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Platelet-Activating Factor: a Role in Preterm Delivery and an Essential Interaction with Toll-Like Receptor Signaling in Mice[1]

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ABSTRACT

Platelet-activating factor (PAF), a potent phospholipid activator of inflammation that signals through its cognate receptor (platelet-activating factor receptor, PTAFR), has been shown to induce preterm delivery in mice. Toll-like receptors (TLRs) are transmembrane receptors that mediate innate immunity. We have shown previously that *Escherichia coli*-induced preterm delivery in mice requires TLR signaling via the adaptor protein myeloid differentiation primary response gene 88 (MyD88), but not an alternative adaptor, Toll/IL-1 receptor domain-containing adapter protein-inducing interferon-beta (TRIF). In the present work, we analyzed the role of endogenously produced PAF in labor using mice lacking (knockout [KO]) PAF acetylhydrolase (PAF-AH; the key degrading enzyme for PAF). PAF-AH KO mice are more susceptible to *E. coli*-induced preterm delivery and inflammation than controls. In peritoneal macrophages, the PTAFR agonist carbamyl PAF induces production of inflammatory markers previously demonstrated to be upregulated during bacterially induced labor, including: inducible nitric oxide synthase (*Nos2*), the chemokine *Ccl5* (RANTES), tumor necrosis factor (*Tnf*), and level of their end-products (NO, CCL5, TNF) in a process dependent upon both IkappaB kinase and calcium/calmodulin-dependent protein kinase II. Interestingly, this induced expression was completely eliminated not only in macrophages deficient in PTAFR, but also in those lacking either TLR4, MyD88, or TRIF. The dependence of PAF effects upon TLR pathways appears to be related to production of PTAFR itself: PAF-induced expression of *Ptafr* mRNA was eliminated completely in TLR4 KO and partially in MyD88 and TRIF KO macrophages. We conclude that PAF signaling plays an important role in bacterially induced preterm delivery. Furthermore, in addition to its cognate receptor, PAF signaling in peritoneal macrophages requires TLR4, MyD88, and TRIF.

cytokines, Escherichia coli*, inflammation, macrophages*

INTRODUCTION

Platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) is a potent phospholipid inflammatory mediator that is known to signal through its cognate G protein-coupled receptor (platelet-activating factor receptor, PTAFR) [1–5]. PAF is synthesized by and acts on a variety of cells, specifically those involved in host defense (e.g., neutrophils, monocytes, macrophages, platelets, and endothelial cells). The initial step in PAF biosynthesis occurs through various phospholipase A_2 (PLA2) isoforms, which catalyze the hydrolysis of the sn-2 acyl bond of glycerophospholipids, releasing the fatty acid from the sn-2 position and resulting in the PAF precursor, lyso-PAF, a molecule with limited biological activity. In a subsequent step, lyso-PAF acetyl-CoA transferase catalyzes the transfer of an acetyl group to the sn-2 position to generate PAF, a potent proinflammatory mediator. PAF is rapidly degraded by PAF-acetylhydrolase (PAF-AH), which exists in three isoforms: two intracellular and one secreted. The latter is known as a ''plasma'' isoform and is considered the main catabolic enzyme for extracellular PAF, which acts on the PTAFR.

These synthetic and catabolic enzymes participate in a tightly regulated homeostatic process that functions to suppress PAF levels at baseline or, in case of need, to activate an inflammatory reaction of sufficient strength to mediate host defense without causing harm. Upregulation of PAF can be stimulated by activating cytoplasmic PLA2 (cPLA2) [6], by inducing secretory PLA2 [7], or by suppressing PAF-AH mRNA and protein levels [8].

There is evidence that PAF plays a role in parturition through stimulation of myometrial contractions, cytokine production, and infiltration of polymorphonuclear leukocytes into the cervix [9–12]. In the rat, systemic levels of PAF-AH decrease and PAF levels increase as parturition nears [13, 14], a phenomenon that may be mediated by estrogen [12]. Bolus intrauterine administration of methyl carbamyl PAF (cPAF; modified to resist degradation) to mice on Day 15 of gestation causes preterm delivery [15], while low-dose continuous intravenous infusion of PAF causes intrauterine fetal growth restriction [16, 17]. PAF is elevated in the amniotic fluid of patients with preterm labor who ultimately deliver preterm [18, 19].

Toll-like receptors (TLRs) are transmembrane receptors that activate the innate immune system by recognizing specific molecular signatures of various pathogens and some endogenous molecules. Once a TLR has been engaged by a ligand and in the presence of appropriate cofactors, a sequence of downstream signaling events occurs to activate the host

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response [20]. These signaling events progress via two main pathways, classified according to the participation of an intracellular adaptor protein known as myeloid differentiation primary response gene 88 (MyD88). All TLRs, with the exception of TLR3, signal via the 'MyD88-dependent' pathway. TLR3 uses the 'MyD88-independent pathway' via an alternate adaptor protein known as Toll/IL-1 receptor (TIR) domain-containing adaptor protein-inducing IFN-β (TRIF). Activation of either of these two pathways leads to expression of distinctive yet partially overlapping sets of inflammatory and other mediators, including nuclear factor (NF)-κB (primarily activated via MyD88 with a late phase mediated by TRIF) and both the chemokine CCL5 (RANTES) and interferon-dependent genes (primarily activated via TRIF) [21–23]. TLR4 is the only TLR that signals via both the MyD88-dependent and the MyD88-independent pathways [24].

We reported previously that mice lacking TLR4 or the adaptor protein MyD88 are completely protected from *Escherichia coli*-induced preterm delivery [25, 26]. In the present study, we sought to examine the role of endogenously produced PAF in bacterially induced preterm delivery using mice deficient in PAF-AH. These animals have been shown to have absent serum PAF-AH activity and increased sensitivity to a PAF-dependent disease model of necrotizing enterocolitis [27]. In the present study, we demonstrate that mice lacking PAF-AH are significantly more sensitive than controls to preterm delivery and production of inflammatory mediators induced by killed *E. coli*. Having found that endogenous PAF plays a role in bacterially induced preterm delivery (paralleling the role previously demonstrated for TLR signaling), we sought to define the extent of interaction between PAF and TLR signaling pathways. To do so, we studied macrophages, considered a critical cell type for inflammation/infection-induced preterm delivery [28]. We report that treatment of peritoneal macrophages with the stable PAF analog cPAF induces the expression of the same inflammatory mediators that are upregulated in bacterially induced premature labor in vivo, and that are similarly induced by the TLR4 ligand lipopolysaccharide (LPS). This PAF-induced inflammatory activation is completely absent in macrophages from gene-targeted mice lacking TLR4, MyD88, or TRIF, suggesting that an intact TLR receptor apparatus is necessary for the induction of inflammatory mediators via the PAF receptor.

MATERIALS AND METHODS

Reagents

Carbamyl-PAF (a modified form of PAF resistant to degradation by PAF-AH) and KN-62 (calcium/calmodulin-dependent protein kinase [CaMK] II inhibitor; Enzo Life Sciences) were dissolved in ethanol and diluted in water. LPS, extracted from *Salmonella enterica* (L2262; Sigma Chemical Co.) and IκB kinase inhibitor peptide (Calbiochem) were resuspended in PBS.

Preparation of Bacteria

A fresh culture of previously frozen *E. coli* (12014; American Type Culture Collection) was grown overnight in 4000 ml Luria-Bertani broth. The overnight culture was concentrated by centrifugation and suspended in 5 ml of PBS. The concentration of this suspension was determined post hoc by plating serial dilutions in triplicate. The bacteria within the suspension were killed by immersion in a boiling water bath for 5 min and then frozen at −20°C. Killing was verified by lack of overnight growth on plates and in broth culture. Once the concentration of the frozen stock was known, it was thawed and diluted to a concentration of 1.4×10^{11} organisms/ml. This latter suspension was frozen at −80°C in aliquots and thawed and diluted as needed prior to each experiment.

