HHS Public Access

Author manuscript

Am J Reprod Immunol. Author manuscript; available in PMC 2024 October 01.

Published in final edited form as:

Am J Reprod Immunol. 2023 October ; 90(4): e13772. doi:10.1111/aji.13772.

Decreased level of TREM like Transcript 1 (TLT-1) is associated with prematurity and promotes the in-utero inflammatory response to maternal lipopolysaccharide (LPS) exposure

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Abstract

Problem: The occurrence of preterm birth is associated with multiple factors including bleeding, infection and inflammation. Platelets are mediators of hemostasis and can modulate inflammation through interactions with leukocytes. TREM like Transcript 1 (TLT-1) is a type 1 single Ig domain receptor on activated platelets. In adults, it plays a protective role by dampening the inflammatory response and facilitating platelet aggregation at sites of vascular injury. TLT-1 is expressed in human placenta and found in cord blood. We thus hypothesized that TLT-1 deficiency is associated with prematurity and fetal inflammation.

Method of study: To test this hypothesis, we examined cord blood levels of soluble TLT-1 (sTLT) in premature and term infants and compared the inflammatory response in C57BL/6 (WT) and TLT-1$^{-/-}$ (*treml1*$^{-/-}$, KO) mice given intraperitoneal LPS mid gestation.

Results: The preterm infant cord blood level of sTLT was significantly lower than that found at term. On exposure to LPS, histology of KO (as compared to WT) placenta and decidua showed increased hemorrhage, and KO decidual RNA expression of IL-10 was significantly lower. KO fetal interface tissues (placenta, membranes, amniotic fluid) over time showed increased expression of inflammatory cytokines such as IL-6, IFN-γ, and TNF, but not MCP-1. However, fetal organs showed similar levels.

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Author Contribution: All authors participated in experimental design, performing experiments, animal care, harvesting of tissue, data analysis and interpretation, and preparation of the manuscript and other forms of communication of the data.

Conflict of Interest:
The authors have nothing to disclose.

Ethics Statement: The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The study conformed to the US Federal Policy for the Protection of Human Subjects.

Conclusion: There is a potential association between insufficient TLT-1 expression and increased fetal inflammatory responses in the setting of prematurity. The data support further study of TLT-1 in the mechanistic link between bleeding, inflammation and preterm birth, and perhaps as a biomarker in human pregnancy.

Keywords

Preterm birth; TLT-1; Inflammation; Bleeding; Cord blood; Mice

1. Introduction:

Preterm birth (PTB), defined in humans as birth of the conceptus before 37 weeks gestational age, is the leading cause of neonatal morbidity and mortality worldwide[1]. Premature infants are at increased risk of respiratory diseases, severe infections, and intraventricular hemorrhage and while it is known that the inflammatory response is often a trigger for preterm birth, its role is poorly understood. PTB can be the outcome of medical intervention in the case of severe fetal or maternal disease. It can also be the result of spontaneous labor leading to premature rupture of membranes, or of premature rupture of membranes followed by uterine contractions. These later, spontaneous, elements are preceded by cervical ripening and dilation, the loss of uterine smooth muscle quiescence and weakening of the fetal membranes. The underlying mechanisms include oxidative stress[2], hormonal,[3,4] or metabolic dysregulation,[5] activation of the hypothalamic-pituitary adrenal (HPA) axis,[6] mechanical overload,[7] infection,[8,9] and bleeding. These all contribute to a final common pathway of inflammation.[10] Maternal (e.g., decidua) or fetal (e.g., membranes, trophoblast) tissue inflammation transmits further inflammatory signals to mother and fetus. On the one hand, inflammation in these tissues leads to further inflammation and uterine contractions and cervical dilation. On the other, this process leads to the fetal inflammatory response syndrome (FIRS).[11] This syndrome also includes the elevation of cytokines such as Interleukin (IL)-6 and inflammatory mediators such as C reactive protein, IL-1species or IL-17. Importantly, in humans, FIRS has been associated with bacterial, viral, fungal, and protozoan infection, and with noninfectious inflammation (reviewed in[11]). FIRS is also associated with the dysfunction or abnormal development of several fetal organs including lung, brain, gut, and thymus.

In humans, bleeding has been associated with a higher risk of preterm birth.[12,13] However, the relationship is likely complex[14] and dependent on the presence of both anti- and pro-coagulant molecules which contribute to the cross talk between bleeding, tissue damage, and inflammation. For example, structural damage to decidual vascular endothelium can lead to increased thrombin generation due to the expression of tissue factor by decidua.[15] High levels of coagulant molecules, such as thrombin, has been found associated with preterm birth.[16] Thrombin can act via G-protein-coupled protease-activated receptors and, as observed in a mouse model, it activates the phosphatidylinositol-signaling pathway which results in the generation of cytosolic calcium oscillations and phasic myometrial contractions.[17] Thrombin generation may also alter local (e.g., decidual) progesterone responsiveness, metalloproteinase expression and increase production of inflammatory

cytokines.[15,18,19] Moreover, this molecule plays an essential role in the clotting cascade and in platelet activation.

Platelets are a-nucleate cells from the megakaryocyte lineage that are classically slated as mediators of hemostasis. However, platelets not only release cytokines from their granules upon activation, but once activated, they adhere to neighboring leukocytes and modulate their immune function which highlights a role in the inflammatory process. Platelets have demonstrated roles in the initiation and exacerbation of cardiovascular disease, cancer and sepsis. Additionally, platelet dysfunction has been associated with intraventricular hemorrhage in preterm infants[20], with severe hypertension-related disease in pregnancy,[21] and with functional progesterone withdrawal.[22] Given the dual role of platelets in clotting and inflammation, modulation of platelet activation is an attractive target that may lower the risk of spontaneous preterm birth.[23]

