$ mice ($n = 10$ per group) were inoculated with 10^4 CFU of GAS in the right uterine horn on day 0, and survival was recorded over time. Vehicle (PBS)–treated mice did not die ($n = 10$; data not shown). Data are expressed as mean ± SEM followed by log-rank (Mantel–Cox) test. ***$p < 0.001$. (**B**) Nonpregnant mice C57BL/6 or 5LO$^{-/-}$ ($n = 10$ per group) were inoculated with 10^4 CFU of GAS in the right uterine horn and then euthanized 24 h postinfection. Bacterial loads from blood, spleen, and uterus were determined as described in *Materials and Methods*. Data shown are from one representative experiment from two independent experiments with similar results. Data are expressed as mean ± SEM followed by unpaired Student *t* test. *$p < 0.05$. (**C**) After 24 h of infection, enzyme immunoassay quantification of LTB_4 concentrations was performed on uterine homogenates obtained from mice that had received either an intrauterine PBS injection (uninfected) or GAS. Data are presented as the mean ± SEM followed by one-way ANOVA and are representative of two independent experiments with similar results ($n = 10$ mice per group). ***$p < 0.001$ compared with wild-type uninfected control. (**D**) Inflammatory mediators in uterine homogenates of C57BL/6 or 5LO$^{-/-}$ mice 24 h after inoculation with 10^4 CFU of GAS. At 24 h after intrauterine inoculation with GAS, cytokines and chemokines were measured by ELISA. **$p < 0.01$, ***$p < 0.001$ by Student *t* test ($n = 10$ mice per group from two combined experiments with similar results).

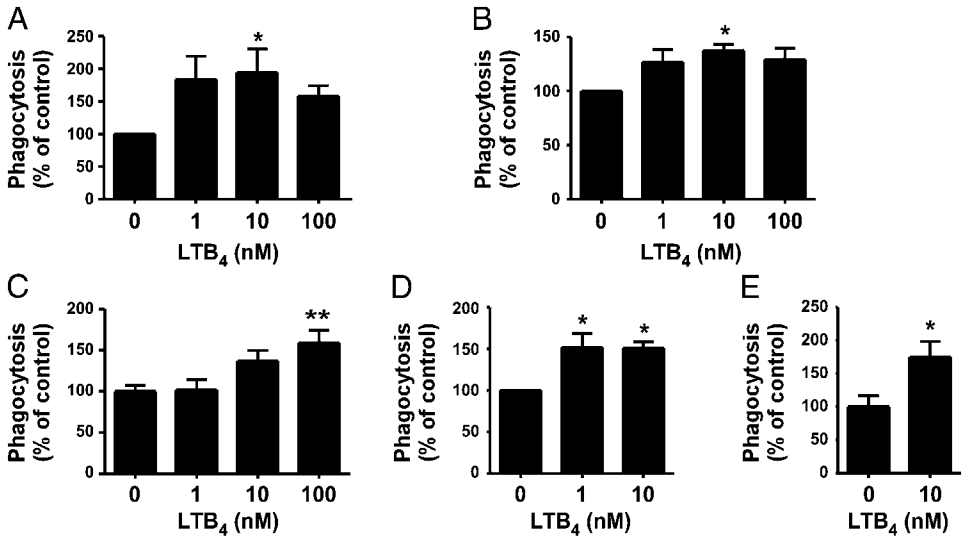

FIGURE 2. LTB_4 increases leukocyte phagocytosis of GAS. Macrophages were exposed for 15 min to LTB_4 before challenge with FITC-labeled HK GAS, and phagocytosis was determined as described in *Materials and Methods*. Data shown are from (**A**) PMA-treated human THP-1 cells, (**B**) human PBMCs, (**C**) human DMs, (**D**) human PMs, and (**E**) C57BL/6 mouse peritoneal macrophages. All data are expressed as a percentage of the phagocytic activity of untreated cells. $*p < 0.05$, $**p < 0.01$ versus untreated, as determined by one-way ANOVA for (A)–(D) and *t* test for (E).

LTB_4. Notably, cAMP levels fell acutely. Within 15 s of exposure, the intracellular levels were undetectable (Fig. 4A). Phagocytosis experiments with PTX (Fig. 4B) confirmed that the capacity for LTB_4 to stimulate THP-1 cell ingestion of GAS was $G\alpha_i$ dependent. Because the BLT1 receptor can signal through a $G\alpha_q$-dependent release of intracellular calcium (53), we tested whether LTB_4 treatment could increase Ca^{2+} in THP-1 cells. As opposed to the positive control compound, the calcium ionophore A23187, LTB_4 failed to significantly alter basal calcium levels (Fig. 4C), consistent with results in lung macrophages (20). This finding was verified over a range of LTB_4 concentrations and incubation times (data not shown).