Mice

All procedures involving animals were approved by the NorthShore University HealthSystem Animal Care and Use Committee and conform to the Guide for Care and Use of Laboratory Animals (National Academy of Sciences, 1996).

For pregnancy outcome experiments, mice deficient in plasma PAF-AH (C57BL/6 PLA2G7 KO, acquired from Professor Diana Stafforini [27]), C57BL/6J and C3HeB/FeJ wild-type (WT) controls (Jackson Laboratories, Bar Harbor, ME) were impregnated naturally by same-genotype males. Mating was confirmed by the presence of a vaginal plug. Intrauterine injections were performed under general anesthesia on Day 14.5 of a 19–20 day gestational period, as previously described [29]. To test for anesthetic-specific effects, three different forms of anesthesia were used: 0.015 ml/g body weight of intraperitoneal avertin (2.5% tribromoethyl alcohol and 2.5% *tert*-amyl alcohol in PBS), inhaled isoflurane, and intraperitoneal ketamine (0.1 mg/g body weight) and xylazine (10 μg/g body weight). Because there were no differences between these three anesthetics, they were combined for reporting the results. A 1.5-cm midline incision was made in the lower abdomen. In the mouse, the uterus is a bicornuate structure in which the fetuses are arranged in a ''beads-on-a-string'' pattern. A 100-μl solution of killed *E. coli* ($1–4 \times 10^6$ cells), cPAF (5–100 μg), or control medium was injected into the midsection of the right uterine horn at a site between two adjacent fetuses, taking care not to inject individual fetal sacs. This inoculum of *E. coli* is well below the threshold for delivery in normal mice. Surgical procedures lasted approximately 10 min. The abdomen was closed in two layers, with 4-0 polyglactin sutures at the peritoneum and wound clips at the skin. Animals recovered in individual, clean cages in the animal facility. Twice-daily observations were made for both preterm delivery and maternal health status. Preterm delivery was defined as the finding of at least one fetus in the cage or in the lower vagina within 48 h of surgery. Necropsies were performed either after delivery, or, at the latest, by 48 h after inoculation. The number of fetuses delivered or remaining in utero and the survival status of these retained fetuses (as determined by cardiac or vascular pulsations in the fetal bodies or membranes) were recorded.

Tissue Collection and Preparation for Ex Vivo Treatment

Uteri were dissected from WT and PAF-AH KO mice on Day 14.5 of pregnancy, and uterine sections from the areas between implantation sites (i.e., lacking decidual caps), decidual caps, and placentas were collected. A biopsy punch (Cooper Surgical) was used to isolate 5-mm-diameter uterine explants, which were cultured individually in RPMI medium in wells of 24-well plates. Decidua and placenta were prepared as single-cell suspensions by mincing in Hanks balanced salt solution (Life technologies), mechanical dispersion through a 100-μm nylon filter, centrifugation at 1500 rpm, and dispersion of the remaining pellet in RPMI medium at 10^7 cells/ml in 24-well plates. Prior to plating, placental suspensions underwent red cell lysis by incubation with red blood cell lysis buffer (BioLegend) according to the manufacturer's instructions. The above specimens were incubated at 37°C in 5% CO_2/95% air for 2 h. Viability of ex vivo cultured cells was >95%, as assessed using the trypan blue dye exclusion test, followed by treatment with killed *E. coli* (2×10^4 cells/ml), LPS (5 ng/ml), or PBS (10 μl) added to culture medium for 5 h. At the end of this incubation period, cells/tissues were collected for RNA extraction (see below). All ex vivo experiments were done thrice and in triplicate.

Macrophage Culture

Thioglycolate-stimulated peritoneal macrophages were obtained as previously described [30] from 10- to 12-wk-old male mice of the following strains: MyD88 KO, TRIF KO (these two mutant strains were acquired from Professor Shizuo Akira [31, 32]), B6129/F2J (WT controls for the above two mutant strains), B6.B10ScN-Tlr4lps-del/JthJ (TLR4 KO), B6.129-Tlr2tm1Kir/J (TLR2 KO) (the latter three strains from Jackson Laboratories), PTAFR KO (acquired from University of Illinois at Chicago with the permission of Professor S Ishii [33]), and C57BL/6J (WT controls for the latter three mutant strains; acquired from Jackson Laboratories).

Mice were injected intraperitoneally with 1 ml of 3% thioglycolate. At 3 days after injection, mice were killed by CO_2 inhalation and the peritoneal cellular exudate was obtained by washing the peritoneal cavity three times with 5 ml ice-cold PBS. These cells were centrifuged, washed, and incubated for 2 h at a density of 1.5×10^6 cells/well in six-well plates in Dulbecco modified Eagle medium High Glucose (11965-092; GIBCO, Grand Island, NY) supplemented with 10% fetal bovine serum, 1% streptomycin, 1% penicillin, and 10 mM Hepes at 37°C in 5% CO_2/95% air. Adherent cells were confirmed as macrophages using the F4/80 macrophage cell marker (ab6640 with rabbit anti-rat IgG-FITC; Abcam, Cambridge, MA) via immunofluorescence. Selected

TABLE 1. Preterm delivery and fetal retention in utero in WT and PAF-AH KO mice.

Inoculum (no. bacteria per mouse)	Genotype	n	Preterm delivery (%)*	Mean no. of pups delivered within 48 h†	Mean no. of fetuses in utero at 48 h†
PBS	PAF-AH-KO	5	0/5 (0)	0	6.6 ± 2.07
E. coli, 1×10^6	WT	10	0/10 (0)	0	7.9 ± 0.99
	PAF-AH-KO	9	4/9 (44.45)[a]	2.7 ± 3.2[b]	4.8 ± 3.8[b]
E. coli, 4×10^6	WT	7	0/7 (0)	0	7.0 ± 1.3
	PAF-AH-KO	8	5/8 (62.2)[a]	2.1± 2.6[b]	2.5 ± 3.1[b]

*Preterm delivery was defined as delivery of at least one pup within 48 h.
†The number of pups per dam is shown as mean ± SD.
[a]$P < 0.05$ compared to WT, Fisher exact test.
[b]$P < 0.05$ compared to WT, Student *t*-test.

wells were pretreated with CaMKII inhibitor KN62 (5 μM) for 30 min or with IκB kinase inhibitor peptide (10 μg/ml), or control solution for 60 min. Cells were then cultured for an additional 5 h in medium supplemented with cPAF (1000 nM), LPS (2.5 or 5 ng/ml), or control solution (PBS or ethanol 0.01%—outcomes for these two control conditions were no different from each other, and were therefore combined). At the end of cell culture experiments, medium was aspirated and cells were washed twice with PBS prior to RNA or protein extraction (see below). All in vitro experiments were done thrice and in triplicate.

Viability of cultured cells was assessed using the trypan blue dye exclusion test. The corresponding viability values were: preplating, 94.5%; after plating, control 92%; LPS, 89.4%; cPAF, 90%. There were no statistically significant differences between postplating values.

RT-PCR Analysis

Activation markers commonly used for TLR signaling pathways were determined using RT-PCR for inducible nitric oxide synthase (*Nos2*), the chemokine *Ccl5* (RANTES), tumor necrosis factor (*Tnf*), and interleukin (*Il*)-1β. These mRNAs were chosen as representative markers for activation of the MyD88-dependent, MyD88-independent, and both pathways, respectively [34]. The transcripts have been previously shown to correlate closely with protein levels in a variety of systems [35, 36].

