



Published in final edited form as:

Reprod Sci. 2020 January ; 27(1): 246–259. doi:10.1007/s43032-019-00011-w.

Protease Amplification of the Inflammatory Response Induced by Commensal Bacteria: Implications for Racial Disparity in Term and Preterm Birth

Scott W. Walsh, Ph.D.^{1,2}, William H. Nugent, Ph.D.¹, S.M. Khorshed Alam, Ph.D.¹, Sonya L. Washington, M.S.¹, Maria Teves, Ph.D.¹, Kimberly K. Jefferson, Ph.D.³, Jerome F. Strauss III, Ph.D., M.D.¹

¹Department of Obstetrics and Gynecology, School of Medicine, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA.

²Department of Physiology and Biophysics, School of Medicine, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA.

³Department of Microbiology and Immunology, School of Medicine, Virginia Commonwealth University, Richmond, VA, 23298-0034, USA.

Abstract

Decidual macrophages secrete proteases that activate protease-activated receptor 1 (PAR-1). We hypothesized that activation of the inflammatory response by bacteria is amplified by proteases, initiating labor. In addition, we hypothesized that commensal bacteria trigger an inflammatory response by activating NF- κ B and tet methylcytosine dioxygenase 2 (TET2), a DNA demethylase, via a protease amplified PAR-1, RhoA kinase (ROCK) pathway. To evaluate these hypotheses, we compared responses of mononuclear cells to *Lactobacillus crispatus*, prevalent in the vaginal microbiome of women of European ancestry, with *L. iners* and *Fusobacterium nucleatum*, which are more prevalent in vaginal samples collected from African-American women. Decidual tissue was collected at term not-in-labor (TNL), term labor (TL), spontaneous preterm labor (sPTL) and infected preterm labor (iPTL) and immunostained for PAR-1, TET2 and CD14. Mononuclear cells and THP-1 macrophage cells were treated with bacteria and elastase, a known activator of PAR-1. The inflammatory response was monitored by confocal microscopy of TET2 and the p65 subunit of NF- κ B, as well as IL-8 production. Decidual staining for PAR-1, TET2 and CD14 increased TNL<TL<sPTL<iPTL. All treatments stimulated translocation of TET2 and p65 from the cytosol to the nucleus and increased IL-8, but *L. iners* and *F. nucleatum* caused more robust responses than *L. crispatus*. Inhibition of PAR-1 or ROCK prevented TET2 and p65 nuclear translocation and increases in IL-8. Our findings demonstrate that proteases amplify the

Corresponding Author: Scott W. Walsh, Ph.D., Virginia Commonwealth University Medical Center, Department of Obstetrics and Gynecology, P.O. Box 980034, Richmond, VA 23298-0034, (804) 828-1772 - Phone; (804) 828-0573 – Fax, Scott.Walsh@vcuhealth.org.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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inflammatory response to commensal bacteria. The more robust response to bacteria prevalent in African-American women may contribute to racial disparities in preterm birth.

Keywords

Lactobacillus iners; *Lactobacillus crispatus*; *Fusobacterium nucleatum*; macrophages; protease-activated receptor 1; tet methylcytosine dioxygenase 2; preterm labor; African-American

Introduction

Preterm birth (PTB), which is defined as delivery before 37 weeks of completed gestation, is the leading cause of neonatal morbidity and mortality worldwide accounting for as many as 75% of perinatal deaths¹⁻³. In the United States 12.5% of births are preterm with the highest rate for non-Hispanic African-Americans at 17.8%. The annual economic burden associated with PTB in the U.S. is approximately \$26.5 billion¹. Both term and preterm labor are associated with inflammation and marked infiltration of leukocytes into intrauterine tissues⁴⁻⁶. Most bacteria found in intrauterine tissues in association with PTB are of vaginal origin, and it is thought the most common pathway of intrauterine infection is the ascending route from the vagina. However bacterial DNA from the oral cavity has also been reported to be present⁷⁻¹⁴. Some studies suggest that both term and preterm intrauterine tissues can have bacteria present without overt infection^{7, 9-11}, so “sub-clinical” colonization by commensal bacteria may convert decidual macrophages into a proinflammatory state, triggering the onset of labor.

The strongest risk factors for PTB are a history of a previous PTB, family history of PTB, and African-American race^{1, 15-18}, consistent with a genetic component. Although a number of genetic variants have been associated with PTB, they can only explain a small portion of preterm births. Our group recently reported that the higher incidence of PTB in African-Americans is associated with a greater environmental contribution than in women of European ancestry¹⁹. Environmental contributions could be amplified by environmental gene interactions or produce their effects by epigenetic mechanisms²⁰⁻²⁴.

The microbiome can be considered an environmental contributor to health, and our group has shown that the vaginal microbiome differs significantly between African-American women and women of European ancestry²⁵. The microbiome of African-Americans is significantly more diverse with a prevalence of bacterial taxa that are associated with microbial invasion and *Lactobacillus* species that are not protective of the vaginal milieu. *L. iners*, the most common species in the vaginal microbiome of African-Americans, allows for a higher diversity of pathologic species because of its low production of lactic acid, which does not maintain a low enough pH to be protective^{26,27}. *L. iners* allows for colonization of species, such as *Fusobacterium nucleatum*, a gram-negative pathologic species associated with infection and preterm premature rupture of the membranes (PPROM)^{7, 28}. *L. crispatus*, which is the most common species in women of European ancestry, is stable, maintains a low pH and protects the vagina from being colonized by pathologic species²⁵⁻²⁷.

Macrophages express protease-activated receptor 1 (PAR-1)²⁹⁻³¹ and release proteases, such as matrix metalloprotease-1 (MMP-1) and MMP-13³²⁻³⁴, that activate PAR-1. Macrophage release of proteases could amplify the bacterial response by activating PAR-1^{35, 36} on adjacent macrophages. We considered the possibility that PAR-1 activation could induce inflammation via the recently discovered TET (Ten-Eleven Translocation) proteins (aka, tet methylcytosine dioxygenases), which enzymatically de-methylate DNA to increased gene expression³⁷⁻⁴¹. TET2 is the main TET protein expressed in leukocytes⁴²⁻⁴⁴. Nuclear factor-kappa B (NF-κB) is a transcription factor that regulates inflammatory response and is activated by protein kinase C (PKC) phosphorylation⁴⁵. RhoA kinase (ROCK) is a downstream effector of PAR-1^{46, 47} and ROCK is in the PKC family of serine/threonine phosphorylases, so activation of TET2 may involve PKC phosphorylation similar to NF-κB because TET2 contains several PKC phosphorylation sites, as well as a nuclear localization signal (PROSITE, prosite.expasy.org).

We developed a model system to test these concepts using mononuclear cells isolated from pregnant women and THP-1 cells, an established cell line used to assess macrophage function. We tested the hypothesis that commensal bacteria initiate an inflammatory response by activating TET2 and NF-κB via a protease amplified PAR-1, ROCK pathway. To evaluate racial disparity, we compared bacterial responses of *L. crispatus* with *L. iners* and *F. nucleatum*.

Materials and Methods

Study Subjects

Term-not-in-labor (TNL) placentas were collected from women who underwent non-emergency caesarian sections between 38-39 weeks of gestation. Term labor (TL) placentas were collected from women with normal pregnancies who gave vaginal birth between 38-40 weeks of gestation. Spontaneous preterm labor (sPTL) placentas were collected from pregnant women who gave birth prior to 37 weeks of gestation and had no clinical signs of infection. Infected preterm labor (iPTL) placentas were collected from pregnant women who gave birth prior to 37 weeks of completed gestation with PPROM and clinical signs of infection. Infection was defined as fever $\geq 100.4^{\circ}\text{F}$, uterine tenderness, malodorous vaginal discharge, maternal or fetal tachycardia. Exclusion criteria were: smokers, HIV/AIDS, drug/alcohol users, pregnancies with stillborn babies, multiple fetuses, preeclampsia, lupus, congenital abnormalities, and hemorrhage. Demographic patient data are given in Table 1.

Immunohistochemistry

For immunohistochemistry, a rectangular section of the fetal membranes (approximately 6 cm x 4 cm) including amnion, chorion and decidua was removed with surgical scissors. A roll with the decidua oriented towards the interior was prepared. Decidual tissue roles were formalin-fixed, paraffin embedded and cut into 7 μm sections with a microtome. Immunohistochemistry was performed as previously described⁴⁸⁻⁵⁰. To quench endogenous tissue peroxidase activity, slides were incubated in 3% hydrogen peroxide in methanol for 30 minutes. For antigen retrieval, slides were heat treated in 10 mM citrate buffer for 5 minutes with a pressure cooker. Slides were blocked for 1 h in 10% goat serum. Tissues were

immunostained with rabbit polyclonal antibody to TET2 (1:50, Proteintech, Rosemont, IL, Cat. #21207-1-AP), rabbit polyclonal antibody to PAR-1 (1:50, Proteintech, Cat. #15607-1-AP), rabbit polyclonal antibody to CD14 (1:200, Proteintech, Cat. #17000-1-AP), or rabbit IgG isotype negative control pre-diluted in phosphate buffered saline (Invitrogen, Cat. #086199) using Vector ABC Kit (Thermo Fisher Scientific, Waltham, MA, Cat. #PK-6101) and ImmPACT DAB (Thermo Fisher Scientific, Cat. #SK-4105). Slides were counterstained with 1:5 dilution of Hematoxylin QS (Vector Laboratories, Burlingame, CA). The staining protocol was the same for all samples with regard to processing, incubation times and temperature. Slides were analyzed with an Olympus BH-2 microscope (Olympus, Center Valley, PA) attached to a digital camera (Olympus QColor5) using image analysis software (cellSens, Olympus). Relative differences in staining were identified by highlighting specific stained areas with yellow overlay using the “Measuring Images” tool. Data were quantified and reported as area stained (μm^2).

