

## Research Article

# ***Neisseria gonorrhoeae* Pilus Attenuates Cytokine Response of Human Fallopian Tube Explants**

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**Background.** A role for pilus during attachment of *Neisseria gonorrhoeae* to epithelia of the female reproductive tract is currently assumed. However, Pil<sup>-</sup> gonococci have been observed during infection of the reproductive tract, which prompted us to examine the effect of pili on the dynamics of infection and the inflammatory responses of mucosal explants of the human Fallopian tube. **Methods.** Mucosal explants were infected in vitro with Opa negative Pil<sup>-</sup> and Pil<sup>+</sup> *N. gonorrhoeae* strains. **Results.** Piliation enhanced gonococcal adherence to the epithelium within 3 h of infection ( $P < 0.05$ ) but thereafter did not offer advantage to gonococci to colonize the epithelial cell surface ( $P > 0.05$ ). No differences were found between the strains in numbers of gonococci inside epithelial cells. Pil<sup>-</sup> bacteria induced higher levels ( $P < 0.05$ ) of IL-1 $\beta$ , TNF- $\alpha$ , GM-CSF, MCP-1, and MIP-1 $\beta$  than Pil<sup>+</sup> bacteria. There were no differences between both strains in LOS pattern, and Pil expression did not change after coinoculation with mucosal strips. **Conclusions.** Results show that gonococcal invasion of the human Fallopian tube can occur independently of pilus or Opa expression, and suggest that pilus, by inhibition of several key elements of the initial inflammatory response, facilitates sustained infection of this organ.

## 1. Introduction

*Neisseria gonorrhoeae* (NGO) is an exclusive pathogen of humans that infects the mucosal epithelia of the male urethra and the female lower genital tract. It is the etiological agent of gonorrhea, and, for women in particular, infection can spread into the upper reproductive tract, including the Fallopian tubes (FTs). Gonococcal interactions with the FT epithelium initiate salpingitis, an inflammatory response that can lead to FT damage and infertility. In the FT, evidence from the classical papers of McGee and colleagues using a culture model of ex vivo FT organ explants suggested that gonococcal interactions were mediated by expression of type IV pilus and the phase-variable colony opacity (Opa) protein adhesins [1, 2]. Moreover, evidence gathered with many isolated human cell lines has also shown that invasion of host cells by gonococci involves initial attachment mediated primarily by pili [3–9] which leads to intracellular

cortical plaque formation [10] with cooperation with Opa proteins leading to bacterial internalization and/or traversal of epithelial barriers [11–15].

In the course of research exploring the influence of contraceptives on gonococcal infection of the human FT [16], we used a clinical isolate that afterwards was phenotypically characterized as a Pil<sup>-</sup>Opa<sup>+</sup> strain [17]. Attachment and internalization of this particular strain into the oviductal epithelium were observed from the first minutes of exposure and were followed by the release of proinflammatory cytokines such as IL-1 $\alpha$ , IL-1 $\beta$ , and TNF- $\alpha$  [17]. It has been suggested that in addition to mediating bacterial interactions, pili also have a role in innate immunostimulation by influencing the cytokine response induced during infection [18]. In the current study, we examined the correlation between pilus expression in gonococcal variants lacking Opa protein on bacterial interactions with FT mucosal explants and the induction of cytokine secretion.

## 2. Materials and Methods

**2.1. *Neisseria gonorrhoeae* Strains.** *Neisseria gonorrhoeae* (NGO) strains P9, variant 1 (Pil<sup>-</sup>Opa<sup>-</sup>), and variant 2 (Pil<sup>+</sup>Opa<sup>-</sup>) [19] were grown on GC agar at 37°C in 5% (v/v) CO<sub>2</sub>. The strains were derived from the original P9 isolate [20, 21], and the strain phenotype was confirmed by examining colony morphology with a stereomicroscope and with SDS-PAGE analysis and immunoblotting with specific monoclonal antipilus antibody SM1 [22] and anti-Opa protein antibody B33 [14], both before and after coinubation with tubal mucosal explants.

