HHS Public Access

Author manuscript

Hum Immunol. Author manuscript; available in PMC 2024 September 01.

Published in final edited form as:

Hum Immunol. 2023 September ; 84(9): 450–463. doi:10.1016/j.humimm.2023.06.005.

Thymic Stromal Lymphopoietin Participates in the Host Response to Intra-Amniotic Inflammation Leading to Preterm Labor and Birth

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Author contributions

NGL conceived and designed the study. TK, LT, RR, YX, JG, DM, MAH, ZL, DL, JMG, JP, and JP performed experiments and/or analyzed data, and provided intellectual input. KRT provided intellectual input and biological materials. NGL, TK, LT, RR, JG, and DM wrote and revised the manuscript. All authors approved the final version of the manuscript.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

The study was conducted at the Perinatology Research Branch, NICHD/NIH/DHHS, in Detroit, Michigan; the Branch has since been renamed as the Pregnancy Research Branch, NICHD/NIH/DHHS

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abstract

The aim of this study was to establish the role of thymic stromal lymphopoietin (TSLP) in the intra-amniotic host response of women with spontaneous preterm labor (sPTL) and birth. Amniotic fluid and chorioamniotic membranes (CAM) were collected from women with sPTL who delivered at term (n=30) or preterm without intra-amniotic inflammation (n=34), with sterile intra-amniotic inflammation (SIAI, n=27), or with intra-amniotic infection (IAI, n=17). Amnion epithelial cells (AEC), *Ureaplasma parvum*, and *Sneathia* spp. were also utilized. The expression of TSLP, TSLPR, and IL-7Rα was evaluated in amniotic fluid or CAM by RT-qPCR and/or immunoassays. AEC co-cultured with *Ureaplasma parvum* or *Sneathia* spp. were evaluated for TSLP expression by immunofluorescence and/or RT-qPCR. Our data show that TSLP was elevated in amniotic fluid of women with SIAI or IAI and expressed by the CAM. TSLPR and IL-7Rα had detectable gene and protein expression in the CAM; yet, *CRLF2* was specifically elevated with IAI. While TSLP localized to all layers of the CAM and increased with SIAI or IAI, TSLPR and IL-7Rα were minimal and became most apparent with IAI. Co-culture experiments indicated that *Ureaplasma parvum* and *Sneathia* spp. differentially upregulated *TSLP* expression in AEC. Together, these findings indicate that TSLP is a central component of the intra-amniotic host response during sPTL.

Keywords

amniotic fluid; chorioamniotic membranes; *Sneathia* ; TSLP; *Ureaplasma*

1. INTRODUCTION

Preterm birth, defined as delivery before 37 weeks of gestation, is the leading cause of perinatal morbidity and mortality worldwide [1, 2]. The global burden of preterm birth is increasing, with its rates rising in most countries including the United States [1, 2]. In 2021, the preterm birth rate in the United States rose to 10.48%, the highest since 2007 [3], with an associated annual healthcare cost of $26.2 billion dollars [4]. Nearly two-thirds of all preterm births are preceded by spontaneous preterm labor [5, 6], a syndrome of multiple pathological processes [6]. Among the putative causes, intra-amniotic inflammation is the most well-established link to preterm birth [5–8]. Such intra-amniotic inflammation can occur in two distinct contexts: the first occurs as the result of microbial invasion of the amniotic cavity, referred to as intra-amniotic infection (IAI), while the second occurs in the absence of microbes and is linked to elevated concentrations of endogenous danger signals or alarmins, a condition known as sterile intra-amniotic inflammation (SIAI) [5–12]. The most frequently identified bacteria in amniotic fluid of women with IAI are *Ureaplasma* spp., with *Ureaplasma parvum* being the most commonly identified species [10, 13–17]. Importantly, recent evidence suggests that a subset of women experiences intra-amniotic

invasion by bacteria that are conventionally difficult to cultivate and detect, such as *Sneathia* spp. [9, 15, 18]. Indeed, reports have indicated that *Sneathia* are one of the most frequently detected bacterial taxa in the amniotic cavity of women with spontaneous preterm birth [9, 17, 19–24]. While the evidence supports *Sneathia* as a notable emerging pathogen associated with obstetrical disease [9, 11, 15, 17–20, 23–27], mechanistic studies exploring the host response triggered by these bacteria are lacking.

The host intra-amniotic inflammatory response triggered by bacteria involves the infiltration of leukocytes into the amniotic cavity [28–39] together with the enhanced release of inflammatory and anti-microbial mediators [40–52]. Thymic stromal lymphopoietin (TSLP) is a known pro-inflammatory cytokine that is rapidly released and perpetuates inflammation [53–57]. TSLP activates JAK1 and JAK2 via its heterodimeric receptor, leading to the activation of STAT5A and STAT5B and subsequently the downstream production of multiple cytokines [58, 59]. Indeed, TSLP has been found to be a critical mediator of the immune response in disease states such as asthma, atopic dermatitis, and cancer [60–62]. Moreover, recent studies have shown that TSLP also participates in functions unrelated to inflammation such as cell maturation, proliferation, survival, and recruitment [60, 63–65], thereby placing TSLP as an important cytokine of interest in the pathological host response. To date, TSLP has largely been investigated outside of reproductive health, although recent studies have begun investigating TSLP in the context of pregnancy maintenance [66–69], endometriosis [70, 71], and cervical cancer [72, 73]. However, the role of TSLP in the intra-amniotic host response is unknown.

In this study, we aimed to establish a role for TSLP in the intra-amniotic host response observed in women with spontaneous preterm labor and birth. First, we investigated whether TSLP concentrations are increased in amniotic fluid of women with spontaneous preterm labor and SIAI or IAI who delivered preterm. Next, we determined whether the expression of TSLP and its receptors, TSLPR and IL-7Rα, was upregulated in the chorioamniotic membranes of women with spontaneous preterm labor and SIAI or IAI, in addition to exploring their localization. Moreover, we explored whether TSLP was expressed by the amniotic fluid neutrophils of women with IAI. Lastly, we sought to elucidate whether the expression of TSLP was upregulated in amnion epithelial cells by *in vitro* inoculation of *Ureaplasma parvum* and *Sneathia* species.

2. METHODS

2.1 Human subjects and clinical specimens

This study included the amniotic fluid samples collected from the following patients: (1) women with spontaneous preterm labor with intact membranes who delivered at term without intra-amniotic inflammation (see Clinical definitions below) (n = 30); (2) women with spontaneous preterm labor who delivered preterm without intra-amniotic inflammation (No SIAI/IAI) (n = 34); (3) women with spontaneous preterm labor who delivered preterm with sterile intra-amniotic inflammation (SIAI) (n = 27); and (4) women with spontaneous preterm labor who delivered preterm with intra-amniotic infection (IAI) (n = 17). Chorioamniotic membrane (CAM) samples from women in each of the study groups were also studied using qRT-PCR for mRNA expression and immunohistochemistry

staining or western blot analysis for protein expression. Human amniotic fluid and CAM samples were retrieved from the Biorepository of the Perinatology Research Branch, an intramural program of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services, Wayne State University (Detroit, MI), and the Detroit Medical Center (Detroit, MI). The collection and use of human materials for research purposes were approved by the Institutional Review Board of Wayne State University. All participating women provided written informed consent prior to sample collection. The clinical and demographic characteristics of the study population are shown in Supplementary Table 1.