LTB_4 enhances bacterial killing of GAS

Along with phagocytosis, intracellular microbicidal activity is an important step in the control of infection. Previous work in other model systems has revealed that PGE_2 can suppress the capacity of macrophages to kill bacteria (13, 18, 54), whereas endogenous and exogenous LTs enhance this action (20, 55). We determined the importance of LTB_4 to macrophage killing of GAS using THP-1 cells. As noted in Fig. 5A, the number of intracellular bacteria present in THP-1 cells following 30 min of incubation was significantly reduced by treating cells with LTB_4 (10 nM). ROIs generated by NADPHox are important for macrophage elimination of GAS (56), and it is known that LTB_4 enhances NADPHox activation and ROI generation in macrophages (25, 55, 57–59). The extent to which the LTB_4 stimulation of ROI production enhanced macrophage GAS killing was evaluated using the selective NADPHox inhibitor apocynin (60) Treatment with apocynin prevented the acute killing of intracellular bacteria during the 30 min of incubation of GAS with THP-1 cells (Fig. 5A). In addition, LTB_4 failed to reduce the number of intracellular bacteria in macrophages pretreated with apocynin, suggesting that the actions of LTB_4 depend upon NAPDHox activation. We then tested whether LTB_4 enhanced intracellular ROI generation in THP-1 cells infected with GAS (Fig. 5B). Notably, live GAS suppressed cellular ROI concentrations, an effect not associated with cell death (data not shown). However, LTB_4 alone significantly increased ROI generation, and this overcame the suppressive action of GAS (Fig. 5B).

The cAMP-reducing actions of LTB_4 antagonize PGE_2 actions on GAS phagocytosis

If the actions of LTB_4 are mediated through suppression of intracellular cAMP, then it can be hypothesized that this lipid would antagonize the capacity for PGE_2 (which stimulates cAMP) to suppress macrophage host defense functions. Indeed, PGE_2 raised intracellular cAMP concentrations and inhibited the phagocytosis of GAS by THP-1 cells (Fig. 6A, Fig. 6B). As noted in Fig. 6A, PGE_2 increased cAMP, LTB_4 reduced it, and the effect of a combination of both lipids was intermediate (Fig. 6A). In addition (Fig. 6B), 10 μM of PGE_2 significantly inhibited GAS phagocytosis by 56.5 ± 3.0%, whereas 100 nM of LTB_4 enhanced it by 44.4 ± 7.3%. However, when cells were exposed to both com-

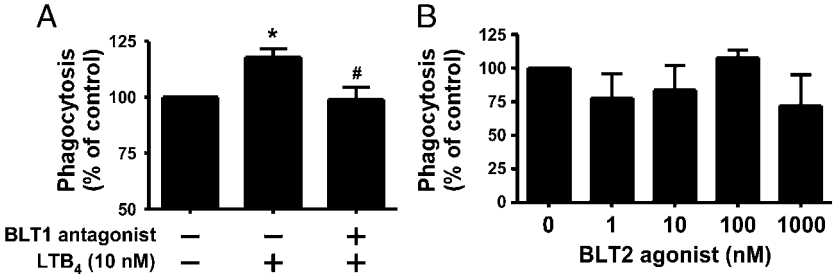

FIGURE 3. LTB_4 enhances GAS phagocytosis in THP-1 cells via the BLT-1 receptor. (**A**) PMA-treated THP-1 cells were incubated with the BLT1 receptor antagonist U75302 (10 μM) or LTB_4 (10 nM) for 15 min before challenge with FITC-labeled HK GAS, as described in *Materials and Methods*. Data shown are mean ± SEM of four independent experiments. (**B**) PMA-treated THP-1 cells were incubated with 0.001–1 μM of BLT2 agonist (12-HHT) for 15 min before challenge with HK FITC-GAS at 150:1. Phagocytosis was quantified after 3 h by fluorometry, as detailed in *Materials and Methods*. $*p < 0.05$ versus untreated, $^{\#}p < 0.05$ versus LTB_4, as determined by one-way ANOVA.

