TLR4 Signaling Is a Major Mediator of the Female Tract Response to Seminal Fluid in Mice 1

Authors: Schjenken, John E., Glynn, Danielle J., Sharkey, David J., and Robertson, Sarah A.

Source: Biology of Reproduction, 93(3)

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.114.125740

BIOLOGY OF REPRODUCTION (2015) **93**(3):68, 1–13
Published online before print 8 July 2015.
DOI 10.1095/biolreprod.114.125740

TLR4 Signaling Is a Major Mediator of the Female Tract Response to Seminal Fluid in Mice[1]

John E. Schjenken, Danielle J. Glynn, David J. Sharkey, and Sarah A. Robertson[2]

Robinson Research Institute and School of Medicine, University of Adelaide, Adelaide, South Australia, Australia

ABSTRACT

Seminal fluid interacts with epithelial cells lining the female reproductive tract to induce expression of proinflammatory cytokines and chemokines, initiating immune tolerance mechanisms to facilitate pregnancy. TGFB cytokines are key signaling agents in seminal plasma but do not fully account for the female response to seminal fluid. We hypothesized that additional molecular pathways are utilized in seminal fluid signaling. Affymetrix microarray was employed to compare gene expression in the endometrium of mice 8 h after mating with either intact males or seminal fluid deficient (SVX/VAS) males. Bioinformatics analysis revealed TLR4 signaling as a strongly predicted upstream regulator activated by the differentially expressed genes and implicated TGFB signaling as a second key pathway. Quantitative PCR and microbead data confirmed that seminal fluid induces endometrial synthesis of several TLR4-regulated cytokines and chemokines, including CSF3, CXCL1, CXCL2, IL1A, IL6, LIF, and TNF. In primary uterine epithelial cells, CSF3, CXCL1, and CXCL2 were strongly induced by the TLR4 ligand LPS but suppressed by TGFB, while IL1A, TNF, and CSF2 were induced by both ligands. TLR4 was confirmed as essential for the full endometrial cytokine response using mice with a null mutation in *Tlr4,* where seminal fluid failed to induce endometrial *Csf3, Cxcl2, Il6,* and *Tnf* expression. This study provides evidence that TLR4 contributes to seminal fluid modulation of the periconception immune environment. Activation of TLR4 signaling by microbial or endogenous components of seminal fluid is thus implicated as a key element of the female tract response to seminal fluid at the outset of pregnancy in mice.

chemokine, cytokine, endometrium, seminal fluid, toll-like receptor

INTRODUCTION

Successful pregnancy requires adaptation of the female immune system to tolerate implantation of embryos expressing paternal alloantigens [1]. Male seminal fluid, made up of sperm and seminal plasma, delivers immune-regulatory factors and paternal antigens that contribute to generating maternal immune tolerance at the time of conception. In particular, seminal fluid promotes activation of paternal antigen-reactive regulatory T cells (Treg cells) that are essential for endometrial receptivity to implantation [2, 3]. Studies in humans, pigs, and other animals show that male-female seminal fluid signaling is common amongst mammalian species [4].

In mice, signaling is initiated after coitus due to the actions of soluble factors present in seminal fluid, which bind to receptors expressed by epithelial cells lining the uterine lumen, to trigger synthesis of colony-stimulating factor-2 (CSF2, also known as granulocyte-macrophage colony-stimulating factor, GM-CSF) [5]. Several other cytokines and chemokines, including interleukin-6 (IL6), TNF, C-X-C motif chemokine ligand-1 (CXCL1, also known as growth-regulated alpha protein, GRO or KC), CXCL2 (macrophage inhibitory protein-2, MIP2), and C-C motif chemokine ligand-3 (CCL3, macrophage inflammatory protein-1 alpha, MIP1A) are also elevated in the uterus during the immediate postcoital phase [6–9].

These cytokines have the capacity to act rapidly on nearby blood vessels to draw inflammatory cells, including macrophages, neutrophils, and dendritic cells, into the superficial endometrial stroma and uterine lumen [5, 7, 10]. The effects of seminal fluid extend the full depth of the reproductive tract to the oviduct [11] and to the ovary [12] where cytokine synthesis and leukocyte numbers also increase. Together, cytokine production and leukocyte recruitment assist in preparing the female reproductive tract for conception and pregnancy by promoting the formation of the corpus luteum [12], supporting the development of the preimplantation embryo [13], inducing endometrial expression of embryo attachment molecules and angiogenic factors to prepare for embryo implantation [14], and clearing the uterine cavity of microorganisms introduced at mating [15]. Cytokines produced in response to seminal fluid also recruit macrophages and dendritic cells that take up antigen and prime the female immune response to paternal antigens [1, 16], leading to expansion of antigen-dependent Treg cells [16]. Depending on the balance of cytokines produced and immune cells recruited as well as the antigenic dissimilarity between the male and the female, the female immune response to seminal fluid may induce tolerance or rejection mechanisms that either facilitate or constrain embryo implantation and progression of the reproductive cycle [17].

Previous studies have begun to identify the active factors present in seminal fluid that contribute to the periconception inflammatory response and generation of maternal immune tolerance. In the mouse and human, the seminal plasma fraction of seminal fluid is identified as containing potent signaling agents that trigger the female response to coitus [5, 18, 19]. Ablation of the seminal vesicle contribution to seminal plasma in mice adversely affects fertility and also alters the growth trajectory and metabolic health of male offspring, partly

[1]This study was supported by an Australian Research Council (ARC) Discovery Grant and NHMRC (Australia) Program and Fellowship grants to SAR. The microarray data discussed in this publication are deposited in the National Center for Biotechnology Information's Gene Expression Omnibus and are accessible through GEO Series accession no. GSE70401 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70401).
[2]Correspondence: Sarah A Robertson, Robinson Research Institute and School of Medicine, University of Adelaide, Adelaide, SA, 5005, Australia. E-mail: sarah.robertson@adelaide.edu.au

Received: 11 October 2014.
First decision: 30 October 2014.
Accepted: 29 June 2015.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

through affecting the expression of embryotrophic cytokines in the oviduct at conception [20]. Analysis of the active factors present in seminal plasma has identified transforming growth factor B (TGFB) as a major component that activates the endometrial response to seminal fluid in mice [21], humans [22], pigs [23], and sheep [24]. In addition, seminal fluid induction of Treg cells depends on seminal plasma TGFB because mouse Treg cells, which require TGFB for their differentiation [25], are not expanded in the absence of seminal plasma [18, 26] and exogenous TGFB administration increases vaginal Treg cells in abortion-prone mice, preventing spontaneous fetal loss [27].

However, experiments in human and mouse reproductive tract epithelial cells show that only some of the cytokines upregulated by seminal fluid are induced by TGFB [21, 22, 28, 29], indicating that other components of seminal fluid contribute to the seminal fluid-signaling cascade. To further define the molecular basis of seminal fluid signaling, we have characterized how seminal fluid alters the gene expression profile in the mouse endometrium following mating and utilized the data to predict novel pathways that are employed by seminal fluid to activate the female tract response. We show that seminal fluid regulates a variety of immune response genes and pathways and that many differentially expressed genes are demonstrated to require TLR4. These data imply that activation of TLR4 signaling by ligands, including pathogen-associated molecular patterns (PAMPS) or danger-associated molecular patterns (DAMPs), in seminal fluid is a key event in the female response to seminal fluid and subsequent immune adaptation for pregnancy.

MATERIALS AND METHODS

Mice and Surgical Treatments

All the animal experiments were performed in accordance with the Guiding Principles for the Care and Use of Research Animals endorsed by the Society for the Study of Reproduction, with approval from the University of Adelaide Ethics Committee (approval numbers M-2010-095 and M-023-14). Mice were housed in a specific pathogen-free facility at the University of Adelaide with controlled temperature and lighting (12L:12D). Food and water were supplied ad libitum. CBA × C57Bl/6 F1 (CBAF1) females, C57Bl/6 males, and Balb/c mice were purchased from the University of Adelaide Central Animal Facility. Mice with a null mutation in the *Tlr4* gene (*Tlr4*$^{-/-}$ mice) back-crossed onto BALB/c for more than 10 generations were sourced from Prof. Akira (Osaka University, Osaka, Japan) [30] and supplied by Prof. Paul Foster (University of Newcastle, Newcastle, Australia).

Seminal fluid-deficient SVX/VAS males were prepared as previously described [5]. Briefly, mice were anesthetized with Avertin (1 mg/ml tribromoethyl alcohol in tertiary amyl alcohol [Sigma-Aldrich, St Louis, MO] diluted to 2.5% [v/v] in saline; 15 μl/g body mass injected intraperitoneally). Mice were vasectomized by bilateral ligation of the vas deferens, and seminal vesicles were excised after ligation and severing of the proximal tubule at the base of the gland, in the one procedure, via a transverse incision in the abdominal wall. The body wall and skin were sutured, and the mice were allowed to recover for at least 2 wk before mating experiments. Successful surgery was confirmed by placement with trial females and remote analysis of video recording (using infrared night filming capability) to ensure competent mating behavior. Trial females mated with SVX/VAS males were examined to confirm the absence of a copulatory plug or ejaculated sperm, and failure to progress to pregnancy.

Mating and Tissue Collection

Mouse estrous cycle was monitored by examination of vaginal lavage cytology. For mated mice, CBAF1 females were placed with intact or SVX/VAS Balb/c stud males, and *Tlr4*$^{-/-}$ or wild-type *Tlr4*$^{+/+}$ Balb/c females were placed with intact or SVX/VAS C57Bl/6 males, at 2300–0100 h on the evening after proestrus was detected. The occurrence and timing of mating events within the subsequent 2 h period were monitored by video recording (for both intact and SVX/VAS males) and confirmed by presence of a copulatory plug (for intact males). At 8 h postmating (0700–0900 h), females were killed and the uterus was excised and processed for either RNA or protein analysis. In some experiments, females were killed 1–2 h postmating (0100–0200 h) for recovery of uterine luminal fluid and endotoxin quantitation. For unmated estrous control mice, CBAF1 females were killed and the uterus excised at 2200–2230 h on the evening after proestrus was detected.

For RNA experiments, the uterus was placed in cold PBS and trimmed of fat, mesentery, and blood vessels under a dissection microscope (Olympus, New York, NY), then transferred into fresh PBS and slit lengthwise. Exposed endometrial tissue was scraped off using a razor blade into Qiazol RNA lysis solution (Qiagen, Venlo, Netherlands), and the myometrium was discarded. RNA was homogenized at a setting of one cycle at 3500 rpm for 10 sec using the Powerlyzer(R) 24 Bench Top Bead Based Homogenizer (Mo Bio Laboratories, Carlsbad, CA).

For protein and endotoxin quantitation experiments, uteri were excised with the cervical end clamped, and then uterine luminal fluid was flushed with 50 μl of PBS containing 1% bovine serum albumin. Endometrial RNA and luminal fluid samples were stored at −80°C until further use.