Cells were lysed in tissue culture wells, and tissues were homogenized with Trizol reagent (Invitrogen) to extract total RNA according to the manufacturer's protocol. Complementary DNA was prepared using random primers and the Moloney murine leukemia virus reverse transcriptase system (Invitrogen). All PCR primers and probes were purchased from Applied Biosystems (Foster City, CA): *Nos2* (Mm00440485); *Ccl5* (RANTES; Mm01302428); *Tnf* (Mm00443258); *Il-1β* (Mm0043422); *Ptafr* (Mm02621061); and mouse *Gapdh* (20×; 4452339E). Use of TaqMan PCR Reagent Kits was in accordance with the manufacturer's manual. Reactions were performed in a 10-μl mixture containing 0.5 μl cDNA. Duplex RT-PCR was performed with one primer pair amplifying the gene of interest and the other an internal reference (*Gapdh*) in the same tube. Thermocycler parameters were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, and 60°C for 1 min. Semiquantitative analysis of gene expression was done using the comparative CT (ΔΔCT) method, normalizing expression of the gene of interest to *Gapdh*. PCR assays were performed in duplicate for each of the triplicate cell culture samples. Values resulting from cPAF exposure were set to 100 for purposes of comparing separate assays.

Protein Extraction and Western Blot Analysis

Cultured macrophages were lysed in ice-cold 1× radioimmune precipitation assay buffer (1× TBS, 1% nonidet P-40, 0.5% Na deoxycholate, 0.1% SDS, 0.004% Na azide, protease inhibitor mixture set [PMSF, Na orthovanadate and protease inhibitor cocktail; Santa Cruz Biotechnology] and phosphatase inhibitors I and II [Santa Cruz Biotechnology]). Lysates were collected and incubated on ice for 1 h and then centrifuged at $10\,000 \times g$ for 10 min at 4°C. Supernatants were collected and used as whole-cell lysates for Western blotting. Protein concentration was measured spectrophotometrically (Nanodrop 2000; Thermo Scientific) at A_{280}. Equal amounts of protein (50 μg) from cell lysates were separated by SDS-PAGE and blotted onto PVDF transfer membranes. The membranes were blocked overnight at 4°C using 5% bovine serum albumin. Blots were incubated with primary antibody for phosphorylated extracellular signal-regulated kinase (ERK; 1:1000 dilution; sc-7383; Santa Cruz Biotechnology) for 2 h at room temperature and secondary antibody (1:2000 dilution; goat anti-mouse IgG-HRP; sc-2031; Santa Cruz Biotechnology) for 1 h at room temperature. Proteins were detected using the ECL Western blot detection system (GE Healthcare Bio-Sciences Corp.) in a Storm Imager (Molecular Dynamics).

After signal detection for pERK, membranes were stripped, redeveloped to check for the removal of pERK signal, and reprobed with primary antibody for ERK (1:1000 dilution; sc-135900; Santa Cruz Biotechnology). A separate membrane was probed with primary antibody for GAPDH (1:2000 dilution; sc-25778; Santa Cruz Biotechnology) as a loading control. Quantification of bands was performed using ImageQuant software (Molecular Dynamics). Background intensity was subtracted from each sample and then fold change was determined by normalizing phosphor-proteins to total protein.

NO and Cytokine/Chemokine Measurement

NO production was analyzed by Griess reagent (Sigma) and secretion of CCL5 and TNF was analyzed by Milliplex map kit (Millipore) in 25 μl of cell culture supernatant. NO production was assayed on fluorometer (Bio-Tek Instruments), and CCL5 and TNF were assayed on a MAGPIX instrument (Millipore), per the instructions provided by manufacturers.

Statistical Analysis

Continuous variables (e.g., differences in gene expression between groups) were assessed using Student *t*-test or one-way ANOVA with Tukey multiple comparison tests. Categorical variables (preterm delivery) were analyzed using Fisher exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Bacterially Induced Preterm Delivery Is Enhanced in Mice Lacking the PAF-Degrading Enzyme, PAF-AH

To determine the effect of endogenously produced PAF on bacterially induced preterm delivery, mice deficient in the PAF-degrading enzyme, PAF-AH, were used. PAF-AH KO mice and WT controls underwent intrauterine injection with killed *E. coli* or PBS on Day 14.5 of gestation (Table 1). In prior experience with this model, WT mice deliver in a dose-dependent fashion after intrauterine inoculation with killed *E. coli* (10^8–10^{10}), but consistently do not deliver following control injections or inoculation with fewer than 10^6 killed *E. coli* [37–39]. Sterile PBS did not produce preterm birth in any of the animals tested. Mice lacking PAF-AH were significantly more susceptible than WT controls to bacterially induced preterm delivery, and delivered with bacterial inocula (10^6) approximately two orders of magnitude below the established threshold for delivery in control mice [39]. PAF-AH KO mice also had lower rates of fetal retention in utero 48 h after surgery.

A dose of intrauterine cPAF leading to preterm delivery could not be identified in two strains of WT mice (C57BL/6J and C3HeB/FeJ) over the range of 5–100 μg/mouse. Intrauterine cPAF at doses reported to be effective for preterm delivery in CD-1 mice (10–40 μg/mouse) [15] caused death within a few hours in C57BL/6J and C3HeB/FeJ mice (most fail to recover from anesthesia), while lower doses produced