**2.2. Miniscale LOS Extraction and Tricine-SDS-PAGE Analysis.** To verify LOS integrity in the two NGO strains, LOS samples were extracted and visualized by subjecting proteinase K digests of whole-cell lysates to Tricine SDS-PAGE [23, 24]. After electrophoresis, the gel was fixed in a solution containing 40% ethanol and 5% acetic acid overnight followed by silver staining [25].

**2.3. Human Fallopian Tube Explants.** Fallopian tubes (FTs) were obtained after informed consent from 13 fertile donors submitted to gynecological surgery for reasons unrelated to this study. Exclusion criteria were sexually transmitted disease during the last year and no history of pelvic inflammatory disease. Protocols were approved by the Ethics Committees of the Universidad de Santiago de Chile and the Hospital San Jose (Santiago, Chile) where the surgeries were carried out. Organs were processed immediately after removal as previously described [16]. Briefly, after muscle dissection, mucosal strips were cut into 1 cm<sup>3</sup> segments and cultured in Dulbecco's modified Eagle's medium (DMEM, Hi Clone) for 3 h before infection with NGO.

**2.4. Infection of Human FT Explants with NGO.** Mucosal explants were infected with 100  $\mu$ L of NGO suspension ( $3 \times 10^6$  cfu/mL) in DMEM as previously described [17]. Adherent and internalized gonococci in the mucosal strips were examined under confocal microscopy (Axiovert 100M Zeiss laser scanning microscope) and counted as described previously [16]. Briefly, tissues for microscopy were fixed in cold 4% (v/v) paraformaldehyde in PBS, pH 7.4 (Winkler, Santiago, Chile) for 1 h before transfer to 10% (w/v) (1 h) and 30% (w/v) (overnight) sucrose. They were mounted in embedding compound (Cryo-M-Bed, Bright Instruments Co. Ltd., Huntingdon, UK) and frozen at -20°C. Sections of 6  $\mu$ m were cut using a Bright Starlet Cryostat, and gonococci were stained with a rabbit antigonococcal antiserum and fluorescein-conjugated anti-rabbit IgG.

**2.5. Cytokine Analysis.** Samples from the culture media were taken at intervals up to 24 h of coinubation and the secretion of cytokines IL-1 $\beta$ , IL-6, TNF- $\alpha$ , IL-10, TGF- $\beta$ , IL-8, GM-CSF, MCP-1, MIP-1 $\beta$ , and RANTES was quantified by sandwich immunoassays as described previously [26].

**2.6. Statistical Analysis.** Statistical analysis was done by ANOVA or *t*-test as required, using Minitab Software

TABLE 1: Attachment of NGO variants to human FT explant epithelium. FT explants were infected with Pil<sup>-</sup>Opa<sup>-</sup> NGO ( $n = 7$ ) and Pil<sup>+</sup>Opa<sup>-</sup> ( $n = 6$ ), and 16 fields were examined for each explant at each time point. In each field, gonococci were counted within a continuous epithelial strip of at least 150 cells. The data are presented as the mean and SEM of the number of gonococci observed, attached, or internalized. \*denotes a significantly ( $P < 0.05$ ) higher number of attached Pil<sup>+</sup>Opa<sup>-</sup> NGO compared to the Pil<sup>-</sup>Opa<sup>-</sup> variant at 3 h.

Time (h)	Attached	
	Pil <sup>-</sup> Opa <sup>-</sup>	Pil <sup>+</sup> Opa <sup>-</sup>
3	2.6 $\pm$ 0.6	7.6 $\pm$ 2.6*
6	4.1 $\pm$ 1.1	6.3 $\pm$ 1.4
12	4.2 $\pm$ 1.1	5.8 $\pm$ 1.3
24	6.8 $\pm$ 0.9	6.8 $\pm$ 2.0
	Internalised	
	Pil <sup>-</sup> Opa <sup>-</sup>	Pil <sup>+</sup> Opa <sup>-</sup>
3	12.4 $\pm$ 1.6	18.0 $\pm$ 3.4
6	14.4 $\pm$ 1.9	16.8 $\pm$ 2.4
12	14.1 $\pm$ 1.8	15.0 $\pm$ 2.4
24	25.1 $\pm$ 1.9	18.3 $\pm$ 3.9

(Minitab 15.1.30.0). Comparison of attached and internalized gonococci between both strains was done by *t*-test at each incubation time using log-transformed data to correct for variance heterogeneity.