Uterine Epithelial Cell Culture

Uteri were harvested from mice identified as estrous by vaginal lavage cytology, and epithelial cell cultures, consisting of >80% epithelial cells, were prepared as previously described [10, 31]. Epithelial cells were resuspended at 1×10^6 cells/ml in RPMI (RPMI-1640, 5% fetal calf serum, 2 mM L-glutamine, 1× antibiotic/antimycotic [Life Technologies, Carlsbad, CA]), and 500 μl aliquots were plated in 4-well multidishes (Nunc, Roskilde, Denmark). Cells were incubated for 4 h at 37°C in 5% CO_2, to permit adherence, then treatment, either 2, 20, or 200 ng/ml of lipopolysaccharide (LPS) (*Salmonella typhimurium;* Sigma-Aldrich) or 1.25, 5, or 20 ng/ml human recombinant TGFB3 (Gropep, Thebarton, Australia), or vehicle (RPMI) alone was added to duplicate wells. Treatment-containing media was replaced with fresh media following overnight (16 h) incubation. Culture supernatants were collected after 24 h further incubation, centrifuged at 244 × *g* to remove cellular material, and stored at −20°C until assay. Adherent cells were quantified following Rose Bengal dye uptake (0.25% in PBS, 5 min at room temperature) (Sigma Aldrich) and cell lysis in 0.1% SDS by measuring absorbance at 570 nm using a Bio-Rad Microplate Reader Benchmark and Microplate Manager 5.2.1 Build 106 (Bio-Rad Laboratories, Hercules, CA) as described previously [10, 31].

Isolation of Total RNA

Total RNA was extracted using the miRNeasy extraction kit (Qiagen) and treated with TURBO DNA-free (Life Technologies) following the manufacturer's instructions. RNA was quantified using a Nano-Drop Spectrophotometer ND-1000 (Thermo Scientific, Waltham, MA), and RNA integrity was analyzed using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) to ensure all RNA preparations had an RNA integrity number of >7.

Microarray Analysis and Bioinformatics

Microarray analysis was performed using Affymetrix Mouse Gene 2.0 ST Arrays at the Adelaide Microarray Centre. Total RNA (300 ng) was labeled using the GeneChip WT PLUS Reagent Kit according to the manufacturer's instructions (Affymetrix, Santa Clara, CA). Microarray data was analyzed using Partek Genomics Suite version 6.6. Briefly, .cel files were imported using RMA background correction, following GC content correction and mean probe summarization. Differentially expressed probes were defined as ≥1.50-fold change, *t*-test $P < 0.05$. To investigate gene pathways and upstream regulators activated by seminal fluid following mating, differentially expressed genes were analyzed using Ingenuity Pathway Analysis (IPA) version 18488943 Build 308606M (Ingenuity Systems, Redwood City, CA). The microarray data discussed in this publication are deposited in the National Center for Biotechnology Information's Gene Expression Omnibus [32] and are accessible through GEO Series accession no. GSE70401 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE70401).

Reverse Transcription and Quantitative Polymerase Chain Reaction

Total cellular RNA was reverse transcribed into cDNA from 1000 ng RNA primed with 150 ng random hexamers employing a Superscript-III Reverse Transcriptase kit (Life Technologies) according to the manufacturer's instructions and stored at −20°C. Primer pairs specific for published cDNA sequences were designed using Primer Express version 2 software (Life Technologies, Table 1). PCR primers were tested for specificity by quantitative PCR (qPCR) amplification (see method below). PCR primer products were

TABLE 1. PCR primers used to quantify mRNA expression.

Target mRNA	Forward primer	Reverse primer	Primer	GenBank accession no.
bAct	5′-TGTGATGGTGGGTATGGGTC-3′	5′-ACACGCAGCTCATTGTA-3′	0.25 μm	NM_007393
Ccl3	5′-TGCCCTTGCTGTTCTTCTCTG-3′	5′-AACGATGAATTGGCGTGGA-3′	0.25 μm	NM_011337
Ccl5	5′-TGCCAACCCAGAGAAGAAGTG-3′	5′-TTACTGAGTGGCATCCCCAAG-3′	0.5 μm	NM_013653
Ccl21	5′-TCCAACTCACAGGCAAAGAGG-3′	5′-GCAGATGTGATGGTTGAAGCA-3′	0.25 μm	NM_011124
Csf2	5′-CCTGGGCATTGTGGTCTACAG-3′	5′-GGCATGTCATCCAGGAGGTT-3′	0.05 μm	NM_009969
Csf3	5′-GCAGACACAGTGCCTAAGCCA-3′	5′-CATCCAGCTGAAGCAAGTCCA-3′	0.25 μm	NM_009971
Cxcl1	5′-ATTGTATGGTCAACACGCACG-3′	5′-TTTGAACGTCTCTGTCCCGAG-3′	0.5 μm	NM_008176
Cxcl2	5′-GAACTGCGCTGTCAATGCCT-3′	5′-CCGCCCTTGAGAGTGGCTAT-3′	0.5 μm	NM_009140
Cxcl10	5′-TCCATCACTCCCCTTTAGCCA-3′	5′-TGTCTCAGGACCATGGCTTGA-3′	0.25 μm	NM_021274
IL1α	5′-CCGACCTCATTTTCTTCTGG-3′	5′-GTGCACCCGACTTTGTTCTT-3′	0.1 μm	NM_010554.4
IL1β	5′-CCCAAGCAATACCCAAAGAA-3′	5′-GCTTGTGCTCTGCTTGTGAG-3′	0.5 μm	NM_008361.3
IL6	5′-ACAACCACGGCCTTCCCTAC-3′	5′-TCCACGATTTCCCAGAGAACA-3′	1 μm	NM_031168
Lif	5′-CGCCAATGCTCTCTTCATTTC-3′	5′-TCCGATGCTCCACCAACT-3′	0.5 μm	NM_008501
Tlr4	5′-TGGCATGGCTTACACCACC-3′	5′-GAGGCCAATTTTGTCTCCACA-3′	0.25 μm	NM_021297
Tnf	5′-GTAGCCCACGTCGTAGCAAAC-3′	5′-CTGGCACCACTAGTTGGTTGTC-3′	1 μm	NM_013693

purified from 2% agarose gels and then sequenced at the Australian Genome Research Facility (AGRF, Adelaide Node, Australia) to confirm primer specificity. Assay optimization and validation experiments were performed using cDNA from murine endometrial tissue to determine the amplification efficiency of each primer pair. All the primers were determined to have a correlation coefficient of >0.95 and an efficiency of 90%–110%.

Quantitative PCR was performed on 20 ng of cDNA containing PCR primers (details in Table 1) and 1× Power SYBR Green PCR master mix (Life Technologies). Negative controls included in each reaction contained H_2O substituted for cDNA or RNA without reverse transcription. PCR amplification was performed in an ABI Prism 7000 Sequence Detection System or QuantStudio12K Flex Real-Time PCR System v1.1 (Life Technologies) using the following conditions: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. The delta C(t) method [33] was then used to calculate messenger RNA abundance normalized to *Actb* mRNA expression.

Cytokine Enzyme-Linked Immunosorbent Assay and Multiplex Microbead Analysis

Cytokines secreted into luminal fluid or culture supernatants were quantified using either Milliplex multi-analyte panels multiplex microbead kit (Merck Millipore, Billerica, MA) or conventional enzyme-linked immunosorbent assay (ELISA) (GCSF DuoSet ELISA; R&D Systems, Minneapolis, MN) following the manufacturer's instructions. The minimum detectable threshold for microbead analytes was <3.2 pg/ml, and samples were diluted in assay buffer (1:5 or 1:200). Analytes examined by microbead assay were CSF2 (GMCSF), CXCL1 (KC), CXCL2 (MIP2), CXCL10 (IP10), IL1B, IL6, LIF, and TNF. Microbead data were analyzed using Luminex 200 and eXponent 3.1 (Luminex Corp, Austin, TX). The minimum detectable threshold for CSF3 (GCSF) DuoSet ELISA was 15.6 pg/ml, and the samples were diluted 1:50. Duoset ELISA data were analyzed using Bio-Rad Microplate Reader Benchmark and Microplate Manager 5.2.1 Build 106 (Bio-Rad Laboratories).

Endotoxin Assay

Endotoxin was quantified in uterine luminal fluid by Pierce LAL Chromogenic Endotoxin Quantification (Pierce Biotechnology, Rockford, IL) following the manufacturer's instructions using sample dilutions proven in spiking experiments to not cause assay inhibition. The minimum detectable threshold was 0.1 endotoxin units/ml, and samples were diluted in endotoxin-free water (1:100 dilution). Endotoxin data was analyzed using the Bio-Rad Microplate Reader Benchmark and Microplate Manager 5.2.1 Build 106 (Bio-Rad Laboratories).

Statistics

SPSS version 17 (SPSS, Chicago, IL) was used to analyze cytokine mRNA and protein data. Data was analyzed by Kruskal-Wallis *H*-test and Mann-Whitney *U*-test because D'Agostino and Pearson omnibus normality test showed most data sets to be not normally distributed. Statistical significance in differences between the groups was concluded when $P < 0.05$.

RESULTS

Seminal Fluid Induces Endometrial Gene Expression

To examine the effect of seminal fluid on the whole genome expression profile of endometrial tissue following mating, RNA was extracted from endometrial tissue collected 8 h after CBAF1 females were mated with intact Balb/c males and compared to RNA from endometrial tissue of females mated with seminal fluid-deficient SVX/VAS Balb/c males. This comparison controlled for ovarian hormone status, exposure to the male and mating activity, and the neuroendocrine response to cervical and vaginal stimulus at mating, so that changes in endometrial gene expression could be attributed specifically to contact with seminal fluid. The endometrial RNA from n = 16 individual females was pooled into four independent biological replicates per treatment group (n = 4 endometrial samples per replicate), and expression profiles were analyzed by Affymetrix microarray.

Seminal fluid exposure induced a clear difference in the profile of genes expressed in the endometrium, as demonstrated by principal component analysis (Fig. 1A). A total of 335 genes were differentially regulated with a fold-change >1.5 and $P < 0.05$. Of these, 190 genes were up-regulated and 145 genes were down-regulated following contact with seminal fluid (Supplemental Table S1; Supplemental Data are available online at www.biolreprod.org). Of the top 10 up-regulated genes, six are associated with the immune response, while 3 of 10 top down-regulated genes are associated with the immune response (Table 2). Amongst the top 15 canonical pathways activated following mating are immune response pathways, including IL6 and IL10 signaling (Table 3).