The Triggering Receptor Expressed on Myeloid cells (TREM) like Transcript-1 (TLT-1)[24] is expressed exclusively in the platelet and megakaryocyte lineage.[25] TLT-1 is stored inside the alpha granules of platelets and, upon activation, it is translocated to the surface of the cell where it binds the plasma clotting protein fibrinogen, a glycoprotein crucial for platelet-platelet interactions[26] While mice deficient in TLT-1 (*treml1*$^{-/-}$, KO) have longer bleeding times, reduced platelet aggregation and increased inflammation associated bleeding compared to controls,[26] TLT-1's role in coagulation remains undefined. TLT-1 mediates transmigration of neutrophils during inflammation and absence of the receptor impedes neutrophil transmigration into tissue. This makes TLT-1 an attractive target for the treatment of syndromes such as lung injury where neutrophil infiltration is the hallmark of the disease. Because TLT-1 deficiency has been shown to exacerbate bleeding and inflammation, we hypothesized that TLT-1 deficiency is associated with prematurity and fetal inflammation. In this study we used a pregnant mouse model of LPS exposure[27] to evaluate the role of TLT-1 in inflammation-induced PTB.

2. Materials and Methods:

2.1 Human samples

This study of stored de-identified cord blood serum from human preterm and term singleton gestations was designated as not requiring a human subject protocol approval by the University of Vermont Institutional Review Board. Birthing persons were otherwise healthy. Samples were thawed and assayed for soluble TLT (s-TLT). Limited clinical data on the samples assayed is presented in Supplemental Table 1.

2.2 TLT-1 ELISAs

Soluble TLT-1 levels for mice and humans were measured by ELISA (R&D Systems cat# DY2424 and DY2394 respectively) according to manufacturer specifications. ELISAs were developed using reagents from R&D Systems and read on a BioTek Synergy HTX multi-mode plate reader.

2.3 Mouse housing and study approval

Mice deficient in TLT-1 (*treml1*$^{-/-}$) have been reported elsewhere.[26] All mice were between 8 and 10 weeks of age and weighed 18 to 21 g. Animal care was provided in accordance with the procedures outlined in Guide for the Care and Use of Laboratory Animals[28] C57BL/6J and *treml*$^{-1/-}$ mice were housed and bred at an ICUAC approved animal care facility at the University of Puerto Rico, Rio Piedras Campus.

2.4 Breeding and induction of preterm birth

Timed mating occurred as has been done previously.[27] Age-matched (8–10-week-old) wild-type or *trem1*$^{-/-}$ male mice were placed in individual cages for one day before being removed and replaced with individual female mice of the same genotype. Synchronized breeding was conducted after the female mice had been in the cage for three days by placing the same male mouse that had been previously removed with the female for 24 hours. The male mouse was subsequently removed, and females were monitored for pregnancy. Preterm birth was induced at day 15 of pregnancy by injecting intraperitoneal 10 μg lipopolysaccharide (LPS, E. coli serotype O111:B4 from Millipore Sigma cat# L2630) /200 μl PBS. Control mice received equivalent amounts of PBS but without LPS. Tissues were collected at 12–16 hours after injection, which is typically just before delivery, approximately 18–24 hours after the dose of LPS used in wild type C57BL/6 mice ([27] and in this study). In other experiments, mice were observed for 24 hours for the delivery of a pup in the cage.

2.5 Tissue and Sample Harvesting

Pregnant females were euthanized, and maternal blood was collected and stored for assay of serum. Uterine draining lymph nodes (Para-aortic, inguinal) were removed for assay. Maternal uteri were removed and opened to reveal the existing implantation sites. The number of total implantation sites, the number of sites with a small discreet area of necrotic tissue (resorption), the sites with a placenta, but not a fetus (delivered) and the implantation sites with intact placenta and associated amniotic sacs with fetuses were counted. Using a 30-guage needle, amniotic sacs were entered and 50–100 μl of amniotic fluid was removed and stored at−80°C. Then the amniotic sac was broken, and the fetal membranes were harvested. The placenta and decidua were removed and the white disc representing the decidua was separated from the placenta. In some cases, implantation (e.g., under the decidua) and non-implantation uterus was also removed. Fetal tissues were isolated from normal-appearing pups and included fetal liver and brain. Tissues were stored in 4% paraformaldehyde, RNA later, or frozen using liquid nitrogen. Decidua, uterine draining lymph nodes, and fetal liver were also used to generate single cell suspensions for flow cytometry.

2.6 RNA extraction and Quantitative PCR

Two protocols were used to determine RNA expression in harvested tissues. In one, total RNA was extracted from 0.5 to 1 mg of tissue using the PrepEase RNA spin kit from USB. The iScript cDNA synthesis kit (Bio-Rad Laboratories, Hercules, CA)] was used to synthesize cDNA from 250 ng of RNA template using a mix of random hexamers and oligo

dTs. From each sample 1μl cDNA was used to amplify the genes of interest. QRT-PCR was performed on an ABI Prism 7000 (Applied biosystems-CA) using Power Sybrgreen master mix. Each sample was run in triplicate and the CTs were averaged. The primers used for amplification are presented in Table 1.

In alternative protocols the Qiagen RNeasy Mini kit (PN 74104) or the Qiagen RNeasy Plus (Qiagen 74134) kit were utilized as recommended by the manufacturer. RNA was quantified using Qubit HS DNA (Thermofisher Q32851). cDNA was synthesized as above. Relative mRNA levels were determined by qRT-PCR using Assays–on–Demand TaqMan Gene Expression platform (FAM-MGB, ThermoFisher Scientific https://www.thermofisher.com) with a Quantstudio 6 Flex (Applied Biosystems): IL-6 (Mm00446190), Ccl2/Mcp-1 (Mm00441242), IFN-γ (Mm01168134), IL-10(Mm01288386), TNF (Mm00443258), TGF-β1 (Mm01337605), and Beta-2 microglobulin (Mm00437762). Values reported are those obtained after normalization to β2–microglobulin and analyzed by the comparative delta CT (Δ-Δ CT) method.