Cell culture for confocal immunofluorescence

Mononuclear cells isolated from blood collected from normal pregnant women before 37 weeks' gestation and THP-1 cells (ATCC, Manassas, VA), a widely used monocytic cell line that can be differentiated into a macrophage cell type, were used to assess inflammatory response to bacteria and elastase and to study the mechanism whereby proteases might amplify the inflammatory response via PAR-1. Both mononuclear cells and macrophages express PAR-1²⁹⁻³¹. Inflammatory response was assessed by the translocation of TET2 and NF- κ B from the cytosol to the nucleus using immunofluorescence staining and confocal microscopy. Mononuclear cells composed of monocytes and lymphocytes, both of which infiltrate the decidua, were separated from granulocytes by Histopaque (1077/1119) density gradient centrifugation according to the manufacturer's protocol (Sigma, St. Louis, MO) and as previously described by our lab⁵¹⁻⁵⁵. Mononuclear cells and THP-1 cells were cultured in RPMI-1640 medium supplemented with 2 mM L-glutamine and 1% antibiotics and antimycotics (100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 25 $\mu\text{g}/\text{ml}$ amphotericin B) at 37°C in a humidified 5% CO₂ atmosphere. For the THP-1 cells, 50 μM 2-mercaptoethanol and 10% fetal bovine serum were added. Cells were seeded at 250,000 cells per ml in 4-chambered slides.

THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA, Abcam, Cambridge, MA), 16 nM, for 24 h to differentiate them into a macrophage cell type and then clean media for 48 h. Mononuclear cells were used fresh. The following treatments were added for 1 h: 1) Control media; and heat killed bacteria (100:1 bacteria to cells): 2) *L. crispatus*; 3) *L. iners*; 4) *F. nucleatum*, and 5) elastase (0.033 U/ml, Sigma, Cat. #E7885). Bacteria were grown in one of our laboratories (KKJ) and characterized by DNA sequencing as to the species. Treatment for 1 h was chosen because nuclear translocation of NF- κ B is maximal at this time⁵⁶ and time course experiments for TET2 showed nuclear localization as early as 15 min. For inhibitor treatments, they were added 30 minutes before activating treatments: 1) PAR-1 inhibitor (2 μM , SCH-79797, Tocris, Minneapolis, MN); 2) ROCK inhibitor (20 μM Y-27632, Tocris). Media were aspirated, cells were washed with PBS and fixed with 4% formalin for 1 h. Cells were washed, then blocked and permeabilized for 1 h in 10% goat serum + 3% BSA + 0.2% Triton X-100. Cells were incubated overnight at 4°C with primary

rabbit antibody specific for TET2 (1:250, Proteintech) or the p65 subunit of NF- κ B (1:50, Proteintech). The next morning, cells were washed and incubated with anti-rabbit IgG secondary antibody green fluorescence dye (Alexa Fluor 488, 1:3000, Jackson ImmunoResearch, Burlingame, CA) for TET2 or red fluorescence dye (Cy3, 1:2000, Jackson ImmunoResearch) for p65 at room temperature for 1 h. Cells were washed and mounted with VectaMount containing DAPI (Vector Laboratories) for nuclear DNA staining. Images were taken using a confocal microscope (Zeiss LSM 710). Percentage of cells with nuclear localization of TET2 or NF- κ B (i.e., negative vs. positive) were determined in 1-2 fields ($\times 63$ lens) for each treatment. Intensity of fluorescence in cytosol vs. nucleus was quantified with Image J software (NIH) using the Freehand tool and Integrated Density measurement. The density of the nucleus was taken as a percentage of the density for the whole cell. Percentage of cells activated was quantified by counting cells with brighter fluorescence in the nucleus than in the cytosol and taking that as a % of the total cells. An average of 27 cells were analyzed in each field for mononuclear cells and an average of 20 cells in each field for THP-1 cells.

Cell culture for interleukin-8 (IL-8)

To further evaluate inflammatory response, media concentrations of IL-8, an NF- κ B regulated gene, were determined. Pregnancy mononuclear cells were isolated by Histopaque density gradient centrifugation as above. Cells were seeded at 250,000 cells per well in 1 ml of RPMI 1640 media in 24 well plates and cultured for 2 hours with *L. crispatus*, *L. iners* or *F. nucleatum* (50:1, bacteria to cells) or *L. iners* (50:1) plus inhibitors of PAR-1 (2 μ M, SCH-79797) or ROCK (20 μ M Y-27632). We used the THP-1 macrophage cell line to evaluate dose responses for bacteria and elastase. THP-1 cells were cultured as above and incubated for 18 h with bacteria (10:1, 50:1, 100:1, bacteria to cells) and elastase (0.0033, 0.033 and 0.33 U/ml). Media were collected and frozen at -20° C until assay. Media concentrations of IL-8 were measured by ELISA (R&D Systems, Minneapolis, MN).

Statistical Analysis

Data were analyzed by one-way ANOVA with Bonferroni multiple comparisons test. Demographic data are presented as mean \pm SD. Quantitative data are presented as mean \pm SE. A probability level of $P < 0.05$ was considered significant. A statistical software application was used (Prism 6.0 for Macintosh, GraphPad Software, Inc., San Diego, CA).

Results

Figure 1 shows representative images for the area of staining highlighted in yellow for TET2, PAR-1 and CD14 in decidual tissue. TET2 and PAR-1 showed similar patterns of staining. There was little expression of either TET2 or PAR-1 in decidual tissue of TNL women. The amount expressed increased in women with TL but was markedly increased in women with sPTL and even more so in women with iPTL. Staining for TET2 and PAR-1 paralleled increases in the infiltration of macrophages as evidenced by staining for CD14. Staining for TET2 and PAR-1 was localized primarily to leukocytes in the tissue and was not present in decidual cells.

Figure 2 shows the area of staining quantified for statistical analysis. Although there was more area stained in TL versus TNL, the increases were not statistically significant. However, the areas of staining for CD14, TET2 and PAR-1 were significantly increased in sPTL as compared to TL and the areas of staining were significantly higher in iPTL as compared to sPTL.

Figure 3 shows representative images for nuclear localization of TET2 (green) and the p65 subunit of NF- κ B (red) induced by bacterial treatments in mononuclear cells isolated from normal pregnant women. DAPI staining (blue) identifies the nucleus. In untreated cells, both TET2 and p65 were primarily localized to the cytosol. *L. crispatus*, *L. iners* and *F. nucleatum* all increased the translocation of TET2 and p65 from the cytosol to the nucleus. *F. nucleatum* caused the most intense nuclear localization.

Figure 4 summarizes the results for % activated cells and % nuclear localization for TET2 and p65 in response to bacteria, as well as the IL-8 response. All three bacterial species increased mononuclear cell activation and the % nuclear localization of TET2 and p65 with a parallel increase in media concentrations of IL-8. In general, the response for *L. iners* was greater than for *L. crispatus* with *F. nucleatum* the most potent.

To evaluate the pathway of protease amplification to bacterial stimulation, mononuclear cells were stimulated with *L. iners* in the presence of inhibitors of PAR-1 or ROCK. *L. iners* was used because it caused activation intermediate between *L. crispatus* and *F. nucleatum*. Representative pictures are shown in Figure 5. Inhibition of either PAR-1 or ROCK prevented the ability of *L. iners* to translocate TET2 and p65 from the cytosol to the nucleus as evidenced by the area of the nuclei being relative absent of green or red fluorescence in cells treated with *L. iners* plus inhibitors.

Figure 6 summarizes results for treatments with *L. iners* plus inhibitors of PAR-1 or ROCK. The significant increases in the % of cells activated and the % nuclear localization for TET2 and p65 induced by *L. iners* were prevented when cells were co-treated with PAR-1 or ROCK inhibitors. Similarly, the increase in IL-8 induced by *L. iners* was significantly attenuated by inhibition of PAR-1 or ROCK. These findings demonstrate that the PAR-1, ROCK pathway amplifies bacterial inflammatory response.