## 3. Results

Human FT explants were infected with Pil<sup>-</sup>Opa<sup>-</sup> and Pil<sup>+</sup>Opa<sup>-</sup> NGO variants, and the numbers of adherent and internalized bacteria were quantified up to 24 h. NGO was found attached to the epithelium and internalized at all time points examined (Table 1), with Pil<sup>+</sup> bacteria showing a significantly higher rate of initial adherence by 3 h than the Pil<sup>-</sup> variant ( $P < 0.05$ ). Thereafter, however, no statistically significant differences were observed between the numbers of attached Pil<sup>+</sup> and Pil<sup>-</sup> NGO ( $P > 0.05$ ). NGO invasion of the FT epithelium was rapid, but there were no statistically significant differences ( $P > 0.05$ ) in the numbers of Pil<sup>+</sup> and Pil<sup>-</sup> bacteria counted within the epithelium at all time points examined (Table 1). Adherence and invasion of the FT epithelium were confirmed by confocal microscopy.

Compared to uninfected FT explants, gonococcal infection resulted in the significant ( $P < 0.05$ ) secretion of key proinflammatory (IL-1 $\beta$ , IL-6, and TNF $\alpha$ ), chemokine (MCP-1, MIP-1 $\beta$ ), and growth-factor related (MCP-1, MIP-1 $\beta$  and GM-CSF) cytokines. By contrast, the secretion of IL-8, RANTES, TGF- $\beta$ , MIP-1 $\alpha$ , or IL-10 was not significantly increased by gonococcal infection compared to uninfected explants (data not shown).

For those cytokines whose secretion was significantly increased by gonococcal infection compared to uninfected FT explants, secretion was influenced by pilus expression. In general, infection with Pil<sup>-</sup> NGO resulted in significantly higher secretion levels compared with Pil<sup>+</sup> NGO. The levels of IL-1 $\beta$  secreted to the culture medium increased with both