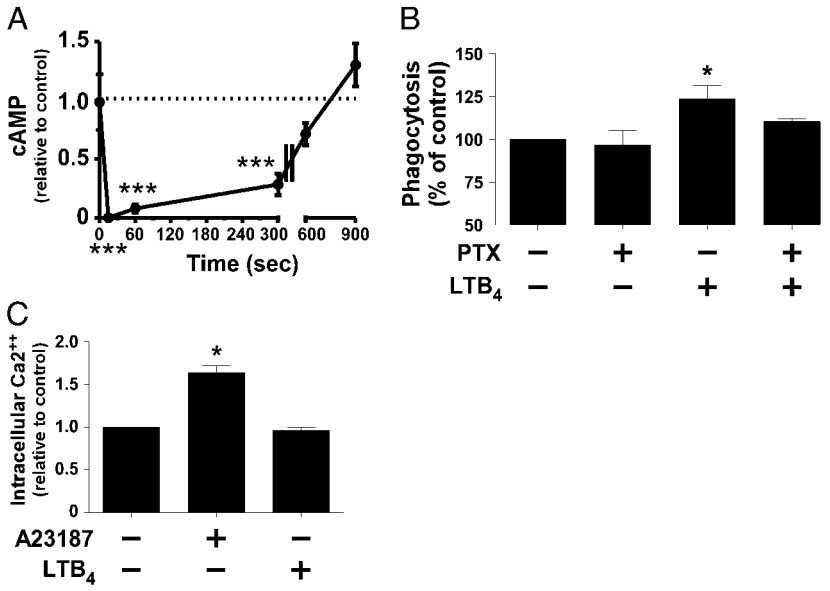

FIGURE 4. Enhancement of phagocytosis by LTB_4 depends on $G\alpha_i$ receptor activation. (**A**) PMA-treated THP-1 cells were cultured for 0, 15, 60, 300, 600, and 900 s with LTB_4 (10 nM), and intracellular cAMP was measured by ELISA. Mean ± SEM data from five independent experiments were combined with $n = 3$ per each condition per experiment. $^{***}p < 0.001$. (**B**) PMA-treated THP-1 cells were cultured overnight with 3 μg/ml of PTX, and 18 h later the cells were incubated for 15 min with LTB_4 (10 nM) before GAS phagocytosis was quantified as detailed in *Materials and Methods*. $^{*}p < 0.05$ versus untreated cells. (**C**) FLUO-4NM–loaded, PMA-treated THP-1 cells were exposed to LTB_4 (10 nM) and A23187 (1 μM), and changes in intracellular Ca^{2+} were measured as described in *Materials and Methods*. Data are expressed as a percentage, relative to untreated cells. $^{*}p < 0.05$ versus untreated cells.

pounds, the net effect was a lack of significant change in phagocytic activity relative to untreated cells (Fig. 6B).

Discussion

S. pyogenes is a leading cause of severe postpartum sepsis worldwide (1, 61). A better understanding of why pregnancy and the postpartum state increase the risk for GAS is needed to develop better preventive or therapeutic strategies. In this article, we report the novel finding that LTB_4 is an endogenous lipid mediator that augments innate immune defenses against invasive GAS infection. Macrophages are important sentinels of host defense against GAS (10, 11). These studies suggest that LTB_4, a major product of the 5LO metabolic pathway, enhances the phagocytosis and bacterial killing of unopsonized GAS by macrophages, including primary human macrophages from the female reproductive tract. The mechanism for these actions involves signaling via the $G\alpha_i$-coupled BLT1 receptor with an acute suppression of cAMP.

Earlier studies established the importance of LTs as immunostimulatory mediators during infection with non-GAS pathogens (21, 25, 42, 49, 62, 63). In fact, the capacity of LTB_4 to regulate host–streptococcal interactions has been shown in a model of lung infection caused by *S. pneumoniae* (21–24). In addition, the administration of aerosolized LTB_4 to mice with pre-existing pneumococcal pneumonia increased mononuclear phagocyte/macrophage accumulation in the lungs, NADPHox subunit expression in pulmonary macrophages, and pulmonary bacterial clearance (25). Although these data provided an important rationale for our hypothesis, little was known about the role of LTB_4 in GAS infections or reproductive tract infections.

