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TGF-β Mediates Proinflammatory Seminal Fluid Signaling in Human Cervical Epithelial Cells

David J. Sharkey,* Anne M. Macpherson,* Kelton P. Tremellen,† David G. Mottershead,* Robert B. Gilchrist,* and Sarah A. Robertson*

The cervix is a major inductive and effector site for immune responses in the female genital tract (1) that are crucial in orchestrating the female tract response to local immune challenges. Through secretion of chemotactic and immune-regulating cytokines, epithelial cells at the cervical surface have a pivotal role in activating defense against bacterial and viral pathogens, and in the generation and maintenance of immune tolerance toward spermatozoa and other antigenic material present in the ejaculate. The responsiveness of cervical epithelial cells to introduced stimuli and their subsequent influence over the cervical immune environment is also implicated as a determinant of neoplasia and development of cervical cancer (2–4).

Recently, we showed that, in women, the male partner’s seminal fluid exerts substantial effects on immune parameters in the cervix after coitus. Although seminal plasma (SP) has conventionally been viewed as simply a transport medium for spermatozoa traversing the female reproductive tissues, it is now known to have broader actions in regulating female fertility. In animals, seminal fluid interacts with epithelial cells lining the female reproductive tract to activate cytokine gene expression and elicit changes in the abundance and behavior of infiltrating leukocyte populations, which, in turn, promote receptivity for embryo implantation (5). In mice, uterine dendritic cells recruited in response to seminal fluid are instrumental in activating local T cell responses (6, 7), including expansion of regulatory T (Treg) cell populations that mediate tolerance of the conceptus in an ensuing pregnancy (8), whereas recruited macrophages influence epithelial cell expression of glycosylated structures required for embryo attachment and invasion (9).

In women, we have recently reported that seminal fluid induces an inflammation-like response in the endocervical tissue with extensive infiltration of macrophages, dendritic cells, and lymphocytes into the epithelial and deeper stromal tissues (10), expanding upon earlier findings that semen induces a neutrophil exudate into the cervical canal (11). Leukocyte recruitment in vivo is accompanied by elevated expression of CSF2, IL6, IL8, and IL1A as well as several other chemokines and cytokine genes (10). The response requires contact between seminal fluid and the female cervical tissues because the characteristic changes in gene expression and leukocyte recruitment are not seen following condom-protected intercourse (10).

Seminal fluid contains a complex array of cytokines, PGs, and other bioactive molecules (5, 12), but, to date, the active signaling agents in human semen responsible for stimulating the female cervical response are unidentified. There is evidence that the plasma fraction of seminal fluid is important, with in vitro experiments utilizing transformed or primary cervical epithelial cells showing that SP induces synthesis of proinflammatory cytokines and chemokines GM-CSF, IL-6, IL-8, CCL2 (MCP-1), CCL20 (MIP-3α), and IL-10 (13). In mice, one agent mediating at least some aspects of the female response to SP is TGF-β, which is synthesized in the male seminal vesicles in the latent form, then activated in the female...
tract after coitus (14). TGF-β is of particular interest as a candidate signaling agent in seminal fluid because of its well-known immune-deviating properties, including induction of immune tolerance mediated by Treg cells (15–17). Because TGF-β1 and TGF-β2 are reported to be highly abundant in human SP (18, 19), we hypothesized that TGF-β may be a signaling agent mediating the actions of human SP on cervical epithelial cells.

The experiments described in this study aimed to define the significance of SP TGF-β in eliciting production of cervical cell cytokines and chemokines after coitus. We used immortalized ectocervical epithelial (Ect1) cells, shown previously to be a faithful in vitro model for cervical epithelial cell immune function and seminal fluid responsiveness (13, 20), as well as primary ectocervical epithelial cells, to evaluate the relative signaling capacity of SP versus the three mammalian isoforms of TGF-β. Two strategies were used, an Affymetrix microarray screen as well as detailed analysis of several cytokines. Our experiments show that all three mammalian isoforms of TGF-β (TGF-β1, TGF-β2, and TGF-β3) contribute to eliciting the cervical cell cytokine response to SP, acting via the conventional TGF-β signaling pathway to induce expression of GM-CSF and IL-6, two cytokines with key roles in establishing pregnancy (21, 22). Together, these results demonstrate that TGF-β is an important male-female signaling agent implicated in regulating the female immune response to seminal fluid at coitus.

Materials and Methods

Cervical tissue and semen samples

Procedures were approved by the human ethics committees of the University of Adelaide and the North West Adelaide Health Service. Human SP was obtained from healthy, proven fertile men at Repromed (Dulwich, Australia). Semen was produced by masturbation and, following liquefaction, was centrifuged at 10,000 × g for 10 min at room temperature within 30 min of ejaculation to recover SP and sperm. Individual SP specimens (n = 20) or pooled SP (from n = 10 donors) was stored frozen in aliquots at −80°C until use. Semen samples were excluded in the event of abnormal sperm number, motility, and morphology parameters (according to World Health Organization criteria), high leukocytes or other symptoms of infection, or donor use of immune-modifying medications such as methotrexate or nonsteroidal anti-inflammatory drugs. For analysis of TGF-β content by ELISA, whole semen and sperm were diluted in ice-cold PBS containing protease inhibitor mixture (Roche) and solubilized using an Ultra-Turrax high-speed homogenizer (Janke and Kunkel, Staufen, Germany), then stored at −80°C until assay.

Cervical human tissue was obtained with consent from three premenopausal women undergoing routine vaginal or total abdominal hysterectomy at the Lyell McEwin Hospital (Elizabeth, South Australia) or Women’s and Children’s Hospital (Adelaide, South Australia), for benign, noncancerous pathology.

Recombinant cytokines, Abs, small molecule inhibitor, and ectodomain receptor antagonist

Recombinant human TGF-β1, TGF-β2, and TGF-β3 were from R&D Systems (240-B, 302-B, and 243-B; Minneapolis, MN). Recombinant human IL-6, IL-1α, IL-1β, IL-12, and TNF-α were purchased from ProspecBio (CYT-213, CYT-253, CYT-208, CYT-362, and CYT-223; Rehovot, Israel). Cytokines were reconstituted to 1 μg/ml in PBS/0.1% BSA/4 mM HCl (TGF-β isoforms), or PBS/0.1% BSA (all others). Neutralizing Abs reactive with TGF-β1, TGF-β2, and TGF-β3 (MAB240, AF-302-NA, and AB-244-NA) were purchased from R&D Systems. The small molecule inhibitor of TGF-β signaling SB-431542 (D8946-5MG; Sigma, Castle Hill, Australia) was reconstituted to 10 mM in DMSO. Recombinant TGF-βRII-Fc receptor antagonist (341-BR; R&D Systems) was reconstituted to 100 μg/ml in sterile PBS/0.1% BSA. All reagents were stored in aliquots at −80°C.