2.2 Amniotic fluid sample collection

Amniotic fluid was retrieved by transabdominal amniocentesis under antiseptic conditions using a 22-gauge needle monitored by ultrasound. All amniotic fluid samples were obtained from women undergoing spontaneous preterm labor to detect intra-amniotic inflammation/infection. Amniotic fluid samples were transported to the clinical laboratory in a capped sterile syringe. Samples were centrifuged at 1 300 x g for 10 minutes (min) at 4°C, and the supernatant was stored at −80°C until use. Additionally, an aliquot of amniotic fluid was transported to the clinical laboratory for culture of aerobic/anaerobic bacteria and genital mycoplasmas. The clinical and research tests also included the determination of amniotic fluid white blood cell (WBC) count and measurement of glucose and interleukin (IL)-6 concentrations.

2.3 Research determination of interleukin-6 concentrations in amniotic fluid

IL-6 concentrations in amniotic fluid samples were measured with a sensitive and specific enzyme immunoassay from R&D Systems (Minneapolis, MN, USA), as previously established [45]. The IL-6 concentrations were determined by interpolation from the standard curves. The inter- and intra-assay coefficients of variation for IL-6 were 8.7% and 4.6%, respectively. The detection limit of the assay was 0.09 pg/mL. A cut-off of 2.6 ng/mL was used to determine intra-amniotic inflammation, as previously established [45].

2.4 Clinical definitions and placental histopathological examination

Gestational age was determined by the last menstrual period and confirmed by ultrasound examination, or by ultrasound examination alone when the sonographic determination of gestational age was inconsistent with menstrual dating. Spontaneous preterm labor was defined as the presence of regular uterine contractions with a frequency of at least two every 10 min and cervical changes between 20 and 36 (6/7) weeks of gestation.

The intra-amniotic inflammatory status of patients was determined by combining the presence or absence of microbes, as determined by microbiological culture and/or molecular test [polymerase chain reaction with electrospray ionization mass spectrometry (PCR/ESI-MS) (Ibis® Technology Athogen, Carlsbad, CA, USA)], together with the evaluation of amniotic fluid IL-6 concentrations [9–12]. A positive microbial signal (either by culture or molecular tests) together with an elevated IL-6 concentration was considered as IAI [9–12], whereas a negative microbial signal (indicated by both culture and molecular tests) together with an elevated IL-6 concentration was considered as SIAI [9–12]. A

negative microbial signal (indicated by both culture and molecular tests), together with a low IL-6 concentration, was considered as confirming the absence of both intra-amniotic inflammation and microbial infection (No SIAI/IAI).

Histopathological examination of the placenta was performed by perinatal pathologists blinded to clinical diagnoses and obstetrical outcomes according to standardized Perinatology Research Branch protocols. Acute inflammatory lesions of the placenta (maternal inflammatory response and fetal inflammatory response) were diagnosed according to established criteria, including staging and grading [74, 75].

2.5 Determination of TSLP concentrations in amniotic fluid

Amniotic fluid samples were assessed for TSLP concentrations. A U-PLEX immunoassay (Meso Scale Discovery, Rockville, MD, USA) was utilized to measure the TSLP concentration in the amniotic fluid samples, according to the manufacturer's instructions. Plates were read using the MESO QuickPlex SQ 120 (Meso Scale Discovery) and analyte concentrations were calculated with the Discovery Workbench 4.0 (Meso Scale Discovery). The sensitivity of the TSLP assay was 0.20 pg/mL.

2.6 Immunoblotting analysis

Tissue lysates of CAM (n = 3 per group) were prepared by mechanically homogenizing snap-frozen tissues in 1X PBS containing a complete protease inhibitor cocktail (Cat# 11836170001; Roche Applied Sciences, Mannheim, Germany). Lysates were centrifuged at 15 700 x g for 5 min at 4°C and the supernatants were stored at −80°C until use. Total protein concentrations of tissue lysates were determined using the Pierce BCA Protein Assay Kit (Cat# 23225; Pierce Biotechnology, Thermo Fisher Scientific, Inc., Rockford, IL, USA) prior to immunoblotting.

Tissue lysates (50 μg) of CAM were subjected to electrophoresis in 4%–12% sodium dodecyl sulphate-polyacrylamide gels (Cat# NP0336BOX; Invitrogen by Thermo Fisher Scientific). Separated proteins were then transferred onto nitrocellulose membranes (Cat# 1620145; Bio-Rad, Hercules, CA, USA). Next, the nitrocellulose membranes were blocked with 5% non-fat dry milk (Cat# 1706404; Blotting-Grade Blocker, Bio-rad) with 1 X Tris-buffered saline containing 0.05% Tween 20 (TBST) for 30 min at room temperature and then probed overnight at 4°C with the following antibodies: anti-human TSLP (Cat# NB110-55234; Novus Biologicals, Littleton, CO, USA), anti-human TSLPR (Cat# PA5-20378; Invitrogen), or anti-human IL-7Rα (Cat# PA5-119230; Invitrogen). Finally, the nitrocellulose membranes were washed with TBST, blocked, and re-probed for 1 h at room temperature with a mouse anti-β-actin (ACTB) monoclonal antibody (Cat# A5441; Sigma-Aldrich). After incubation with each primary antibody, the membranes were incubated with HRP-conjugated anti-mouse IgG (Cat# 7076S; Cell Signaling, Danvers, MA, USA) or HRP-conjugated anti-rabbit IgG (Cat# 7074S; Cell Signaling) for 1 h at room temperature. Chemiluminescence signals were detected with the ChemiGlow West Chemiluminescence Substrate Kit (Cat# 60-12596-00; ProteinSimple, San Jose, CA, USA) and images were acquired using the ChemiDoc Imaging System (Bio-Rad). The specificity of the TSLP signal was confirmed with a blocking peptide against the TSLP antibody. Quantification was

performed with ImageJ software [76]. Each individual protein band on the blot image was automatically quantified by the software. The target protein expression in each individual sample of the fetal membranes was normalized using the internal control, β-actin, in the same sample to obtain relative quantification.

2.7 Immunohistochemistry

Formalin-fixed paraffin-embedded CAM (n = 5 per group) tissues were cut in 5-μm-thick sections. Slides were deparaffinized in xylene and hydrated with decreasing concentrations of ethanol. Immunohistochemistry staining for TSLP, TSLPR, and IL-7Rα was performed using the Leica Bond Max automatic staining system (Leica Microsystems; Wetzlar, Germany). The Bond™ Polymer Refine Detection Kit (Leica Microsystems) was used to detect the chromogenic reaction of horseradish peroxidase upon oxidation of 3'3-Diaminodenzidine (DAB). Isotypes were used as negative controls. Brightfield images were taken using the Vectra Polaris Multispectral Imaging System and inForm software version 2.5.1. Representative images were taken at 200X magnification.

2.8 Primary cell culture of amnion epithelial cells

Immediately after collection of the CAM from women without labor at term (n = 3), the amnion membrane was manually peeled from the underlying chorion layer of the chorioamniotic membranes and dissected into small pieces. The amnion fragments were rinsed in 0.05% (w/v) trypsin/EDTA (Life Technologies) and incubated in 25 mL of fresh trypsin/EDTA at 37°C with gentle shaking for 10 min. The trypsin digestion supernatant was discarded, and the amnion fragments were placed into fresh trypsin/EDTA solution. Amnion epithelial cells (AEC) were obtained by digesting at 37°C for 40 min with gentle shaking. The total digestion/incubation process was repeated twice. Fetal bovine serum (FBS; Thermo Fisher Scientific) was added to the supernatant to stop digestion between each incubation period. Finally, digested tissues were filtered through a 100 μm cell strainer (Fisher Scientific, Durham, NC, USA). The resulting cell suspensions were centrifuged at 300 x g for 10 min and the cells were cultured in DMEM (Life Technologies) containing 10% FBS and 100 U/mL penicillin and streptomycin (Thermo Fisher Scientific) at 37°C with 5% CO_2. The absence of mycoplasma contamination in AEC cultures was confirmed by qPCR using the MycoSensor QPCR Assay Kit (Cat# 302107, Agilent Technologies, Santa Clara, CA, USA) and the Mycoplasma Detection Kit (Cat# rep-mys-20; MycoStrip™; InvivoGen, San Diego, CA, USA) according to the manufacturer's instructions. Cells between passages two – five were used for incubation with *Ureaplasma* isolates.