A significant finding includes the critical importance of the enzyme 5LO in supporting immune containment of intrauterine *S. pyogenes* infection. Unlike wild-type mice, mice lacking the 5LO enzyme did not produce meaningful quantities of LTB_4 following infection, they were significantly more susceptible to death from GAS endometritis, they were less capable of preventing dissemination of bacteria from the uterus to distant sites, and they displayed a significantly enhanced inflammatory response to infection. These findings suggest that an overwhelming inflammatory response, due to poor innate immune containment of a local GAS infection, played a role in the mortality of this infection in $5LO^{-/-}$

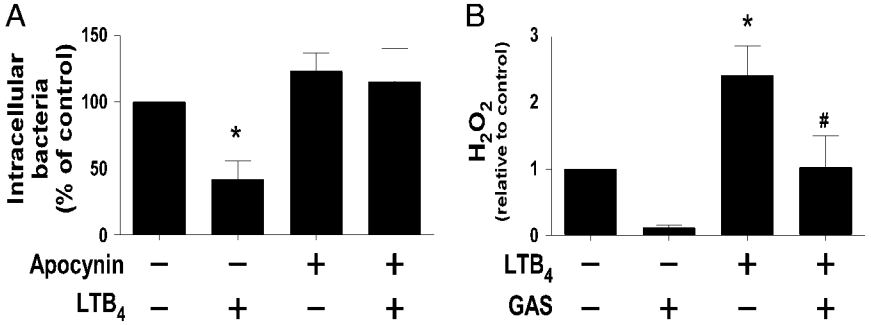

FIGURE 5. LTB_4 enhances macrophage killing of GAS through activation of reactive oxygen species production. (**A**) PMA-treated THP-1 cells were exposed to apocynin (100 μM) for 30 min and/or LTB_4 (10 nM) for 15 min prior to inoculation with live unopsonized GAS (10 bacteria per cell). Phagocytosis was determined as detailed in *Materials and Methods*; after 30 min THP-1 cells were washed to remove extracellular bacteria and lysed to release intracellular bacteria, which were enumerated on solid agar. Data are expressed as the percent of bacterial CFU relative to the untreated control and are presented as the mean ± SEM of five independent experiments. $^{*}p < 0.05$ compared with untreated control, as determined by one-way ANOVA. (**B**) PMA-treated THP-1 cells were incubated with H2DCFDA (10 μM) for 30 min followed by LTB_4 (10 nM) and/or live unopsonized GAS (10 bacteria per cell) for another 30 min, and H_2O_2 production was measured as described. Data are expressed relative to untreated cells and display the mean ± SEM of eight independent experiments. $^{*}p < 0.05$ compared with untreated cells, $^{\#}p < 0.05$ compared with LTB_4, as determined by one-way ANOVA.

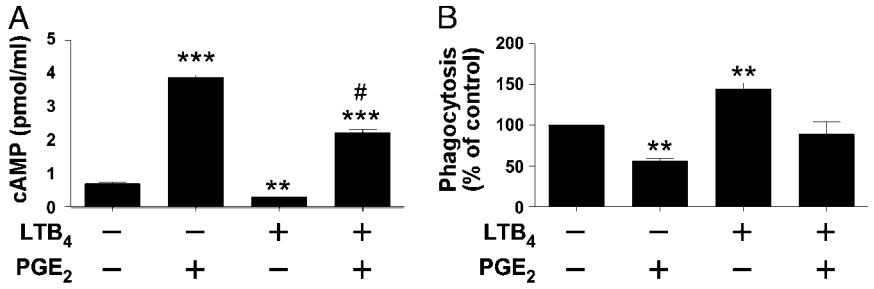

FIGURE 6. Effects of exogenous PGE_2 and LTB_4 on phagocytosis and cAMP production in macrophages. (**A**) PMA-treated THP-1 cells were exposed for 1 min to LTB_4 (10 nM) or vehicle followed by PGE_2 (1 μM) for 3 min. Intracellular cAMP was determined as described in the text. Data are expressed as the mean ± SEM from seven independent experiments. $^{**}p < 0.01$ compared with untreated cells and $^{***}p < 0.001$, $^{\#}p < 0.001$ versus PGE_2 treatment. (**B**) PMA-treated THP-1 cells were cultured with LTB_4 (100 nM) and PGE_2 (10 μM) for 15 min before challenge with HK FITC-GAS. Phagocytosis was quantified after 3 h, as detailed in *Materials and Methods*. Data are expressed as the mean ± SEM relative to untreated cells for four independent experiments. $^{**}p < 0.01$ compared with untreated.

mice. Establishing a causal relationship between the exaggerated inflammatory response to GAS in $5LO^{-/-}$ mice and their mortality will require further study.