Primary ectocervical epithelial cell culture

Ectocervical epithelial cells were prepared, as described (23). Cervical tissue biopsies were washed twice in ice-cold HBSS (Invitrogen, Mount Waverley, Australia), and then incubated overnight at 4°C in 5 ml DMEM (Invitrogen) containing 5 U dispase (Boehringer Mannheim, Mannheim, Germany). Next morning, after 1-h further incubation at room temperature, intact sheets of epithelial cells were separated from stromal tissue using forceps and a scalpel, and then disaggregated in DMEM containing 0.25% trypsin and 0.01% collagenase type I (Sigma-Aldrich, Castle Hill, Australia) at 37°C for 30 min, with the aid of trypsin digestion by hypodermic needles of incrementally decreasing gauge. Epithelial cells were then treated with 0.01% EDTA in DMEM at 37°C for 10 min, and plated at 1 × 10^5 cells/well in 70% DMEM/22% Ham’s F12/7% FCS/1% Nutridoma-SP (Boehringer Mannheim) with 0.02 mM glutamine and 5 mM hydrocortisone (Upjohn, Rydalmore, Australia) (epithelial cell culture medium) in 1.5-ml culture wells (Nunc, Weisbaden, Germany). Wells were seeded 24 h prior with mitomycin C-inactivated murine 3T3 fibroblast cells (4 × 10^3 cells/well) to provide extracellular matrix components required for epithelial cell attachment.

Ect1 cells were allowed to attach and displace the 3T3 fibroblasts for 5–7 d, when unattached epithelial cells and 3T3 cells were aspirated, and media was replaced with 500 μl fresh EpCM/2% FCS. This pretreatment supernatant was collected 12 h later and replaced with 500 μl EpCM/2% FCS containing TGF-β1, TGF-β2, or culture media alone. After 12 h the treatment was replaced with EpCM/2% FCS, which in turn was collected 24 h later (posttreatment). Supernatants were centrifuged and stored at −80°C until ELISA.

Ect1 cervical epithelial cell culture

Ect1 cells were propagated, as described (20, 24), in keratinocyte serum-free media (KSFm; Life Technologies, Mount Waverley, Australia) supplemented with 0.1 ng/ml recombinant human epidermal growth factor (Life Technologies), 0.05 mg/ml bovine pituitary extract (Life Technologies), and 0.4 mM CaCl2. For experiments, 1 × 10^5 cells in 500 μl KSFm were seeded in 1.5-ml culture wells (Nunc) and incubated at 37°C 5% CO2 for 12 h, to generate a confluent monolayer; then the medium was replaced with 500 μl fresh KSFm. This pretreatment supernatant was collected 12 h later and replaced with 500 μl KSFm containing TGF-β1, TGF-β2, or TGF-β3 (0.5–50 ng/ml), other cytokines (all 1 ng/ml), 10% pooled human SP (v/v), or medium alone. For TGF-β neutralization, confluent Ect1 cells were cultured with 10% SP (v/v), or 10% SP (v/v) plus neutralizing Ab specific to TGF-β1, TGF-β2, or TGF-β3 (all 15 μg/ml). For small molecule inhibition of TGF-β signaling, confluent Ect1 cells were pretreated for 30 min with SB431542 (0.5 or 5 μM), and then washed and cultured with inhibitor alone or 10% SP (v/v) plus inhibitor. For inhibition of TGF-β signaling using the TGF-βRII-Fc receptor antagonist, confluent Ect1 cells were cultured with TGF-βRII-Fc (2 μg/ml), 10% SP (v/v), or 10% SP (v/v) preincubated for 30 min with TGF-βRII-Fc (2 μg/ml). For all experiments, the treatment was replaced with fresh KSFm at 12 h, which in turn was collected 24 h later (posttreatment) for cytokine analysis by ELISA or Luminalex multiplex micro bead assay. For Ect1 cell mRNA preparation, cultures were terminated at 4 or 10 h after application of treatments.

Affymetrix GeneChip microarray

Quadruplicate wells of Ect1 cells were incubated in KSFm alone (control), KSFm, and 10% pooled SP, or KSFm and 5 ng/ml TGF-β3 for 10 h. Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions, and purified using RNeasy micro kits (Qiagen, Doncaster, Australia). RNA (10 μg per sample) from four biological replicates, each comprising pooled material from separate sets of replicative wells, was sent to the Australian Genome Research Facility (Melbourne, Australia) for single-cycle labeling and hybridization to Affymetrix GeneChip Human Genome U133 plus 2 microarrays (Affymetrix, Santa Clara, CA) using 12 μg RNA integrity, no DNA contamination, and washing were performed, according to the manufacturer’s instructions. GCOS 1.4 software (Affymetrix) using MAS 5.0 was used to generate Celestia script files for each chip. Quality control was assessed using the affyQC-Report package in R (v2.6.1) (25). The array data were initially examined using Partek Genomics Suite (Partek), after Celestia script files were imported using robust multiairway average background correction, guanine/cytosine content correction, and mean probe summarization, to generate dendrogram hierarchical clustering analysis and principal components analysis. Non-log2–transformed expression values for each chip were submitted to IlluminaGUI (26) (free software implemented by R 2.6.1) after substituting illumina probe IDs for Affymetrix IDs. Two contrasts were analyzed, as follows: SP versus control and TGF-β3 versus control. In both cases, differentially expressed probes were identified using default high stringency parameters (≥2.0-fold change, test p < 0.05, >100 difference between mean expression intensity) as well as reduced stringency parameters (≥1.4-fold change, test p < 0.10, >50 difference between mean expression intensity). The Kyoto Encyclopedia of Genes and
Genomics (KEGG) database (27) and Pathway Express and OntoExpress analysis software (28, 29) were used to assign genes to cytokine-cytokine receptor interaction, TGF-β signaling, JAK/STAT signaling, or vascular endothelial cell growth factor (VEGF) signaling pathways. The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information’s Gene Expression Omnibus (30) and are accessible through GEO Series Accession GSE35830.

Quantitative RT-PCR

Ect1 cell total RNA was extracted using TRizol solution (Invitrogen), and, following treatment with RNase-free DNase I (500 IU/ml; 60 min/37°C) (Roche, Basel, Switzerland), first-strand cDNA was reverse transcribed from 5 μg RNA employing a SuperScript-III Reverse Transcriptase kit (10 min/30°C, 45 min/42°C; Invitrogen). Primer pairs specific for cytokine and chemokine cDNA sequences were designed and PCR conditions were optimized for each primer pair, as described (31). Primer sequences, product sizes, and GenBank accession numbers are as published (13).