2.7 Slide preparation and staining of mouse placenta, decidua and fetal brain.

Isolated mouse mid-gestation (~day 16) tissues were fixed in 4% paraformaldehyde for 24 hours and sent to Reveal Biosciences (San Diego, California) for paraffin embedding, sectioning and staining with Hematoxylin and Eosin. Heat induced antigen retrieval was performed using the Leica Bond Epitope Retrieval system. Non-specific antibody binding was blocked using Novolink Protein Block (Leica, cat#RE7280-CE, lot#6071120). Tissue sections were incubated with an antibody to fibrinogen (Abcam, ab92572, lot# GR29760-10) at a concentration of 1:250 versus a no-antibody control. Endogenous peroxidase activity was prevented using Vector Labs BloxAll (cat#SP-6000, lot#ZF1007). Sections were then incubated with anti-rabbit Poly-HRP-IgG antibody (Leica Novolink Polymer, cat#RE7280-CE, lot#6071120). Staining was detected and visualized with Novocastra Bond Refine Polymer Detection and 3'3-diaminobenzidine (DAB; brown).

To visualize TLT-1 in mouse placental tissue, tissues were dissected out and fixed in 4% paraformaldehyde for 24 hours then incubated in increasing concentrations of sucrose (4% and 30%) before placing in OTC solution and frozen. Samples were then sectioned at 7mm and incubated with2-(4-Amidinophenyl)-6-indolecarbamidine dihydrochloride, 4′, 6-Diamidino-2-phenylindole dihydrochloride (DAPI, # D9542-SIGMA Aldrich), mouse anti-Cytokeratin 7 (Clone RCK105BD Pharmingen) followed by Rhodamine (TRITC) donkey anti mouse IgG (# 711-025-152-Jackson Immuno Research), and/or Rabbit anti TLT-1 Voldemort – polyclonal rabbit antibody developed by our laboratory[29] followed by Alexa 647 anti-rabbit secondary (Jackson Immunoresearch, West Grove, PA). Antibody incubations were for 1 hour in dark at room temperature. Slides were mounted in Fluoromount-G™ Mounting Medium (Invitrogen™ Cat # 00–4958-02). Confocal analysis was completed with a NIS-Elements Confocal inverted microscope (Nikon Melville, NY) equipped with 4X, 10X, 20X, 40X oil, 60X oil, 100X oil objectives.

2.8 Serum and Amniotic Fluid analysis

Cytokine analysis of serum and amniotic fluid was done by multiplex and ELISA. Cytokines measured by multiplex (BD Sciences mouse inflammation kit cat# 552364) included IL-6, IL10, IFN-γ, MCP-1, TNF and IL-12p70 and results were obtained according to manufacturer's instructions.

2.9 Flow cytometry

Single cell suspensions of maternal decidua were prepared by gently straining the tissue through a sterile 70 μm filter utilizing the plunger from a sterile syringe. Uterine samples were first cut into small pieces and incubated crude Collagenase (Sigma, Cat #SCR103) 2 mg/ml in Hanks Buffered Saline Solution for 20 minutes at 37° C before straining. Samples were washed in FACS buffer (PBS, 0.5% BSA, 0.5% Sodium Azide) and then incubated with mouse serum to block nonspecific staining. Samples were then incubated with a panel of antibodies including CD3-PEcy5 (clone 17A2 BD Sciences #555276) CD45PEcy5 (30-F11 (RUO), BD-Pharmingen #553082) or CD45 PERcpcy5.5 (104, eBioscience #45045482), CD4-FITC (RM4.4 BD Sciences #553055) or CD4-APC (RM4.5, BD #561091) Ly-6G/Ly-6C-FITC (GR1-RB6-8C5 #563127), CD14-PE (clone emC5-3, BD Science #564145) CD8-PE or APC (clone 53–6.7, BD Sciences #s 553032 or 553035) or CD41-PE (MWReg30 BD Sciences cat#558040S), Ter119-FITC (BD Sciences #557915) and MHC A^b −647 (M5/114/15/2, BD 562367) samples were run on a Acuri cytometer (BD Sciences) and analyzed with FlowJo (Version 8.8.7. or 9.9.6 Ashland, OR).

2.10 Statistical Analyses

The data was analyzed by unpaired two-tailed t-tests, one-way or two-way ANOVA and Bonferroni multiple comparisons. All analyses were done on GraphPad PRISM 9.0.0 for Windows and Mac OS. A p-value smaller than 0.05 was considered statistically significant for t-tests, one-way or two-way ANOVA and multiple comparisons.

3. Results:

3.1 Analysis of human samples suggests a potential role of TLT in human PTB

To understand if TLT-1 may play a role in preterm birth we measured sTLT-1 levels by gestational age from human cord blood samples. Fig. 1 suggests that plasma sTLT-1 increases with gestation and presents the possibility that TLT-1 may play a role in normal or abnormal gestation.[30]

3.2 Analysis of TLT-1 deficiency in maternal tissue response to LPS in mice

The exact source of sTLT-1 in human cord blood is unknown. However, to begin to examine this issue, immunohistochemical analysis of the mouse placenta (Supplemental Fig. 1) suggested that TLT-1 is related to trophoblast lining the intervillous space or cells (e.g., platelets) associated with that layer.

To begin to evaluate if TLT-1 plays a role in preterm birth we used the *treml1*$^{-/-}$ mouse model to compare birth at 12 or 16 hours after lipopolysaccharide (LPS) treatment with wild type mice. On average, unmanipulated *treml1*$^{-/-}$ mice have slightly larger litters than

wild type mice (Fig. 2A; 7.5 vs 6 pups) but this is not statistically significant (p=0.8). At sixteen hours after injection of PBS, no mice were delivered (data not shown). Sixteen hours after injection of LPS, 2 of 4 *treml1*$^{-/-}$ mothers delivered at least one pup (proportion or non-resorbed implantation sites delivered 0.6 and 1, Fig. 2B) while no WT mothers delivered. No mice delivered by 12 hours after LPS injection (data not shown). Histological examination of the placenta in response to LPS in *treml1*$^{-/-}$ pregnancies as opposed to wild type pregnancies is shown in Fig. 2C (top panels), where the absence of TLT-1 is associated with increased bleeding as potentially consistent with other studies,[26] and a tendency for increased deposition of fibrinogen as seen in Fig. 2C (bottom).