For the initial studies to test our hypotheses, we used primary cultures of mononuclear cells isolated from pregnant women. Mononuclear cells are composed of both monocytes and lymphocytes, both of which infiltrate the decidua during pregnancy. We were particularly interested in macrophages as first responders to bacterial invasion, and because of their extensive infiltration into the decidua of women with sPTL and iPTL. To determine if the responses observed for mononuclear cells were also present in macrophages, we repeated the experiments using THP-1 cells, a monocytic cell line that can be differentiated into a macrophage cell phenotype. Figure 7 shows representative pictures for nuclear localization of TET2 and p65. Results were similar to those for mononuclear cells. TET2 and p65 were primarily localized to the cytosol in control cells. *L. crispatus* slightly increased nuclear localization, whereas *L. iners* and *F. nucleatum* caused strong localization of TET2 and p65 to the nucleus with little remaining in the cytosol. To demonstrate protease activation,

THP-1 cells were treated with elastase, a known activator of PAR-1. Similar to *L. iners* and *F. nucleatum*, elastase caused strong nuclear localization of TET2 and p65.

To quantify the inflammatory response of THP-1 macrophages, the p65 subunit of NF- κ B, an accepted hallmark of inflammation^{45, 57}, was used (Figure 8). In control cells, few cells were activated with p65 primarily localized to the cytosol. All bacterial treatments activated THP-1 macrophages with translocation of p65 from the cytosol to the nucleus indicating activation of NF- κ B. *L. crispatus* activated the fewest cells with 15% of cells activated, however *L. iners* activated over 60% of cells and *F. nucleatum* activated almost all cells similar to protease treatment with elastase.

To evaluate protease amplification and the PAR-1, ROCK pathway in THP-1 macrophages, *L. iners* was again tested as a representative commensal bacteria and elastase was tested as a known activator of PAR-1. Figure 9 shows representative images. Inhibition of PAR-1 or inhibition of ROCK prevented the translocation of TET2 and p65 from the cytosol to the nucleus. In cells co-treated with *L. iners* or elastase plus inhibitors, TET2 and p65 were primarily localized to the cytosol.

Figure 10 shows summarized results for treatment with *L. iners* and elastase plus inhibitors of PAR-1 or ROCK for THP-1 macrophage cells. Inhibition of PAR-1 or ROCK significantly inhibited cell activation and nuclear translocation of TET2 and p65 induced by *L. iners* or elastase. These results confirm the findings with primary culture of mononuclear cells that proteases amplify the bacterial inflammatory response via a PAR-1, ROCK pathway.

By using THP-1 cells it was possible to grow a sufficient number of cells to test dose responses for IL-8 production. Figure 11 shows that bacteria and elastase induced dose dependent increases in IL-8. All doses of *L. iners* and *F. nucleatum* significantly increased IL-8, whereas only the highest dose of *L. crispatus* did. The responses for *L. iners* and *F. nucleatum* were very robust with the lowest doses increasing IL-8 as much as the highest dose of *L. crispatus*. Elastase significantly increased IL-8 at the medium and high doses.

Discussion

In this study, we provide evidence that proteases amplify the inflammatory response induced by commensal bacteria via a PAR-1, ROCK pathway. Treatment of pregnancy mononuclear cells or THP-1 macrophage cells with commensal bacteria or elastase, a protease known to activate PAR-1⁵⁸, caused translocation of TET2 and the p65 subunit of NF- κ B from the cytosol to the nucleus coincident with increased production of IL-8, an NF- κ B regulated gene. Inhibition of PAR-1 or ROCK prevented these changes. Immunohistochemical examination of decidual tissue obtained from women at delivery revealed a progressive increase in the expression of TET2 and PAR-1 that paralleled the infiltration of macrophages in the order TNL < TL < sPTL < iPTL. TET2 and PAR-1 staining was localized to leukocytes and not present in decidual cells.

The concurrent translocation of TET2 and NF- κ B from the cytosol to the nucleus upon activation suggests enzymatic de-methylation may be necessary for inflammatory response.

It makes sense that during pregnancy inflammatory genes would be silenced and a mechanism could be DNA methylation to block transcription factor binding sites. Upon activation, TET2 moves into the nucleus to erase methylation tags masking transcription factor binding sites, which allows NF- κ B to bind and increase expression of inflammatory genes. Consistent with this idea is our recent finding that increased expression of toll-like receptors is associated with reduced DNA methylation in sPTL⁵⁹.

We studied decidual tissue because it is in a key location to be first impacted by invasion of commensal microorganisms of vaginal origin and to, thus, act as a trigger of inflammatory response. As resident sentinels, macrophages are a first alert system to warn the body of danger. In vivo, protease amplification could occur as a consequence of release of proteases from activated macrophages, triggering PAR-1 signaling on adjacent macrophages, as well as other decidual leukocytes that express PAR-1, such as lymphocytes. Protease amplification may be particularly important in sPTL and iPTL because of the extensive infiltration of macrophages into the decidua and their close proximity to each other. Although bacteria produce proteases, we were not able to find any evidence that bacterial proteases activate PAR-1, whereas proteases released by macrophages do³²⁻³⁴. In our experiment, bacterial proteases cannot be responsible because the bacteria were heat-inactivated. To our knowledge, this is the first report that proteases amplify the inflammatory response to bacteria and is necessary for a robust response.

We were interested in how the vaginal microbiome might be implicated in the racial disparity of preterm birth of African-Americans. That is why we compared *L. crispatus* which is prevalent in European ancestry women with *L. iners* which is prevalent in African-American women. *L. crispatus* maintains a stable, healthy vaginal environment, whereas *L. iners* allows a higher diversity of pathologic species, such as *F. nucleatum*. Both *Lactobacillus* species initiated an inflammatory response, but *L. iners* initiated a more robust response than *L. crispatus*. The strongest response was induced by *F. nucleatum*. These results suggest that the greater prevalence of *L. iners* and *F. nucleatum* in the vaginal microbiome of African-American women might initiate an inflammatory response prematurely resulting in their higher incidence of PTB.

The results of this study raise an interesting question. If a beneficial bacterium like *L. crispatus* can cause an inflammatory response when in direct contact with immune cells, could vaginal bacteria be the initiating agents in term, as well as preterm labor. This could come about as the cervix effaces and dilates and the fetal membranes are exposed to the vaginal microbiome. This would provide a route for vaginal bacteria to enter intrauterine tissues where they could initiate an inflammatory response to initiate the onset of labor.

We measured IL-8 production, which is encoded by an NF- κ B regulated gene. IL-8 has additional significance with regard to labor as it is a potent neutrophil chemokine, and neutrophils infiltrate reproductive tissues with the onset of labor⁴⁻⁶. We have shown that neutrophil proteases are potent stimulators of uterine contractility via PAR-1⁶⁰, and neutrophil matrix metalloproteinases, such as MMP-1 and MMP-8, have been recognized as important contributors to labor because of their ability to degrade extracellular matrix and, as such, have been implicated in cervical ripening and preterm premature rupture of fetal

membranes^{4, 27, 61-63}. The significant increases we observed in IL-8 induced by vaginal bacteria lends support to the idea that commensal bacteria may be initiators of both term and preterm labor.

Figure 12 summarizes the implications of this study. Commensal bacterial stimulate decidual macrophages to release proteases that amplify the inflammatory response by activating PAR-1 on adjacent macrophages and other decidual leukocytes that express PAR-1. Activation of PAR-1 leads to downstream signaling whereby ROCK phosphorylates TET2 and NF- κ B causing their translocation from the cytosol to the nucleus. Enzymatic demethylation of DNA by TET2 opens up transcription factor binding sites for NF- κ B to increase inflammatory gene expression initiating labor. The more robust inflammatory responses initiated by *L. iners* and *F. nucleatum*, which are prevalent in the vaginal microbiome of African-American women, as compared to *L. crispatus*, which is prevalent in the vaginal microbiome of women of European ancestry, might explain the higher incidence of PTB in African-Americans. However, in both cases commensal bacteria could play a role in the initiation of labor.

Acknowledgments

Microscopy was performed at the VCU Department of Anatomy & Neurobiology Microscopy Facility, supported, in part, by funding from the NIH-NINDS Center Core Grant 5 P30 NS047463 and, in part, by funding from the NIH-NCI Cancer Center Support Grant P30 CA016059.

Funding: The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article. This work was supported by grants from NHLBI, R01 HL069851 (SWW), NICHD 5R01 HD088386 (SWW), and NCMHD P60 MD002256 (JFS, KKJ, SWW).