PCR amplification employed SYBR Green PCR Master Mix kits (Applied Biosystems), with 3 μl cDNA and 5’ and 3’ primers at concentrations of 0.1–1.0 μM, as described (31). PCR used a ABI Prism 7000 Sequence Detection System (Applied Biosystems) to allow ampiclon quantification using the Equation 2^(-ΔΔCt) x 100 K^-1 (Applied Biosystems User Bulletin #2), where Ct is the cycle number at which 50% maximum ampiclon synthesis occurs. Products were analyzed by dissociation curve profile and 2% agarose gel electrophoresis, and were sequenced using Big Dye version 2 or 3 (Applied Biosystems) to confirm primer specificity.

Cytokine ELISAs and Luminex microbead assays

IL-8, IL-6, GM-CSF, CCL2, CCL20, and IL-1α in culture supernatants were measured in ELISA kits (all R&D Systems) or, in some experiments as specified, by Luminex microbead assay (Millipore). Minimum detectable thresholds were 15.6 pg/ml (GM-CSF, CCL2, and CCL20), 9 pg/ml (IL-6), 7.8 pg/ml (IL-1α), and 31.2 pg/ml (IL-8) for ELISA and 9.5 pg/ml or less for Luminex assays. Intra- and interassay coefficients of variation were all <10%. The TGF-β content of semen, sperm, and seminal fluid was measured by ELISA (TGF-β1 and TGF-β2 from R&D Systems, and TGF-β3 from Promega, Madison, WI). Minimum detectable thresholds were 15.6 pg/ml (TGF-β1 and TGF-β2) and 31.2 pg/ml (TGF-β3). Intra- and interassay coefficients of variation for all TGF-β ELISAs were <8%. All samples were assayed in duplicate, according to the manufacturers’ instructions.

Data analysis and statistics

Cytokine data were expressed as pg/10⁵ cells/24 h. Data were excluded from the analysis when comparable values (within 10% of control) were not obtained for pretreatment supernatants from control and treatment wells. SPSS version 15.0 (SPSS, Chicago, IL) was used to analyze complete data sets. Student t test preceded by one-way ANOVA for multiple groups was used to compare differences between treatment groups. Statistical significance was concluded when p < 0.05. Gene symbols and protein names are according to nomenclature specified by HUGO Gene Nomenclature Committee.

Results

SP contains high concentrations of TGF-β1, TGF-β2, and TGF-β3

TGF-β1 and TGF-β2 are known to be present in human SP (18, 19), but the third isoform TGF-β3 has not previously been evaluated. Quantification of the total TGF-β1, TGF-β2, and TGF-β3 content of SP by isofrom-specific ELISA in SP samples collected from 20 proven fertile men revealed high levels of TGF-β1, TGF-β2, and TGF-β3 in all of 20 samples, with a total TGF-β content of (mean ± SEM) 397 ± 53 ng/ml, comprising on average 55% TGF-β1, 1% TGF-β2, and 44% TGF-β3 (Table 1). All three TGF-β isoforms were present predominantly in the latent, as opposed to

Table 1. Relative concentrations of each of the three mammalian isoforms of TGF-β in human SP from n = 20 proven fertile men

<table>
<thead>
<tr>
<th></th>
<th>TGF-β1 (ng/ml)</th>
<th>TGF-β2 (ng/ml)</th>
<th>TGF-β3 (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>219.3 ± 13.4</td>
<td>5.3 ± 0.7</td>
<td>172.2 ± 32.8</td>
</tr>
<tr>
<td>Range</td>
<td>113.1–314.2</td>
<td>1.0–13.5</td>
<td>4.7–462.0</td>
</tr>
<tr>
<td>Bioactive (% total)</td>
<td>2.3 ± 0.4 (1.2%)</td>
<td>0.25 ± 0.04 (5.3%)</td>
<td>3.5 ± 1.2 (1.8%)</td>
</tr>
<tr>
<td>Range</td>
<td>0.3–6.9</td>
<td>0.01–0.80</td>
<td>0.16–15.9</td>
</tr>
</tbody>
</table>

aTGF-β1, TGF-β2, and TGF-β3 were measured by isofrom-specific ELISA in SP samples from n = 20 individual healthy, proven fertile men.

bTotal TGF-β1, TGF-β2, and TGF-β3 were determined by analysis after transient acid activation of SP to activate latent cytokine. Bioactive TGF-β1, TGF-β2, and TGF-β3 were determined by analysis without prior acid activation.

FIGURE 1. The relative abundance of TGF-β isoforms in whole semen, SP, and sperm, and the correlation between SP TGF-β content and SP capacity to induce GM-CSF, IL-6, and IL-8 production in Ect1 cells. (A–C) Ect1 cell production of GM-CSF, IL-6, and IL-8 in response to incubation with SP (10%) from 10 healthy, proven fertile men was determined in a previous study, as described (13). The total TGF-β content (ng/ml) was determined as the sum of total TGF-β1, TGF-β2, and TGF-β3 (Table 1). The correlation between SP TGF-β content and Ect1 cytokine production, including GM-CSF (D), IL-6 (E), and IL-8 (F), is shown. Each SP sample is plotted as an individual data point, and the Pearson’s correlation coefficient R² and corresponding p values are shown for each pair.
active form (98.8% TGF-β1, 94.7% TGF-β2, and 98.2% TGF-β3) (Table I). When the relative amount of each TGF-β isoform associated with the plasma and sperm fractions of semen was quantified, the majority was present in the plasma fraction, and only 1, 15, and 1% of the total TGF-β1, TGF-β2, and TGF-β3, respectively, were associated with sperm (Fig. 1A–C).

### Table II. Genes of interest identified as differentially expressed using high stringency criteria in Affymetrix microarray analysis of mRNA expression in Ect1 ectocervical epithelial cells treated with SP, and their regulation by TGF-β3