2.9 Preparation of *Ureaplasma parvum* isolates for *in vitro* experiments

Ureaplasma parvum isolates from −80°C stock were cultivated in SP4 broth as previously described [77] for co-culture experiments. The culture media was collected and centrifuged at 3 000 x g for 30 min at room temperature. The resulting *Ureaplasma parvum* pellet was resuspended in 1 mL of SP4 broth and kept on ice. The *Ureaplasma parvum* cell count in the inoculation suspension was measured using flow cytometry, following a modified version of a previously described method for counting mycoplasmas using a bacteria counting kit (Cat# L34856, LIVE/DEAD *Bac*Light Bacterial Viability and Counting Kit for flow cytometry; Invitrogen), the BD LSR II flow cytometer (BD Bioscience), and BD FACSDiva

6.0 software (BD Bioscience). The absolute number of cells was determined using the counting beads included in the kit. The *Ureaplasma parvum* suspension was diluted in SP4 broth according to the obtained count. SP4 broth was used as the vehicle control treatment in all *Ureaplasma parvum* experiments.

2.10 Preparation of *Sneathia* for *in vitro* experiments

Frozen stocks of *Sneathia vaginalis* (type strain CCUG 51846) and *Sneathia sanguinegens* (type strain CCUG 41628T) were utilized for *Sneathia* co-culture experiments. *Sneathia* stock cultures were kept on dry ice and transferred to the anaerobic culture chamber (5% CO_2, 10% H_2, 85% N_2) for recovery of isolates in Brain Heart Infusion broth supplemented with human serum (10%), yeast extract (1%), glycogen (0.1%), hemin (0.01%), and Vitamin K1 (0.01%). Supplemented BHI broth (hereafter referred to as sBHI broth) was given 24 h to acclimate to the anaerobic chamber prior to inoculation, and non-inoculated sBHI broth samples were included as negative controls. Inoculated broth was incubated for 48 h at 37 °C before use in the experiments. The *Sneathia* cell count in the inoculation suspension was measured using flow cytometry, following a modified version of the method described above for counting *Ureaplasma parvum*. Briefly, an aliquot of the inoculation suspension (10 μL) was diluted 100X in 1X PBS and then stained with SYTO9 (final concentration of 10 μM) for 15 min at room temperature in the dark. The stained *Sneathia* were counted using the BD Fortessa flow cytometer (BD Bioscience) and BD FACSDiva 9.0 software (BD Bioscience). The absolute number of cells was determined using counting beads included in the kit. The *Sneathia* suspension was centrifuged at 10 000 x g for 2 min and subsequently diluted in sBHI broth according to the obtained count. sBHI broth was used as the vehicle control treatment in all *Sneathia* experiments.

2.11 *In vitro* co-culture experiments

Primary amnion epithelial cells or an amnion epithelial cell line (Cat# T0531, Applied Biological Materials Inc, Richmond, BC, Canada) were cultured in 6-well tissue culture plates (Corning, Inc., Corning, NY, USA) at a density of 1.5 x 10^5 cells/well or 1 x 10^6 cells/well at 37°C with 5% CO_2. The cells were incubated with *Ureaplasma parvum* (1.3 x 10^5 cells/well) or *Sneathia* spp. (1.0 x10^6 cells/well). Serum-free Opti-MEM (Life Technologies), SP4 broth, or sBHI broth with serum-free Opti-MEM were used as negative controls. After 24 h of treatment, cells were collected for RNA isolation.

2.12 RNA isolation, cDNA synthesis, and reverse transcription quantitative polymerase chain reaction analysis

Total RNA was isolated from the CAM (n = 6 per group), amnion (n = 6 per group), primary AEC, or the AEC line using RNase-Free DNase Sets and RNeasy Mini Kits (Qiagen), according to the manufacturer's instructions. RNA concentrations and integrity were assessed using NanoDrop 1000 (Thermo Scientific, Wilmington, DE, USA) and Bioanalyzer 2100 (Agilent Technologies, Wilmington, DE, USA). Complementary (c)DNA was synthesized using SuperScript IV VILO master mix (Thermo Fisher Scientific). Gene expression profiling was performed on the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies) for *TSLP*, *CRLF2* and *IL7R*, while *GAPDH* was used as internal control.

2.13 Immunofluorescence

Cytospin slides of human amniotic fluid samples from women with IAI were prepared using Fisherbrand Superfrost microscope slides (Thermo Fisher Scientific) and a Shandon Cytospin 3 cytocentrifuge (Thermo Fisher Scientific) at 800 rpm for 5 min. After rinsing with PBS, the slides were fixed with 4% paraformaldehyde (Electron Microscopy Sciences Hatfield, PA, USA) for 20 min at room temperature. AEC were cultured in four-well Lab-Tek chamber slides (Thermo Fisher Scientific) at 1 x 10^4 cells/well. After 24 h of treatment, the cells were fixed using 4% paraformaldehyde. For immunofluorescence of TSLP, cells were permeabilized using 0.25% Triton X-100 (EMD Millipore, Billerica, MA, USA) for 5 min at room temperature. Non-specific antibody interactions were blocked using antibody diluent/block (Cat# ARD1001EA; PerkinElmer, Boston, MA, USA) for 30 min at room temperature. Cells were then stained with a mouse anti-human TSLP antibody at room temperature for 1 h. Mouse IgG (Thermo Fisher Scientific) was used as a negative control. Following staining, cells were washed with 1X PBS containing 0.1% Tween 20 (PBST). After the wash, cells were incubated with secondary goat anti-mouse IgG–Alexa Fluor 594 (Cat# A11032, Life Technologies) for 1 h at room temperature in the dark. Finally, cells were washed with PBST and mounted using ProLong Diamond Antifade Mountant with DAPI (Life Technologies). Immunofluorescence was visualized using a KEYENCE BA-X800 Microscope (KEYENCE, Itasca, IL, USA).

2.14 Statistical analysis

Statistical analyses were performed using Prism v9 (GraphPad, San Diego, CA, USA). For patient demographics information, the Kruskal-Wallis test was used for continuous variables and the Fisher's exact test was used for non-continuous variables. TSLP concentrations were compared using the Kruskal-Wallis test followed by Dunn's post-hoc test. Gene expression in the CAM and co-culture experiments was compared using Mann-Whitney U-tests. For qPCR analysis in the CAM and AEC, relative fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method and shown as $\log_2$(fold change). A p-value < 0.05 was considered statistically significant.

3. RESULTS

3.1 Amniotic fluid concentrations of TSLP are disrupted by the intra-amniotic inflammatory status in women with spontaneous preterm labor and birth.

We first evaluated the concentrations of TSLP in the amniotic fluid of women who underwent spontaneous preterm labor with SIAI or IAI (Figure 1A and Supplementary Table 1). Amniotic fluid samples from women who underwent spontaneous preterm labor and delivered at term or delivered preterm without SIAI or IAI were included as controls (Supplementary Table 1). Notably, women who underwent spontaneous preterm labor with SIAI or IAI had elevated concentrations of TSLP compared to those without inflammation or those who delivered at term; yet, there was no difference in TSLP concentrations between the SIAI and IAI groups (Figure 1B). These data indicate that TSLP is elevated in the amniotic cavity of women who undergo spontaneous preterm labor and birth with intra-amniotic inflammation, regardless of the nature of the stimuli.