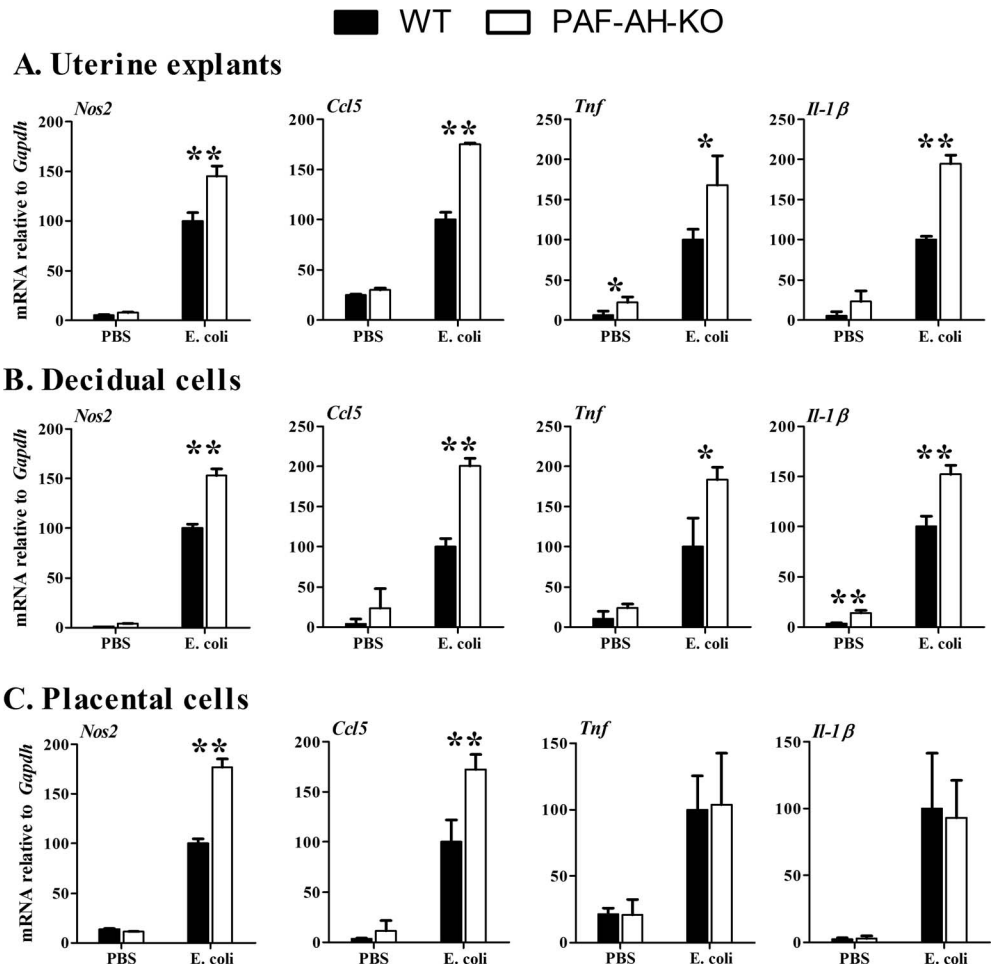

FIG. 1. Effect of killed *E. coli* on the mRNA expression of inflammatory mediators in WT and PAF-AH KO mice. Relative quantitative mRNA expression of *Nos2*, *Ccl5*, *Tnf*, and *Il-1β* in uterine explants (**A**), decidual cells (**B**), and placental cells (**C**) from WT and PAF-AH KO mice after treatment with killed *E. coli* or control medium for 5 h. The mRNA for the gene of interest was normalized to the housekeeping gene *Gapdh* and arbitrarily set to 100 for the WT group treated with killed *E. coli*. Concentrations of reagents are provided in the text (see *Materials and Methods*; n = 3 replicates per condition per experiment). Depicted is a representative figure from three repeat experiments. Error bars = SD. *P* values were calculated by *t*-test. $^{*}P < 0.05$, $^{**}P < 0.01$, comparing mutant animals to WT.

either death by the next day or no apparent effect (i.e., no illness and no preterm delivery) (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org).

Tissue Inflammation Is Enhanced in PAF-AH KO Mice

To determine whether the above phenotype of enhanced susceptibility to bacterially induced preterm delivery is paralleled by enhanced inflammatory responsiveness in PAF-AH KO mice, gestational tissues were collected on Day 14.5 of pregnancy. Uterine explants and single-cell suspensions of decidua and placenta from WT and PAF-AH KO mice were treated with killed *E. coli*, LPS, or PBS ex vivo for 5 h. Killed *E. coli* (Fig. 1) and LPS (Supplemental Fig. S1) induced the expression of inflammatory markers (*Nos2*, *Ccl5*, *Tnf*, and *Il-1β*) in gestational tissues from WT mice, an effect significantly enhanced in PAF-AH KO mice (except for *Tnf* and *Il-1β* in placental cells). Thus, mice lacking PAF-AH are significantly more sensitive than controls for the production of inflammatory mediators.

The Downstream Effects of cPAF in Cultured Peritoneal Macrophages Are Completely Dependent on PAF Receptor

To verify that treatment with cPAF has the expected effects on signal transduction in our culture system, peritoneal macrophages were isolated from WT control mice and plated. Adherent cells were confirmed as macrophages using the F4/80 macrophage cell marker (Supplemental Fig. S2). As expected, cPAF induced phosphorylation of ERK-1 and ERK-2 within 60 min (Supplemental Fig. S3). PAF signaling is known to require its cognate G protein-coupled receptor (PTAFR). To confirm that PAF's effects are mediated by PTAFR in our culture system, peritoneal macrophages from PTAFR KO and WT mice were incubated with cPAF, LPS, or control medium for 5 h. Carbamyl PAF and LPS each induced the expression of inflammatory markers *Nos2*, *Ccl5*, and *Tnf* in WT macrophages, with correlation between mRNA and levels of end-product (NO, CCL5, and TNF; Fig. 2). As expected, cPAF had no effect in PTAFR KO peritoneal macrophages. In addition to demonstrating an absolute requirement for PTAFR for cPAF actions, this result demonstrates the absence of endotoxin or other contaminants in the cPAF preparation that might induce inflammation via alternative pathways in PTAFR-deficient animals. In contrast

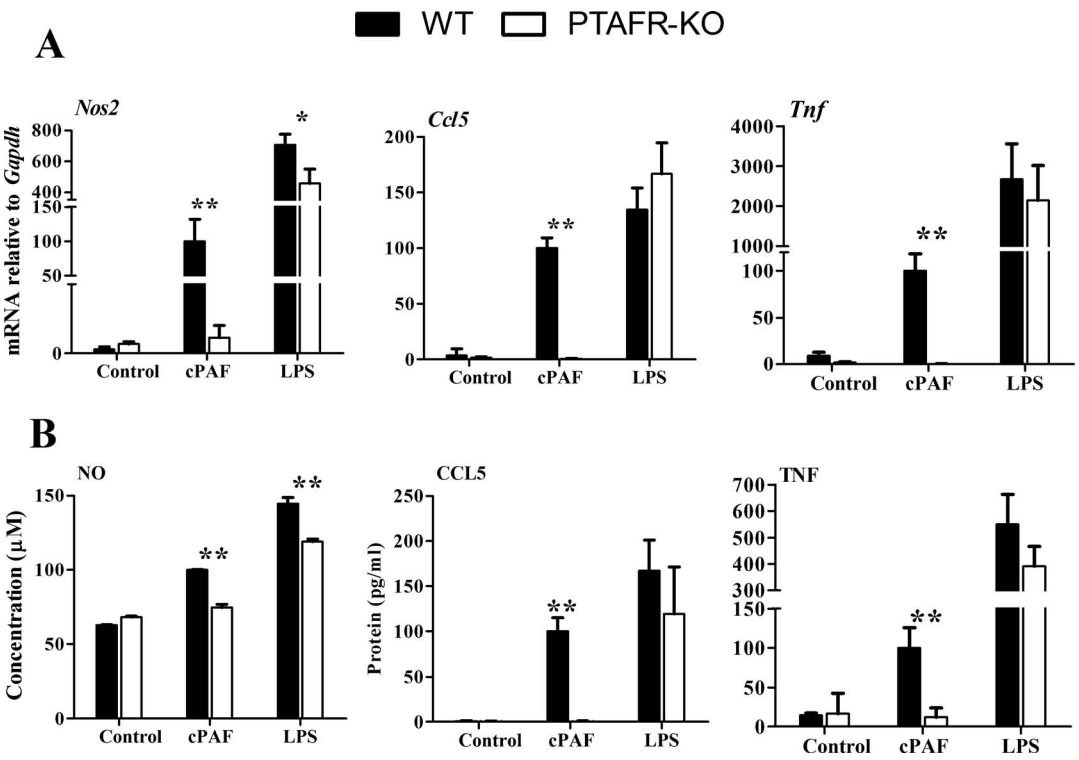

FIG. 2. Effect of cPAF and LPS on the expression of inflammatory mediators in WT and PTAFR KO peritoneal macrophages. WT and PTAFR KO peritoneal macrophages were treated for 5 h with cPAF, LPS, or control medium. Production of *Nos2*, *Ccl5*, and *Tnf* mRNA (**A**) and end-products (**B**) were determined. The mRNA for each gene of interest was normalized to the housekeeping gene *Gapdh*. The average value for treatment with cPAF was set to 100 in each case. Concentrations of reagents are provided in the text (see *Materials and Methods*; n = 3 replicates per condition per experiment). Depicted is a representative figure from three repeat experiments. Error bars = SD. *P* values were calculated by *t*-test. $*P < 0.05$, $**P < 0.01$, compared to WT.

to cPAF, LPS-induced cytokine expression was not affected by the absence of PTAFR, while NOS and NO were minimally affected. Thus, LPS, which signals via the TLR4 receptor, does not require the PAF receptor for efficient induction of inflammatory cytokine production and has at most a partial requirement for NO production.