<table>
<thead>
<tr>
<th>Accession No.</th>
<th>Gene Symbolb</th>
<th>Control Mean ± SEM</th>
<th>SP Mean ± SEM</th>
<th>Fold Change</th>
<th>p Value</th>
<th>TGF-β3 Mean ± SEM</th>
<th>Fold Change</th>
<th>p Value</th>
<th>TGF-β Regulationc</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_004591</td>
<td>CCL20</td>
<td>504.8 ± 35.8</td>
<td>2344.1 ± 690.1</td>
<td>4.6</td>
<td>0.008</td>
<td>1702.2 ± 562.4</td>
<td>3.4</td>
<td>0.034 Y</td>
<td></td>
</tr>
<tr>
<td>NM_002889</td>
<td>CCL2</td>
<td>524.6 ± 58.1</td>
<td>30179.9 ± 868.2</td>
<td>5.8</td>
<td>0.004</td>
<td>734.9 ± 207.9</td>
<td>1.4</td>
<td>0.001 N</td>
<td></td>
</tr>
<tr>
<td>NM_002909</td>
<td>CCL3</td>
<td>172.8 ± 39.2</td>
<td>12332.3 ± 327.2</td>
<td>7.1</td>
<td>&lt;0.001</td>
<td>369.6 ± 112.1</td>
<td>2.1</td>
<td>0.098 ?</td>
<td></td>
</tr>
<tr>
<td>NM_0019452</td>
<td>HBEGF</td>
<td>247.6 ± 39.2</td>
<td>10597.3 ± 306.0</td>
<td>4.3</td>
<td>0.009</td>
<td>286.1 ± 54.8</td>
<td>1.2</td>
<td>0.002 N</td>
<td></td>
</tr>
<tr>
<td>NM_0008743</td>
<td>IFNAR2</td>
<td>395.7 ± 33.1</td>
<td>928.7 ± 251.6</td>
<td>2.4</td>
<td>0.036</td>
<td>370.9 ± 45.6</td>
<td>1.1</td>
<td>0.017 N</td>
<td></td>
</tr>
<tr>
<td>NM_001961</td>
<td>IL1F9</td>
<td>69.0 ± 31.7</td>
<td>214.0 ± 58.4</td>
<td>3.1</td>
<td>0.029</td>
<td>174.0 ± 55.7</td>
<td>2.5</td>
<td>0.006 N</td>
<td></td>
</tr>
<tr>
<td>NM_004633</td>
<td>IL1R2</td>
<td>231.3 ± 42.6</td>
<td>480.9 ± 108.2</td>
<td>2.1</td>
<td>0.032</td>
<td>140.1 ± 63.9</td>
<td>1.7</td>
<td>0.009 N</td>
<td></td>
</tr>
<tr>
<td>NM_003456</td>
<td>IL1R1L1</td>
<td>86.9 ± 14.3</td>
<td>2075.7 ± 790.9</td>
<td>23.7</td>
<td>0.013</td>
<td>1110.0 ± 4.9</td>
<td>1.3</td>
<td>0.003 N</td>
<td></td>
</tr>
<tr>
<td>NM_000660</td>
<td>IL6</td>
<td>218.1 ± 13.2</td>
<td>863.8 ± 95.7</td>
<td>4.0</td>
<td>&lt;0.001</td>
<td>884.1 ± 276.0</td>
<td>4.1</td>
<td>0.016 Y</td>
<td></td>
</tr>
<tr>
<td>NM_000584</td>
<td>IL8</td>
<td>230.8 ± 43.7</td>
<td>3330.3 ± 1340.3</td>
<td>14.4</td>
<td>0.020</td>
<td>1520.3 ± 743.6</td>
<td>6.6</td>
<td>0.008 Y</td>
<td></td>
</tr>
<tr>
<td>NM_006641</td>
<td>IL11</td>
<td>19.6 ± 7.9</td>
<td>170.6 ± 48.7</td>
<td>8.7</td>
<td>0.002</td>
<td>196.9 ± 58.4</td>
<td>10.0</td>
<td>0.003 Y</td>
<td></td>
</tr>
<tr>
<td>NM_00185156</td>
<td>IL24</td>
<td>95.2 ± 27.9</td>
<td>408.7 ± 77.7</td>
<td>4.3</td>
<td>&lt;0.001</td>
<td>124.7 ± 29.8</td>
<td>1.3</td>
<td>0.006 Y</td>
<td></td>
</tr>
<tr>
<td>NM_0026802</td>
<td>PDGFB</td>
<td>121.4 ± 30.4</td>
<td>287.3 ± 18.8</td>
<td>2.4</td>
<td>&lt;0.001</td>
<td>401.6 ± 97.9</td>
<td>3.3</td>
<td>0.006 Y</td>
<td></td>
</tr>
</tbody>
</table>

*Fold Change = Mean of fold change in expression between treated and control samples.*

Data are mean ± SEM for n = 4 biological replicates from Ect1 ectocervical epithelial cells, each prepared from pools of 3 to 4 replicate wells, for three treatments: SP (10%, 1 h), TGF-β3 (5 ng/ml, 10 h), or medium alone (control).

Genes are classified as high confidence of TGF-β regulation (Y) if high stringency criteria are met for differential expression in response to 5 ng/ml TGF-β; low confidence of TGF-β regulation (?) if reduced stringency criteria (defined as: fold change > 1.4; t test p < 0.10; difference between means > 0.5) but not high stringency criteria are met, or not regulated by TGF-β (N) if reduced stringency criteria are not met.

Genes are regulated in opposite direction by TGF-β as indicated.

**SP TGF-β content correlates with Ect1 cytokine-inducing activity**

Next, we investigated the association between SP TGF-β and capacity to elicit proinflammatory cytokine production in Ect1 ectocervical epithelial cells. Previously, we quantified GM-CSF, IL-6, IL-8, and CCL2 production in Ect1 in response to SP treatment.

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*a* Listed are genes of interest identified as differentially expressed using high stringency criteria in Affymetrix microarray analysis of mRNA expression in Ect1 ectocervical epithelial cells treated with SP, and their regulation by TGF-β3.

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samples from 10 individual fertile men (13). For these same 10 SP samples, there was a positive correlation between total TGF-β content (sum of TGF-β1, TGF-β2, and TGF-β3) and the response of some, but not all, cytokines elicited in Ect1 cells. Total seminal fluid TGF-β content correlated with Ect1 GM-CSF output (Pearson correlation \( r = 0.720, p = 0.019 \)) and IL-6 output (\( r = 0.829, p = 0.003 \)) (Fig. 1D, 1E). There was a weaker correlation with Ect1 IL-8 production (\( r = 0.643, p = 0.045 \)) (Fig. 1F), but no correlation with CCL2 output (data not shown).

**TGF-β and SP both activate cytokine signaling and related pathways in Ect1 cells**

To explore whether TGF-β can induce inflammatory cytokine gene transcription in Ect1 cells, and the extent to which TGF-β mimics changes induced by SP, a microarray experiment was undertaken. The profile of gene expression in Ect1 cells incubated with TGF-β3 (5 ng/ml, 10 h) was compared with expression in cells exposed to SP (10%, 10 h). MAS 5.0 (GCOS, Gene Chip Operating Software; Affymetrix) analysis of \( n = 4 \) biological replicates for

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**Table III. Effect of TGF-β3 and SP on cytokine and chemokine production by Ect1 ectocervical epithelial cells**