3.2 TSLP and its receptors are expressed by the chorioamniotic membranes and modulated by the intra-amniotic inflammatory status

We then sought to determine whether TSLP signaling was targeted towards the chorioamniotic membrane (CAM). TSLP signaling is initiated by the establishment of a complex between this protein and its receptor, TSLPR, as well as the IL-7 receptor-α (IL-7Rα) [78–80]. Therefore, we evaluated the gene and protein expression of TSLP and its co-receptors in CAM samples from each of our study groups (Figure 2A). The expression of *TSLP* by the CAM was reduced in women who underwent spontaneous preterm labor and birth with SIAI (Figure 2B); yet, immunoblotting revealed a tendency for increased protein levels of TSLP in the CAM of women with SIAI or IAI (Figure 2C). Given that intra-amniotic infection involves the infiltration of leukocytes into the amniotic cavity [28–34, 36–39], we also evaluated TSLP expression by amniotic fluid neutrophils and found that such cells express this protein (Figure 2D). Next, we examined the expression of TSLP receptors by the CAM and showed that the expression of *CRLF2* (gene encoding the TSLPR protein) was upregulated in women who underwent spontaneous preterm labor and birth with IAI compared to the other study groups (Figure 2E), which was not reflected by the protein levels of this receptor (Figure 2F). Furthermore, the expression of *IL7R* displayed no differences between study groups (Figure 2G); however, IL-7Rα protein levels were enhanced in the CAM of women who underwent spontaneous preterm labor and birth with IAI in comparison to the other study groups (Figure 2H). Together, these data suggest that the inflammatory status of the amniotic cavity can influence the expression of TSLP receptors by the CAM at the gene and protein level.

We then performed immunohistochemistry to localize TSLP and its receptors to the layers and cell types of the CAM (Figure 3). Consistent with our findings of gene and protein expression within whole tissue extracts, TSLP was consistently detected across all study groups and seemed to primarily localize to the chorion layer of the CAM, with some amnion and decidual cells also showing expression (Figure 3A). Importantly, the overall expression of TSLP seemed to increase depending on the intra-amniotic inflammatory status (Figure 3A). By contrast, TSLPR seemed to be predominantly found in the decidual layer of the CAM (decidua parietalis) (Figure 3A), suggesting that the sender and receiver cells of the TSLP signaling pathway are different. The expression of the IL-7Rα tended to increase with the severity of the intra-amniotic inflammatory response, being most highly detected in the SIAI and IAI study groups (Figure 3A). The latter finding indicates that the leukocytes infiltrating the CAM as part of the intra-amniotic inflammatory response may be the predominant IL-7Rα-expressing cells.

To further validate the expression of TSLP and its receptors within the different compartments of the CAM, we then separated the amnion layer to evaluate gene expression, which we compared to gene expression within the entire CAM (Figure 3B). *TSLP* and *IL7R* were detected within all CAM and amnion samples regardless of study group; yet, their expression was similar among groups (Figure 3B). Notably, *CRLF2* was consistently expressed by all amnion samples from women with IAI, whereas several amnion samples from the other study groups displayed *CRLF2* expression below the detection limit (Figure 3B).

Taken together, these data indicate that cells present in the CAM can express the receptors for TSLP, which may respond to protein present in the amniotic fluid or released by neighboring cells within the CAM itself.

3.3. TSLP is a component of the host response to *Ureaplasma parvum* and *Sneathia* spp.

Given that TSLP and IL-7Rα appeared to exhibit a modest dose-response relationship with the inflammatory status of the amniotic cavity (Figure 3A), we next explored whether the *in vitro* exposure to microbes commonly found in women with IAI could induce the expression of TSLP (Figures 4 & 5). We isolated amnion epithelial cells (AEC) from the CAM and performed co-culture experiments using two isolates of *Ureaplasma parvum* that were previously collected from the amniotic fluid of women who underwent spontaneous term or preterm labor with IAI [77] (Figure 4A). Amnion epithelial cells were chosen given their direct contact with the amniotic fluid, thereby positioning these cells as some of the first to participate in the host response to alarmins or microbes present in amniotic fluid of women with SIAI or IAI, respectively. Both *Ureaplasma parvum* isolates caused a dramatic upregulation of *TSLP* expression by AEC (>10-20-fold change), thereby supporting the involvement of this cytokine in the host response to *Ureaplasma parvum* present in the amniotic cavity (Figure 4B). Next, we performed co-culture experiments using the two known *Sneathia* species, *Sneathia vaginalis* (formerly known as *Sneathia amnii* [81]) and *Sneathia sanguinegens*, together with a human AEC cell line (Figure 5A). While both *Sneathia* spp. induced *TSLP* expression by the AEC compared to unstimulated controls, *Sneathia vaginalis* exerted greater upregulation compared to *Sneathia sanguinegens* (Figure 5B). Given the observed moderate increase in *TSLP* only with *Sneathia vaginalis* (two-fold change), we performed immunofluorescence imaging of AEC co-cultured with *Sneathia* spp. to confirm the results of gene expression and demonstrated protein expression of TSLP by such cells (Figure 5C). Together, these data support the involvement of TSLP in the host response to microbial invasion of the amniotic cavity.

4. DISCUSSION

Herein, we utilized a multifaceted approach to characterize the expression and role of TSLP and its receptors in the intra-amniotic host response associated with SIAI or IAI. First, we showed that amniotic fluid concentrations of TSLP are elevated in women who underwent spontaneous preterm labor and birth with SIAI or IAI. Next, we demonstrated that this cytokine is expressed by the CAM as well as by amniotic fluid neutrophils, providing two potential sources of TSLP in the amniotic cavity. Both of the known receptors for TSLP, TSLPR and IL-7Rα, had detectable gene and protein expression in the CAM; yet, *CRLF2* expression was specifically elevated in women with IAI. Immunohistochemistry revealed that TSLP was constitutively localized to the amnion, chorion, and decidua, where its expression increased with intra-amniotic inflammation. By contrast, the expression of TSLPR and IL-7Rα was minimal and became most apparent in the presence of IAI. Last, the *in vitro* co-culture of AEC with IAI-relevant bacteria demonstrated that *Ureaplasma parvum* and *Sneathia* spp. differentially upregulated *TSLP* expression in these cells. Together, these data provide insight into the participation of TSLP in the local host response to intra-amniotic infection.

The amniotic fluid comprises numerous cellular and soluble components as physiological constituents that provide baseline protection against potential microbial threats [82–89]. The cellular component comprises both innate (i.e., neutrophils, monocytes/macrophages, NK cells, and innate lymphoid cells) and adaptive (i.e., T cells and B cells) immune cells [33, 90], whereas the soluble component includes a diverse array of molecules, many of which display anti-microbial properties [82, 86, 91–105]. During intra-amniotic infection, the resulting local inflammatory response is characterized by the bolstering of neutrophil and monocyte/macrophage numbers to fight off invading microbes [28–34, 36–39] together with the enhanced release of inflammatory and anti-microbial mediators [40–52, 106]. Herein, we have provided novel evidence for the involvement of TSLP in the host response to microbes by demonstrating that the amniotic fluid concentrations of this cytokine are released in women who underwent spontaneous preterm labor with intra-amniotic infection. The participation of TSLP in anti-microbial responses is an area of ongoing research, with multiple studies reporting the induction of this cytokine by bacteria [107–113], fungi [114–122], viruses [123–135], and parasites [136–139]. Notably, a distinct mechanism whereby TSLP drives the killing of methicillin-resistant *Staphylococcus aureus* (MRSA) by human and murine neutrophils was reported [111], suggesting that this mediator serves an important role by amplifying the bactericidal functions of leukocytes and could thus be a key component of neutrophil-mediated host defense in the amniotic cavity. However, TSLP has also been shown to display context-dependent functions in the murine female genital tract, as progesterone induced a TSLP-dependent suppression of neutrophil influx and Th17-associated gene expression in response to gonococcal infection [140]. Thus, TSLP may undergo additional hormonal regulation, particularly during pregnancy, which could influence its effectiveness in promoting microbial clearance.