Induction of Inflammatory Mediators by cPAF in Peritoneal Macrophages Is Completely Dependent upon TLR4, MyD88, and TRIF, and Is Partially Dependent upon TLR2

Peritoneal macrophages freshly obtained from control mice and TLR4 KO, TLR2 KO, MyD88 KO, and TRIF KO mice were incubated with cPAF, LPS, or control medium for 5 h (Figs. 3 and 4). We analyzed markers primarily governed by alternate TLR signal transduction pathways NOS2 (the MyD88-dependent pathway), CCL5 (the MyD88-independent [TRIF-dependent] pathway), and TNF (both pathways). The absence of TLR proteins (TLR4 and TLR2) or adaptor proteins (MyD88 and TRIF) had the expected impact upon the effects of LPS. For example, in the absence of TLR4, *Nos2*, *Ccl5*, and *Tnf* become undetectable or nearly so with LPS stimulation, while the absence of TLR2 has minimal impact upon the effects of LPS. In another example, we confirm that LPS-induced production of CCL5 is dependent mostly upon TRIF. Surprisingly, however, cPAF-induced expression of *Nos2*, *Ccl5*, and *Tnf* mRNA (Fig. 3) and end-product (Fig. 4) is abolished or nearly abolished by the absence of any one of TLR4, MyD88, or TRIF, and is diminished by the absence of TLR2 for *Ccl5* and *Tnf*, but not *Nos2*. Thus, cPAF-induced activation of inflammatory mediator gene expression in peritoneal macrophages requires TLR4, MyD88, and TRIF and is partially dependent upon TLR2. Because we were unable to identify a dose of cPAF that reliably produces preterm delivery in mice without causing maternal death (see above and Supplemental Table S1), this observation cannot be confirmed in vivo.

PAF-Induced PTAFR mRNA Expression in Peritoneal Macrophages Requires TLR4, MyD88, TRIF, and PTAFR

To test whether the dependence of PAF effects on intact TLR signaling components is related to regulation of the expression of the PAF receptor (PTAFR), peritoneal macrophages from WT, PTAFR KO, TLR4 KO, MyD88 KO, and TRIF KO mice were treated with cPAF or control medium for 5 h (Fig. 5). As expected, *Ptafr* mRNA was undetectable in PTAFR KO macrophages. In WT macrophages, cPAF induced the expression of *Ptafr*, while LPS exposure did not. However, this induction by cPAF of its own receptor was abolished in macrophages lacking TLR4 and was significantly suppressed in macrophages lacking MyD88 and TRIF. Thus, the dependence of PAF effects upon components of TLR signaling pathways (demonstrated in Figs. 3 and 4) may be related in part to a role for these TLR elements in the autoinduction of the PAF receptor. This action exists despite the fact that direct stimulation of TLR4 by LPS (and downstream activation MyD88 and TRIF) does not lead to upregulation of PTAFR.

Attempts to study PTAFR protein in immunoblots and in tissues were unsuccessful due to the absence of a suitable murine antibody.

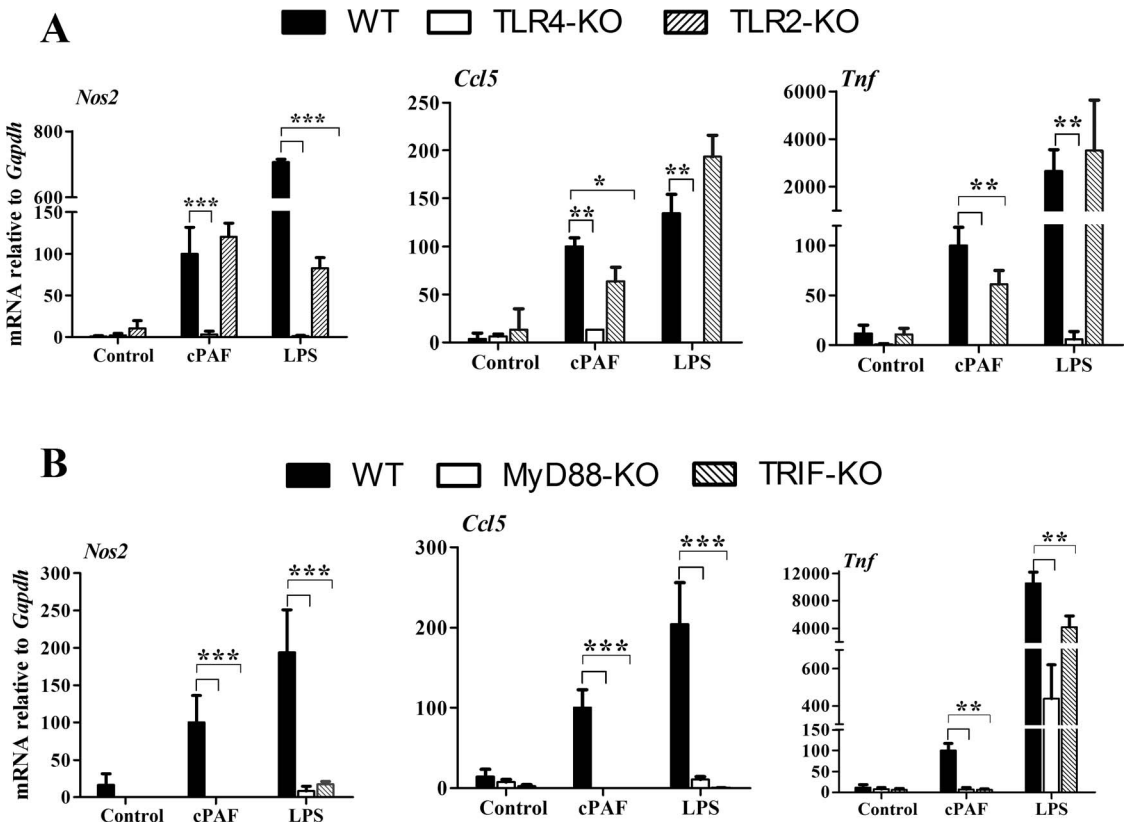

FIG. 3. Effect of cPAF and LPS on expression of inflammatory mediators in WT, TLR4 KO, TLR2 KO, MyD88 KO, and TRIF KO peritoneal macrophages. Relative quantitative mRNA expression of *Nos2*, *Ccl5*, and *Tnf* in TLR4 KO and TLR2 KO (**A**) and MyD88 KO and TRIF KO (**B**) peritoneal macrophages with WT controls after treatment with cPAF, LPS, or control for 5 h. The mRNA for the gene of interest was normalized to the housekeeping gene *Gapdh* and arbitrarily set to 100 for the cPAF-treated WT group. Concentrations of reagents are provided in the text (see *Materials and Methods*; n = 3 replicates per condition per experiment). Depicted is a representative figure from three repeat experiments. Error bars = SD (where not visible, the size of the error bars is smaller than the icon depicting the mean). *P* values were calculated by one-way ANOVA with Tukey multiple comparison test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, comparing mutant animals to WT.

Inflammatory Gene Expression Induced by cPAF Is Mediated in Part by NF-κB and CaMKII

The transcription factor NF-κB is a master regulator of expression of proinflammatory genes. In the unactivated state, NF-κB is bound to an inhibitor, IκB. Phosphorylation and subsequent degradation of IκBα leads to activation and nuclear translocation of NF-κB, a main mechanism by which TLR ligands induce inflammatory cytokines [40]. To investigate whether cPAF-induced expression of inflammatory markers (previously demonstrated to be dependent upon TLR4, MyD88, and TRIF; Figs. 3 and 4) is mediated via NF-κB, we pretreated peritoneal macrophages with an IκB kinase inhibitory peptide (resulting in suppression of NF-κB activation) for 1 h followed by incubation with cPAF, LPS, or control medium for 5 h. Pretreatment with IκB kinase inhibitory peptide decreases LPS-induced expression of all the inflammatory markers examined, as expected. This treatment also inhibited cPAF-induced expression of the same inflammatory markers by a similar or greater magnitude (Fig. 6). Thus, cPAF-induced inflammatory responses in peritoneal macrophages are mediated in part via NF-κB.