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Synonym</th>
<th>Control*</th>
<th>TGF-β3 (0.5 ng/ml)$^a$</th>
<th>TGF-β3 (5 ng/ml)$^a$</th>
<th>TGF-β3 (50 ng/ml)$^a$</th>
<th>10% SP*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eotaxin</td>
<td>CCL11</td>
<td>3.2 ± 0.1</td>
<td>2.9 ± 0.2</td>
<td>2.9 ± 0.1</td>
<td>2.9 ± 0.1</td>
<td>6.7 ± 1.5</td>
</tr>
<tr>
<td>G-CSF</td>
<td>CSF3</td>
<td>3.3 ± 0.8</td>
<td>2.4 ± 0.4</td>
<td>4.7 ± 1.7</td>
<td>3.2 ± 1.9</td>
<td>232.9 ± 70.7</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>CSF2</td>
<td>5.8 ± 0.13</td>
<td>19.9 ± 0.8</td>
<td>190.7 ± 21.5</td>
<td>255.8 ± 34.0</td>
<td>320.0 ± 66.3</td>
</tr>
<tr>
<td>CXCL1</td>
<td>GRO</td>
<td>143.7 ± 7.5</td>
<td>155.6 ± 3.3</td>
<td>243.5 ± 24.7</td>
<td>186.0 ± 16.8</td>
<td>3802.8 ± 919.8</td>
</tr>
<tr>
<td>IL-1α</td>
<td></td>
<td>15.2 ± 0.7</td>
<td>7.5 ± 0.2</td>
<td>13.1 ± 2.0</td>
<td>14.0 ± 1.5</td>
<td>239.08 ± 24.5</td>
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<td>161.5 ± 3.6</td>
<td>194.9 ± 11.1</td>
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<td>8.6 ± 0.6</td>
<td>28.4 ± 2.6</td>
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<td>BDL</td>
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<td>BDL</td>
</tr>
<tr>
<td>RANTES</td>
<td>CCL5</td>
<td>8.2 ± 0.5</td>
<td>7.5 ± 0.4</td>
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<td>8.1 ± 0.6</td>
<td>28.4 ± 2.6</td>
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<td>2161.2 ± 50.4</td>
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<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
<td>BDL</td>
</tr>
</tbody>
</table>

$^a$Data are mean ± SEM of triplicate wells (pg/10⁵ cells/24 h).

$^b$BDL, concentration of analyte is below level of detection for the assay.

GRO, Growth-related oncogene; IP-10, IFN-γ-inducible protein 10; MDC, macrophage-derived chemokine.
each treatment identified an average of 42.6% of probe sets as present in untreated Ect1 ectocervical epithelial cells, whereas 36.2 and 41.4% of probe sets were present in Ect1 cells incubated with SP or TGF-β3, respectively. Dendrograms obtained using Partek software showed clustering of the array data according to treatment group, with greater similarity between the control and TGF-β3 treatment groups than between the SP and other treatment groups (Supplemental Fig. 1A). Principal component analysis, which displays the data after transformation and reduction to a small number of key coordinates, also showed clustering according to treatment group. SP and (to a lesser extent) TGF-β3 arrays both showed separation from the control arrays along the first principal component, and TGF-β3 arrays showed separation from both control and SP arrays along the second principal component (Supplemental Fig. 1B).

Exposure of Ect1 cells to SP resulted in differential expression of a total of 3955 probe sets, identified using high stringency criteria with MAS 5.0 analysis. These corresponded to 1338 genes upregulated and 1343 genes downregulated by SP. TGF-β3 treatment of Ect1 cells resulted in differential expression of 884 probe sets, corresponding to 346 upregulated genes and 229 downregulated genes. The genes differentially regulated by SP included 47 genes of interest, relevant to the current study by virtue of their association with cytokine–cytokine receptor interaction, TGF-β signaling, JAK/STAT signaling, or VEGF signaling pathways, as specified by the KEGG database. Of these 47 genes, 17 (36.1%) were similarly regulated by both SP and TGF-β3 (Table II). Genes found to be upregulated by both SP and TGF-β3 include cytokine genes IL6 (4.0-fold and 4.1-fold for SP and TGF-β3, respectively), CCL20 (4.6-fold and 3.4-fold), IL11 (8.7-fold and 10.0-fold), VEGFA (both 2.4-fold), PDGFb (2.4-fold and 3.3-fold), FST (10.0-fold and 2.2-fold), FSTL3 (3.7-fold and 5.0-fold), INHBA (8.5-fold and 5.8-fold), and cytokine signaling pathway genes SMURF1 (2.9-fold and 2.2-fold), CBL (4.6-fold and 4.8-fold, respectively), PIK3CD (2.7-fold and 3.1-fold), SOCS2 (3.1-fold and 2.1-fold), and PTGS2 (9.1-fold and 3.6-fold, respectively). Genes downregulated by both SP and TGF-β3 include the TGF-β receptor gene TGFBR3 (3.0-fold and 2.9-fold) and cytokine signaling pathway gene PIK3R3 (5.8-fold and 2.6-fold, respectively). Two genes, the TGF-β receptor TGFBR1 and TNF family member TNFSF10, were downregulated by SP (8.7-fold and 2.0-fold, respectively) and upregulated by TGF-β3 (2.1-fold and 2.7-fold, respectively).

When reduced stringency criteria for regulation by SP (≥1.4-fold change, t test p < 0.10) were applied to the array data set, another 79 genes of interest were identified as potentially regulated by SP. Of the total 146 genes, 51 (40.5%) met the same reduced stringency criteria for regulation by TGF-β3 (Supplemental Table I). Among these, 8 additional genes met high stringency criteria for upregulation by TGF-β3, including cytokines CSF2, IL1A, and PDGFC; chemokine CXCL1, TNF family member TNFAIP8; and cytokine signaling pathway genes CBLB, SOCS1, and SOCS3 (Supplemental Table I). Additional genes previously shown to be induced by SP in Ect1 cells (13) either showed no indication of regulation by TGF-β3 (CCL2), or did not meet high stringency criteria for differential expression (IL8).

TGF-β stimulates cytokine production in Ect1 cells

To further evaluate whether seminal TGF-β elicits a cytokine response in Ect1 cells, we used cytokine-specific ELISAs to analyze production of GM-CSF, IL-6, IL-8, CCL2, CCL20, and IL-1α by Ect1 cells after incubation with each of the three TGF-β isoforms. These cytokines were chosen for detailed study on the basis of the microarray experiment as well as previous in vitro experiments in primary cervical epithelial cells and Ect1 cells (13), together with studies in cervical tissue biopsies that demonstrate that CSF2, IL6, IL8, and IL1A are induced in vivo after coitus (10). Ect1 cells were incubated with TGF-β1, TGF-β2, or TGF-β3 at concentrations of 0.5, 5.0, and 50 ng/ml, approximating the total TGF-β content of 0.1, 1, and 10% SP. All three TGF-β isoforms elicited increased production of GM-CSF in a generally dose-dependent manner (Fig. 2A), with 50 ng/ml inducing a 4.6- to 7.1-fold increase, which is 53–85% of the increase induced by 10% SP [a dilution shown previously to induce maximal cytokine responses from Ect1 cells (13)]. Similarly, all three TGF-β isoforms stimulated IL-6 production in a dose-dependent manner (Fig. 2B), with 50 ng/ml inducing a 2.7- to 6.7-fold increase, which is 40–96% of the increase induced by 10% SP. In contrast, none of the three TGF-β isoforms significantly altered secretion of IL-8 (Fig. 2C), CCL2 (Fig. 2D), or IL-1α (Fig. 2F), and CCL20 production was inhibited by all TGF-β isoforms (Fig. 2E).