In an effort to predict novel signaling pathways that may be activated by seminal fluid, the upstream regulator function component of IPA was employed. This function utilizes known information on the differentially regulated genes to predict which upstream molecules are likely regulators. Top upstream regulators predicted to be activated by seminal fluid were components of the TLR4-signaling pathway, including the surface receptor TLR4 (Z score = 4.021, $P = 3.58E{-}20$) (Fig. 1B, and Supplemental Table S2), the adaptor molecule MYD88 (Z score = 4.07, $P = 3.09E{-}21$), and the archetypal TLR4 ligand LPS (Z score = 5.795, $P = 3.12E{-}28$) (Supplemental Fig. S1A, S1B and Supplemental Table S2). Consistent with our previous studies, TGFB ligand was also a predicted upstream regulator of the gene expression changes induced by seminal fluid, although with a weaker probability score (Z score = 1.18, $P = 3.6E{-}15$) (Supplemental Fig. S1C

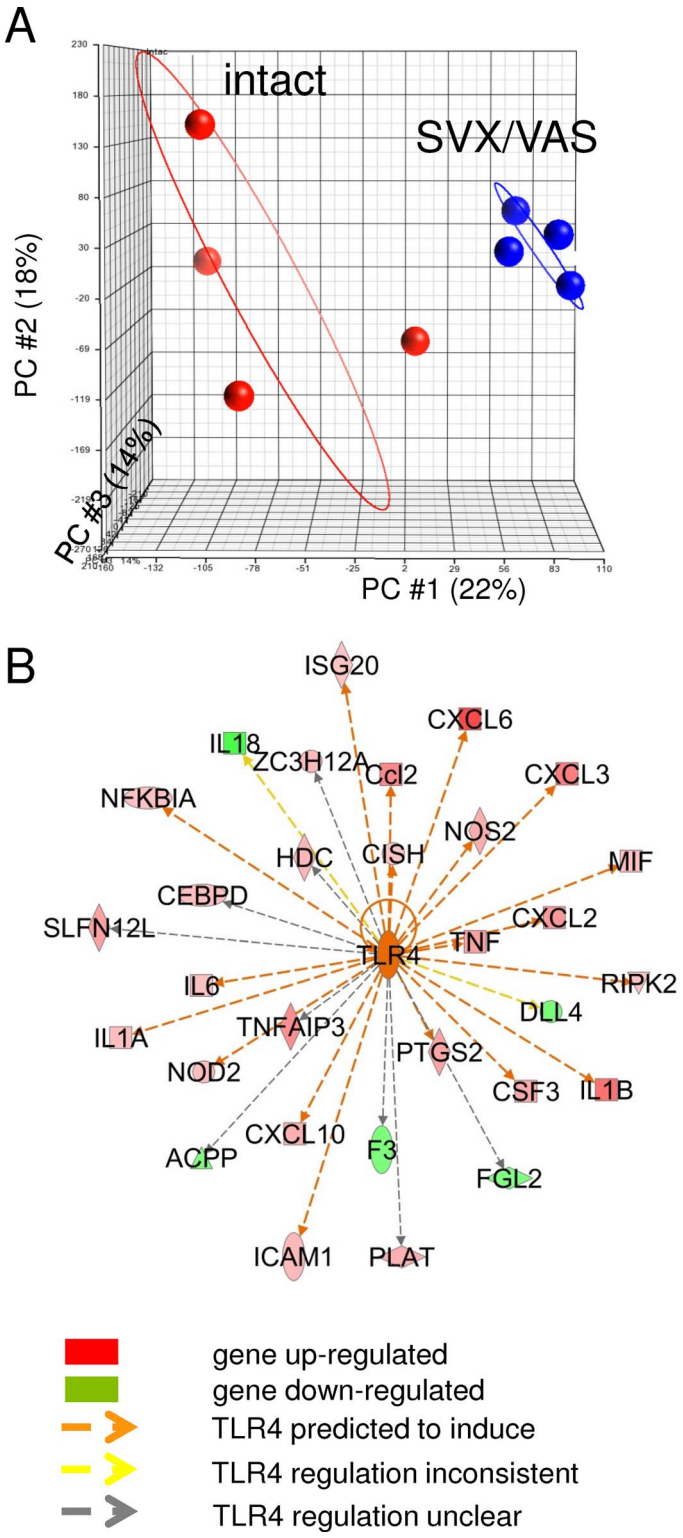

FIG. 1. Seminal fluid exposure at coitus alters endometrial gene expression profile. Endometrial tissue was collected 8 h after mating with either SVX/VAS males or intact males. RNA was extracted and gene expression measured by Affymetrix Mouse Gene 2.0 ST arrays (n = 4 per group from a total of 16 samples). Microarray data was analyzed by Partek Genomics Suite. **A**) Principal component analysis of microarray data. **B**) Ingenuity Pathway Analysis (IPA) was used to predict upstream regulators of the differentially expressed genes, and TLR4 was predicted with a high degree of confidence to be activated by seminal fluid. Results are presented as gene interaction networks where the central gene (upstream regulator) is predicted to be involved in the regulation of all the peripheral genes. Red peripheral genes = genes up-regulated by seminal fluid; green peripheral genes = genes down-regulated by seminal fluid; orange arrow = activation of TLR4 causes gene up-regulation, as predicted; yellow arrow = activation of TLR4 causes gene regulation in opposition to predicted direction; gray arrow = gene is predicted to be regulated by TLR4, but the direction of effect is not ascertained.

and Supplemental Table S2). The TGFB-signaling pathway ($P = 0.031$) was also predicted to be activated by seminal fluid (data not shown).

Endometrial Cytokine/Chemokine Gene Expression Is Induced by Seminal Fluid

To validate the microarray data, several of the genes identified as both differentially regulated and associated with immune response pathways (Table 4) were analyzed further by qPCR. Endometrial samples from both intact and SVX/VAS mating groups were analyzed, and endometrial samples from a third control group of unmated estrous mice were also included, as an additional comparator (n = 15–22 per group). Quantitative PCR confirmation showed that genes *Csf3, Cxcl1, Cxcl2, Cxcl10, Il1a, Il1b, Il6, Lif,* and *Tnf* were all induced following exposure to seminal fluid at coitus (Fig. 2A–I) while *Ccl21* expression was inhibited following exposure to seminal fluid at coitus (Fig. 2J).

An additional gene *Csf2* previously identified as up-regulated in the endometrium after mating in mice [5], was also shown to be induced by seminal fluid in the current study despite not reaching criteria for differential regulation in the microarray (Fig. 2K). Two other chemokine genes *Ccl3* and *Ccl5* shown previously to be induced in the immediate postmating phase in mice [7–9] were not differentially regulated in the microarray, and qPCR confirmed they were not regulated by seminal fluid. In the case of *Ccl3*, there was a >2.0-fold increase in both mated groups, but no discernable difference induced by intact as opposed to SVX/VAS males (Fig. 2L), suggesting that the increase at 8 h after mating is due to factors other than sperm or seminal vesicle-derived agents. For *Ccl5*, expression in both mating groups was not different to the unmated control group (Fig. 2M).

For some genes induced by seminal fluid, including *Csf3, Cxcl1, Cxcl2,* and *Cxcl10*, there was partial induction of expression after mating with SVX/VAS males compared to unmated control mice. This shows that factors other than sperm or seminal vesicle-derived agents contribute to induction of these genes after mating. This was particularly evident for *Cxcl1* and *Cxcl10*, where approximately 50% and 30%, respectively, of the relative increase was seen after mating in the absence of seminal fluid stimulus (Fig. 2, B and D). For the other genes *Csf2, Il1a, Il1b, Il6, Lif, Tnf,* and *Ccl21*, where there was no difference between expression in unmated mice and mice mated with SVX/VAS males, the vast majority of the effect was fully attributable to seminal fluid.

Endometrial Cytokine/Chemokine Protein Synthesis Is Induced by Seminal Fluid

To further confirm the impact of seminal fluid on cytokine and chemokine synthesis, proteins were quantified in uterine luminal fluid using multiplex microbead technology. Although polarized secretory behavior in uterine epithelial cells [34] implies the cytokine profile of luminal fluid likely has only partial overlap with the profile in the subepithelial stroma, inflammatory leukocytes are recruited into both compartments

TABLE 2. The top 20 differentially expressed endometrial genes regulated by seminal fluid following coitus, identified by microarray.

Gene symbol	Accession number	*P* value	Fold-change	Immune role
Up-regulated				
Sprr2d	NM_011470	8.74E−5	6.12	–
Peg10	NM_130877	8.83E−5	5.25	Negative regulation of TGFb signaling
Sprr2e	NM_011471	6.92E−5	5.08	–
Cxcl5	NM_009141	3.61E−4	4.66	Chemokine-LIX
Tnfaip2	ENSMUST00000102745	0.003	4.46	Induced by TNFα
Chi3l1	NM_007695	0.004	3.76	Inflammation and tissue remodeling
Clca4	NM_139148	0.001	3.71	–
Il1b	NM_008361	0.001	3.62	Cytokine
Cxcl2	ENSMUST00000075433	0.013	3.34	Chemokine-MIP2α
Cyp51	ENSMUST00000001507	0.001	3.07	–
Down-regulated				
Klk1b5	NM_008456	0.043	−3.39	–
Abcb1b	NM_011075	0.005	−2.69	Cellular response to TNFα
Mettl7a1	NM_027334	2.35E−4	−2.46	–
Akr1c18	NM_134066	0.001	−2.37	–
Wfdc16	NM_001012723	0.007	−2.21	–
Mir30e	NR_029602	0.022	−2.19	Regulates NK cell cytotoxicity
Il18	NM_008360	0.001	−2.18	Cytokine
Rasa4	NM_133914	3.23E−4	−2.07	–
Sult1d1	NM_016771	0.021	−2.05	–
Sv2b	NM_153579	5.74E−05	−2.04	–

in the female postmating response [5, 7, 10]. Luminal fluid was collected from CBAF1 females 8 h after mating with intact or SVX/VAS males and compared to fluid from unmated estrous controls. At 8 h postcoitus, luminal fluid levels of CSF3, CXCL1, CXCL2, CXCL10, IL6, LIF, and TNF (Fig. 3A–G) were significantly increased following exposure to seminal fluid at coitus. In contrast, IL1B levels were not significantly changed, but notably the concentration of IL1B in luminal fluid was close to the limit of detection in all groups (Fig. 3H). We are confident that these changes are due to endometrial synthesis of cytokines, consistent with the gene expression changes seen in vivo because minimal or substantially lower levels of these cytokines are detectable in murine seminal fluid even before dilution in the uterus [35].

Tlr4 *Is Down-Regulated by Seminal Fluid*

Because TLR4 was identified as a candidate upstream regulator of seminal fluid signaling, we evaluated the possibility that seminal fluid factors activate the TLR4-signaling pathway. TLR4 has previously been shown to be expressed by epithelial and stromal cells in the mouse endometrium [36]. The microarray data showed *Tlr4* expression was 1.4-fold down-regulated in the endometrium following exposure to seminal fluid. Altered expression was confirmed by qPCR where *Tlr4* was elevated 1.5-fold in endometrium of females mated with SVX/VAS group compared to the unmated estrous control, but decreased by 25% in endometrium of females mated with intact males, resulting in a 2.0-fold difference in expression between the intact and SVX/VAS groups (Fig. 2N).