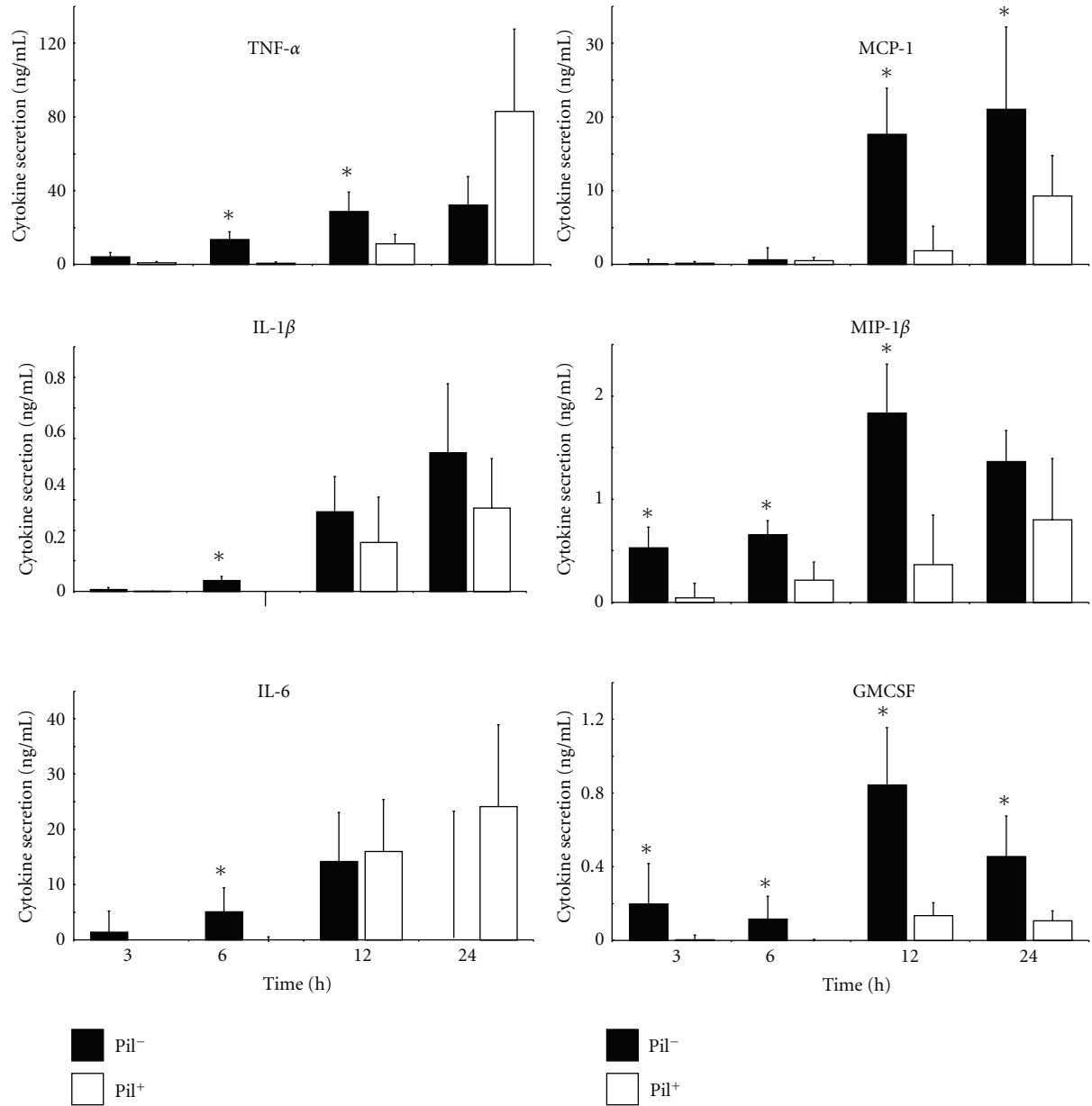


FIGURE 1: Expression of pilus attenuates cytokine production by human FT explant epithelium. FT explants were infected with Pil<sup>-</sup>Opa<sup>-</sup> NGO ( $n = 7$ ) and Pil<sup>+</sup>Opa<sup>-</sup> ( $n = 6$ ) and cytokine secretion measured over time. The columns represent mean cytokine levels and the standard error for the sample group. \*denotes statistically significant ( $P < 0.05$ ) increase in cytokine secretion induced by Pil<sup>-</sup> bacteria compared to Pil<sup>+</sup> bacteria at given time point.

strains of NGO over time, but by 24 h they were significantly higher in explants infected with Pil<sup>-</sup> bacteria compared with Pil<sup>+</sup> bacteria ( $P < 0.05$ ; Figure 1). Secretion of TNF- $\alpha$  also increased over time with both NGO strains, with the levels induced by Pil<sup>-</sup> bacteria by 12 hr significantly higher than those observed with Pil<sup>+</sup> bacteria ( $P = 0.006$ ), although this difference disappeared by 24 h (Figure 1). IL-6 secretion induced by Pil<sup>-</sup> NGO was significantly higher at the earlier time points (3 and 6 h) compared with Pil<sup>+</sup> NGO ( $P < 0.05$ ), but these differences were not significant by 12 h onwards ( $P > 0.05$ ). For MCP-1, MIP-1 $\beta$ , and GM-CSF, infection with

Pil<sup>-</sup> NGO induced significantly higher amounts of secretion when compared with Pil<sup>+</sup> NGO at all time points examined ( $P < 0.05$ ; Figure 1).