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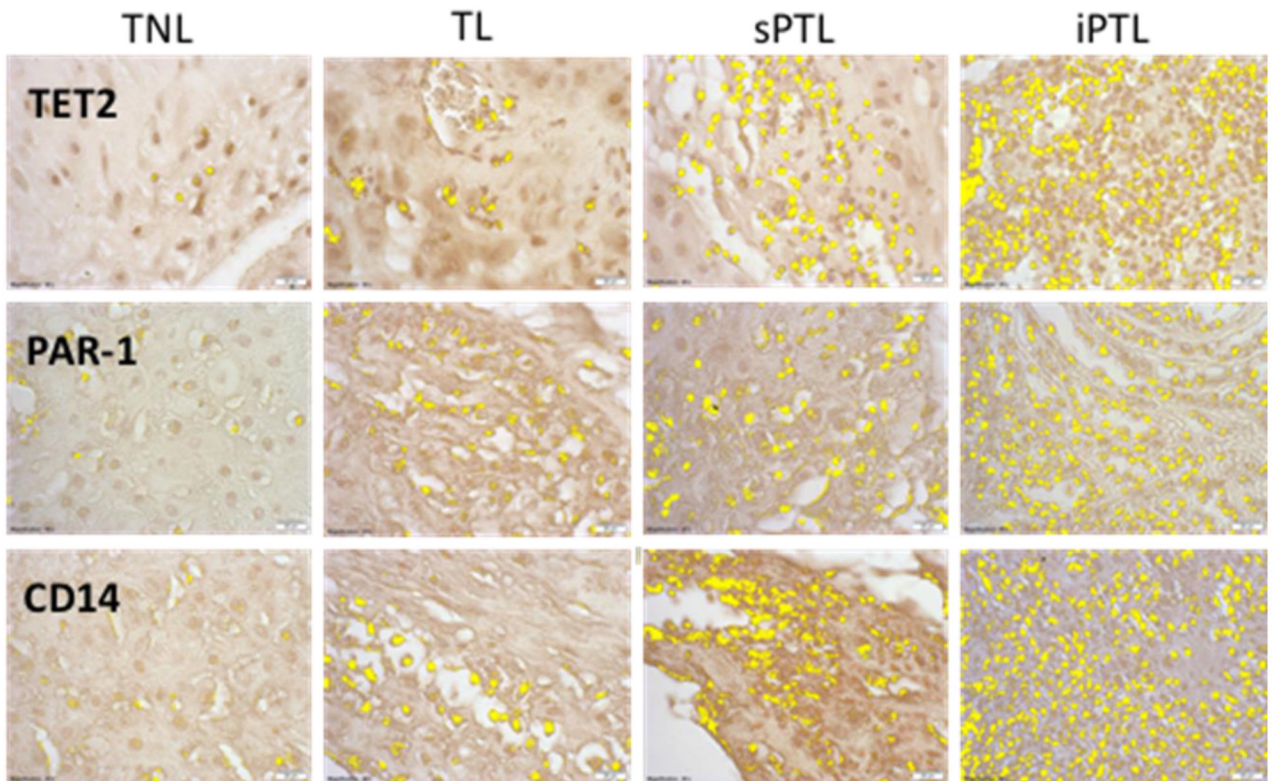


Figure 1.

Representative sections of decidual tissue immunostained for TET2, PAR-1 and CD14 from women who were at term not-in-labor (TNL), women who were at term in labor (TL), women who delivered spontaneously preterm with no clinical signs of infection (sPTL) and women who delivered preterm with PPROM and clinical signs of infection (iPTL). Specific antigen staining was highlighted in yellow using the Measuring Images tool in the cellSens software. There was little staining for TET2, PAR-1 or CD14 in women with TNL. Staining increased slightly for TL but was markedly increased in sPTL and iPTL. The increasing pattern of staining for TET2 and PAR-1 correlated with the macrophage marker, CD14. Decidual cells did not expression TET2 or PAR-1. All pictures and analyses were done with a 40X lens.

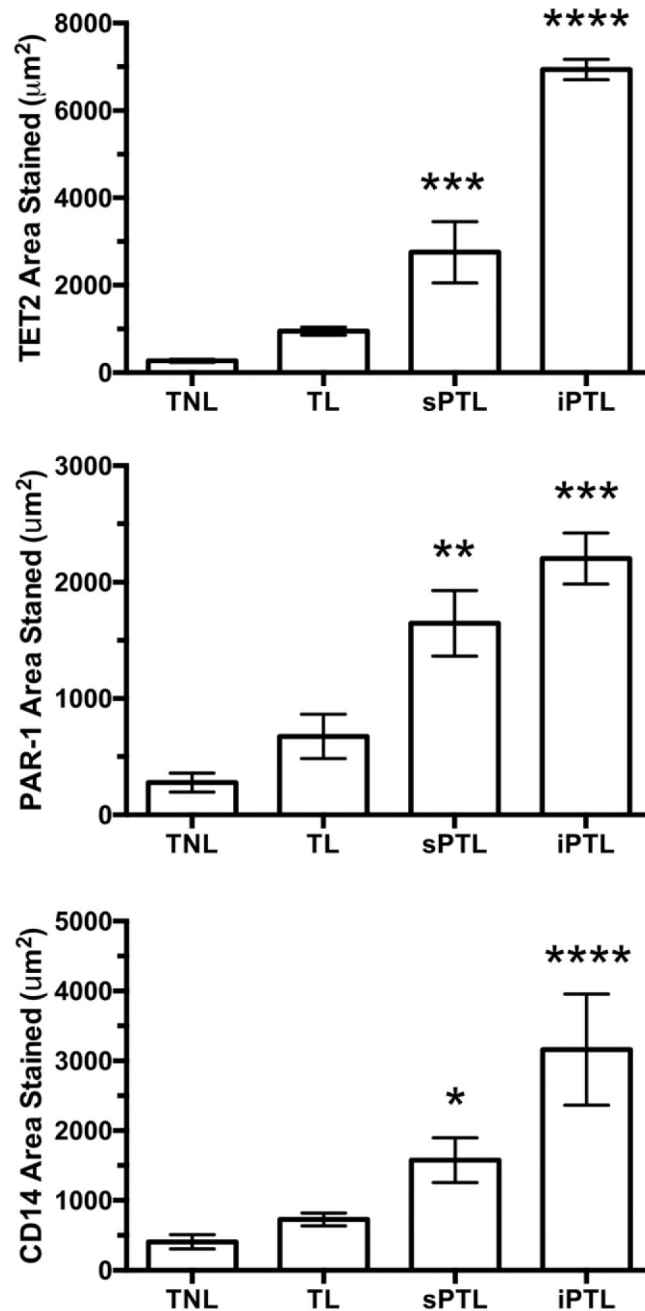


Figure 2.

Area of staining in decidual tissue for women who were TNL (n = 6), women who were TL (n = 5), women who were sPTL (n = 4), and women who were iPTL (n = 3). The areas of staining for TET2 and PAR-1 were small in women with TNL or TL. However, there was a marked and significant increase in the areas stained for both TET2 and PAR-1 in women with sPTL and an even greater increase in women with iPTL. The increase in areas stained for TET2 and PAR-1 correlated with the staining for macrophages, CD14. The Measuring Images tool in the cellSens software was used to determine the area of staining for statistical analysis. Data represent mean \pm SE, * P < 0.05, ** P < 0.01, **** P < 0.001.