A large body of TSLP investigation has focused on the role of mediator in non-infectious or sterile inflammatory processes, such as allergy and asthma, due to its close relationship with other type 2 cytokines such as IL-33 [141]. In such a context, TSLP has been largely reported to propagate or exacerbate inflammation [142–148], in part by promoting Th2 responses [56, 148–155]. Yet, whether TSLP was also participating in the sterile inflammatory response in the amniotic cavity of a subset of women undergoing spontaneous preterm labor had not been reported. Here, we show that TSLP is increased in the amniotic fluid of such women, potentially indicating its involvement in sterile intra-amniotic inflammation. This observation is strengthened by the fact that women with SIAI included in the current study were diagnosed using PCR/ESI-MS that can detect a large number of microbes, including fastidious bacteria such as *Sneathia* spp. [9–12], and thus were confirmed to be truly sterile. Moreover, TSLP and IL-33 have known roles as potent alarmins and participate in the mechanisms leading to sterile inflammation [65, 141]. The wide variety of stimuli that have been reported to induce the expression and release of TSLP, including mechanical injury, infection, and inflammatory mediators [65], provide further support for the participation of this cytokine in sterile inflammation. Importantly, TSLP seems to act upstream of multiple Th2-associated signaling pathways and interacts with various immune and non-immune cell types [156], giving promise to therapeutics targeted at TSLP for the treatment of atopic disease [65, 157–159]. While the targeting of TSLP may not necessarily be effective for the treatment of SIAI-associated preterm labor, further

research pointed at uncovering the signaling mechanisms of this cytokine in the amniotic cavity may be worthwhile.

Independent of the underlying insult, herein we showed that TSLP is produced by the chorioamniotic membranes, including the fetal layers (chorion and amnion) and the attached maternal layer (decidua parietalis), in women with intra-amniotic inflammation. Overall, our findings support the responsiveness of the chorioamniotic membranes to intra-amniotic inflammation. As observed herein, the correlation between mRNA and protein expression levels in mammals is variable and, in some cases, can be relatively low [160–163]. Yet, whether TSLP production was further modified in the current study via post-transcriptional and/or post-translational modification is unclear. It is worth noting that TSLP is released as a soluble protein, and thus the determination of its concentrations in the amniotic fluid collected at the time of diagnosis with SIAI or IAI represents the best readout of its participation in such conditions. By contrast, the chorioamniotic membranes collected after delivery provide retrospective information regarding the expression of TSLP and its receptors that may not reflect the state of these tissues during the onset and propagation of SIAI or IAI, necessitating careful interpretation.

Herein, we also showed that TSLP was expressed by the innate immune cells (e.g., neutrophils and monocytes/macrophages) invading the amniotic cavity, which can be predominantly of fetal origin in cases of intra-amniotic inflammation associated with spontaneous preterm labor [164, 165]. Thus, the release of TSLP into this compartment at least partially represents a fetal response to a local insult, whether it is microbial invasion or sterile injury. We also found that the receptors for TSLP were expressed throughout the CAM, including the maternal decidual layer, suggesting that TSLP signaling in this tissue is enabled regardless of the inflammatory status of the amniotic cavity. Moreover, we showed that *CRLF2* expression was specifically upregulated in the context of IAI. Such an increase is likely due to the infiltration of the chorioamniotic membranes by maternal neutrophils and monocytes/macrophages [75, 166], which are among the multiple cell types that express these receptors [111, 167, 168]. Receptor expression within the layers of the CAM suggests that fetal TSLP signaling in response to intra-amniotic inflammation drives both a positive feedback loop on the fetal side as well as communicates with cells on the maternal side (i.e., decidua parietalis), potentially aiding in the activation and recruitment of maternal immune cells. Of note, the induction of TSLP during fetal life could have long-term consequences, as a recent murine investigation demonstrated TSLP-altered chromatin accessibility in dendritic cells resulting in activation of pathogenic gene programs in response to neonatal respiratory viral infection [135]. Therefore, TSLP may drive trained immunity resulting in potentially harmful alterations of the immune response later in life; yet, this concept requires further exploration.

Herein, we demonstrated that an established pathogen frequently detected in the amniotic cavity of women with IAI, *Ureaplasma*, and an emerging pathogen implicated in obstetric disease, *Sneathia*, both induce the release of TSLP by amniocytes. The role of *Ureaplasma* spp. in triggering intra-amniotic inflammation leading to spontaneous preterm labor is well established [13–15, 17, 169–172]. Indeed, we have shown that *Ureaplasma parvum* isolated from the amniotic fluid of women with IAI induces a similar intra-amniotic inflammatory

response in mice, including the induction of inflammatory gene expression by amniocytes [77]. Moreover, we demonstrated that the macrolide clarithromycin prevents *Ureaplasma*-induced preterm birth and adverse neonatal outcomes in mice [77], indicating that this antibiotic is an important component of the optimal treatment regimen for women diagnosed with IAI. By contrast, the role of *Sneathia* in the intra-amniotic inflammatory response has been much less explored, due in part to the difficulty involved in its cultivation [18]. However, the emergence of sensitive molecular techniques has allowed for the detection of *Sneathia* in clinical samples [18], even if bacterial viability cannot be firmly established. The mechanisms of virulence whereby *Sneathia* exerts its adverse effects in the context of intra-amniotic infection remain an ongoing area of investigation, with reports showing that *Sneathia vaginalis* can damage the chorioamniotic membranes through the release of a pore-forming cytotoxin [173]. While a direct link between *Sneathia* and TSLP remains to be established, previous studies have reported that synthetic ligands for multiple TLRs (TLR3, TLR5, and TLR2/TLR6) and *Staphylococcus aureus* drove the expression of TSLP by human keratinocytes [174], which is consistent with our findings in amniocytes. Thus, while the current study supports the induction of TSLP by *Sneathia* spp., continued mechanistic investigation is required to establish the specific pathogenic factors and signaling pathways implicated in this process.

Collectively, our findings have generated novel evidence of a role for TSLP in the intra-amniotic inflammation triggered by alarmins or microbial infection. The release of TSLP into the amniotic cavity may represent a key element of the fetal response to insult by amplifying the functions of infiltrating neutrophils as well as signaling to maternal cells in the surrounding decidua. Moreover, the induction of TSLP expression by an emerging obstetrical pathogen, *Sneathia*, provides further understanding of the underexplored virulence mechanisms whereby these bacteria exert their harmful effects. Taken together, these data generate new insight into the molecular mechanisms underlying intra-amniotic host response leading to spontaneous preterm labor and birth.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors would like to thank Rona Wang and Gregorio Martinez III for their help in carrying out some of the assays. The authors also thank the physicians, nurses, and research assistants from the Center for Advanced Obstetrical Care and Research, Intrapartum Unit, and Perinatology Research Branch Clinical Laboratory for help with collecting samples.