When the cytokine profile induced in Ect1 cells by TGF-β3 was examined by Luminex microbead analysis, similar increases in GM-CSF and IL-6, with no change in IL-8, CCL2, and IL-1α production, were obtained (Table III). This approach revealed additional cytokines G-CSF, CXCL1 (growth-related oncogene), and CCL5 (RANTES) to be induced by 10% SP in Ect1 cells, but none of these were influenced by TGF-β3.

### Table IV. Effect of GM-CSF, IL-1α, IL-1β, IL-6, IL-12, LIF, and TNF-α on cytokine and chemokine production by Ect1 ectocervical epithelial cells

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Synonym</th>
<th>Control</th>
<th>GM-CSF (1 ng/ml)</th>
<th>IL-1α (1 ng/ml)</th>
<th>IL-1β (1 ng/ml)</th>
<th>IL-6 (1 ng/ml)</th>
<th>IL-12 (1 ng/ml)</th>
<th>LIF (1 ng/ml)</th>
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aData are mean of duplicate wells (pg/10⁵ cells/24 h).
bBDL, concentration of analyte is below level of detection for the assay.

GRO, Growth-related oncogene; IP-10, IFN-γ-inducible protein 10.
Seminal fluid cytokines IL-6, IL-1α, IL-1β, IL-12, GM-CSF, and TNF-α do not stimulate cytokine production in Ect1 cells

SP contains several cytokines in addition to TGF-β. Some of these, including IL-6, IL-1α, IL-1β, IL-12, GM-CSF, and TNF-α (32–34), were evaluated for their capacity to induce inflammatory cytokines in Ect1 cells. Luminescence analysis showed that none of these induced any substantial production of GM-CSF, IL-6, IL-8, CCL2, or IL-1α in Ect1 cells (Table IV). TGF-β stimulates cytokine production in primary ectocervical epithelial cells

Next, the cytokine response of primary ectocervical epithelial cells to TGF-β was examined by incubation with 5.0 ng/ml TGF-β1, TGF-β2, or TGF-β3. Each TGF-β isoform elicited increased GM-CSF and IL-6 release in cells recovered from each of three women, which was similar in scale to changes seen in Ect1 cells (Fig. 3A, 3B) and comparable to the response to 10% SP in our previous experiments (13). Similar to the response in Ect1 cells, each TGF-β isoform induced moderate suppression of CCL20 production in primary ectocervical cells (Fig. 3E). However, the response of primary ectocervical cell preparations to TGF-β was different to that seen in Ect1 cells for three cytokines, with relatively small but significant increases in CCL2 and IL-1α (Fig. 3D, 3F) and moderate inhibition of IL-8 (Fig. 3C).

TGF-β neutralizing Abs, signaling inhibitors, and TGF-βRII receptor antagonist inhibit SP-induced cytokine production in Ect1 cells

TGF-β neutralizing Abs were added to Ect1 cells incubated with SP to determine the proinflammatory activity attributable to each TGF-β isoform. Concentrations of each Ab sufficient to completely neutralize the quantities of TGF-β1, TGF-β2, and TGF-β3 present in SP were determined in preliminary experiments (data not shown). Neutralizing Abs reactive with TGF-β1, TGF-β2, or TGF-β3 inhibited SP-induced Ect1 cell synthesis of GM-CSF by 37, 36, and 56%, respectively. Neutralization of all three isoforms completely neutralized the quantities of TGF-β in SP to determine the proinflammatory activity attributable to each TGF-β isoform.

SP-induced increases in IL-6 were decreased by 13, 20, and 52% after neutralization of TGF-β1, TGF-β2, and TGF-β3, respectively, and neutralization of all three isoforms of TGF-β reduced IL-6 production by 77% (Fig. 4D). In contrast, neutralization of TGF-β1, TGF-β2, TGF-β3, or all three TGF-β isoforms had no effect on SP-induced production of IL-8 (Fig. 4G), or CCL2, CCL20, or IL-1α (data not shown). Irrelevant mouse and goat Ab did not change GM-CSF or IL-6 production (data not shown).

When Ect1 cells were preincubated with SB-431542, a specific inhibitor of the activin receptor-like kinase (ALK) receptor ALK5 (TGF-βRII receptor) and related ALK4 and ALK7 receptors required for TGF-β and activin signaling (35), SP-induced increases in both GM-CSF and IL-6 were significantly impaired. The increase in GM-CSF induced by 10% SP was reduced by 67 and 91% when cells were pretreated with 0.5 or 5 μM SB-431542, respectively (Fig. 4B). Likewise, the SP-induced increase in IL-6 production was reduced by 51 and 67%, respectively, by SB-431542 inhibitor (Fig. 4E). SB-431542 did not alter SP-induced production of IL-8 (Fig. 4H), CCL2, CCL20, or IL-1α (data not shown).

The effect of rTGF-βRII-Fc receptor ectodomain antagonist on SP-induced Ect1 cell production of cytokines was also investigated. Preincubation of SP with rTGF-βRII-Fc prior to addition to Ect1 cells reduced GM-CSF and IL-6 production by 73 and 77%, respectively (Fig. 4C, 4F). TGF-βRII-Fc receptor ectodomain antagonist did not affect production of IL-8 (Fig. 4I), CCL2, CCL20, or IL-1α (data not shown).

FIGURE 3. Effect of TGF-β on GM-CSF, IL-6, IL-8, CCL2, CCL20, and IL-1α production by primary ectocervical epithelial cells. Primary cervical epithelial cells were incubated with TGF-β1, TGF-β2, or TGF-β3 (5.0 ng/ml) for 12 h, and supernatants were collected 24 h later for cytokine analysis by ELISA. (A) GM-CSF, (B) IL-6, (C) IL-8, (D) CCL2, (E) CCL20, and (F) IL-1α output in pg/10⁵ cells/24 h. Data are the mean ± SEM of triplicate wells in each experimental group, and are representative of three replicate experiments with cells from three patients. Data were analyzed by Kruskal-Wallis and Mann-Whitney U test to compare differences between the control and treatment groups. *p < 0.05 versus control.