Pregnancy Mononuclear Cells

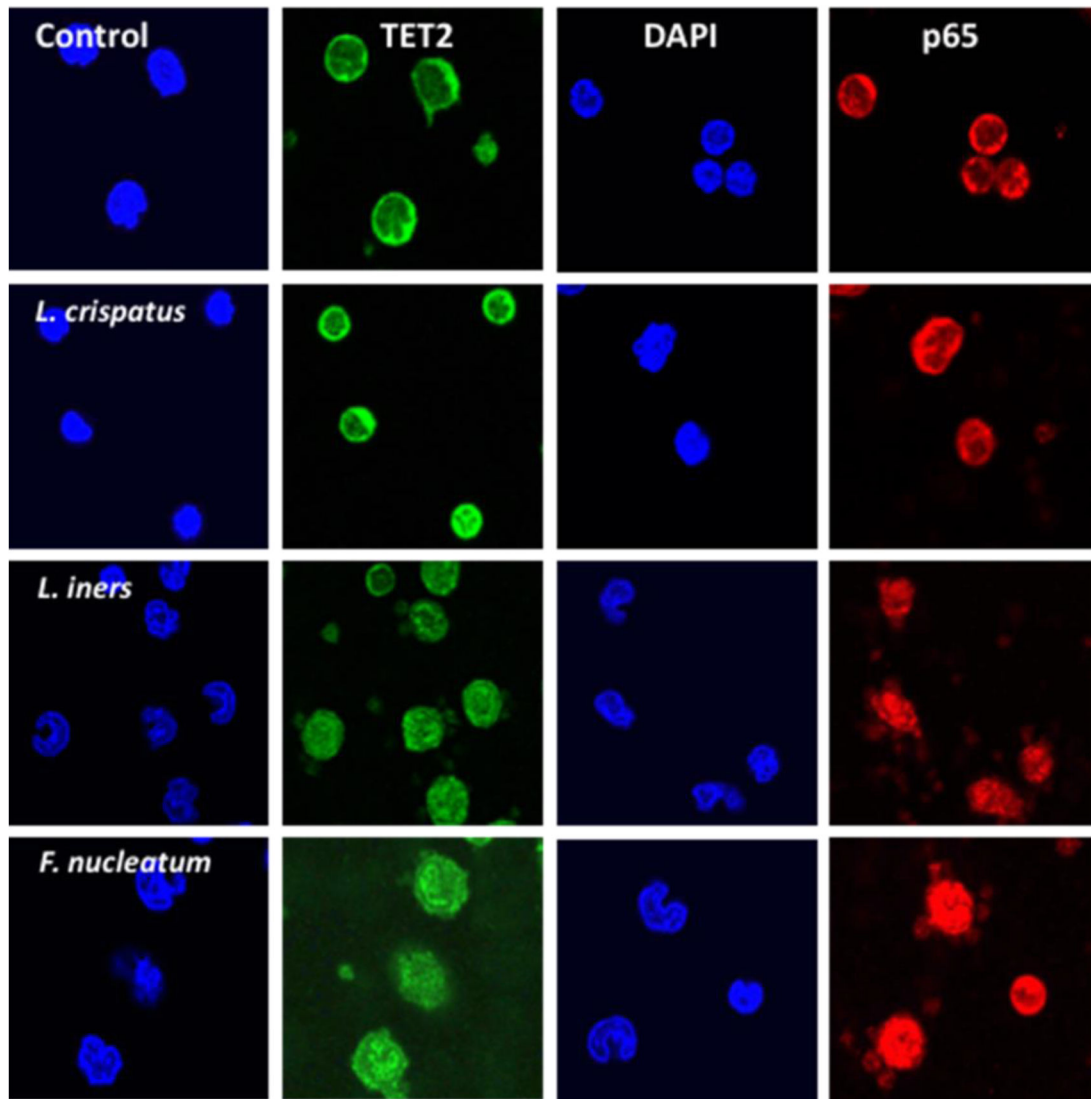


Figure 3.

Representative images of mononuclear cells isolated from normal pregnant women treated with commensal bacteria. Green fluorescence identifies TET2, red identifies the p65 subunit of NF- κ B and DAPI blue identifies the nucleus. In untreated cells, both TET2 and p65 were primarily localized to the cytosol. *L. crispatus*, *L. iners* and *F. nucleatum* all increased the translocation of TET2 and p65 from the cytosol to the nucleus, with *F. nucleatum* causing the most intense nuclear localization. The dose for bacteria was 100:1 (bacteria to cells).

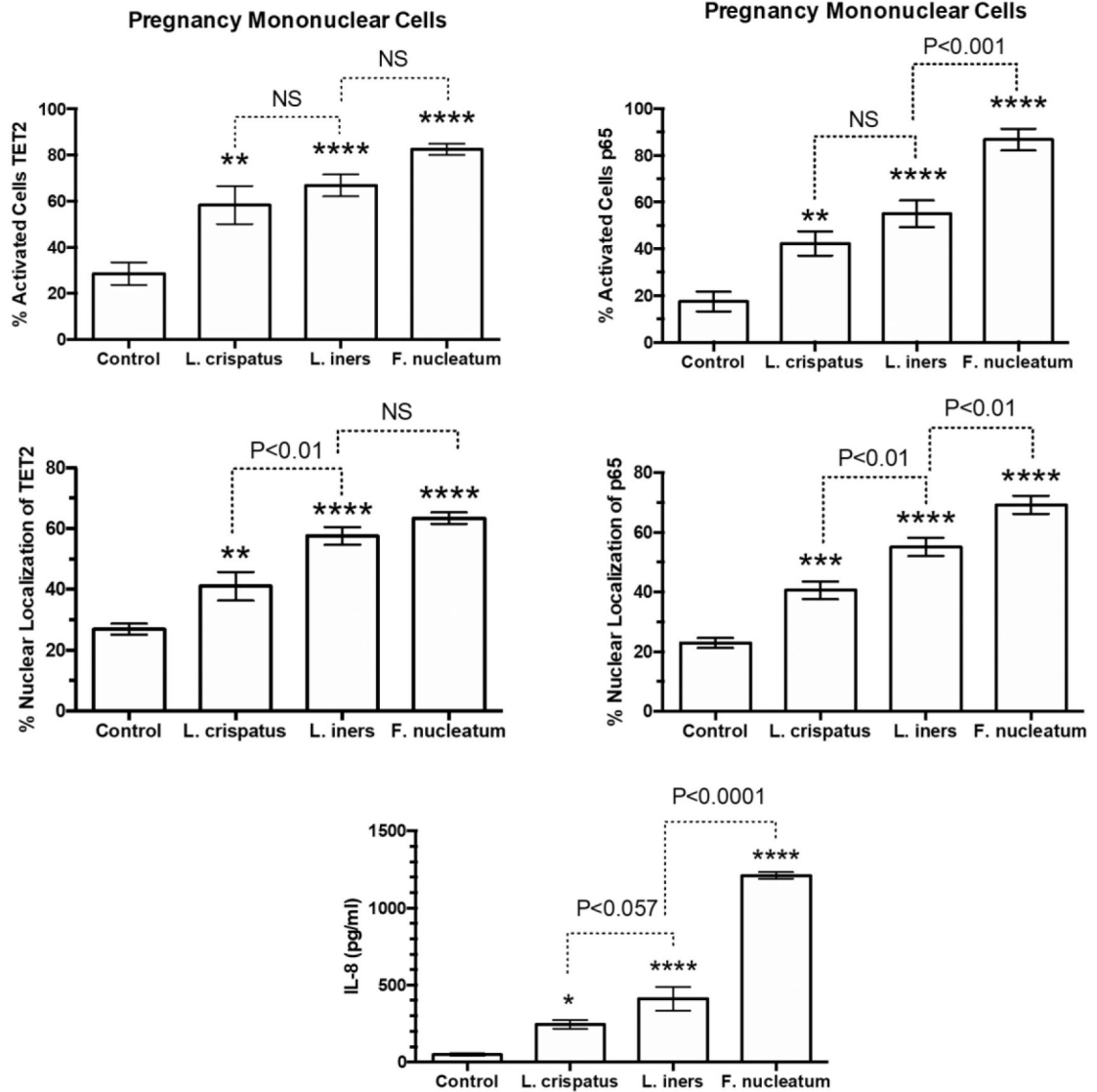


Figure 4.

Percent of pregnancy mononuclear cells activated, % nuclear localization for TET2 and p65, and media concentrations of IL-8 in response to treatments with bacteria. Nuclear localization was quantitated by the density of immunofluorescence in the area of the nucleus as a percentage of the density of the entire cell using Image J software. Percentage of cells activated was quantified by counting the number of cells with brighter fluorescence in the nucleus than in the cytosol and taking that as a % of the total cells. The average number of cells counted per field was 27. *L. crispatus*, *L. iners* and *F. nucleatum* all activated the cells causing translocation of TET2 and p65 from the cytosol to the nucleus with a parallel increase in IL-8 in a general order of *L. crispatus* < *L. iners* < *F. nucleatum*. (n = 8 for TET2, n = 10 for p65, n = 11 for IL-8, data represent mean \pm SE, * P < 0.05, ** P < 0.01, *** P < 0.001, **** P < 0.0001 as compared to control. P values are for comparisons between bacterial treatments. NS, non-significant)

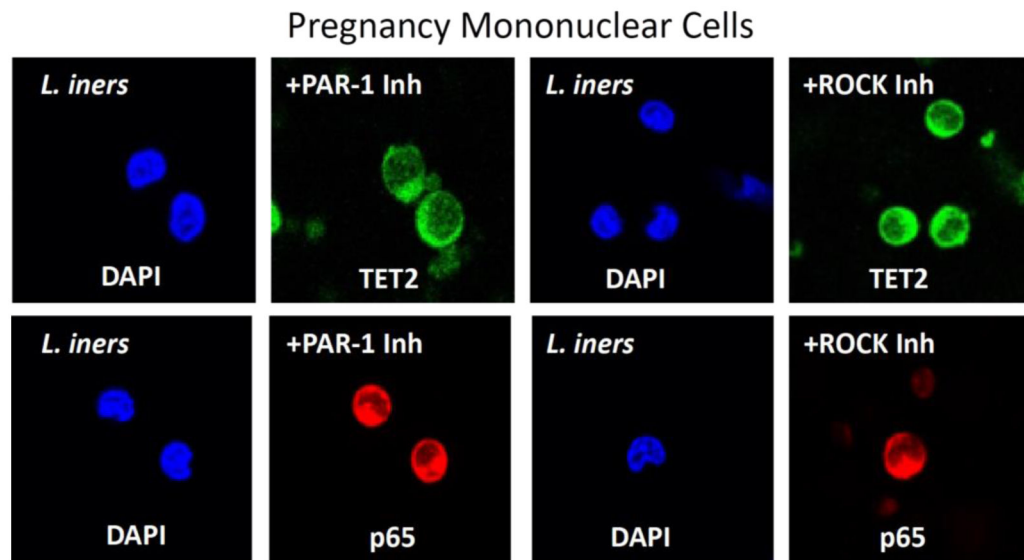


Figure 5. Representative images of the cellular localization of TET2 (green) and p65 (red) in pregnancy mononuclear cells after treatment with *L. iners* plus inhibitors of PAR-1 or ROCK. Co-treatment with either PAR-1 inhibitor or ROCK inhibitor prevented the ability of *L. iners* to move TET2 or p65 from the cytosol into the nucleus.