It is important that LTB_4 has been shown to augment macrophage immune defenses against several pathogens in vivo (49, 51, 55, 62, 63) and to amplify the ability of leukocytes to phagocytose and kill microbes (43, 49, 51, 62–65). Our results support the generalizability of the previous reports and add evidence that one causal mechanism by which LTB_4–BLT1 coupling regulates macrophage behaviors is by reducing cAMP production through a PTX-sensitive signaling cascade. How the acute lowering of basal cAMP levels impairs host defense against GAS requires more exploration, but both phagocytosis and ROI-dependent bacterial killing are certainly involved. cAMP is a critical intracellular immunoregulatory molecule that can rapidly dampen macrophage antimicrobial responses (19). The lipid mediator PGE_2, for example, has been shown to suppress macrophage–GAS interactions via this second messenger (18). Our results confirm that report and extend them to demonstrate that a significant interaction is possible when LTB_4 and PGE_2 costimulate a macrophage at the time of GAS infection (summarized in Fig. 7). Such crosstalk between these lipid mediators has been shown in other systems (17), adding generalizability to this finding.

This work has limitations, which underscore areas requiring investigation in future studies. For example, $5LO^{-/-}$ mice not only failed to generate increased LTB_4 during infection but also lacked the capacity to produce the cysteinyl LTs, such as LTC_4, D_4, and E_4. The extent to which such compounds affect survival or bacterial clearance during infection will require further study. Preliminary studies in our laboratory suggest that exogenously added cysteinyl LTs also have positive immunomodulatory actions on macrophage–GAS interactions (data not shown), and their contribution to the in vivo phenotype of infected mice remains to be determined. Another limitation was our use of a human macrophage–like cell line to build a model of LTB_4 immunoregulation. We used this THP-1 cell line because it demonstrated responsiveness to LTB_4 in the regulation of GAS phagocytosis similar to that of several other primary macrophage types, including human and mouse macrophages. A longer term plan is to validate our complete model in primary cells.

The mouse model of intrauterine infection that we have newly developed for this study involved nonpregnant mice that were also not immediately postpartum. It is possible that different results would be obtained if experiments were conducted in such animals, particularly given the hormonal and immunological differences associated with pregnancy and the puerperium (6, 66). Experiments to assess the impact of pregnancy and the postpartum state on this infection are planned.

In summary, this study newly establishes a role for LTs in positively regulating innate immune defenses against the puerperal sepsis pathogen GAS. These findings confirm those in other model systems and suggest that exploiting this endogenous signaling system pharmacologically might be a robust mechanism for improving therapeutic approaches to this infection. For example, amplifying the synthesis and signaling LTB_4, while limiting the production and responsiveness to PGE_2, might significantly enhance the efficacy of existing medical treatments of severe GAS infections. Future studies to assess such approaches are in order. The global problem of increasing antimicrobial resistance further highlights the ongoing need for new treatment modalities for life-threatening infections. Reducing maternal mortality requires ongoing studies of fundamental mechanisms of disease, with a goal of identifying novel targets for treatment and prevention.

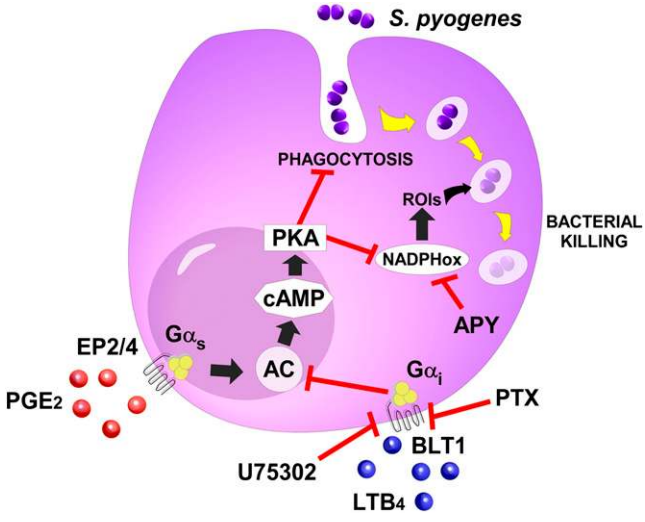

FIGURE 7. Summary of immunoregulation of macrophages by LTB_4 during *S. pyogenes* infection. Ligation of BLT1 by LTB_4 results in the activation of $G\alpha_i$ protein, with a subsequent decrease in intracellular cAMP levels. This, in turn, limits the activation of protein kinase A (PKA), an endogenous suppressor of both phagocytosis and NADPHox-dependent ROI generation. These actions oppose those of the endogenous lipid mediator PGE_2, which stimulates cAMP production via EP2 and/or EP4 receptors in macrophages. Compounds used in our studies to modulate signaling are shown. AC, Adenylate cyclase; APY, apocynin.

Acknowledgments

We thank Jill Mogle, RN, for technical assistance.

Disclosures

The authors have no conflicts of interest.

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