Funding

This research was supported by the Perinatology Research Branch, Division of Obstetrics and Maternal-Fetal Medicine, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services (NICHD/NIH/DHHS) under Contract No. HHSN275201300006C (R. Romero). This research was also supported by the Wayne State University Perinatal Initiative in Maternal, Perinatal and Child Health (K.R. Theis and N. Gomez-Lopez). R. Romero has contributed to this work as part of his official duties as an employee of the United States Federal Government.

Availability of Data and Materials

All data generated or analyzed during this study are included in this published article.

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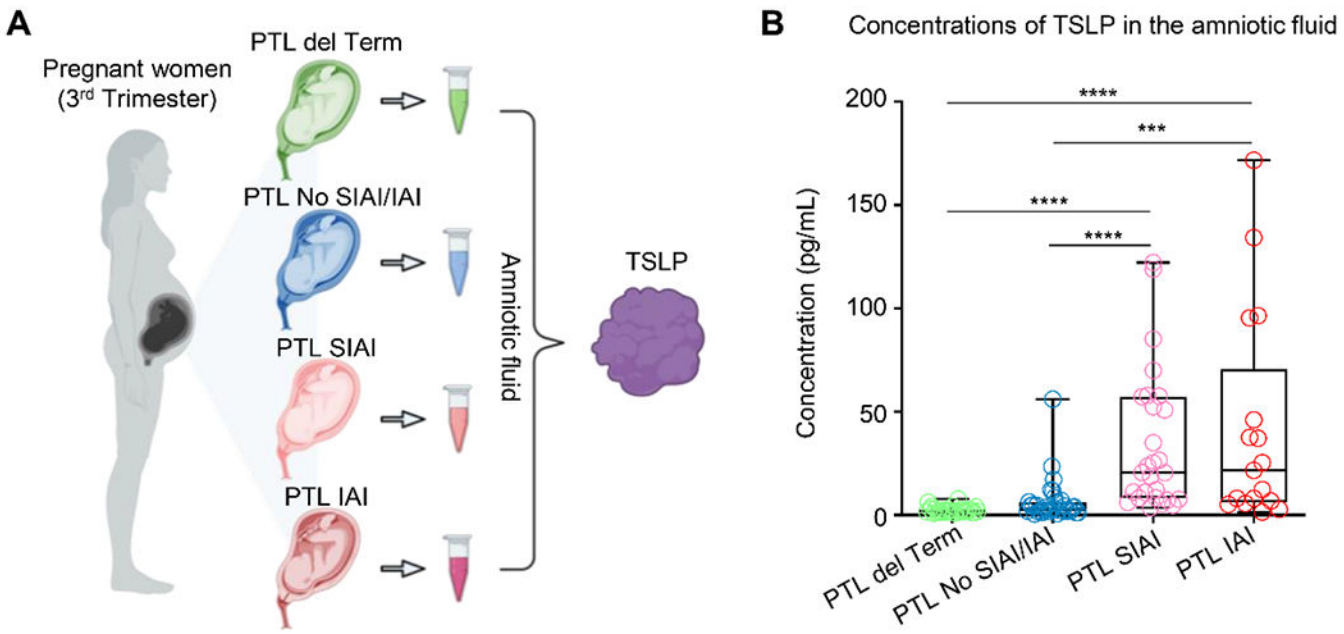

Figure 1. Concentrations of TSLP in amniotic fluid of women with spontaneous preterm labor.

(A) Representative diagram showing the study groups of women who underwent spontaneous preterm labor and delivered at term (PTL del Term) and those who delivered preterm without intra-amniotic inflammation (PTL-No SIAI/IAI), with sterile intra-amniotic inflammation (PTL-SIAI), or with intra-amniotic infection (PTL-IAI) from whom amniotic fluid was collected to measure TSLP. **(B)** TSLP concentrations in the amniotic fluid of women with PTL del Term (n = 30), PTL No SIAI/IAI (n = 34), PTL SIAI (n = 27), and PTL IAI (n = 17). Data are shown as box and whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum values. P-values were determined using the Kruskal-Wallis test followed by Dunn's post-hoc test. ***$p < 0.001$, ****$p < 0.0001$.

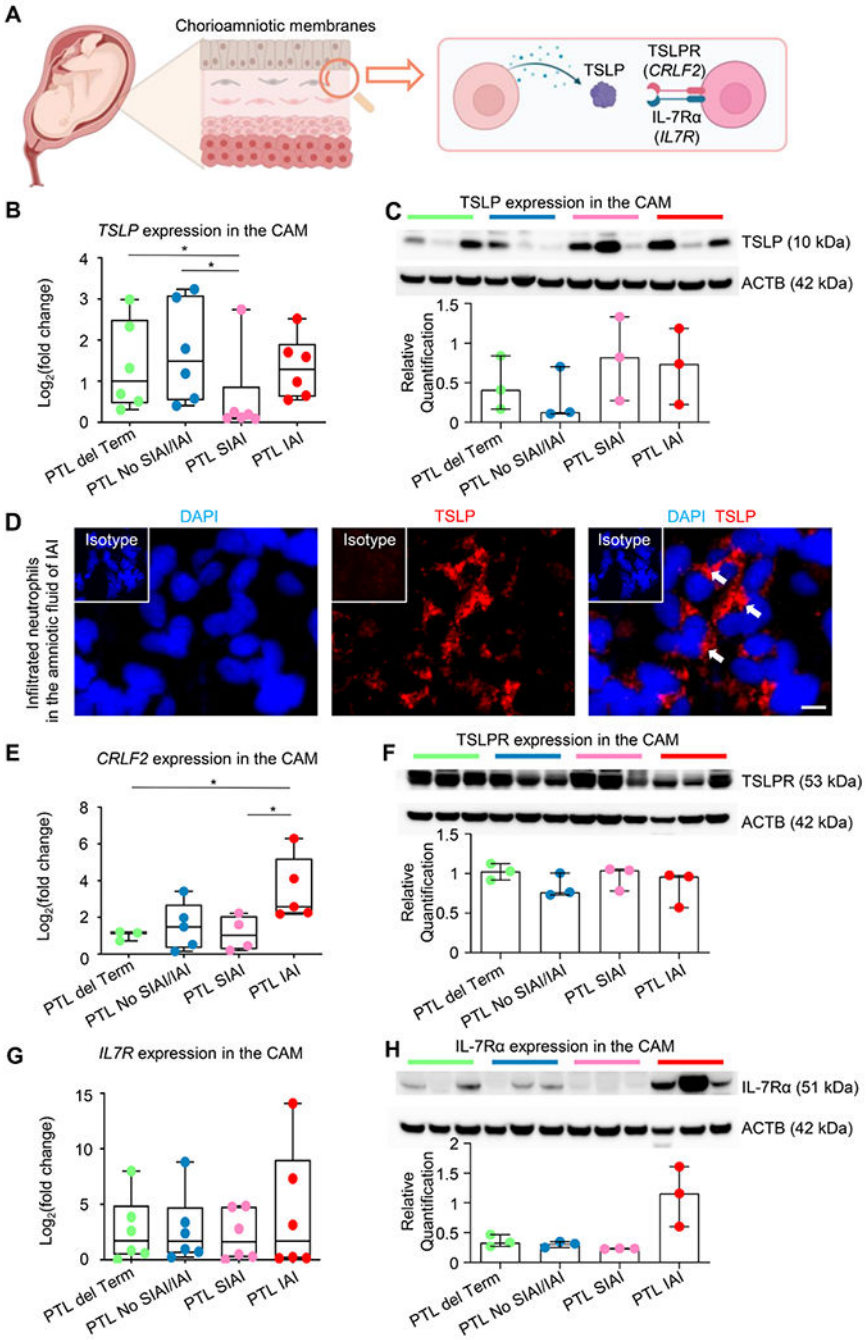

Figure 2. The expression of TSLP and its receptors in the chorioamniotic membranes.