Because inflammation is a driver of poor birth outcomes, and because of the role played by maternal decidua in pregnancy success,[31,32] we next evaluated the decidua for the infiltration of lymphocyte and granulocytic population by flow cytometry. Our gating scheme is shown in Supplemental Fig. 2. While there were no significant differences in the overall size of the lymphocyte pool in this tissue (data not shown) there were proportionately less Gr1+ (p=0.008) and Gr1+/platelet conjugates (p=0.01) in the decidua of *treml1*$^{-/-}$ mice (Fig. 3A). Platelet or neutrophil conjugates with T cells was similar in WT and KO decidua (Supplemental Fig. 5) This may suggest trafficking to the myometrium, which would be consistent with an increase in preterm birth[33] or to the fetal membranes.[34,35] Alternatively, it may represent a delay in granulocyte arrival that would have been observed at a later time point. Because inflammation has been implicated as a risk factor in preterm birth, we evaluated RNA expression levels of inflammatory cytokines at 12 and 16 hours post exposure in the decidua Fig. 3B. We observed that in the decidua of KO mothers exposed to LPS, there was a relative decrease in IL-10 at 12 hours, suggesting a possible significant early deregulation of anti-inflammatory responses in the absence of TLT-1 as compared to that present in WT decidua. This difference however was no longer present by 16 hours, suggesting other mechanisms might be important.

Fetal membranes promote entry into the parturition pathway and dysregulation of this tissue drives premature rupture, labor, and preterm birth.[36] We thus examined the effect of early induction in both KO and wild type mouse fetal membranes (Fig. 4 A. and B.). We observed that between 12 and 16 hours post LPS exposure there was an increase in IL-6, IFN-γ and TNF which trended towards significance in IFN-γ and TNF. This suggests that the fetal membranes in *trem1*$^{-/-}$ mice may have a great capacity for an inflammatory response to LPS.

The placenta is anchored in the maternal decidua basalis and placental trophoblast is bathed in maternal blood. Therefore, this tissue could be a conduit for both infectious and inflammatory responses initiated in the uterus and the maternal systemic circulation. To understand if TLT1 plays a role in the inflammatory processes generated in response to intraperitoneal injection of LPS, we isolated this tissue at 12 hours post injection (Fig. 4C). Placental tissue showed higher IL-6 expression (p=0.0365) and higher expression of TNF-α (p=0.011) in KO as compared to WT pregnancies. This early difference resolved, however, by 24 hours post injection when the remaining fetal placental units had similar levels of expression of several pro-inflammatory cytokines (Supplemental Fig. 3).

In human studies, amniotic fluid inflammation and or the presence of inflammatory cytokines is a biomarker of PTB[37,38] and in mouse studies, injection of “alarmins” generate PTB.[39] We hypothesized that there would be a TLT-1- related response to LPS in amniotic fluid. We observed that soluble TLT-1 was increased in the amniotic fluid of pups whose mothers were injected with LPS as compared to pups of PBS- injected mothers (Fig. 5A). In addition, we observed that both IFN-γ and IL-6 were elevated in the amniotic fluid of *treml1*$^{-/-}$ pups whose mothers were exposed to LPS (Fig. 5B). However, the cytokines IL-10, MCP-1, TNF and IL-12p70 demonstrated no significant differences between the two groups (Fig. 5B).

Inflammatory mediators in the amniotic cavity can trigger signaling pathways which extend outward, but they can also be taken up by the developing fetus. We next examined the bodies of pups exposed to LPS via maternal injection. Firstly, because of the potential[40] but complex[41] role of TNF in dysregulation of the fetal brain, and because of data suggesting that amniotic inflammation rapidly generates a signal in this tissue,[40,42,43] we isolated fetal brain and measured RNA expression of TNF. We observed that exposure to LPS, as opposed to PBS, during pregnancy generated increased expression of TNF in the fetal brain of both WT and KO pups by 16 hours post injection (Fig. 6). However, the level of expression was not different in pups of either type. Because of the potential role of inflammatory cytokines IFN-γ[44,45] and IL-6[46,47] in shaping the developing immune system, we also examined the expression of these cytokines in the fetal liver, but we did not observe a difference between the pups of WT and KO pregnancies (Supplemental Fig. 4).

Discussion:

In this study we began an investigation of the potential role of TLT-1 in inflammation-induced adverse pregnancy outcomes. First, we used cord blood from samples of birth from 25 to 40 weeks of gestation and found that levels of soluble TLT-1 were significantly lower in preterm than term babies suggesting that TLT-1 may be a confounding factor in prematurity. We further found low levels of TLT-1 in the intervillous space. Traditionally, changes in the levels of plasma TLT-1 are associated with activated platelets post hemostasis and/or in modulation of inflammation. These results suggest that over the course of gestation, the presence of sTLT-1 and platelet activation by sTLT-1 may protect against neonatal bleeding and modulate the fetal experience of the increased inflammatory profile of the maternal fetal interface leading to parturition. The increase of sTLT-1 with gestational age may reflect the priming of the fetus for partition and maintenance of the maternal fetal homeostasis until birth.