TGF-β activates CSF2 and IL6 cytokine mRNA expression in Ect1 cells

To confirm microarray data indicating that TGF-β regulates cytokine expression at the transcriptional level, additional preparations of Ect1 cells were incubated with 10% SP or 5 ng/ml TGF-β1, TGF-β2, or TGF-β3 for 4 or 10 h, and cytokine mRNA expression was quantified by RT-PCR. SP induced substantial expression of CSF2, IL6, and IL8 with evidence of a later peak in IL6 compared with CSF2 and IL8 (Fig. 5). All three isoforms of TGF-β induced increased CSF2 expression at both time points, showing that elevated GM-CSF production is regulated at the mRNA level (Fig. 5A). TGF-β2 and TGF-β3, but not TGF-β1, also stimulated increased IL6 mRNA expression, although this was evident only at 10 h, not 4 h (Fig. 5B). Conversely, none of the three TGF-β isoforms induced expression of IL8 mRNA (Fig. 5C). Similarly, whereas SP clearly induced CCL2, CCL20, and IL1A expression in Ect1 cells consistent with the microarray data, this effect was not replicated by any of the TGF-β isoforms (data not shown).
Discussion

Seminal fluid elicits an inflammation-like response in the cervix after coitus by inducing a surge in proinflammatory cytokine and chemokine synthesis (10). The experiments described in this study identify TGF-β as a key factor in human SP that regulates inflammatory cytokine production in cervical epithelial cells. With isoform-specific assays, we showed that all three mammalian isoforms, TGF-β1, TGF-β2, and TGF-β3, are present in high concentrations in human SP. Using microarray analysis, we demonstrated that TGF-β3 induces a pattern of gene expression in Ect1 cells sharing features with the Ect1 cell response to SP, including several gene pathways linked with progression of the cervical inflammatory response to seminal fluid in vivo (10). We then focused on six cytokines identified previously as central to the cervical cell response (10, 13) and found that TGF-β1, TGF-β2, and TGF-β3 each promote synthesis of GM-CSF and IL-6 in both transformed Ect1 cells and primary ectocervical epithelial cells. This is consistent with the strong correlation between the differential capacity of SP samples to induce GM-CSF and IL-6 and their TGF-β content, and the finding that neutralizing any or all of the TGF-β isoforms in SP, or inhibition of TGF-β signaling, impairs SP induction of these cytokines. Because GM-CSF and IL-6 are identified as centrally involved in the physiological response to seminal fluid after coitus in women (10), these results strongly implicate seminal fluid TGF-β as responsible, at least in part, for triggering the postcoital inflammatory cascade in the human cervix.

Our findings extend earlier reports of TGF-β1 and TGF-β2 in human seminal fluid (18, 36) to show that TGF-β3 is present at levels comparable to TGF-β1 and substantially greater than TGF-β2. The TGF-β content of semen is extraordinarily high, ~5-fold that of serum, and similar to colostrum, which is the most potent biological source of TGF-β known (17). TGF-β is abundant in the seminal fluid of other animals, including pigs (37) and mice (14), indicating the likely conservation of this signaling pathway across mammalian species. As in the mouse and pig, where TGF-β is synthesized in male accessory glands in the latent form, the majority of TGF-β present in human SP is latent and would require activation to bind to receptors on cervical cells. Together with proteolytic enzymes endogenous to seminal fluid (38, 39), activation after coitus would be facilitated by the acid pH of the vaginal environment, proteases such as plasmin and metalloproteinases produced in the female reproductive tissues (40, 41), and the expression...

FIGURE 4. Effect of TGF-β neutralization or inhibition of TGF-β signaling with SB431542 or TGF-βRII ectodomain receptor antagonist on SP-induced production of GM-CSF (A–C), IL-6 (D–F) or IL-8 (G–I) by Ect1 cells. Ect1 cells were incubated with 10% SP (v/v) in the presence or absence of neutralizing Abs specific for TGF-β1, TGF-β2, or TGF-β3 (all 15 μg/ml) or a combination of all three Abs (A, D, G); the ALK4/ALK5/ALK7 inhibitor SB431542 (0.5 or 5 μM) (B, E, H); or TGF-βRII-Fc receptor antagonist (2 μg/ml) (C, F, I) for 12 h, and supernatants were collected 24 h later for cytokine analysis by ELISA or Luminex multiplex microbead assay. Data are the mean ± SEM of triplicate wells in each experimental group, and are representative of three replicate experiments. Data were analyzed by Kruskal-Wallis and Mann-Whitney U test to compare differences between the 10% SP and treatment groups. *p < 0.05 versus control.
The three isoforms of TGF-β share similar target cell lineages, and each signals through heterotrimeric complexes of type I and type II receptor serine-threonine kinases (TGF-βRI and TGF-βRII), resulting in signal transduction to the nucleus through the action of specific members of the SMAD family of transcriptional regulators (44). The different isoforms display differential affinities for type II receptors, with TGF-β2 requiring colocalization of additional transmembrane-docking molecules to facilitate receptor binding. In the current experiments, all three TGF-β isoforms exerted qualitatively similar GM-CSF and IL-6 responses in Ect1 and primary ectocervical cells, but TGF-β3 was consistently found to be moderately more potent than TGF-β1 and TGF-β2, and correlations between this isoform and the GM-CSF and IL-6 response in Ect1 cells were stronger than the other isoforms (data not shown). This may be due to differences in the relative expression of TGF-β receptors by ectocervical epithelial cells, because TGF-β1 and TGF-β3 have high affinity for TGF-βRII, whereas TGF-β2 binds with low affinity and requires coexpression of TGF-βRI for successful signal transduction (45). TGF-βRII is highly expressed in the cervix and endometrium, whereas TGF-βRI is less abundant (46, 47). TGF-β receptor expression in the endometrium is regulated by ovarian steroid hormones (47), but whether similar regulation occurs in cervical tissue is not known.

Consistent with the self-limited TGF-β response in other cell lineages (48), the microarray data indicate that Ect1 cells down-regulate expression of all three TGF-β receptors, TGF-βRI, TGF-βRII, and TGF-βRIII, after exposure to SP, and, at least for TGF-βRII, TGF-β contributes to this response (Supplemental Table I). Downregulation of the TGF-β response would be further facilitated by induction of SMURF1, which encodes Smad ubiquitination regulatory factor-1, a homologous to E6-AP carboxy terminus-type ubiquitin ligase that regulates quantity of TGF-β receptors and various Smads via the ubiquitin–proteasome pathway (49).

In the physiological setting, the interaction between seminal fluid and female tract epithelial cells is important in inducing tolerance of paternal Ags required for sperm survival and adaptation for pregnancy (50). The cytokine environment prevailing when these Ags are first encountered is pivotal in controlling APC recruitment, which in turn determines the strength and quality of the ensuing T cell response, and thus capacity to tolerate sperm and the implanting embryo (51). Because sexually transmitted infectious agents are introduced in the context of seminal fluid, the cytokine environment in the tract after coitus will also impact the course of infection and immune defense to these organisms.