(A) Representative diagram showed the detection of TSLP and its receptors (TSLPR and IL-7Rα) in the chorioamniotic membranes (CAM). **(B)** RT-qPCR assay showing the gene expression of *TSLP* in the CAM of women with PTL del Term, PTL No SIAI/IAI, PTL SIAI, and PTL IAI (n = 6 per group). **(C)** Representative western blot image and relative quantification showing the protein expression of TSLP (10 kDa) in the CAM (n = 3 per group). **(D)** Representative immunofluorescence staining showing the expression of TSLP (red signal) by amniotic fluid neutrophils. White arrows indicate positive TSLP staining.

DAPI staining (blue) shows cell nuclei. The isotype (negative control) staining images in each group are shown at the left corner. All images taken at 400X magnification. Scale bar represents 5 μm. RT-qPCR assays showing the gene expression of **(E)** *CRLF2* and **(G)** *IL7R* in the CAM (n = 6 per group). Representative western blot images and relative quantification showing the protein expression of **(F)** TSLPR (53 kDa) and **(H)** IL-7Rα (51 kDa) in the CAM (n = 3 per group). RT-qPCR data are shown as box and whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum values. β-actin (ACTB) (42 kDa) was used as internal control for western blot analysis. P-values were determined by Mann-Whitney U-tests. *$p < 0.05$.

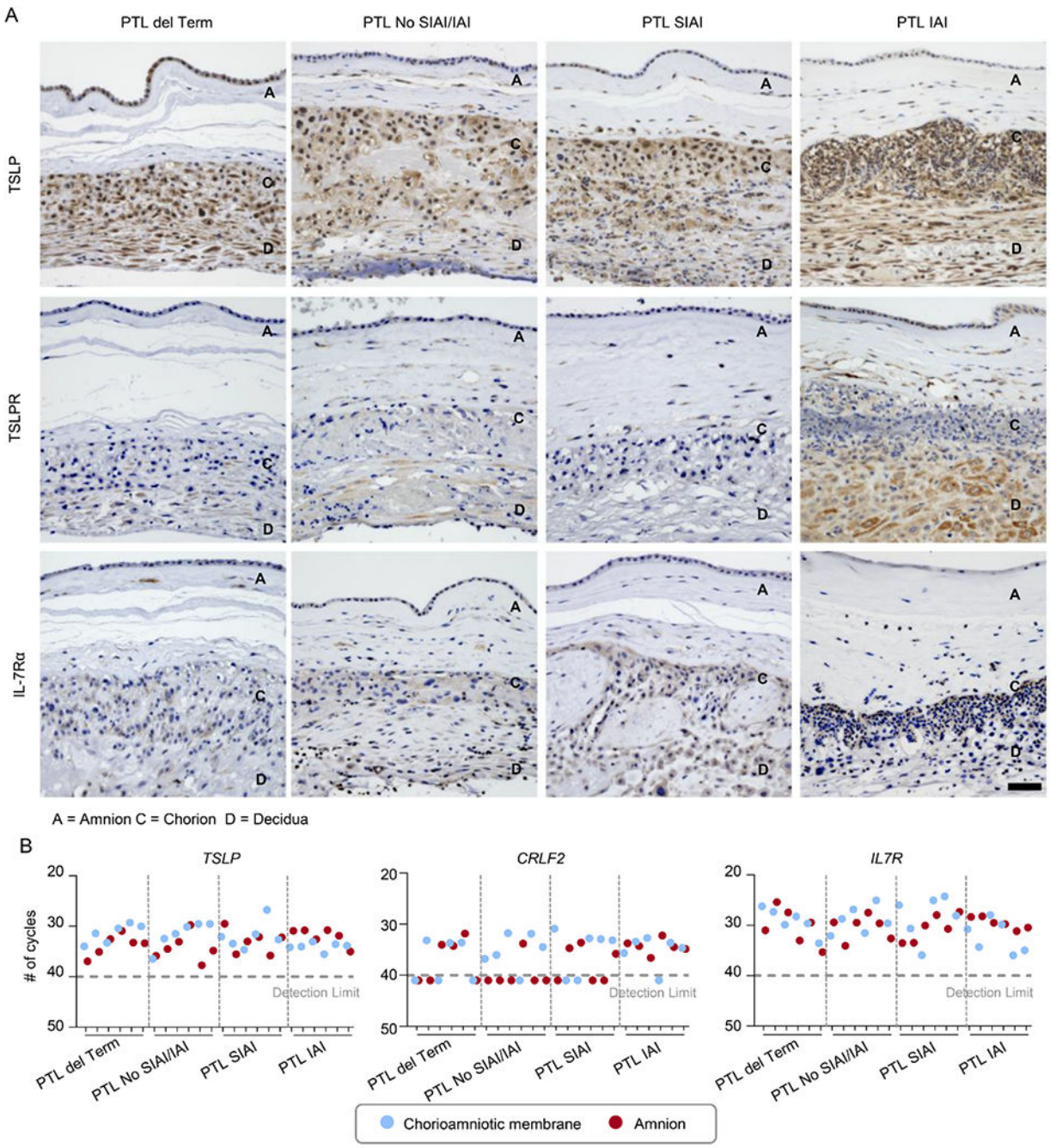

Figure 3. Spatial expression of TSLP and its receptors in the chorioamniotic membranes.

(A) Representative immunohistochemistry images showing the expression of TSLP, TSLPR, and IL-7Rα (brown staining) in the amnion, chorion, or decidua of the CAM from women with PTL del Term, PTL No SIAI/IAI, PTL SIAI, and PTL IAI (n = 5 per group). All images were taken at 200X digital magnification. Scale bar represents 50 μm. Tissue layers are indicated as A: Amnion; C: Chorion; D: Decidua. **(B)** Dot plots representing the expression of *TSLP*, *CRLF2*, and *IL7R* in the CAM and amnion of women with PTL del Term, PTL No SIAI/IAI, PTL SIAI, and PTL IAI (n = 6 per group). Dotted horizontal lines

correspond to 40 cycles, which was considered as the detection limit threshold. Blue dots represent CAM, red dots represent amnion.