TABLE 3. Signaling pathways predicted as activated in vivo in the endometrium following exposure to seminal fluid at coitus.

Ingenuity Canonical Pathways
Superpathway of cholesterol biosynthesis
LXR/RXR activation
Granulocyte adhesion and diapedesis
IL10 signaling
Role of hypercytokinemia/hyperchemokinemia in the pathogenesis of influenza
Role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis
Role of cytokines in mediating communication between immune cells
PPAR signaling
Role of osteoblasts, osteoclasts, and chondrocytes in rheumatoid arthritis
Atherosclerosis signaling
Graft-versus-host disease signaling
Altered T cell and B cell signaling in rheumatoid arthritis
IL6 signaling
Acute phase response signaling

Tlr4 *Ligation in Uterine Epithelial Cells Elicits Cytokine Synthesis*

An in vitro uterine epithelial cell culture experiment was then undertaken to further explore whether TLR4 activation

TABLE 4. Differentially expressed (>1.5-fold) endometrial cytokine and chemokine genes regulated by seminal fluid following coitus, identified by microarray.

Gene symbol	Accession number	*P* value	Fold-change
Up-regulated			
Cxcl5	NM_009141	3.61E−04	4.66
Il1b	NM_008361	6.06E−04	3.62
Cxcl2	ENSMUST00000075433	0.013	3.34
Il1f6	NM_019450	0.001	3.07
Ccl2	NM_011333	0.017	3.06
Il17c	NM_145834	0.014	2.85
Il1f9	NM_153511	0.002	2.77
Cxcl1	NM_008176	0.004	2.61
Cxcl3	NM_203320	0.014	2.49
Tnf	NM_013693	0.004	2.4
Csf3	ENSMUST00000038886	2.58E−06	2.19
Lif	NM_008501	0.001	2.04
Cxcl10	NM_021274	0.019	1.96
Il1a	NM_010554	0.024	1.64
Il6	NM_031168	0.011	1.6
Csf1	NM_007778	0.016	1.57
Down-regulated			
Tgfb3	NM_009368	0.001	−1.66
Ccl21b	NM_011335	0.004	−1.67
Il18	NM_008360	0.001	−2.18

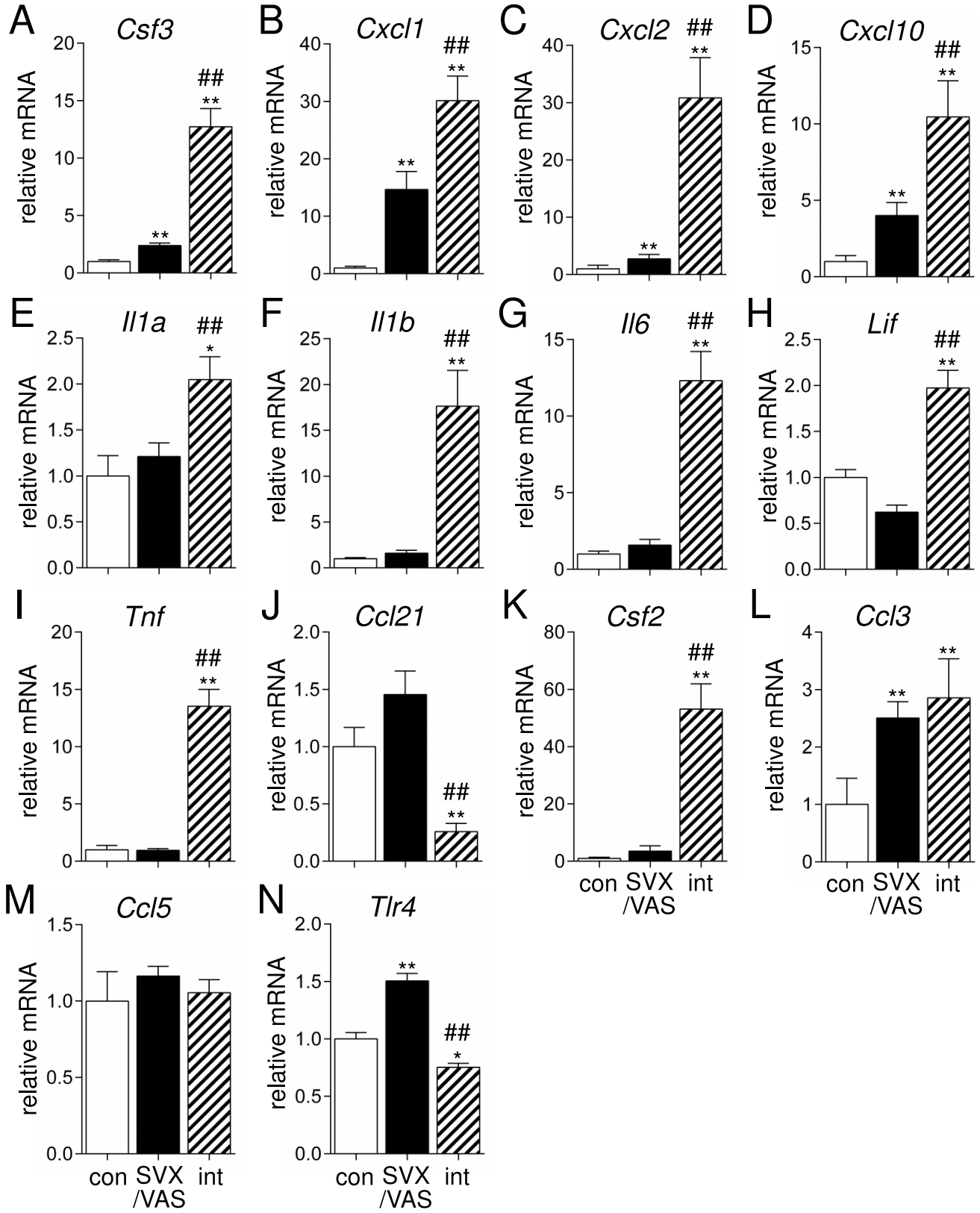

FIG. 2. Effect of exposure to seminal fluid at coitus on cytokine and chemokine mRNA expression in the endometrium. Endometrial tissue was collected from female mice 8 h following mating with either seminal vesicle deficient and vasectomized (SVX/VAS) or intact (int) Balb/c males. As an additional control, endometrial tissue was recovered prior to ovulation from unmated virgin estrous females (con). Expression levels were calculated by qPCR using the delta C(t) method with *Actb* as housekeeping gene. *Csf3* (**A**), *Cxcl1* (**B**), *Cxcl2* (**C**), *Cxcl10* (**D**), *Il1a* (**E**), *Il1b* (**F**), *Il6* (**G**), *Lif* (**H**), *Tnf* (**I**), *Ccl21* (**J**), *Csf2* (**K**), *Ccl3* (**L**), *Ccl5* (**M**), and *Tlr4* (**N**). Data are mean ± SEM expression relative to the unmated control from n = 15–22 mice per group and were analyzed using Kruskal-Wallis *H*-test and Mann-Whitney *U*-test. $^{*}P < 0.05$ compared to control, $^{**}P < 0.01$ compared to control, $^{\#\#}P < 0.01$ compared to SVX/VAS.

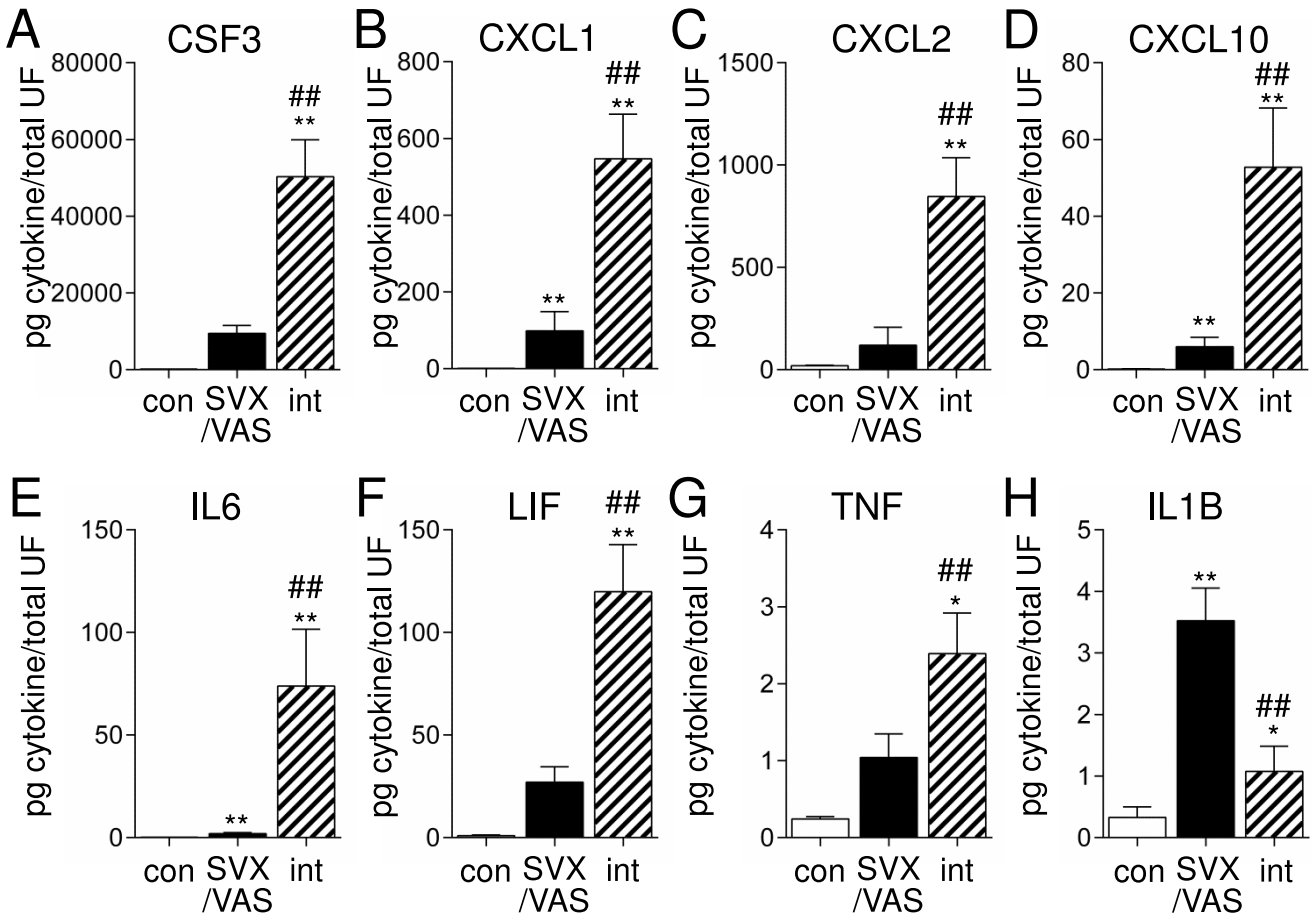

FIG. 3. Effect of exposure to seminal fluid at coitus on cytokine and chemokine protein in mouse uterine luminal fluid. Luminal fluid was collected from female mice 8 h following coitus with either seminal vesicle deficient and vasectomized (SVX/VAS) or intact (int) Balb/c males. As an additional control, luminal fluid was recovered prior to ovulation from unmated virgin estrous females (con). Cytokine/chemokine levels were calculated by multiplex microbead assay or ELISA. CSF3 (**A**), CXCL1 (**B**), CXCL2 (**C**), CXCL10 (**D**), IL6 (**E**), LIF (**F**), TNF (**G**), and IL1B (**H**). Data are mean ± SEM pg cytokine/total uterine fluid from n = 8–13 per group and were analyzed using Kruskal-Wallis *H*-test and Mann-Whitney *U*-test. *$P < 0.05$ compared to control. **$P < 0.01$ compared to control, ##$P < 0.01$ compared to SVX/VAS.