CaMKII is a serine/threonine kinase activated in response to increased intracellular calcium following activation of the PTAFR and other G protein-coupled receptors [41]. CaMKII has a role in mediating several important functions of PAF signaling, including potentiation of ERK1/2 phosphorylation [41] and activation of Ca^{2+}-dependent Cl^- channel 3 [42]. CaMKII has been shown to be involved in TLR signaling, and can mediate signaling by both Myd88 and TRIF [43]. KN62, a selective inhibitor of CaMKII, can effectively block its activation by interfering with calmodulin binding. To investigate the role of CaMKII in PAF signaling, we examined the effects of KN62 on PAF-induced expression of inflammatory mediators in peritoneal macrophages from WT mice. Peritoneal macrophages were pretreated with CaMKII inhibitor for 30 min followed by incubation with cPAF, LPS, or control medium for 5 h. Pretreatment with CaMKII inhibitor decreases cPAF-induced, but not LPS-induced, expression of inflammatory markers (Fig. 7). Thus, cPAF-induced expression of inflammatory markers in peritoneal macrophages is dependent upon CaMKII, a mechanism different from that of LPS.

DISCUSSION

In the present study, we report three novel findings. First, elimination of the key PAF-degrading enzyme PAF-AH results in increased susceptibility to *E. coli*-induced preterm delivery by two orders of magnitude and an exaggerated inflammatory response in the gestational compartment, suggesting that PAF is an important mediator of inflammation- and infection-induced preterm labor. While previous reports showed that exogenous PAF can induce labor [15], this is the first to show an essential role for endogenously produced PAF. Second, the induction of proinflammatory mediators by PAF in peritoneal macrophages in vitro is completely dependent upon TLR4 and

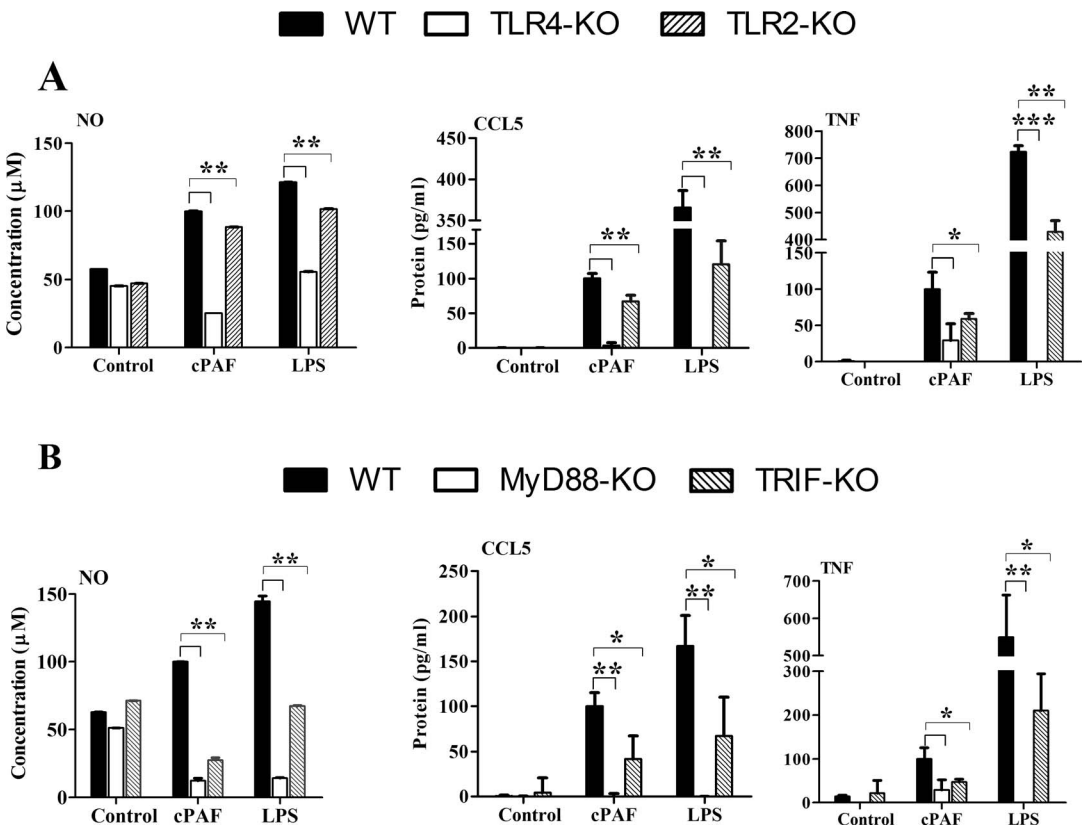

FIG. 4. Effect of cPAF and LPS on the expression of inflammatory mediators in WT, TLR4 KO, TLR2 KO, MyD88 KO and TRIF KO peritoneal macrophages. NO and protein concentrations of CCL5 and TNF in TLR4 KO and TLR2 KO (**A**) and MyD88 KO and TRIF KO (**B**) peritoneal macrophages in comparison to WT controls after treatment with cPAF, LPS, or control for 5 h. Concentrations were arbitrarily set to 100 for the cPAF-treated WT group. Concentrations of reagents are provided in the text (see *Materials and Methods*; n = 3 replicates per condition per experiment). Depicted is a representative figure from three repeat experiments. Error bars = SD (where not visible, the size of the error bars is smaller than the icon depicting the mean). *P* values were calculated by one-way ANOVA with Tukey multiple comparison test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, comparing mutant animals to WT.

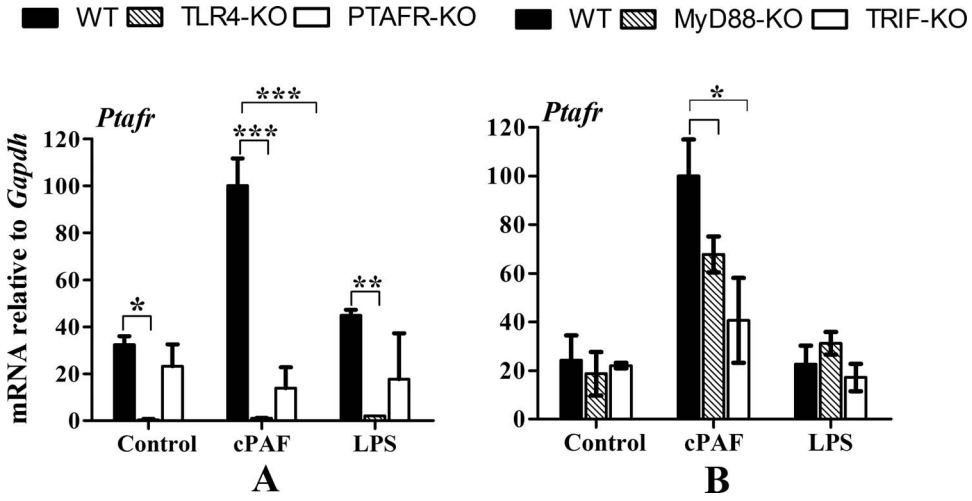

FIG. 5. Effect of cPAF on the expression of PAF receptor in WT, TLR4 KO, PTAFR KO, MyD88 KO, and TRIF KO peritoneal macrophages. Relative quantitative mRNA expression of *Ptafr* in TLR4 KO, PTAFR KO (**A**), MyD88 KO, and TRIF KO (**B**) peritoneal macrophages after treatment with cPAF or control medium for 5 h. The mRNA for the gene of interest was normalized to the housekeeping gene *Gapdh* and arbitrarily set to 100 for the cPAF-treated WT group. Concentrations of reagents are provided in the text (see *Materials and Methods*; n = 3 replicates per condition per experiment). Depicted is a representative figure from three repeat experiments. Error bars = SD (where not visible, the size of the error bars is smaller than the icon depicting the mean). *P* values were calculated by one-way ANOVA with Tukey multiple comparison test. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, comparing mutant animals to WT.