We utilized a well-known mouse model of inflammation-induced preterm delivery in WT and our *treml1*$^{-/-}$ mice to delineate the pre-delivery physiology of the interface between mother and fetus, as this might point to novel interventions. Another principal finding was that in WT mothers, systemic injection of LPS in WT mothers led to increased sTLT-1 in the amniotic fluid, suggesting a potential regulatory role for this molecule. In fact, while we found that WT and KO mothers had similar systemic responses to LPS (Supplemental Fig. 2B), in KO pregnancies as compared to WT there was an accelerated maternal-to-fetal inflammatory signature that began with alteration of the maternal decidual immune cell

pool and decreased anti-inflammatory cytokines such as IL-10. This was accompanied by an increased expression of inflammatory cytokines, such as IL-6 and IFN-γ in the amniotic fluid of *treml1*$^{-/-}$ pups when compared to control pups, consistent with a role for TLT-1 in regulating inflammation. Here we found an inverse correlation between the presence of TLT-1 and IL-6 and surprisingly, γ-interferon levels in the amniotic fluid. While we have seen increased levels of IL-6 in various TLT-1 studies, this is the first association we have seen with IFN-γ. Increases in IL-6 may represent perturbation of the endothelium not protected by TLT-1 mediated platelet function. IFN−γ, however, represents a potentially new direction in TLT-1 regulated aspects of preterm birth.

In the same time frame as increased amniotic fluid cytokines, we observed a trend to increased inflammatory cytokines in the fetal membranes of LPS-exposed KO as compared to WT pregnancies. Inflammation in the fetal membranes is a driver of preterm birth,[38] and in animal models, direct injection of inflammatory molecules into the amniotic cavity leads to preterm birth,[39] It is interesting that deficiency in TLT-1 may uncover a maternal-to-fetal-to-maternal inflammatory feed forward loop that could augment inflammation-induced preterm birth. Our data (Fig. 2) does not argue against this hypothesis. Moreover, recent data raises speculation that the immune regulatory effects of TLT-1 may extend to adaptive immunity at the maternal-fetal interface.[48]

The finding that amniotic fluid demonstrated increased sTLT-1 levels after LPS treatment suggested that in addition to its activation- regulatory role,[49] TLT-1 may help to maintain vascular integrity in the face of inflammation. Histological analysis of the placenta showed a significant amount of extravascular red blood cells in the treml*1*$^{-/-}$ mice compared to controls. This is consistent with what has been seen in the lungs of *treml1*$^{-/-}$ mice. This bleeding may drive preterm birth if it is associated with thrombin generation which itself may drive uterine contractions. However, the biology of hemostasis at the maternal interface is complex.

Interestingly, despite these data, there was no clear pattern in fibrinogen deposition in the placenta, differing between pups of the same mother. In fact, there was a slight overall increase in samples from KO mice that received LPS. TLT-1 has been shown to bind fibrinogen and in our published and unpublished studies TLT-1 deficiencies have been associated with reduced fibrinogen deposition. It would be interesting to investigate the placenta in TLT-1/αIIbβ3 double null mice in which both of the fibrinogen binding receptors on platelets are absent. Based on our results we could speculate that systemic inflammatory stress could affect the fetal system in adverse ways that involve platelet activation. While maternal LPS exposure increased brain cytokines, such as TNF,[40,43,50,51] we were not able to detect differences in the pups of LPS-exposed WT versus LPS-exposed KO pregnancies. Further, we could not detect cytokine expression differences in fetal liver. However, we cannot rule out developmental differences which may be revealed by examination of surviving pups.

To our knowledge, this is the first study evaluating TLT-1 function the context of inflammation-induced preterm birth. The study highlights the potential interaction between bleeding, hemostasis, and inflammation in normal and abnormal pregnancy and underlines

the potential importance of platelets as well as immune and inflammatory cells. The definitive impact and relationship of both platelet and TLT-1 function in the context of PTB remains unclear. We however speculate that these studies suggest the potential to assay of the presence of sTLT-1 in the peripheral blood or in amniotic fluid of human pregnancies to utilize as a diagnostic tool to assess the risk of PTB. This may also shed light on the biology underlying normal and preterm parturition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

This work was supported by The March of Dimes Prematurity Research Initiative, the University of Vermont Department of Obstetrics, Gynecology and Reproductive Sciences NIH R01HL090933, NIH P30 GM118228 to the Vermont Center for Immunology and Infectious Disease, and the University of Puerto Rico and the Research Initiative for Scientific Enhancement (RISE) NIH 5R25GM061151-18.

The campus of the University of Vermont sits within a place of gathering and exchange, shaped by water and stewarded by ongoing generations of Indigenous peoples, in particular the Western Abenaki. The University of Puerto Rico sits within the ancestral homeland of the Jíbaro and Taíno, who have served as guardians of the land and water for thousands of years.

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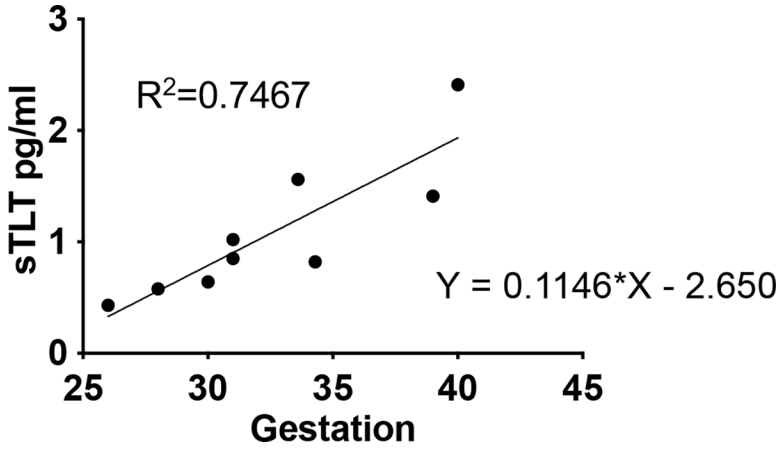

Fig 1. Soluble TLT in human cord blood by gestational age.

Concentrations present in cord blood at delivery were assayed by ELISA and analyzed by linear regression. A significant relationship between concentration and gestational age (p=0.0027) was observed.