may contribute to seminal fluid signaling. The capacity of TLR4 activation to elicit cytokine secretion was examined following addition of the archetypal TLR4 ligand LPS to uterine epithelial cells recovered from estrous CBAF1 mice. As a comparison, uterine epithelial cells were treated with TGFB, shown previously to at least partly mediate the effect of seminal fluid in inducing cytokine release from mouse and human uterine epithelial cells [21, 22].

Ligation of TLR4 with LPS induced dose-dependent increases in several of the same cytokines induced by seminal fluid and predicted to be regulated by TLR4 ligation, including CSF3, CXCL1, CXCL2, IL1A, and TNF (Fig. 4, A–E). Additionally, TLR4 ligation induced secretion of CSF2 (Fig. 4F). As expected, TGFB treatment elicited increased production of CSF2 from uterine epithelial cells in a dose-dependent manner (Fig. 4F) and also elevated IL1A and TNF moderately (Fig. 4, D and E). In contrast, TGFB substantially suppressed secretion of CSF3, CXCL1, and CXCL2 (Fig. 4, A–C). IL6, CXCL10, and LIF secretion were not significantly altered by treatment with either TGFB or LPS (Fig. 4, G–I).

The Female Tract Response to Seminal Fluid Is Disrupted in Tlr4$^{-/-}$ *Mice*

To confirm a role for TLR4, the effect of *Tlr4* null mutation on the endometrial cytokine and chemokine response to seminal fluid was examined. When endometrial gene expression was compared by qPCR in *Tlr4*$^{-/-}$ and wild-type *Tlr4*$^{+/+}$ mice 8 h after mating with intact or SVX/VAS C57Bl/6 males, a significant effect of TLR4 deficiency was seen, with impaired induction of cytokines shown in the previous in vitro experiment to be regulated by TLR4 activation. Comparison between expression after mating with SVX/VAS males versus intact males shows that *Csf3, Cxcl2, Il6,* and *Tnf* were all induced following exposure to seminal fluid in control mice, but were not induced in *Tlr4*$^{-/-}$ mice (Fig. 5, A–D). This was also reflected as significantly lower expression in the absence of TLR4, with 2.1-fold lower C*sf3* ($P = 0.020$), 46.0-fold lower *Cxcl2* ($P = 0.003$), 7.2-fold lower *Il6* ($P = 0.007$), and 6.4-fold lower *Tnf* ($P = 0.005$) observed in *Tlr4*$^{-/-}$ compared with *Tlr4*$^{+/+}$ mice mated with intact males. In contrast, TLR4 deficiency did not alter the capacity of seminal fluid to induce endometrial expression of other cytokine genes identified in the in vitro experiment as induced by both LPS and TGFB, including *Csf2* and *IL1a* (data not shown).

Additionally, *Cxcl10* and *Lif* were induced following exposure to seminal fluid in control mice, but were not

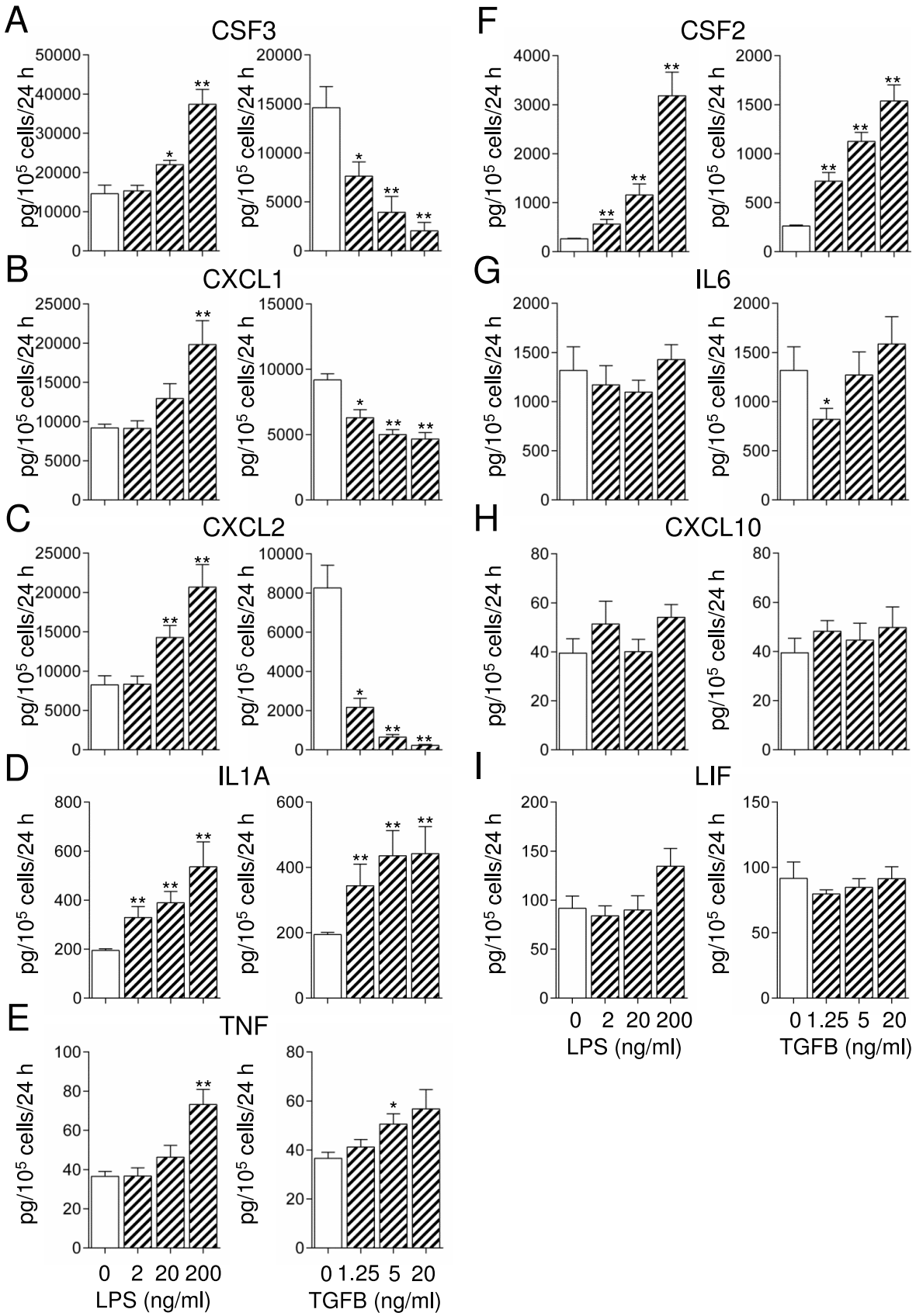

FIG. 4. Effect of the TLR4 agonist, LPS, and TGFB on cytokine and chemokine secretion in mouse uterine epithelial cells in vitro. Uterine epithelial cells were stimulated with LPS (2, 20, or 200 ng/ml), TGFB (1.25, 5, 20 ng/ml), or control (medium alone), and supernatants were recovered for cytokine analysis. CSF3 (**A**), CXCL1 (**B**), CXCL2 (**C**), IL1A (**D**), TNF (**E**), CSF2 (**F**), IL6 (**G**), CXCL10 (**H**), and LIF (**I**). Data are mean ± SEM cytokine output in pg/10^5 cells from n = 5 wells per group and were analyzed using Kruskal-Wallis *H*-test and Mann-Whitney *U*-test. *$P < 0.05$, **$P < 0.01$ compared to control.

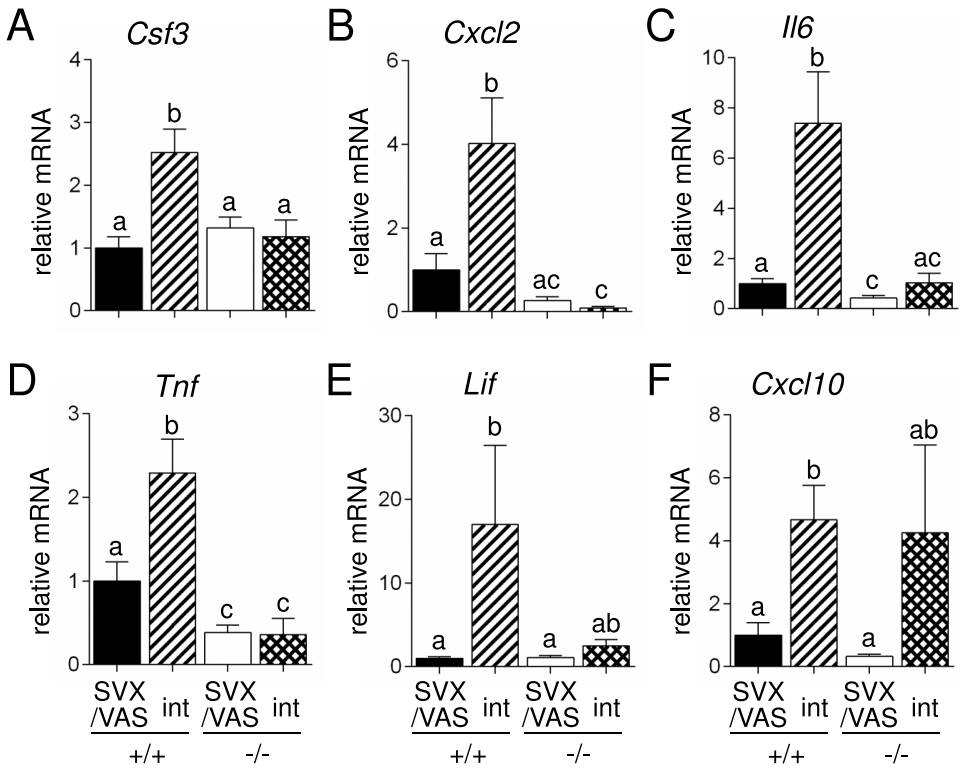

FIG. 5. TLR4 is a major mediator in the female tract response to seminal fluid. Endometrial tissue was collected from wild-type *Tlr4*$^{+/+}$ (+/+) or *Tlr4*$^{-/-}$ (–/–) females 8 h following mating with either seminal vesicle deficient and vasectomized (SVX/VAS) or intact (int) C57Bl/6 males. RNA was extracted and gene expression was measured by qPCR. Expression levels were calculated using the delta C(t) method with *Actb* as housekeeping gene. *Csf3* (**A**), *Cxcl2* (**B**), *Il6* (**C**), *Tnf* (**D**), *Lif* (**E**), and *Cxcl10* (**F**). Data are mean ± SEM expression relative to the wild-type SVX/VAS mated control from n = 5–9 samples per group and were analyzed using Kruskal-Wallis *H*-test and Mann-Whitney *U*-test. Different superscripts represent different statistical significance between groups.