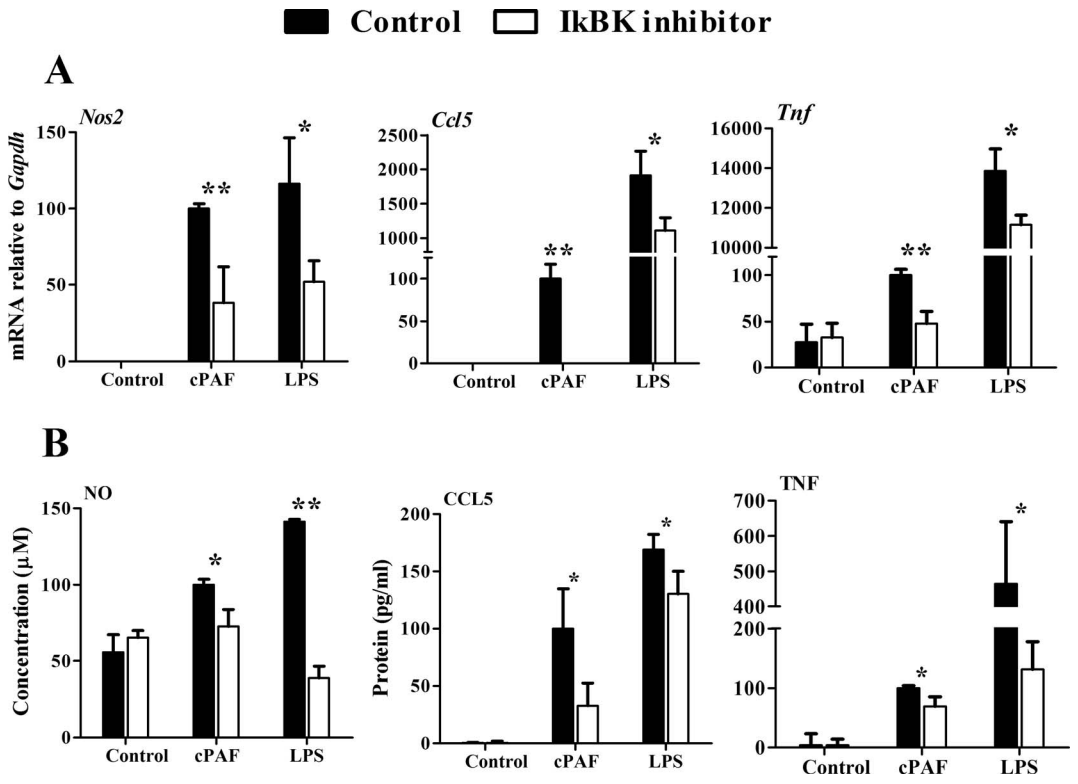

FIG. 6. Effect of IκB kinase inhibitor on cPAF- and LPS-induced expression of inflammatory mediators in peritoneal macrophages. Relative quantitative mRNA expression of *Nos2*, *Ccl5*, and *Tnf* (**A**) and NO and protein concentration of CCL5 and TNF (**B**) in WT peritoneal macrophages after pretreatment with either IκB kinase inhibitor peptide or control for 60 min followed by cPAF, LPS, or control for 5 h. The mRNA for the gene of interest was normalized to the housekeeping gene *Gapdh*. The mRNA and protein values were arbitrarily set to 100 for the cPAF-treated control group. Concentrations of reagents are provided in the text (see *Materials and Methods*; n = 3 replicates per condition per experiment). Depicted is a representative figure from three repeat experiments. Error bars = SD. *P* values were calculated by *t*-test. $*P < 0.05$, $**P < 0.01$, compared to control.

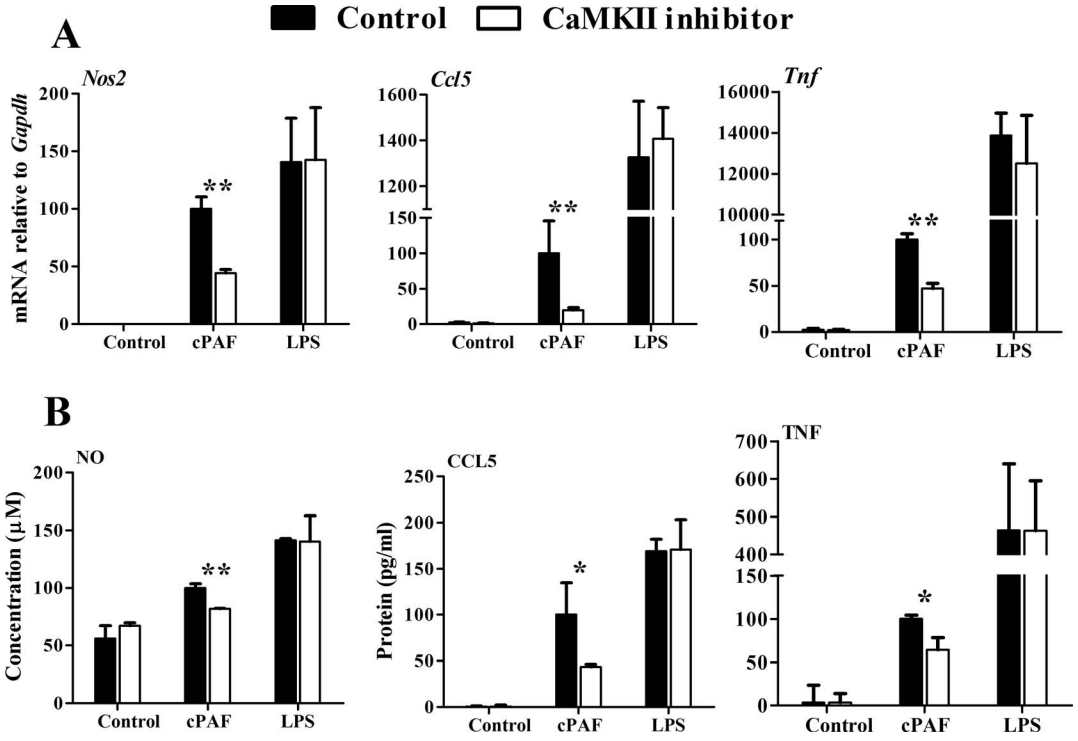

FIG. 7. Effect of CaMKII inhibitor on cPAF- and LPS-induced expression of inflammatory mediators in peritoneal macrophages. Relative quantitative mRNA expression of *Nos2*, *Ccl5*, and *Tnf* (**A**) and NO and protein concentration of CCL5 and TNF (**B**) in WT peritoneal macrophages after pretreatment with either CaMKII inhibitor or control for 30 min followed by cPAF, LPS, or control for 5 h. mRNA for the gene of interest was normalized to the housekeeping gene *Gapdh*. The mRNA and protein values were arbitrarily set to 100 for the cPAF-treated control group. Concentrations of reagents are provided in the text (see *Materials and Methods*; n = 3 replicates per condition per experiment). Depicted is a representative figure from three repeat experiments. Error bars = SD. *P* values were calculated by *t*-test. $*P < 0.05$, $**P < 0.01$, compared to control.