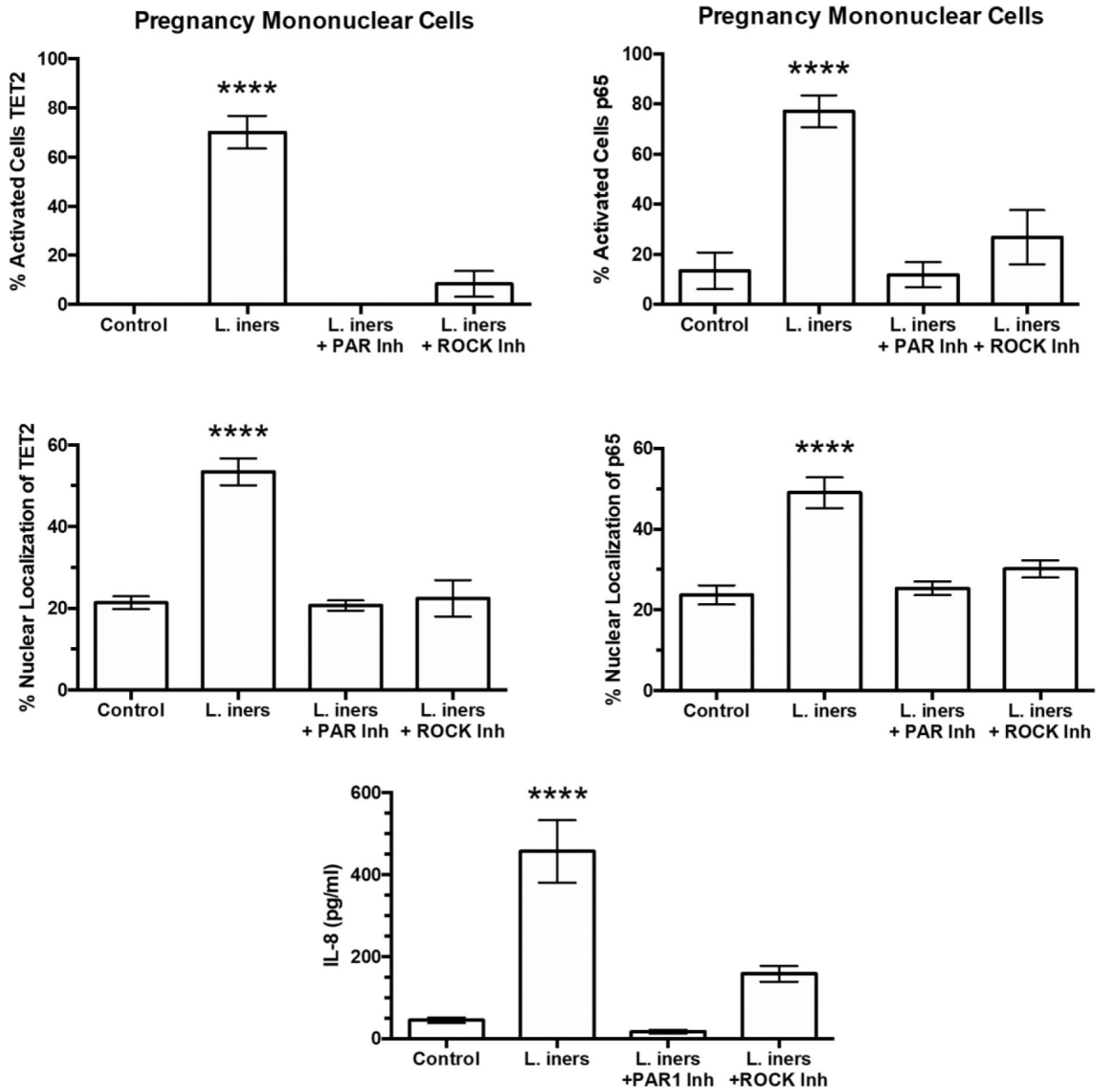


Figure 6. Percent of pregnancy mononuclear cells activated in response to treatment with *L. iners* or *L. iners* plus inhibitors of PAR-1 or ROCK. Inhibition of PAR-1 or ROCK prevented *L. iners* from causing nuclear translocation of TET2 and p65 and increasing IL-8 concentrations. (n = 7, mean ± SE, **** P < 0.0001)

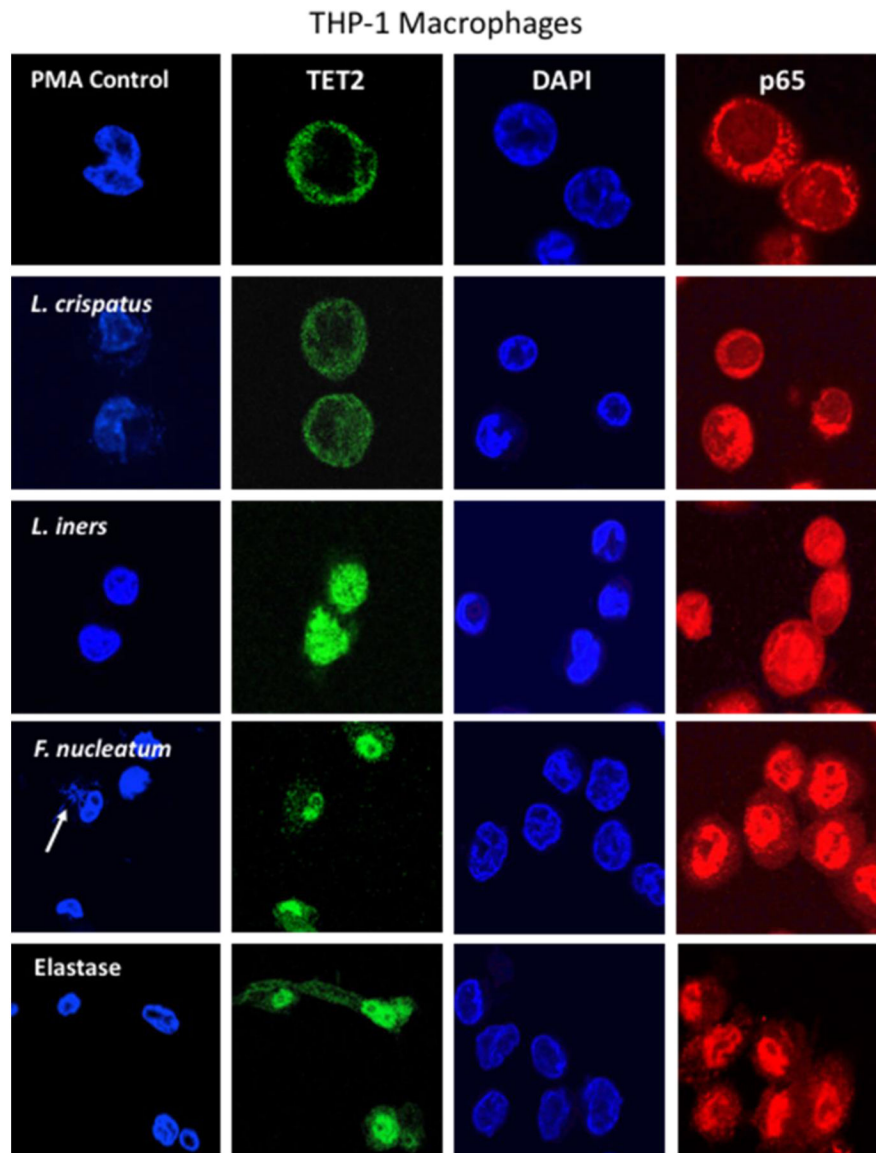


Figure 7. Representative images of the nuclear translocation of TET2 (green) and the p65 subunit of NF- κ B (red) in THP-1 macrophage cells in response to bacteria (100:1, bacteria to cells) or elastase (0.033 U/ml). DAPI blue indicates the location of the nucleus. In control cells, TET2 and p65 were primarily localized to the cytosol indicating TET2 and NF- κ B were not activated in most cells. In cells treated with *L. crispatus*, there was some movement of TET2 and p65 from the cytosol to the nucleus, however in cells treated with *L. iners*, *F. nucleatum* or elastase, TET2 and p65 were primarily localized to the nucleus in most cells. White arrow for *F. nucleatum* points to nuclei of bacteria engulfed by a THP-1 macrophage.