induced (*Lif*) or more variably induced (*Cxcl10*) in *Tlr4*$^{-/-}$ mice (Fig. 5, E and F). These two cytokines, neither of which responded to TLR4 activation in the in vitro experiment, did not show a significant difference between genotypes after mating with intact males (Fig. 5, E and F), suggesting that while TLR4 signaling may contribute to their induction, other factors may have at least a partial compensatory role.

Bacterial Endotoxin in Uterine Fluid after Mating

Finally, we examined whether bacterial LPS accessing the female reproductive tract might be responsible for activation of TLR4 after coitus. Uterine luminal fluid containing deposited seminal fluid was collected from Balb/c females 1–2 h after mating with C57Bl/6 males and endotoxin levels were measured. The mean ± SEM level of endotoxin detected in luminal fluids from n = 8 mice was 2.3 ± 1.0 ng/ml (range: 0.1–8.1 ng/ml).

DISCUSSION

Following mating in mice, the uterus undergoes an inflammation-like response characterized by recruitment of granulocytes and monocytes, and accompanied by synthesis of several proinflammatory cytokines and chemokines, in a process that facilitates immune adaptation and development of receptivity for pregnancy. Studies in mice and in women implicate seminal fluid factors, including TGFB in seminal plasma, as instrumental in activating this process [21, 22]. However whether seminal fluid TGFB is responsible for induction of all of the genes involved in the postmating inflammatory response seems unlikely [22]. The experiments in this study aimed to confirm that seminal fluid factors are indeed responsible for gene induction and to identify novel signaling pathways that are utilized in addition to TGFB.

Our results show that seminal fluid is required to elicit the increased expression of a wide range of cytokine genes induced during the postmating response. *Csf2*, *Csf3, Cxcl1, Cxcl2, Cxcl10*, *Il1a, Il1b, Il6, Lif,* and *Tnf* were all identified as induced between 2- and 50-fold following coitus with intact males, but not induced or substantially less induced when males were rendered surgically deficient in seminal fluid by vasectomy and excision of seminal vesicles. A similar pattern of requirement for contact with seminal fluid in cytokine proteins CSF3, CXCL1, CXCL2, CXCL10, IL6, LIF, and TNF confirms that endometrial gene expression is accompanied by increased protein synthesis.

For other genes, including *Ccl3*, *Csf3, Cxcl1, Cxcl2,* and *Cxcl10,* complete or partial induction in females mated with SVX/VAS males compared with estrous mice killed 8–9 h earlier in the estrous cycle immediately prior to ovulation suggests that a shift in ovarian steroid hormone levels, the neuro-endocrine stimulus of male contact and the mating event, and/or the introduction of microorganisms at mating may also contribute to gene regulation. An action of factors in residual fluid intromitted by the male, potentially derived from the prostate or bulbourethral glands, cannot be excluded but seems unlikely given the small fraction of seminal fluid derived from these tissues in the mouse [37]. However, the substantially greater expression evident in the direct comparison between females mated with intact versus SVX/VAS males for all of these genes other than *Ccl3* clearly demonstrates that seminal

fluid-independent effects do not fully account for cytokine gene induction and instead show that contact with seminal fluid is essential to maximally induce expression after mating.

Analysis for upstream regulators of the differentially regulated genes identified the TLR4-signaling pathway as activated by seminal fluid following coitus. Using LPS as a model TLR4 ligand in uterine epithelial cell cultures as well as *Tlr4*$^{-/-}$ null mutant mice, we confirmed that endometrial induction of *Csf3, Cxcl2, Il6,* and *Tnf* expression after mating requires activation of TLR4 and that TLR4 may also contribute to induction of *Cxcl10* and *Lif*. These studies are the first to provide evidence for the TLR4-signaling pathway contributing to the postmating inflammatory response and thus the consequent immune adaptation for pregnancy.

The findings presented in this study extend earlier reports implicating seminal fluid in regulating the periconception cytokine environment [5–7]. Previous studies have suggested in vivo induction in the uterus at 12 h after mating of *Csf2, Cxcl1, Cxcl2,* and *Ccl3* using qualitative or semiquantitative PCR [5, 7], and *Ccl2*, *Ccl3, Ccl5,* and *Ccl6* using semiquantitative Northern blot [8, 9]. Additionally, the uterus has been shown to express *Csf1, Il1, Il6,* and *Tnf* on the day after mating [6, 8]. However, other than for *Csf2,* where in vitro experiments show epithelial cell gene induction with seminal plasma and reduced expression following mating with seminal vesicle excised males [5], these early studies did not formally demonstrate a specific role for seminal fluid in gene induction. More recent studies show uterine *Ccl19* is induced on Day 3.5 postcoitus when exposed to the complete ejaculate but not seminal vesicle-excised males [18], and the type 1 interferon *Ifne* is induced following exposure to seminal fluid on Day 0.5 postcoitus [38].

The current study confirms previous observations of inflammatory cytokine induction after mating [5–9], although notably one chemokine thought previously to be induced, CCL5 [7, 8], was not identified as differentially regulated in the microarray experiment. These data are also consistent with a recent study showing that cytokines induced by seminal plasma extend to the oviduct, with oviduct *Csf2, Lif,* and *Il6* induced by seminal plasma [20].

Comparison with studies in human cervical tissue biopsies taken after coitus or in cervical cells incubated with seminal fluid in vitro [22, 39] shows extensive similarity in the responses induced by seminal fluid in mice and women. In women, CSF2, IL6, IL8, and IL1A were confirmed by qPCR to be induced in the cervix by seminal fluid, while microarray implicated several other genes overlapping with those identified in the current mouse study, including *CXCL1, CXCL2,* and *IL1B,* while *CCL5* was not regulated by seminal fluid [22, 39].

The current study identifies some cytokines induced by seminal fluid not previously reported to be elevated in the female tract during the postmating phase. Notably CSF3 was >4-fold higher at the mRNA level and >5-fold higher at the protein level when seminal fluid was present. CSF3 is emerging as a potentially important player in early pregnancy, related to its capacity to generate T cell tolerance [40]. In human, CSF3 promotes the generation of regulatory immune responses through expanding tolerogenic dendritic cells and T regulatory cells [41, 42]. In mouse models of pregnancy, CSF3 produced by invading trophoblast cells is postulated to recruit macrophages and modulate their activity, inducing a switch toward a tolerogenic M2-like phenotype [43]. A randomized controlled study indicates CSF3 has efficacy in the treatment of primary unexplained recurrent miscarriage [44]. Further work is required to determine the extent to which CSF3 is involved in programming maternal immune tolerance at conception.

The identification of TLR4 as a key upstream regulator adds to our existing knowledge of TGFB as a major signaling factor in seminal fluid [21–24]. Other components in seminal fluid that are postulated to play a role in seminal fluid signaling include prostaglandins [45, 46], IL8 [47], cysteine-rich secretory protein 3 [48], and in pig, porcine-sperm-adhesion proteins [49].

TLR4 has previously been identified to be expressed in the uterus of both mice [34] and human [50, 51] as well as in the human cervix [52]. In the current study, we observed up-regulation of *Tlr4* expression in endometrium of females mated to SVX/VAS males compared to unmated females, suggesting that factors other than seminal fluid such as hormone changes around ovulation induce *Tlr4* at the mRNA level. However exposure to seminal fluid at coitus decreased *Tlr4* expression, suggesting a possible negative feedback after ligation of TLR4 by seminal fluid constituents. This is consistent with previous reports of *Tlr4* down-regulation after ligation in macrophages and neural tissue [53–55].

The TLR4 expressed by uterine epithelial cells is able to bind ligands and transmit signals delivered in seminal fluid because the in vitro experiment in uterine epithelial cell cultures and in vivo experiment in *Tlr4* null mutant mice provide clear evidence that synthesis of seminal fluid-induced cytokines CSF3, CXCL2, IL6, and TNF is elevated at the transcriptional level by TLR4 activation, in agreement with previous studies showing LPS induces CSF3, IL6, and TNF in mouse uterine epithelial cells in a polarized fashion [34]. For TNF, CSF2, and IL1A, the in vitro experiment suggests both TLR4 ligands and TGFB can induce synthesis, which explains why CSF2 and IL1A were not inhibited after mating in *Tlr4*$^{-/-}$ mice (data not shown). The anomaly between TLR4-mediated induction of *Il6* in vivo and absence of induction by LPS in our in vitro assays is likely to reflect effects of dose or timing of exposure in vitro because IL6 release from mouse uterine epithelial cells in response to LPS has been previously reported [34].

The precise components of seminal fluid capable of ligating TLR4 are yet to be identified. The presence of TLRs on uterine epithelial cells has been predicted to activate cytokines and chemokines to aid in pathogen defense [51]. TLRs are commonly recognized as interacting with PAMPS such as LPS, a component of the cell membrane of gram negative bacteria. Bacterial LPS is low but detectable in human seminal fluid and varies between individuals [56] (Sharkey and Robertson, manuscript in preparation). In the current study, the level of LPS detectable in the female reproductive tract shortly after coitus was very low. LPS of male origin or from bacteria redistributed in the female tract is unlikely to explain TLR4 activation after coitus because a similar 2 ng/ml concentration of LPS had no effect on TLR4-regulated cytokines in uterine epithelial cells in vitro. These findings suggest that TLR4 ligands in seminal fluid other than LPS may mediate the female tract response.

In addition to TLR4 activation by foreign molecules, the presence of endogenous DAMPs in seminal fluid provides alternative ligands for TLR4 signaling. DAMPs, including beta defensin-2 [57], fibronectin [58], heat shock proteins [59, 60], hyaluronan and hyaluronanidase [61, 62], and S100 proteins [59, 63], are known to activate TLR4 [64], and some have been identified in seminal fluid of various species. Further studies are required to identify the critical TLR4 ligands present in seminal fluid, and whether they are of endogenous or microbial origin.