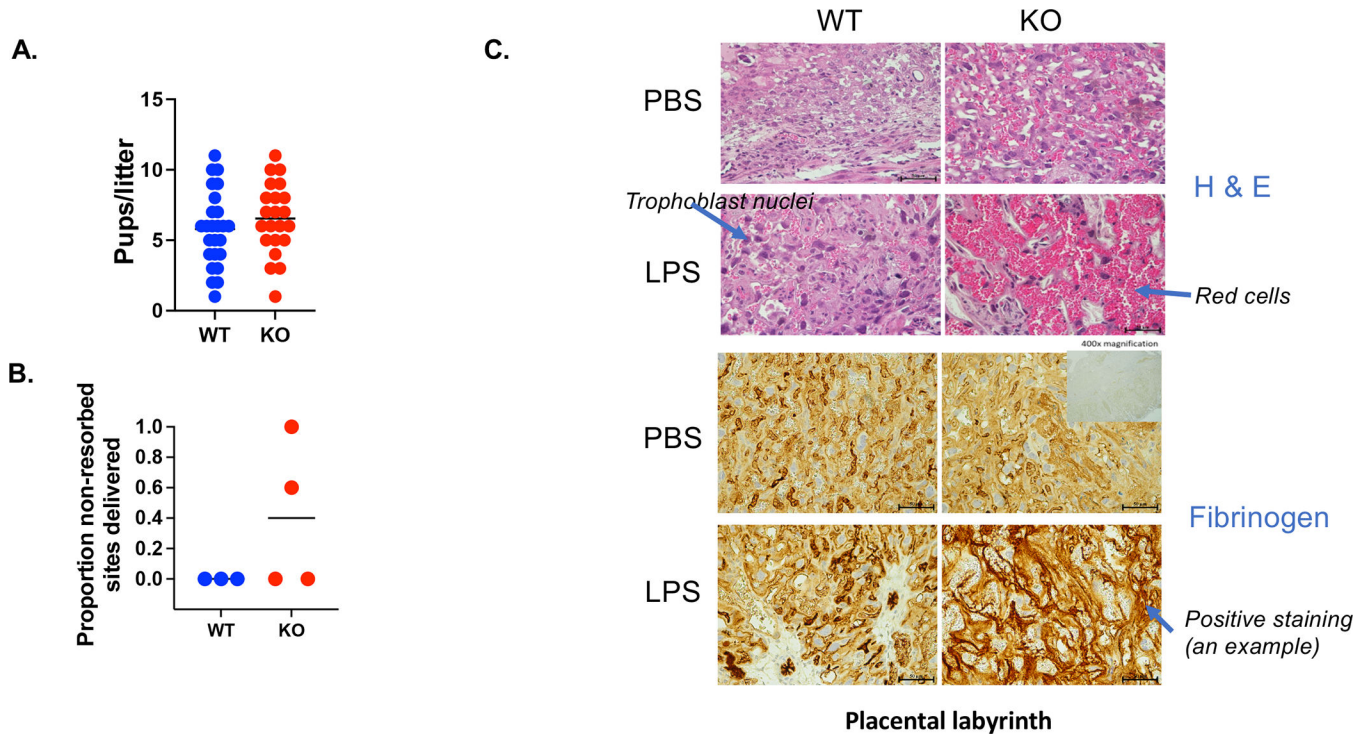

Fig. 2. Pregnancy outcome in WT and *treml1*$^{-/-}$ (KO) mice.

A. Pregnant mice were allowed to progress to delivery and numbers of pups per litter were not significantly different (t test p=0.8). **B.** Outcome sixteen hours after LPS exposure (not significant by Mann Whitney test). **C.** Representative histological examination of placenta/decidua sixteen hours after LPS exposure. Top panels: H and E stain, Bottom: Fibrinogen. Inset, lower- negative control. Arrows point to trophoblast nuclei, red cells and an example of positive staining.

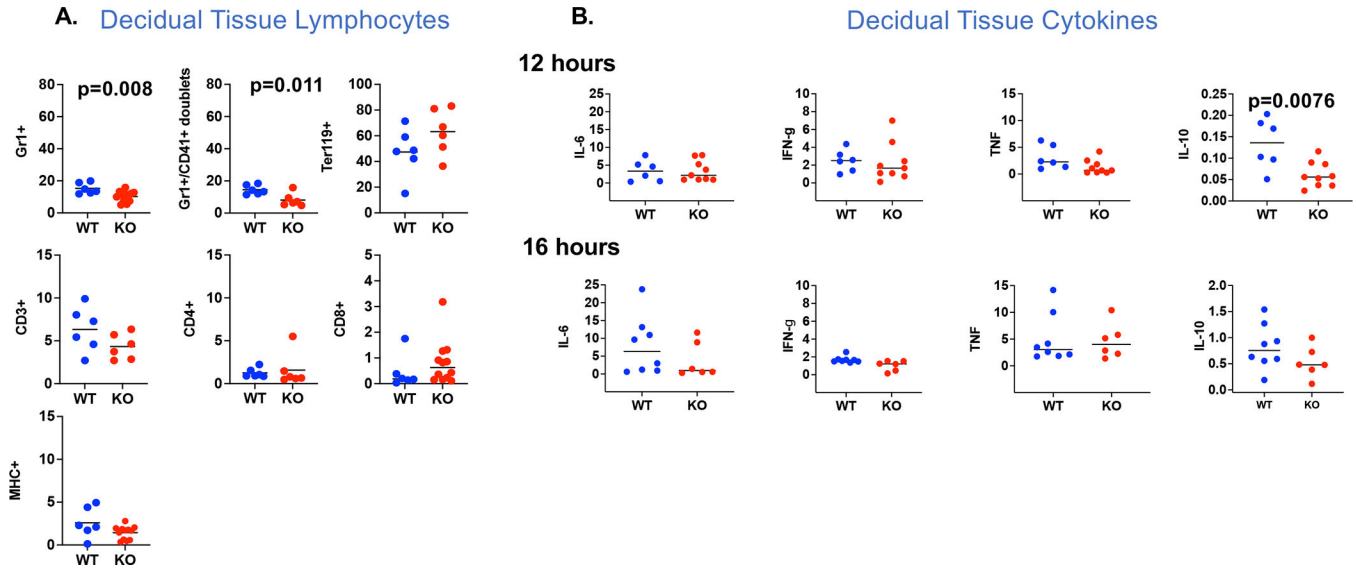

Fig. 3. Immune cell and cytokine profile of decidua from WT and KO pregnancies after exposure to LPS.