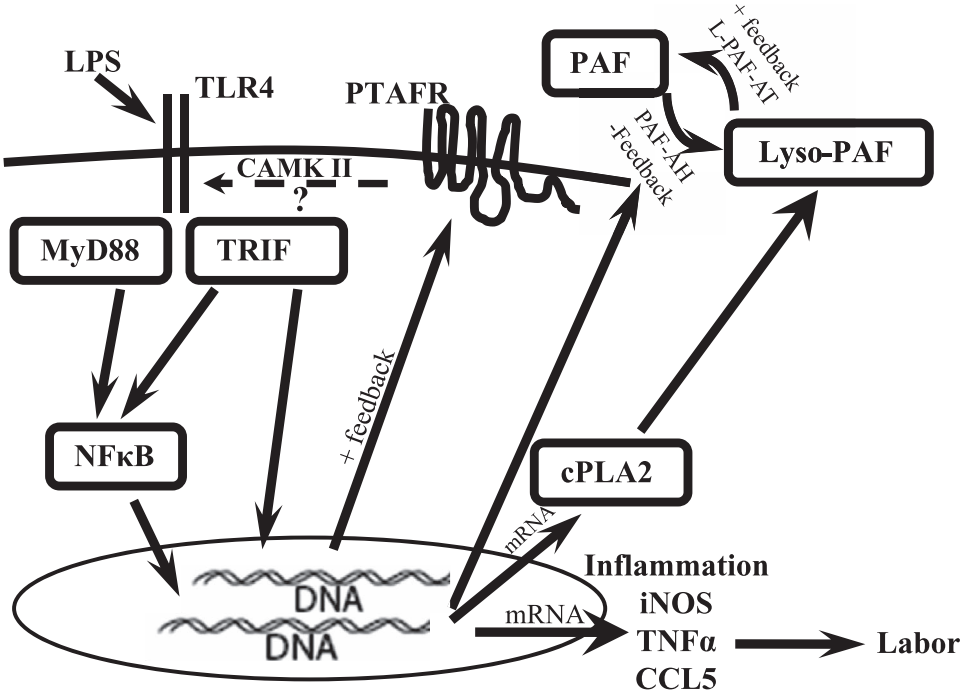

FIG. 8. Model of PAF signaling and interaction with TLR4.

its adaptor proteins MyD88 and TRIF, and is partially dependent upon TLR2. This second novel finding is partially explained by the third: autoinduction of PAF receptor by its ligand appears in part dependent upon TLR4, MyD88, and TRIF. Additional observations of this study are that PAF effects are mediated in part by NF-κB and CaMKII.

A role for PAF in processes related to parturition has been previously described [44]. PAF levels are higher in the amniotic fluid of women in labor compared to women at a similar gestational age not in labor [45, 46]. PAF content increases within the uterus near term and stimulates synthesis of prostaglandins through upregulation of COX-2 in the chorio-decidua [47], perhaps in synergism with other stimulatory factors present in the human fetal membranes [13, 48]. In human tissues, PAF contributes to cervical ripening by stimulating cytokine production [10, 11, 49]. Local PAF application in pregnant rats induces cervical ripening [9]. Antagonism of PAF with a PTAFR antagonist inhibits LPS-induced, but not spontaneous, cervical ripening in the rat [50]. PAF stimulates Ca^{2+} and myometrial contractile activity in human myometrial strips [51, 52]. Intrauterine administration of exogenous methyl cPAF to mice on Day 15 of gestation causes preterm delivery [15].

Our prior observation that *E. coli*-induced preterm delivery is completely dependent upon TLR4 [26] and MyD88, but not TRIF [25], combined with the finding that PAF plays an important role in the same process, led us to hypothesize that there was a point of interaction between PAF and TLR signaling pathways. Our data support this hypothesis: induction of inflammatory mediator gene expression by PAF is absolutely dependent on both PAF receptor and an intact TLR4, and is partially dependent upon TLR2. Additionally, the two major signaling pathways downstream of TLR4 (i.e., the MyD88- and TRIF-dependent pathways) are each required for PAF-induced gene expression. Interestingly, the converse is not true: the TLR4 ligand LPS requires TLR4, but is unaffected by absence of the PAF receptor.

The mechanisms through which members of TLR signaling pathways (TLR4, MyD88, and TRIF) are involved in mediating PAF-induced inflammatory gene expression appear to be complex. Unlike LPS-induced inflammatory gene expression, PAF-induced TLR4-dependent inflammatory gene expression does not conform fully to the canonical TLR4 signaling pathway. In the canonical TLR4 signaling pathway, ligand-induced inflammatory gene expression should be either dependent on MyD88, or TRIF, or could be partially dependent on each. This is what we observed for LPS-induced *Nos2*, *Ccl5*, and *Tnf* gene expression and their end-products. In contrast, we find that PAF-induced inflammatory gene expression has an absolute requirement for the simultaneous presence of both MyD88 and TRIF. Furthermore, while PAF-induced stimulation of PTAFR production is dependent upon TLR4, direct stimulation of TLR4 by LPS does not affect PTAFR levels.

We reasoned that most of these findings might be explained by a mechanism by which PTAFR engagement elicits both direct transcriptional regulation of downstream elements and activation of the canonical TLR4 signaling pathway (NOS2, CCL5, and TNF) via transactivation. For the autoinduction of PTAFR (which does not occur with direct activation of TLR4), transactivation of the TLR4 pathway and an additional TLR4-independent signaling step by the PTAFR would be necessary.

We hypothesized that CaMKII may play a transactivating role in PAF/TLR interdependence for the following reasons: 1) CaMKII has been implicated in transactivation of the epidermal growth factor receptor by multiple GPCRs [53–55]; 2) CaMKII has a role in mediating several important functions of PAF signaling, including potentiation of ERK1/2 phosphorylation [41] and activation of Ca^{2+}-dependent Cl^- channel 3 [42]; 3) CaMKII can mediate signaling by both Myd88 and TRIF by binding and activating transforming growth factor beta-activated kinase 1 (TAK1) and IFN regulatory factor 3 (IRF3) [43]; and 4) inhibition of CaMKII suppresses TLR4-triggered production of cytokines in macrophages [43]. Our results suggest that cross-talk between the PAF and TLR

signaling pathways plays an important role in the pathogenesis of bacterially induced preterm delivery. CaMKII-mediated TLR4 transactivation might provide the mechanistic link underlying the dependence of PAF receptor signaling on the presence of an intact TLR4 receptor complex (see Fig. 8 for a model of these interactions).

TLR4 activation has been shown to induce cPLA2 activity [56] and generation of both lyso-PAF acetyltransferase [57] and PAF-AH [58]. This simultaneous induction of the substrate and enzymes responsible for both PAF synthesis and degradation allows for self-limiting PAF biosynthesis in the presence of LPS. PAF is then available to activate PTAFR, which transactivates TLR4 via CaMKII, providing a positive feedback loop leading to a further increase in the production of PTAFR. The key to the self-limited nature of this pathway is the simultaneous activation of negative feedback via the upregulation of PAF-AH, as evidenced by the two orders of magnitude increase in sensitivity to bacterially induced premature labor in PAF-AH KO mice.

In summary, we have demonstrated that control of PAF activity is critically important in limiting the severity of bacterially induced preterm labor and the inflammatory response. The actions of PAF in macrophages (a key cell for inflammation-induced labor) are dependent upon a functional interaction between PTAFR and TLR4 and its adaptor proteins MyD88 and TRIF.

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