TLR4 activation is generally considered as a mechanism to potently activate the innate immune response to remove foreign pathogens or deal with tissue injury, which might seem at odds with the requirement for immune tolerance in early pregnancy. However, there is evidence that activation of TLR4 can generate protolerance as well as inflammatory responses to limit damage during inflammation [65]. Indeed TLR4 has been suggested to act directly or indirectly to induce the differentiation, activation, and proliferation of Treg cells [66–68]. In mice, $CD45RB^{low}$ $CD25^{+}$ regulatory T cells, which function to control inflammatory responses to commensal bacteria and pathogens, selectively express TLR4 and enhance their suppressive function following exposure to LPS [66]. Dendritic cells from TLR4-deficient mice have reduced capacity to produce IL10 in response to *Bordetella pertussis* LPS, impairing expansion of inducible Treg cells [67]. Further, LPS-treated Treg cells cultured in the presence of dendritic cells can induce tryptophan catabolism through a cytokine-dependent pathway, suggesting that TLR-activated Treg cells can enhance tolerogenic dendritic cell production [68]. In the context of seminal fluid exposure, TLR4 activation by PAMPs or DAMPs may thus plausibly contribute to the establishment of maternal immune tolerance induced by seminal fluid [18].

Despite the role for TLR4 in the female response to seminal fluid, *Tlr4*$^{-/-}$ mice are reproductively competent [54], so female TLR4 deficiency at coitus does not result in complete infertility, at least in syngeneic pregnancy. However, our recent studies demonstrate that *Tlr4*$^{-/-}$ mice mated with genotype-matched males exhibit impaired reproductive outcomes with delayed labor and a 35% reduction in litter size, attributable to late gestation and early postnatal loss of pups [69]. It is possible that an altered immune environment from conception contributes to this late gestation fetal loss in *Tlr4*$^{-/-}$ mice because seminal fluid signaling affects not only the periconception immune environment but also has consequences for later fetal growth and offspring health [20]. When conception occurs in the absence of seminal plasma, placental hypertrophy is evident in late gestation and offspring exhibit an altered growth trajectory and metabolic dysfunction [20]. It remains to be determined whether and how seminal fluid-activated TLR4 signaling at conception contributes to later gestation events and offspring health, and whether any effects of perturbing this pathway are increased in allogeneic pregnancy, when the requirement for maternal immune tolerance is elevated.

Clinical studies provide consistent evidence for a beneficial effect of seminal plasma exposure on outcomes of human pregnancy. Limited exposure to partner's seminal fluid has been correlated with immunological disorders of pregnancy particularly preeclampsia [70–75]. Exposure to semen around the time of embryo transfer improves embryo viability 6–8 wk posttransfer [76]. The absence of seminal plasma in in vitro fertilization may be one of several factors contributing to altered birthweights and health outcomes for in vitro fertilized children [77]. Understanding the mechanisms by which seminal fluid establishes this favorable periconception immune environment, and the contribution of TLR4 to this process, will be informative in understanding how disruptions to seminal fluid signaling may influence the outcomes of pregnancy.

In conclusion, this study advances knowledge of the immune changes induced in the mouse by seminal fluid and provides evidence that TLR4 is a key agent mediating seminal fluid signaling. This expands our understanding of the mechanism by which seminal fluid interacts with female tissues to initiate the periconception immune response and generate maternal immune tolerance to paternal antigens. Future studies will elucidate the signaling molecules present in seminal fluid that ligate TLR4 and examine how interfering with TLR4-mediated signaling may contribute to impaired reproductive outcomes in *Tlr4*$^{-/-}$ mice.

REFERENCES

1. Robertson SA, Petroff MG, Hunt JS. Immunology of Pregnancy. In: Plant TM, Zeleznik AJ (eds.), Knobil and Neill's Physiology of Reproduction, 4th ed. Oxford, UK: Elsevier; 2015:1835–1874.
2. Aluvihare VR, Kallikourdis M, Betz AG. Regulatory T cells mediate maternal tolerance to the fetus. Nat Immunol 2004; 5:266–271.
3. Robertson SA, Prins JR, Sharkey DJ, Moldenhauer LM. Seminal fluid and the generation of regulatory T cells for embryo implantation. Am J Reprod Immunol 2013; 69:315–330.
4. Schjenken J, Robertson S. Seminal fluid and immune adaptation for pregnancy–comparative biology in Mammalian species. Reprod Domest Anim 2014; 49(Suppl 3):27–36.
5. Robertson SA, Mau VJ, Tremellen KP, Seamark RF. Role of high molecular weight seminal vesicle proteins in eliciting the uterine inflammatory response to semen in mice. J Reprod Fertil 1996; 107: 265–277.
6. Sanford TR, De M, Wood GW. Expression of colony-stimulating factors and inflammatory cytokines in the uterus of CD1 mice during days 1 to 3 of pregnancy. J. Reprod. Fertil 1992; 94:213–220.
7. Robertson SA, Allanson M, Mau VJ. Molecular regulation of uterine leukocyte recruitment during early pregnancy in the mouse. Placenta 1998; 19:101–119.
8. Pollard JW, Lin EY, Zhu L. Complexity in uterine macrophage responses to cytokines in mice. Biol Reprod 1998; 58:1469–1475.
9. Wood GW, Hausmann EH, Kanakaraj K. Expression and regulation of chemokine genes in the mouse uterus during pregnancy. Cytokine 1999; 11:1038–1045.
10. Robertson SA, Mayrhofer G, Seamark RF. Uterine epithelial cells synthesize granulocyte-macrophage colony-stimulating factor and interleukin-6 in pregnant and nonpregnant mice. Biol Reprod 1992; 46: 1069–1079.
11. Jasper MJ, Bromfield JJ, Skinner R, Robertson SA. 242. LIF expression is induced in the mouse oviduct following activation by seminal factors. Reprod Fertil Dev 2005; 17:96–96.
12. Gangnuss S, Sutton-McDowall ML, Robertson SA, Armstrong DT. Seminal plasma regulates corpora lutea macrophage populations during early pregnancy in mice. Biol Reprod 2004; 71:1135–1141.
13. Robertson SA, Sjoblom C, Jasper MJ, Norman RJ, Seamark RF. Granulocyte-macrophage colony-stimulating factor promotes glucose transport and blastomere viability in murine preimplantation embryos. Biol Reprod 2001; 64:1206–1215.
14. Jasper MJ, Care AS, Sullivan B, Ingman WV, Aplin JD, Robertson SA. Macrophage-derived LIF and IL1B regulate alpha(1,2)fucosyltransferase 2 (Fut2) expression in mouse uterine epithelial cells during early pregnancy. Biol Reprod 2011; 84:179–188.
15. Robertson SA, Roberts CT, Farr KL, Dunn AR, Seamark RF. Fertility impairment in granulocyte-macrophage colony-stimulating factor-deficient mice. Biol Reprod 1999; 60:251–261.
16. Moldenhauer LM, Diener KR, Thring DM, Brown MP, Hayball JD, Robertson SA. Cross-presentation of male seminal fluid antigens elicits T cell activation to initiate the female immune response to pregnancy. J Immunol 2009; 182:8080–8093.
17. Robertson SA. Immune regulation of conception and embryo implantation–all about quality control? J Reprod Immunol 2010; 85:51–57.
18. Guerin LR, Moldenhauer LM, Prins JR, Bromfield JJ, Hayball JD, Robertson SA. Seminal fluid regulates accumulation of FOXP3(+) regulatory T cells in the preimplantation mouse uterus through expanding the FOXP3(+) cell pool and CCL19-mediated recruitment. Biol Reprod 2011; 85:397–408.
19. Sharkey DJ, Macpherson AM, Tremellen KP, Robertson SA. Seminal plasma differentially regulates inflammatory cytokine gene expression in human cervical and vaginal epithelial cells. Mol Hum Reprod 2007; 13: 491–501.
20. Bromfield JJ, Schjenken JE, Chin PY, Care AS, Jasper MJ, Robertson SA. Maternal tract factors contribute to paternal seminal fluid impact on metabolic phenotype in offspring. Proc Natl Acad Sci U S A 2014; 111: 2200–2205.
21. Tremellen KP, Seamark RF, Robertson SA. Seminal transforming growth factor beta1 stimulates granulocyte-macrophage colony-stimulating factor production and inflammatory cell recruitment in the murine uterus. Biol Reprod 1998; 58:1217–1225.