A. Decidua was removed 16 hours after LPS exposure and stained with antibodies for the specificities listed and examined by flow cytometry. Data is expressed as % of cells falling into the leukocyte gate. Gating scheme is as described in Supplemental Fig. 2. **B. Relative cytokine RNA expression of maternal and fetal tissues of WT and KO pregnancies.** Differences in relative cytokine expression in the decidua at 12 hrs vs 16 hrs were analyzed by t test or Mann Whitney test.

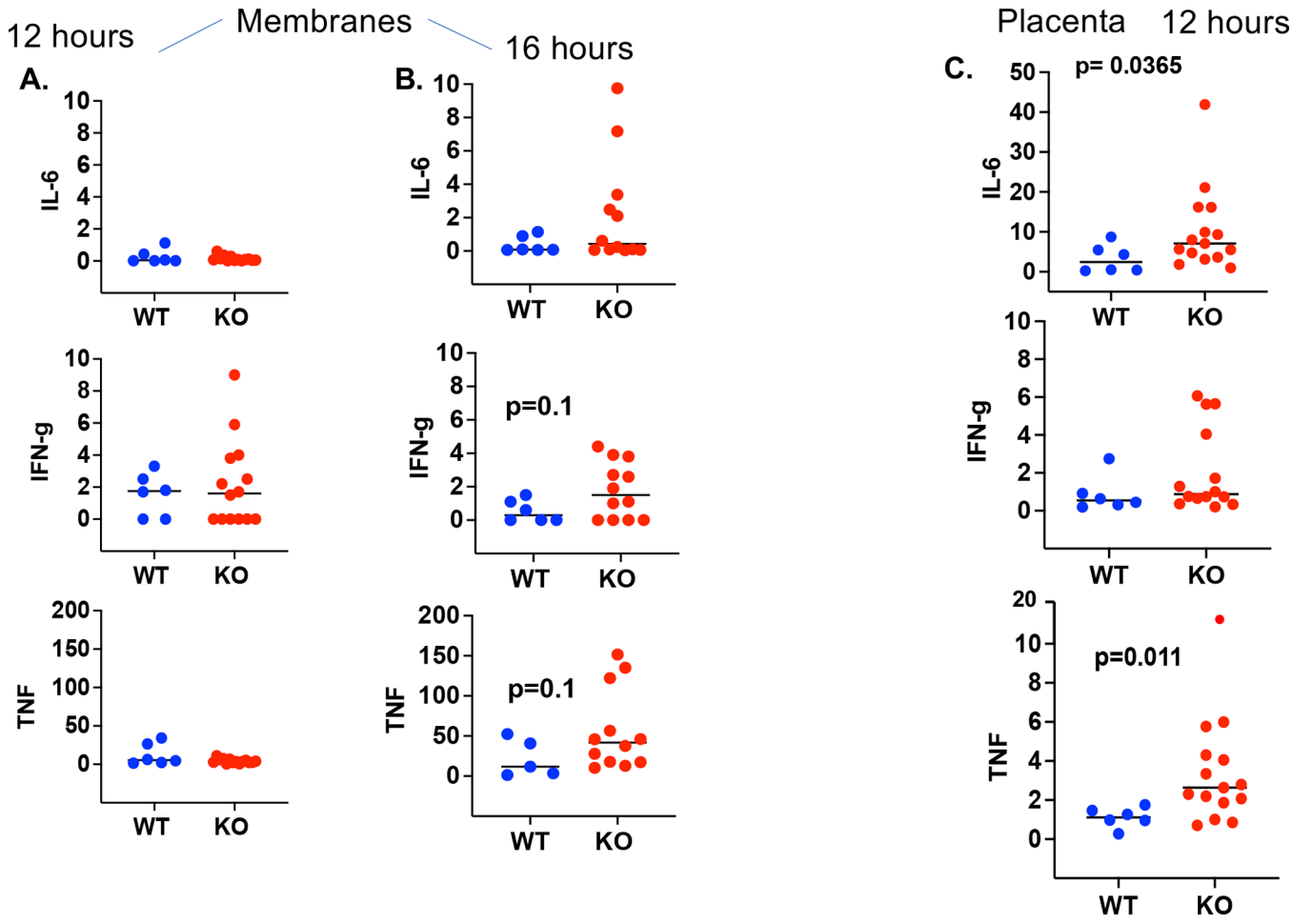

Fig. 4. Relative cytokine RNA expression of fetal "border" tissues of WT and KO pregnancies

A and B. Fetal membrane cytokines 12 and 16 hours after exposure to LPS. **C.** Placental cytokines 12 hours after LPS exposure. Analysis by t test or Mann Whitney test with significance or trend as noted.