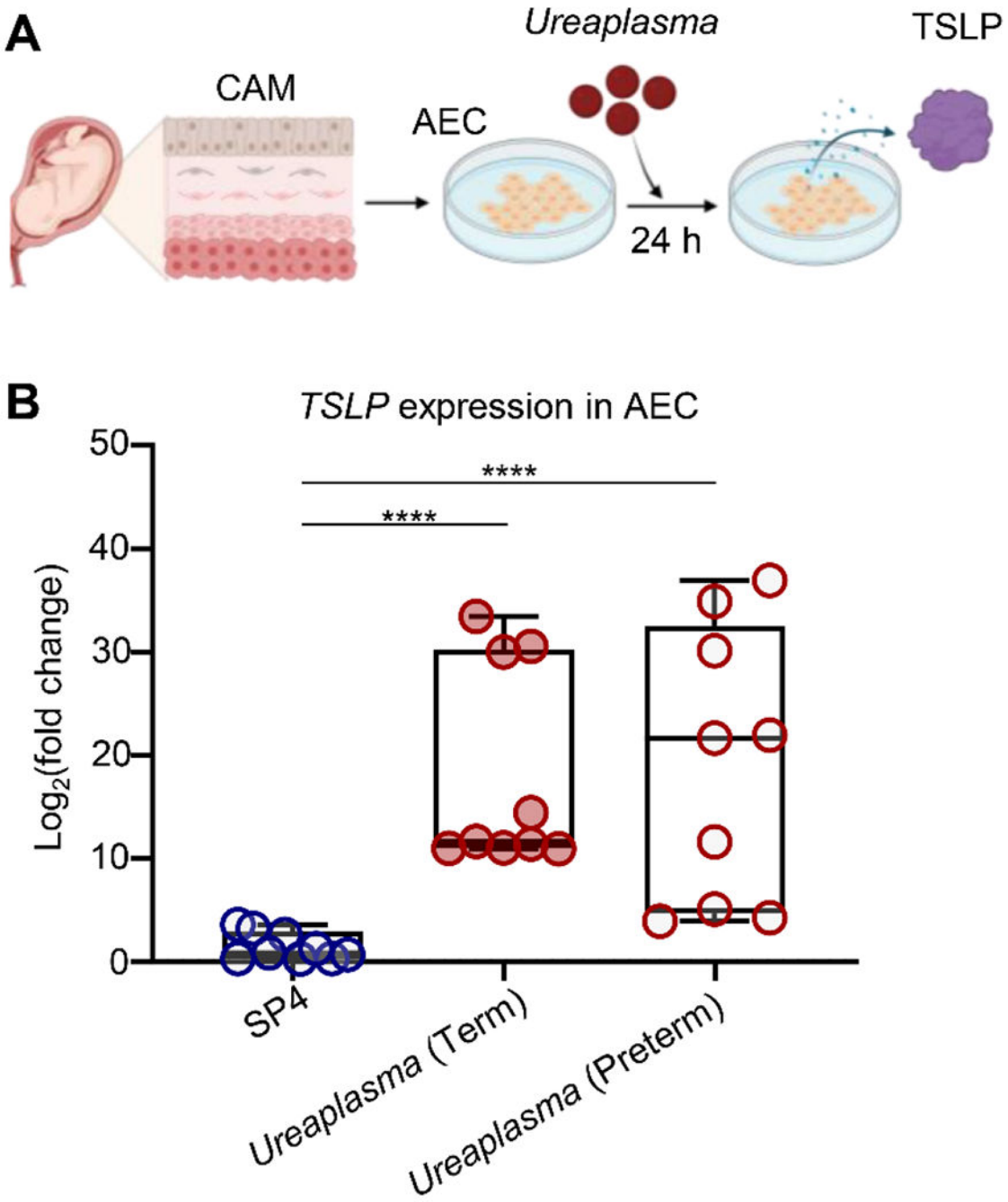

Figure 4. ***Ureaplasma parvum*** **stimulates** ***TSLP*** **expression in human amnion epithelial cells.**
(A) Representative diagram showing the co-culture of human amnion epithelial cells (AEC) (n = 3 samples with 3 technical replicates each) with two isolates of *U. parvum* (from a woman with term or preterm labor) or vehicle control (SP4 broth) for 24 h, after which AEC were collected to determine *TSLP* expression. **(B)** *TSLP* expression in AEC incubated with SP4 borth or *U. parvum* isolates. Data are shown as box and whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate

minimum/maximum values. P-values were determined by Mann-Whitney U-tests. ****p <0 .0001.

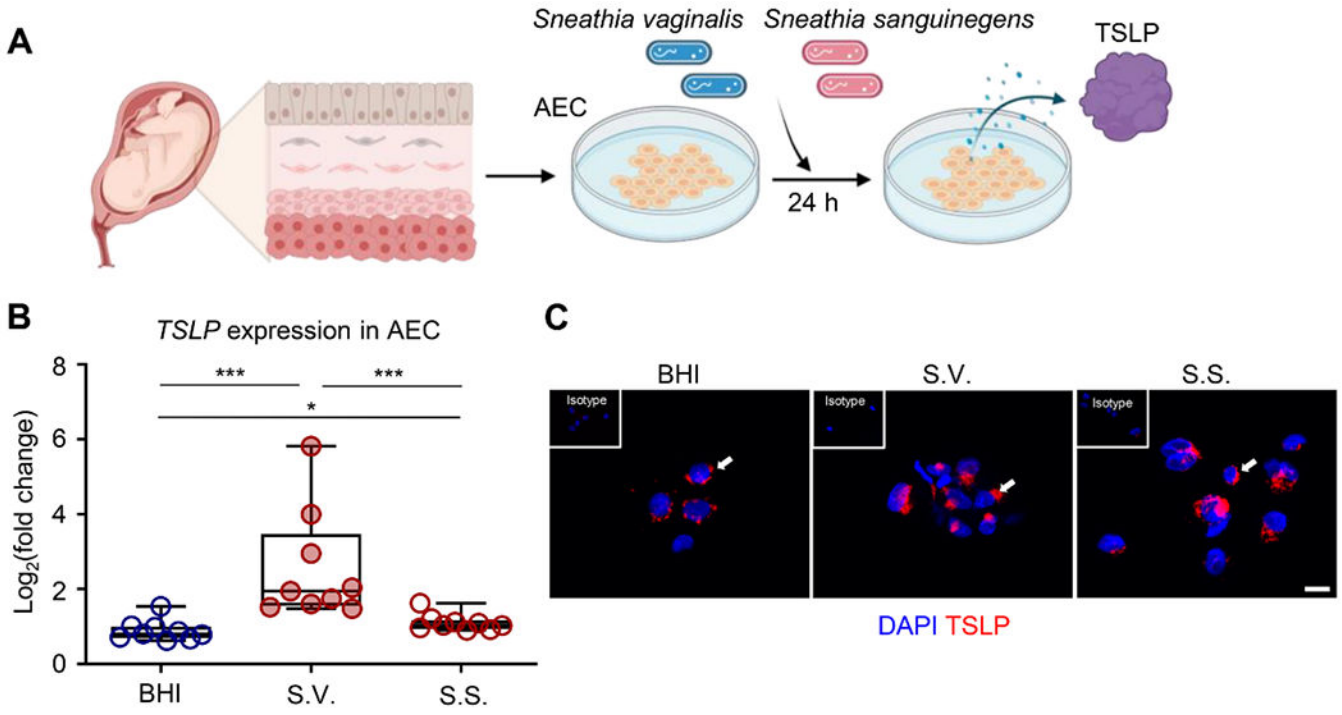

Figure 5. *Sneathia* spp. stimulate *TSLP* expression in amnion epithelial cells.

(A) Representative diagram the co-culture of human amnion epithelial cells (AEC) (n = 3 samples with 3 technical replicates each) with *Sneathia vaginalis* (S.V.), *Sneathia sanguinegens* (S.S.), or vehicle control [supplemented (s)BHI broth, see Methods] for 24 h, after which the AEC were collected to determine *TSLP* expression. **(B)** *TSLP* expression in AEC incubated with sBHI broth, S.V., or S.S. Data are shown as box and whisker plots where midlines indicate medians, boxes indicate interquartile ranges, and whiskers indicate minimum/maximum values. P-values were determined by Mann-Whitney U-tests. *$p < 0.05$, ***$p < 0.001$. **(C)** Representative immunofluorescence images showing the expression of TSLP (red signal) in AEC treated with sBHI broth, S.V., or S.S. White arrows indicate positive TSLP staining. DAPI staining (blue signal) shows cell nuclei. The isotype (negative control) staining images in each group are shown at the left corner. All images taken at 400X magnification. Scale bar represents 20 μm.