It is known that gonococcal phase and antigenic variation occur during in vitro growth [27] as well as during experimental infection [28], so phenotypic stability of the Pil<sup>-</sup>Opa<sup>-</sup> and Pil<sup>+</sup>Opa<sup>-</sup> strains used in the current study was examined during infection of FT explants. Western blotting for pilus and Opa protein expression of NGO recovered from the FT explants demonstrated no changes in phenotype during infection (Figure 2). There were no differences in LOS

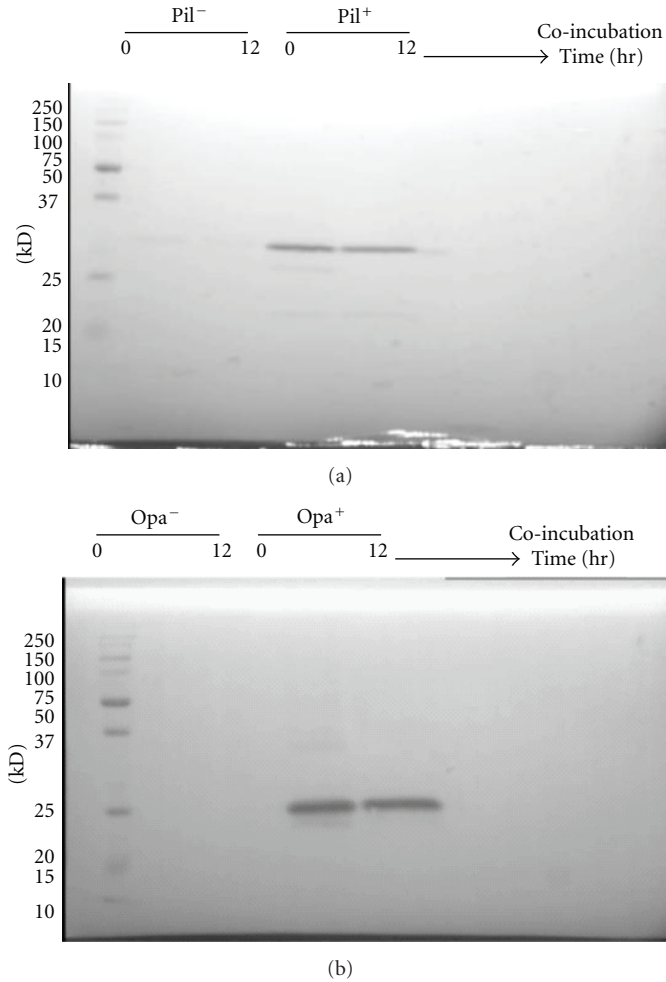


FIGURE 2: Western blot for Pil and Opa proteins in Pil<sup>+</sup>Opa<sup>+</sup> and Pil<sup>-</sup>Opa<sup>-</sup> gonococcal strains incubated for 12 h with Fallopian tube mucosal strips. (a): Western blot for Pil protein. (b): Western blot for Opa protein. The two dark bands corresponding to Pil (a) and Opa (b) appear in lanes from the Pil<sup>+</sup>Opa<sup>+</sup> strain only. Densitometry analysis showed no change in Pil and Opa expression after the co-incubation.

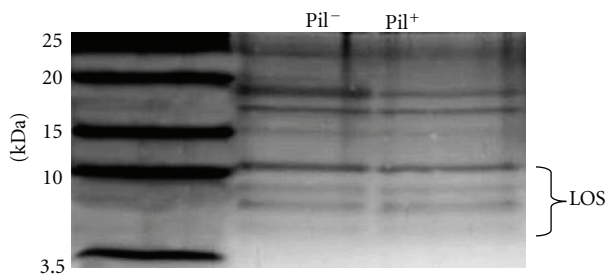


FIGURE 3: Lipooligosaccharide profiles of NGO strains used in this study. Whole-cell lysates of gonococcal strains were treated with proteinase K overnight and then boiled prior to Tricine SDS-PAGE (16%). Protein molecular weight marker: Novex Sharp Standard (Invitrogen).

expression between both NGO strains (Figure 3). Therefore the patterns of adhesion/internalization and cytokine production in these experiments can safely be correlated with the presence or absence of pili.