22. Sharkey DJ, Macpherson AM, Tremellen KP, Mottershead DG, Gilchrist RB, Robertson SA. TGF-beta mediates proinflammatory seminal fluid signaling in human cervical epithelial cells. J Immunol 2012; 189: 1024–1035.
23. O'Leary S, Armstrong DT, Robertson SA. Transforming growth factor-beta (TGFbeta) in porcine seminal plasma. Reprod Fertil Dev 2011; 23: 748–758.
24. Scott JL, Ketheesan N, Summers PM. Presence of TGFbeta but not IL-8 or GM-CSF in ram seminal plasma. In: Society of Reproductive Biology, Gold Coast, Queensland, 2006: abstract 49.
25. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 2003; 198:1875–1886.
26. Robertson SA, Guerin LR, Bromfield JJ, Branson KM, Ahlstrom AC, Care AS. Seminal fluid drives expansion of the CD4+CD25+ T regulatory cell pool and induces tolerance to paternal alloantigens in mice. Biol Reprod 2009; 80:1036–1045.
27. Clark DA, Fernandes J, Banwatt D. Prevention of spontaneous abortion in the CBA x DBA/2 mouse model by intravaginal TGF-beta and local recruitment of CD4+8+ FOXP3+ cells. Am J Reprod Immunol 2008; 59: 525–534.
28. Robertson SA, Ingman WV, O'Leary S, Sharkey DJ, Tremellen KP. Transforming growth factor beta-a mediator of immune deviation in seminal plasma. J Reprod Immunol 2002; 57:109–128.
29. Robertson SA, O'Connell AC, Hudson SN, Seamark RF. Granulocyte-macrophage colony-stimulating factor (GM-CSF) targets myeloid leukocytes in the uterus during the post-mating inflammatory response in mice. J Reprod Immunol 2000; 46:131–154.
30. Hoshino K, Takeuchi O, Kawai T, Sanjo H, Ogawa T, Takeda Y, Takeda K, Akira S. Cutting edge: Toll-like receptor 4 (TLR4)-deficient mice are hyporesponsive to lipopolysaccharide: evidence for TLR4 as the Lps gene product. J Immunol 1999; 162:3749–3752.
31. Robertson SA, Mayrhofer G, Seamark RF. Ovarian steroid hormones regulate granulocyte-macrophage colony-stimulating factor synthesis by uterine epithelial cells in the mouse. Biol Reprod 1996; 54:183–196.
32. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res 2002; 30:207–210.
33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25:402–408.
34. Soboll G, Shen L, Wira CR. Expression of Toll-like receptors (TLR) and responsiveness to TLR agonists by polarized mouse uterine epithelial cells in culture. Biol Reprod 2006; 75:131–139.
35. Gopichandran N, Ekbote UV, Walker JJ, Brooke D, Orsi NM. Multiplex determination of murine seminal fluid cytokine profiles. Reproduction 2006; 131:613–621.
36. Sheldon IM, Roberts MH. Toll-like receptor 4 mediates the response of epithelial and stromal cells to lipopolysaccharide in the endometrium. PLoS One 2010; 5:e12906.
37. Mann T. The Biochemistry of Semen and the Male Reproductive Tract. New York, NY: John Wiley and Sons, Inc.; 1964.
38. Fung KY, Mangan NE, Cumming H, Horvat JC, Mayall JR, Stifter SA, De Weerd N, Roisman LC, Rossjohn J, Robertson SA, Schjenken JE, Parker B, et al. Interferon-epsilon protects the female reproductive tract from viral and bacterial infection. Science 2013; 339:1088–1092.
39. Sharkey DJ, Tremellen KP, Jasper MJ, Gemzell-Danielsson K, Robertson SA. Seminal fluid induces leukocyte recruitment and cytokine and chemokine mRNA expression in the human cervix after coitus. J Immunol 2012; 188:2445–2454.
40. Rutella S, Zavala F, Danese S, Kared H, Leone G. Granulocyte colony-stimulating factor: a novel mediator of T cell tolerance. J Immunol 2005; 175:7085–7091.
41. Rutella S, Pierelli L, Bonanno G, Sica S, Ameglio F, Capoluongo E, Mariotti A, Scambia G, d'Onofrio G, Leone G. Role for granulocyte colony-stimulating factor in the generation of human T regulatory type 1 cells. Blood 2002; 100:2562–2571.
42. Rutella S, Bonanno G, Pierelli L, Mariotti A, Capoluongo E, Contemi AM, Ameglio F, Curti A, De Ritis DG, Voso MT, Perillo A, Mancuso S, et al. Granulocyte colony-stimulating factor promotes the generation of regulatory DC through induction of IL-10 and IFN-alpha. Eur J Immunol 2004; 34:1291–1302.
43. Litwin S, Lagadari M, Barrientos G, Roux ME, Margni R, Miranda S. Comparative immunohistochemical study of M-CSF and G-CSF in feto-maternal interface in a multiparity mouse model. Am J Reprod Immunol 2005; 54:311–320.
44. Scarpellini F, Sbracia M. Use of granulocyte colony-stimulating factor for the treatment of unexplained recurrent miscarriage: a randomised controlled trial. Hum Reprod 2009; 24:2703–2708.
45. Claus R, Dimmick MA, Gimenez T, Hudson LW. Estrogens and prostaglandin F2alpha in the semen and blood plasma of stallions. Theriogenology 1992; 38:687–693.
46. Templeton AA, Cooper I, Kelly RW. Prostaglandin concentrations in the semen of fertile men. J Reprod Fertil 1978; 52:147–150.
47. Gutsche S, von Wolff M, Strowitzki T, Thaler CJ. Seminal plasma induces mRNA expression of IL-1beta, IL-6 and LIF in endometrial epithelial cells in vitro. Mol Hum Reprod 2003; 9:785–791.
48. Doty A, Buhi WC, Benson S, Scoggin KE, Pozor M, Macpherson M, Mutz M, Troedsson MH. Equine CRISP3 modulates interaction between spermatozoa and polymorphonuclear neutrophils. Biol Reprod 2011; 85: 157–164.
49. Rodriguez-Martinez H, Saravia F, Wallgren M, Martinez EA, Sanz L, Roca J, Vazquez JM, Calvete JJ. Spermadhesin PSP-I/PSP-II heterodimer induces migration of polymorphonuclear neutrophils into the uterine cavity of the sow. J Reprod Immunol 2010; 84:57–65.
50. Krikun G, Lockwood CJ, Abrahams VM, Mor G, Paidas M, Guller S. Expression of Toll-like receptors in the human decidua. Histol Histopathol 2007; 22:847–854.
51. Schaefer TM, Desouza K, Fahey JV, Beagley KW, Wira CR. Toll-like receptor (TLR) expression and TLR-mediated cytokine/chemokine production by human uterine epithelial cells. Immunology 2004; 112: 428–436.
52. Wang Y, Weng Y, Shi Y, Xia X, Wang S, Duan H. Expression and functional analysis of Toll-like receptor 4 in human cervical carcinoma. J Membr Biol 2014; 247:591–599.
53. Laflamme N, Rivest S. Toll-like receptor 4: the missing link of the cerebral innate immune response triggered by circulating gram-negative bacterial cell wall components. FASEB J 2001; 15:155–163.
54. Poltorak A, He X, Smirnova I, Liu MY, Van Huffel C, Du X, Birdwell D, Alejos E, Silva M, Galanos C, Freudenberg M, Ricciardi-Castagnoli P, et al. Defective LPS signaling in C3H/HeJ and C57BL/10ScCr mice: mutations in Tlr4 gene. Science 1998; 282:2085–2088.
55. Nomura F, Akashi S, Sakao Y, Sato S, Kawai T, Matsumoto M, Nakanishi K, Kimoto M, Miyake K, Takeda K, Akira S. Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. J Immunol 2000; 164:3476–3479.
56. Fujita Y, Mihara T, Okazaki T, Shitanaka M, Kushino R, Ikeda C, Negishi H, Liu Z, Richards JS, Shimada M. Toll-like receptors (TLR) 2 and 4 on human sperm recognize bacterial endotoxins and mediate apoptosis. Hum Reprod 2011; 26:2799–2806.
57. Narciandi F, Lloyd A, Meade KG, O'Farrelly C. A novel subclass of bovine beta-defensins links reproduction and immunology. Reprod Fertil Dev 2014; 26:769–777.
58. Lilja H, Oldbring J, Rannevik G, Laurell CB. Seminal vesicle-secreted proteins and their reactions during gelation and liquefaction of human semen. J Clin Invest 1987; 80:281–285.
59. Rego JP, Crisp JM, Moura AA, Nouwens AS, Li Y, Venus B, Corbet NJ, Corbet DH, Burns BM, Boe-Hansen GB, McGowan MR. Seminal plasma proteome of electroejaculated Bos indicus bulls. Anim Reprod Sci 2014; 148:1–17.
60. Pilch B, Mann M. Large-scale and high-confidence proteomic analysis of human seminal plasma. Genome Biol 2006; 7:R40.
61. Geipel U, Kropf J, Krause W, Gressner AM. The concentration pattern of laminin, hyaluronan, and aminoterminal propeptide of type III procollagen in seminal fluid. Andrologia 1992; 24:205–211.
62. Shimada M, Yanai Y, Okazaki T, Noma N, Kawashima I, Mori T, Richards JS. Hyaluronan fragments generated by sperm-secreted hyaluronidase stimulate cytokine/chemokine production via the TLR2 and TLR4 pathway in cumulus cells of ovulated COCs, which may enhance fertilization. Development 2008; 135:2001–2011.
63. Donato R, Cannon BR, Sorci G, Riuzzi F, Hsu K, Weber DJ, Geczy CL. Functions of S100 proteins. Curr Mol Med 2013; 13:24–57.
64. Yu L, Wang L, Chen S. Endogenous toll-like receptor ligands and their biological significance. J Cell Mol Med 2010; 14:2592–2603.
65. Conroy H, Marshall NA, Mills KH. TLR ligand suppression or enhancement of Treg cells? A double-edged sword in immunity to tumours. Oncogene 2008; 27:168–180.
66. Caramalho I, Lopes-Carvalho T, Ostler D, Zelenay S, Haury M, Demengeot J. Regulatory T cells selectively express toll-like receptors and are activated by lipopolysaccharide. J Exp Med 2003; 197:403–411.
67. Higgins SC, Lavelle EC, McCann C, Keogh B, McNeela E, Byrne P, O'Gorman B, Jarnicki A, McGuirk P, Mills KH. Toll-like receptor 4-

mediated innate IL-10 activates antigen-specific regulatory T cells and confers resistance to Bordetella pertussis by inhibiting inflammatory pathology. J Immunol 2003; 171:3119–3127.
68. Fallarino F, Pallotta MT, Matino D, Gargaro M, Orabona C, Vacca C, Mondanelli G, Allegrucci M, Boon L, Romani R, Talesa VN, Puccetti P, et al. LPS-conditioned dendritic cells confer endotoxin tolerance contingent on tryptophan catabolism. Immunobiology 2015; 220:315–321.
69. Wahid HH, Dorian C, Chin PY, Hutchinson MR, Rice KC, Olson DM, Moldenhauer LM, Robertson SA. Toll-like receptor 4 is an essential upstream regulator of on-time parturition and perinatal viability in mice. Endocrinology (in press). Published online ahead of print 7 July 2015; DOI: 10.1210/EN.2015-1089.
70. Dekker GA, Robillard PY, Hulsey TC. Immune maladaptation in the etiology of preeclampsia: a review of corroborative epidemiologic studies. Obstet Gynecol Surv 1998; 53:377–382.
71. Robillard PY, Hulsey TC, Alexander GR, Keenan A, de Caunes F, Papiernik E. Paternity patterns and risk of preeclampsia in the last pregnancy in multiparae. J Reprod Immunol 1993; 24:1–12.
72. Trupin LS, Simon LP, Eskenazi B. Change in paternity: a risk factor for preeclampsia in multiparas. Epidemiology 1996; 7:240–244.
73. Klonoff-Cohen HS, Savitz DA, Celafo RC, McCann MF. An epidemiologic study of contraception and preeclampsia. JAMA 1989; 262: 3143–3147.
74. Robillard PY, Hulsey TC, Perianin J, Janky E, Miri EH, Papiernik E. Association of pregnancy-induced hypertension with duration of sexual cohabitation before conception. Lancet 1994; 344:973–975.
75. Saftlas AF, Rubenstein L, Prater K, Harland KK, Field E, Triche EW. Cumulative exposure to paternal seminal fluid prior to conception and subsequent risk of preeclampsia. J Reprod Immunol 2014; 101–102: 104–110.
76. Tremellen KP, Valbuena D, Landeras J, Ballesteros A, Martinez J, Mendoza S, Norman RJ, Robertson SA, Simon C. The effect of intercourse on pregnancy rates during assisted human reproduction. Hum Reprod 2000; 15:2653–2658.
77. Hart R, Norman RJ. The longer-term health outcomes for children born as a result of IVF treatment: part I–general health outcomes. Hum Reprod Update 2013; 19:232–243.