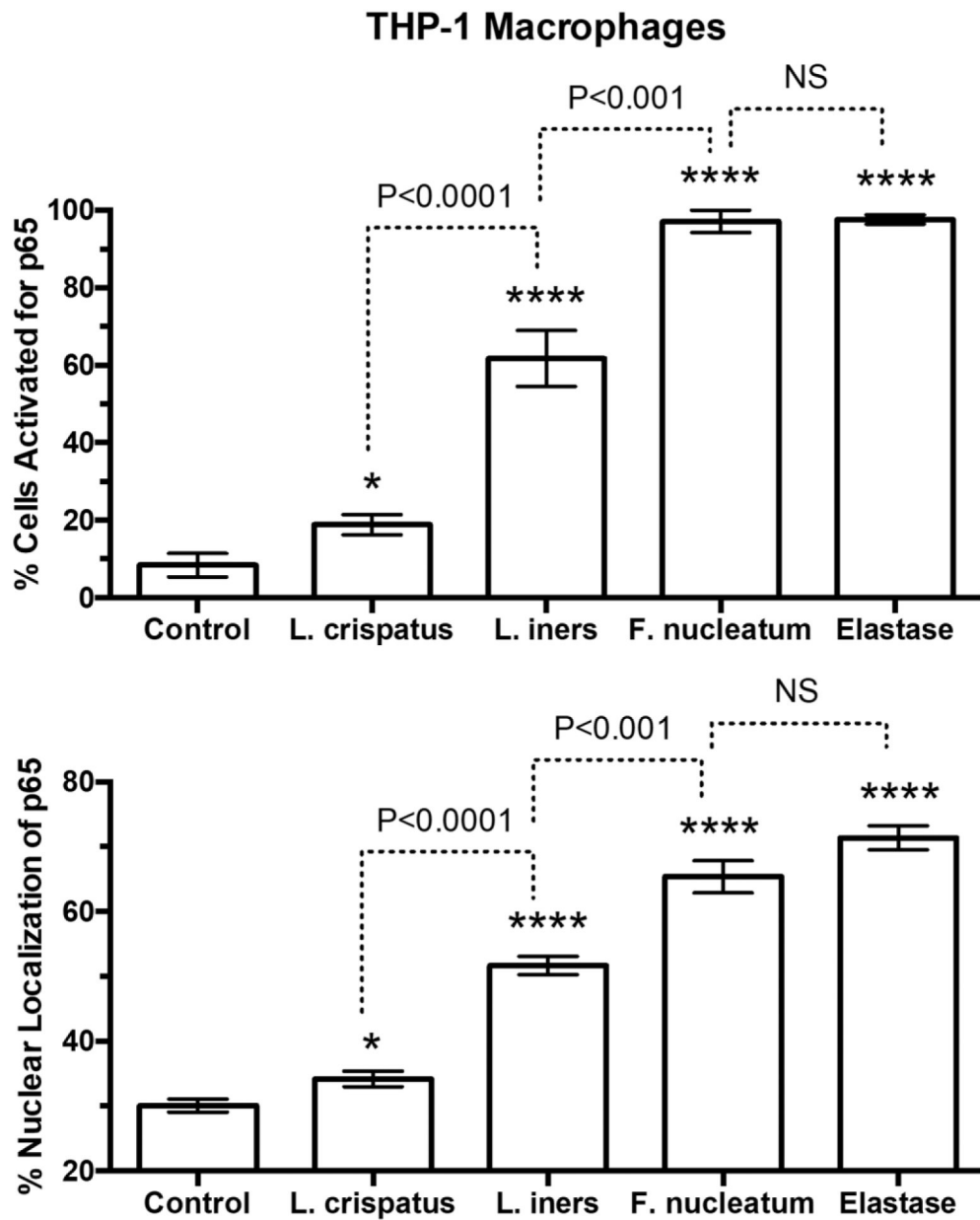


Figure 8. Percent of cells activated and % of nuclear fluorescence for the p65 subunit of NF- κ B in THP-1 cells in response to treatments with bacteria or elastase. Percent of cells activated and % nuclear localization was quantitated as described for Figure 4. The average number of cells counted per field was 20. *L. crispatus* activated 15% of cells, however *L. iners* activated over 60% of cells and *F. nucleatum* activated almost all cells similar to protease treatment with elastase. (n = 5 for bacteria, n = 3 for elastase, mean \pm SE, * P < 0.05, **** P < 0.0001 as compared to control. P values are for comparisons between bacterial treatments. NS, non-significant)

THP-1 Macrophages

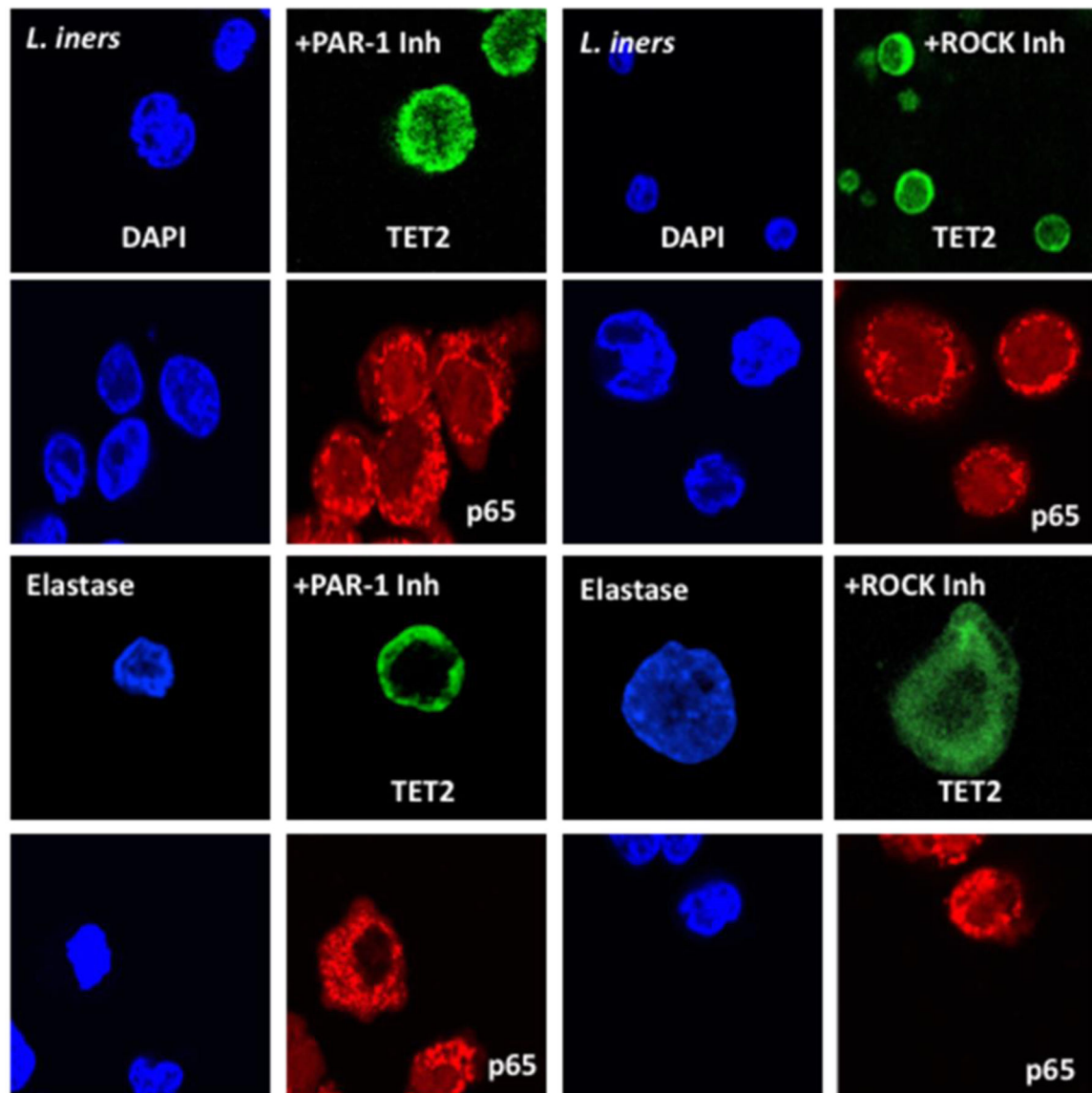


Figure 9. Representative images of the cellular localization of TET2 (green) and p65 (red) in THP-1 macrophage cells after treatment with *L. iners* or elastase plus inhibitors of PAR-1 or ROCK. Co-treatment with either PAR-1 inhibitor or ROCK inhibitor prevented the ability of *L. iners* or elastase to move TET2 or p65 from the cytosol into the nucleus.