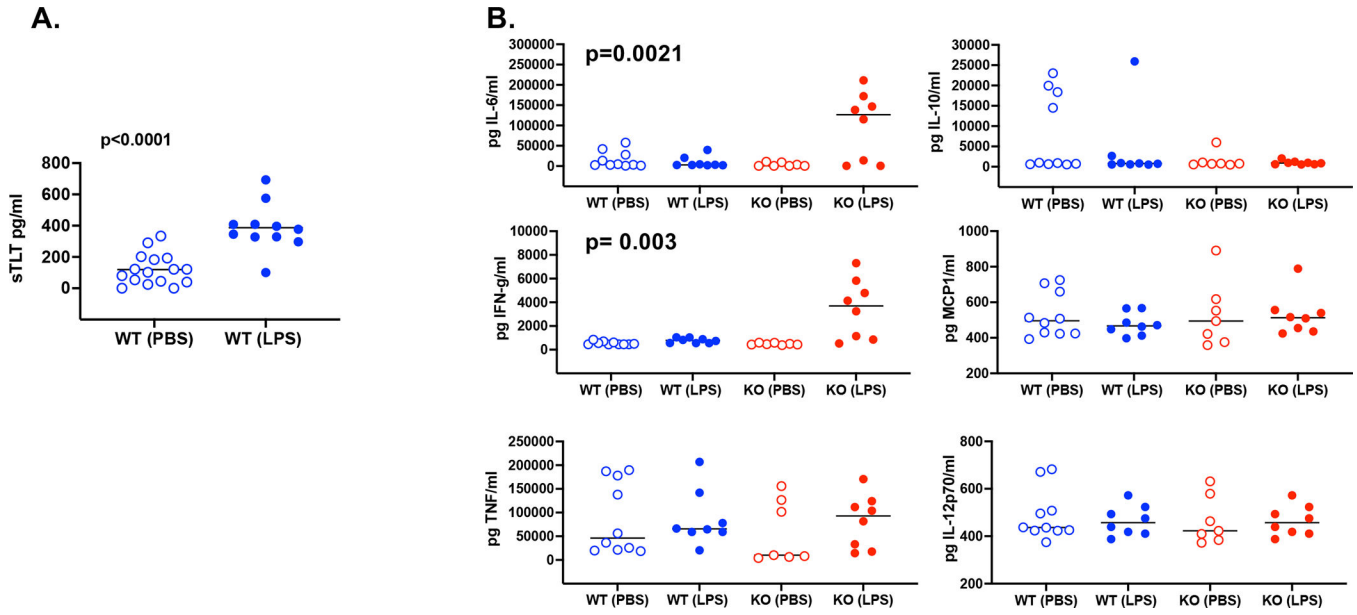

Fig. 5. Amniotic fluid response 16 hours after response to LPS in WT and KO pups.

A. Soluble TLT-1 in WT amniotic fluid in response to LPS (closed circles) versus PBS (open circles); significant difference by Mann Whitney test. **B.** Pro-inflammatory cytokines are elevated in amniotic fluid from KO mice. Amniotic fluid was collected from pups of at least three mothers per type and per exposure. Analysis by 2-way ANOVA suggested an effect of exposure (p=0.0058) and genotype (p=0.0148) with significant interaction (p=0.0021) in IL-6 response as was true for IFN-g (exposure p=0.008, genotype p=0.0048, interaction 0.003). Other cytokine responses were not significantly different.

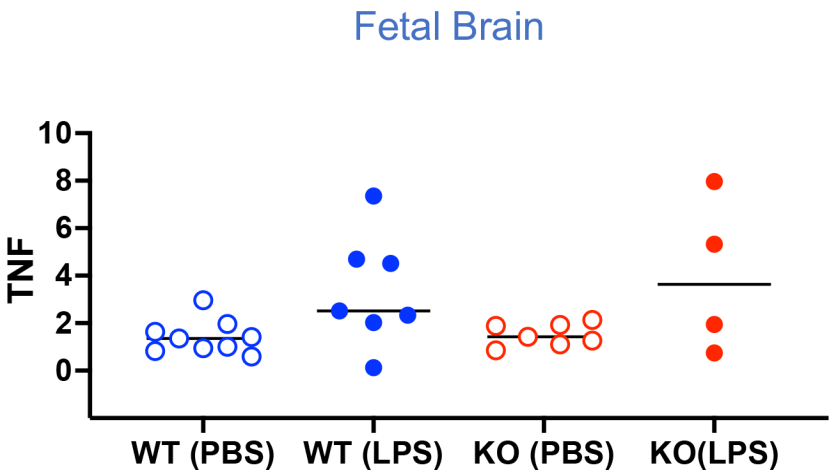

Fig. 6. RNA expression of Fetal brain TNF 16 hours after LPS exposure in WT and KO pregnancies.

Tissues were collected and analyzed from mothers who were exposed to LPS (closed circles) and those who were only given PBS (open circles). Analysis by 2-way ANOVA suggested an effect of exposure (p=0.0046) but not of genotype (p=0.6) with no significant interaction (p=0.7).

Table 1.

Primer Sequences used in these studies

Gene	Name	Forward Primer	Reverse Primer
β2m	Beta-2 microglobulin	ATGCTATCCAGAAAACCCCTCAAA	CAGTTCAGTATGTTCGGCTTCCC
Tgf-β	Transforming growth factor beta	CGCAACAACGCCATCTATGAG	TGCTCCACACTTGATTTTAATCTCTGC
Ifng	Interferon gamma	CCTCATGGCTGTTTCTGGCTGTTA	CATTGAATGCTTGGCGCTGGACC
Ifngr	Interrferon gamma receptor	CAGGTAAAGGTGTATTCGGGTTCC	CCAGGCAGATACATCAGGATACATAAT
IL10	Interleukin-10	TTACTGACTGGCATGAGGATCA	GAAAGAAAGTCTTCACCTGGCTGA
IL17	Interleukin-17	GACTCTCCACCGCAATGAAGACC	CCCACACCCACCAGCATCT
IL6	Interleukin-6	AGAAAGACAAAGCCAGAGTCCTTCAG	GTCCTTAGCCACTCCTTCTGTGACT
Ifnb	Interferon beta	TGTCCTCAACTGCTCTCCAC	CCTGCAACCACCACTCATTC
Mcp1	Monocyte chemoattractant protein-1	TGATCCCAATGAGTAGGCTGGAG	TCCGCATAGGTGGTAACTTGT
IL23a	Interleukin 23 alpha	GACCCACAAGGACTCAAGGACAAC	TGGCTGGAGGAGTTGGCTGA
Cxcl1/KC	Chemokine (C-X-C) ligand 1	CATGGCTGGGATTCACCTCA	TTACTTGGGGACACCTTTTAGCAT
Gmcsf/Csf2	Granulocyte- Macrophage Colony Stimulating Factor	CGTTGAATGAAGAGGTAGAAGTCG	TCCGCATAGGTGGTAACTTGT
IL1	Interleukin-1	TGCCACCTTTTGACAGTGATG	TGATGTGCTGCTGCGAGATT