The cytokines induced by TGF-β in human cervical cells are consistent with contributing to reproductive function and fit with observations in the mouse, where seminal fluid TGF-β induces GM-CSF production after mating (14). GM-CSF is a key factor regulating fertility that targets both reproductive tract dendritic cells and the developing embryo (21). CSF2 null mutant mice have reduced expression of uterine dendritic cell MHCII, scavenger receptor, and costimulatory molecules, causing stunted CD4+ and CD8+ T cell responses to seminal fluid Ags (7), linked with impaired capacity to clear bacteria from the reproductive tract after coitus, depressed fertility, and elevated fetal loss (52). It is not known whether GM-CSF exerts similar actions in women, but reduced GM-CSF production in early pregnancy is linked with recurrent miscarriage (53). The significance of seminal fluid induction of IL-6 in cervical cells is less clear, but a function in controlling progression of the inflammatory response through regulation of chemokine-mediated monocyte recruitment seems likely (54). Several studies show reduced IL6 expression in the reproductive tract tissues of women experiencing miscarriage (31, 55), and IL6-deficient mice are fertile, but show elevated rates of fetal loss in midgestation (56).

Seminal fluid induction of other cytokines in Ect1 cells, IL-8, CCL2, CCL20, and IL-10, occurred independently of TGF-β. This implies that other seminal fluid agents contribute to the cervical cell cytokine response and that the array of cytokines and chemokines produced is differentially regulated. A factor likely to interact with TGF-β is PGE, which is present in high concentrations in SP and mediates immune regulatory functions (12, 57). PGE can induce IL-8 in cervical explants (58) as well as VEGF in cervical carcinoma cells (3). TLR-mediated signaling is likely to be an important pathway whereby seminal fluid microorganisms influence the cervical cytokine response (59). Another possibility is a sequential cascade of induction, whereby endogenous cytokines amplify the cascade. The later onset of IL6 expression compared with CSF2 suggests the possibility of indirect induction, and, in turn, IL-6 trans-signaling is a candidate for regulating chemokine synthesis (54, 60). Evidence supporting this includes the array data showing elevated IL6ST (GP130) expression in response to seminal fluid (Supplemental Table I), increased CCL2 produc-
tion in Ect1 cells induced by IL-6 (Table IV), and delayed onset of CCL2 expression compared with other factors (13).

A limitation in this study is the use of Ect1 cervical cells, generated by transformation with human papilloma virus, as a model for primary ectocervical cells. Ect1 cells have a comparable morphology and gene expression profile to their primary parent cells (24) and are more responsive to SP than endocervical or vaginal epithelial cell lines (13). The similarity between Ect1 cells and primary cells in GM-CSF and IL-6 induction by TGF-β supports the validity of conclusions concerning those factors, and their relevance to the cervical response in vivo. However, there are important differences in the cytokine response of Ect1 cells and primary cells that caution against complete extrapolation. In primary cells, but not Ect1 cells, TGF-β moderately inhibited IL-8 and induced modest increases in IL-1α and CCL2. Additionally, primary cells show considerably higher IL-8 and CCL2 output, consistent with reports that immortalizing cervical keratinocytes with human papilloma virus DNAs can suppress cytokine secretion (61). In particular, reduced CCL2 expression in Ect1 cells has been attributed to active suppression by viral oncoproteins E6 and E7 (62). Thus, in the in vivo setting, cervical epithelial cells may respond to seminal fluid TGF-β stimulation with a wider range of cytokine response than observed in this study in Ect1 cells.

In addition to cytokine production, the microarray experiment revealed that TGF-β accounts for other aspects of the Ect1 cell response to seminal fluid, which warrant further detailed evaluation. These include effects on the VEGF pathway involved in angiogenesis and neovascularization, with compelling evidence for upregulation of genes VEGFA and PTGS2 (COX-2). This extends earlier findings that SP induces these genes (3, 4), and indicates that TGF-β might contribute to possible effects of seminal fluid exposure on neoplastic transformation and tumor progression in the cervix of sexually active women.

Additionally, the microarray experiments showed clear effects of TGF-β in mediating the SP-induced upregulation of FST and FSTL3 and INHBA encoding follistatin, follistatin-like-3 glycoprotein, and activin A, respectively. Activin A modulates expression of inflammatory cytokines and directs development of M2 macrophages and dendritic cells, as well as T cell differentiation, to promote type 2 and regulatory immune responses (63). Activin A signaling is negatively regulated by follistatin and follistatin-like-3 (63). This pathway is implicated in curtailing the female response to inflammation, with follistatin-deficient mice experiencing an unrestrained cervical immune response after coitus (64).

TGF-β is a potent immune-deviating cytokine with pivotal roles in regulating the inflammatory response and inducing active immune tolerance in mucosal and peripheral tissues. Its copious quantity in SP would, together with the PGs, largely explain the immunosuppressive properties attributed to this fluid (12). TGF-β cytokines exert profound effects on lymphocytes, macrophages, and dendritic cells (15, 16), but these depend on microenvironmental context and vary according to phase of the inflammatory response, such that initially TGF-β exerts proinflammatory activity, before later acting to promote the resolution phase (65). There may be commonality across epithelial tissues in pathways through which TGF-β signaling to epithelial cells strengthens and amplifies direct effects of TGF-β in leukocytes. Similar to Ect1 cells, human retinal pigment epithelial cells respond to all three TGF-β isoforms with potent induction of GM-CSF (66) and VEGF (67). In airway epithelial cells, TGF-β induces GM-CSF, but not IL-8 or CCL5 synthesis (68), whereas in prostate carcinoma cells, TGF-β upregulates IL-6 expression (69). IL11, another gene induced in Ect1 cells by both SP and TGF-β, is induced in several cell lineages, including A549 alveolar epithelial cells by TGF-β moieties (70).

The immune-regulatory properties of TGF-β would also contribute to the known actions of seminal fluid in promoting maternal immune tolerance for pregnancy. Our studies in mouse models indicate that seminal fluid exposure is essential for promoting uterine Treg cells prior to embryo implantation (8, 71), and experiments administering exogenous TGF-β to mice after coitus show effects on Treg cells that support a central role for this cytokine (72). Ag delivery in the presence of TGF-β favors development of Treg cells (73, 74), and naive CD4+CD25+ T cells express FOXP3 when TGF-β is present (75). TGF-β is also implicated in proliferation of mature Treg cells through modifying the function and signaling capabilities of dendritic cells (76). It remains to be determined whether TGF-β in seminal fluid acts on cervical lymphocytes and dendritic cells to induce tolerogenic phenotypes in women, and whether this has consequences for immune tolerance in pregnancy.

In summary, this study advances understanding of the mechanisms by which seminal fluid interacts with cervical cells in women. TGF-β is identified as a key signaling agent for inducing cytokines that control cervical tissue immune responses to seminal fluid Ags, and influence the quality of the ensuing effector phase of the response to skew the balance toward tolerance as opposed to immunity. Depending on whether the female tract has evolved mechanisms to discriminate seminal Ags from opportunistic pathogens, there may be a detrimental cost of seminal TGF-β in inhibiting protective immunity to agents of sexually transmitted disease, including HIV. Whereas SP TGF-β content appears unaltered when sperm parameters are abnormal (19), whether TGF-β abundance is different in the SP of individuals with unexplained infertility warrants further investigation.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