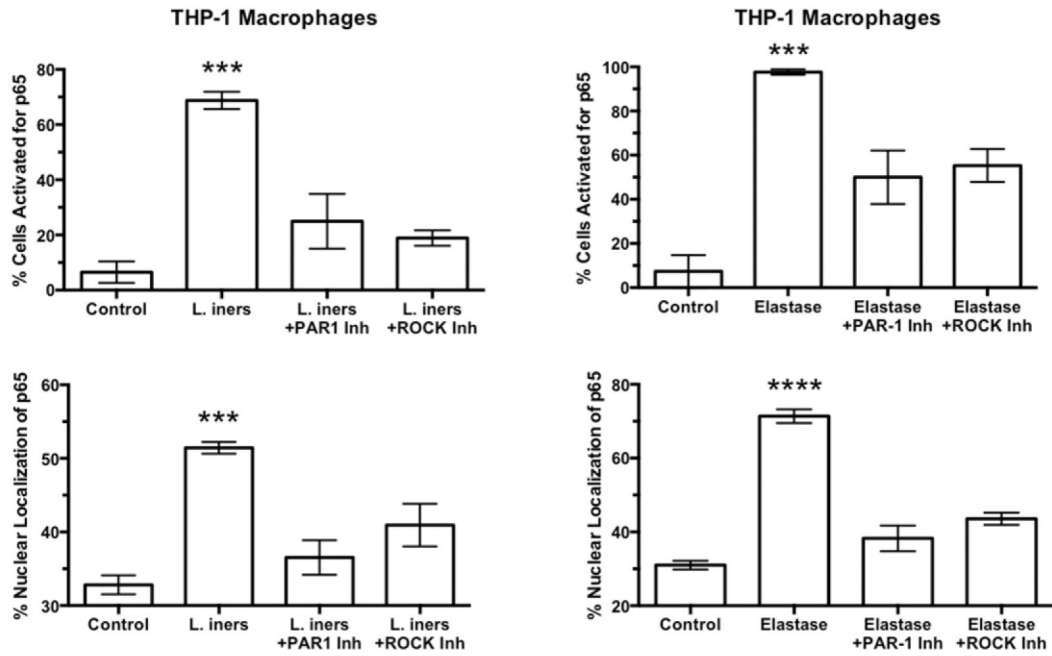


Figure 10.

Percent of THP-1 macrophage cells activated and % of nuclear fluorescence localization for p65 in response to treatments with *L. iners* or elastase plus inhibitors of PAR-1 or ROCK. Inhibition of PAR-1 or ROCK prevented the nuclear translocation of p65 induced by *L. iners* or elastase. (n = 5 for *L. iners*, n = 4 for elastase, mean \pm SE, *** P < 0.001, **** P < 0.0001)

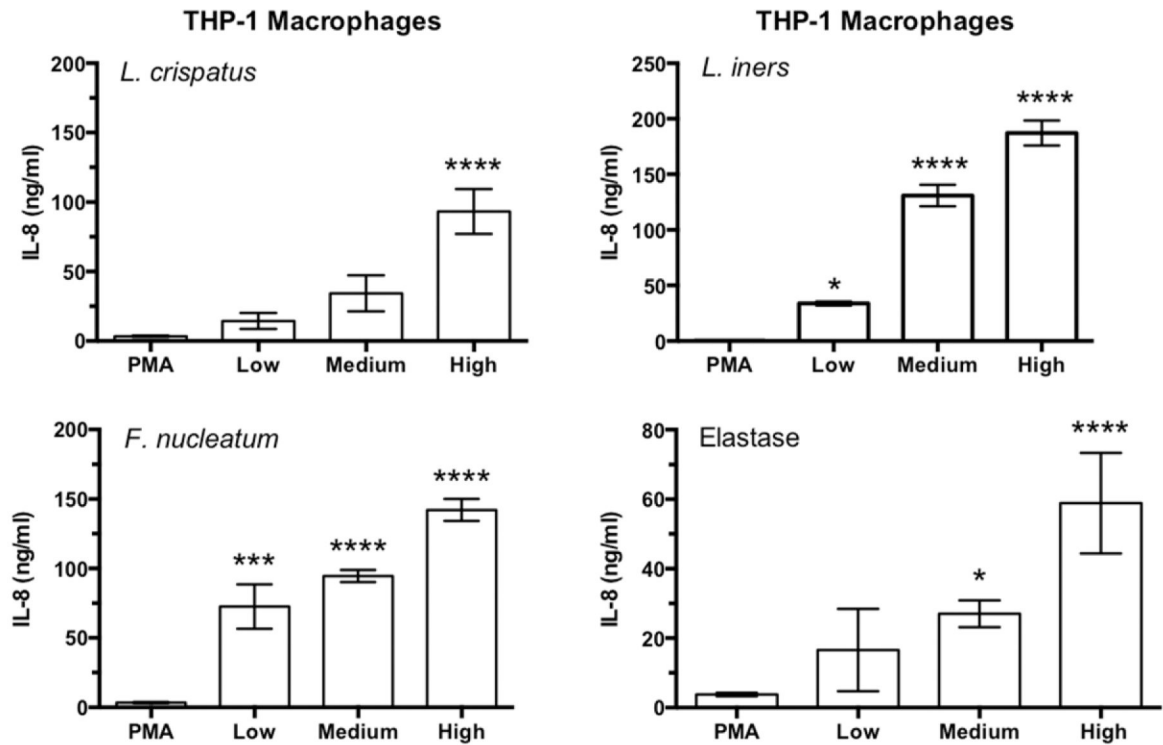


Figure 11.

Dose response effect of bacteria and protease treatment on media concentrations of IL-8 in THP-1 macrophage cells. Doses for bacteria were: Low, 10:1; Medium, 50:1; High, 100:1 bacteria to cells. Doses for elastase were: Low, 0.0033 U/ml; Medium, 0.033 U/ml; High, 0.33 U/ml. *L. iners* and *F. nucleatum* significantly increased IL-8 at all doses as opposed to *L. crispatus*, which significantly increased IL-8 only at the highest dose. Elastase stimulated significant increases in IL-8 at the medium and highest doses. (n = 6 for bacteria, n = 4 for elastase, mean ± SE, * P < 0.05, *** P < 0.001 **** P < 0.0001)

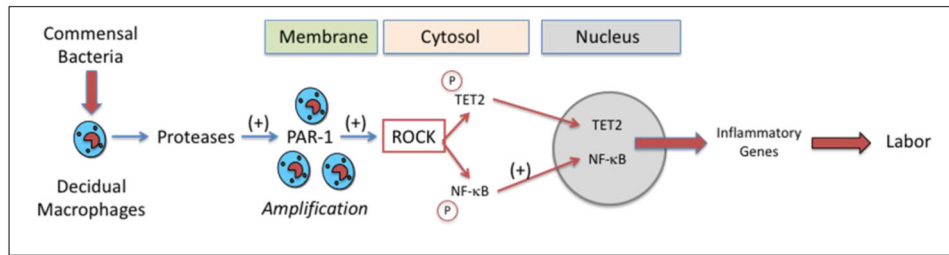


Figure 12.

Proposed mechanism for protease amplification of the inflammatory response induced by commensal bacteria that could trigger the onset of labor. Commensal bacteria present in decidual tissue stimulate resident macrophages to release proteases that act in an autocrine manner to amplify the response by activating PAR-1 on adjacent macrophages and other decidual leukocytes that express PAR-1. Activation of PAR-1 leads to down-stream signaling to ROCK that phosphorylates TET2 and NF- κ B causing their translocation from the cytosol to the nucleus. In the nucleus, TET2 enzymatically de-methylates DNA opening up transcription factor binding sites for NF- κ B resulting in increased expression of inflammatory genes to initiate labor. Commensal bacteria tested were *L. crispatus*, which is prevalent in the vaginal microbiome of women of European ancestry, and *L. iners* and *F. nucleatum*, which are prevalent in the vaginal microbiome of African-American women. We observed a more robust inflammatory response for *L. iners* and *F. nucleatum* than for *L. crispatus* which might explain the higher incidence of PTB in African-Americans, however commensal bacteria may be the initiators of labor in both term and preterm labor.

Table 1:

Clinical Characteristics of Patient Groups for Immunohistochemistry

Variable	TNL n = 6	TL n = 5	sPTL n = 4	iPTL n = 3
Maternal age (y)	30.0 ± 5.9	26.8 ± 10.6	27.3 ± 8.1	23.3 ± 1.7
Pre-pregnancy BMI (kg/m ²)	26.9 ± 5.7	25.1 ± 5.8	21.6 ± 3.6	23.7 ± 1.5
BMI at sample collection (kg/m ²)	32.0 ± 5.9	30.2 ± 6.0	26.3 ± 3.4	27.3 ± 0.6
Race				
White	2		1	
Black	3	3	2	3
Asian			1	
Hispanic				
Other	1	2		
Primiparous		3	1	
Multiparous	6	2	3	3
Gestational age (wk)	38.8 ± 0.4	39.2 ± 0.8	33.3 ± 1.7 [*]	30.3 ± 6.7 ^{**}
Infant birth weight (g)	3341 ± 508	3139 ± 506	2346 ± 496	1723 ± 1092 [*]
Delivery Method				
Vaginal		3	2	3
C-section	6	2	2	

Values are mean ± SD

^{*} p < 0.05

^{**} p < 0.01 compared to TNL and TL