#### 4. Discussion

In the current study, we report an experimental *in vitro* examination of the role of gonococcal pili during epithelial invasion and cytokine production at the initial stages of infection of the human Fallopian tube. Experimental infection based on cell lines and cultures has provided a theoretical model for gonococcal infection in which pili have a prominent role in the initial attachment to the host cell [18, 29]. We observed in our study that the presence of pili enhanced gonococcal adherence to the tubal epithelium within 3 h of infection compared with the corresponding nonpilated variant but thereafter did not appear to offer a significant advantage to gonococci to colonize the epithelial cell surface. We have shown previously that a clinical Pil<sup>-</sup>Opa<sup>+</sup> gonococcal strain attached and invaded the epithelium of tubal explants [17] and the appearance of Pil<sup>-</sup> bacteria during experimental infection has also been reported [28]. In our study, pilus expression also did not confer any advantage to the gonococcus for invasion. Moreover, because the strains we used in our experiments were both Opa

negative, our data also suggested an Opa-independent mechanism for invasion of the tubal epithelium. The redundancy of Opa is consistent with reports demonstrating that Opa<sup>-</sup> variants were as infective as Opa<sup>+</sup> gonococci in human experimental infections [30], and adhesion and phagocytosis of Pil<sup>-</sup> Opa<sup>-</sup> gonococcal strains by human dendritic cells were influenced by differences in their lipooligosaccharides [31].

Taken together with the reported literature, our findings therefore suggest that mechanisms other than pilus or Opa-mediated binding to surface molecules of the epithelial cells operate with high efficiency during gonococci invasion of the Fallopian tube. The exact nature of these mechanisms is unclear, although it has been reported, using recombinant *E. coli* expressing specific gonococcal components, that invasion of FT epithelia depended on the expression of combinations of Opa, porin, and lipooligosaccharide [32]. However, it is also likely that the endocytic properties of the oviductal epithelium [33] probably contribute to uptake of gonococci.

In the current study, expression of pili appeared to have a role in the regulation of cytokine responses following gonococcal infection. Although both Opa<sup>-</sup> gonococcal strains induced secretion of IL-1 $\beta$ , TNF- $\alpha$ , GM-CSF, MCP-1, and MIP-1 $\beta$ , the stimulatory effects were significantly higher with the Pil<sup>-</sup> bacteria compared to Pil<sup>+</sup> bacteria. Thus, expression of gonococcal pili appeared to attenuate cytokine-chemokine responses in the mucosal epithelial cells of the human Fallopian tube, although the contribution of other uncharacterized genetic differences cannot be categorically ruled out. Or, alternatively, the absence of the large numbers of surface-located pili structures results in remodeling of the OM of the gonococcus to allow increased interactions with the epithelial cell LOS receptors, leading to increased cytokine production. These findings are in contrast to other studies that have investigated the involvement of pilus expression in determining the magnitude of the cytokine response. For example, during infection of a human monocytic cell line with *Salmonella typhi*, Pil<sup>+</sup> bacteria induced higher levels of IL-6 production and NF- $\kappa$ B activation than nonpiliated bacteria [34]. However, we should stress that the cytokine pattern observed in our experiments might not necessarily represent the in situ infection, because in vitro mucosal explants are devoid of blood and/or tissue-associated immune effector cells [35], whose contribution to the local cytokine pool is to be expected during the advanced stages of salpingitis when bacteria reach deep into the subepithelial tissue.

By contrast to pilus expression, the presence of Opa protein was not a contributory factor to cytokine attenuation in the FT epithelium itself. However, it has been shown with human dendritic cells that Opa proteins can modulate innate and adaptive immune responses, by directly suppressing T-cell receptor signaling through engagement of coinhibitory CD66a/CEACAM1 receptor, thereby raising the threshold for CD4<sup>+</sup> T cell activation and proliferation to activating stimuli [36, 37] and interfering with antibody production by promoting human B-cell death [38]. Thus, Opa expression may play an indirect role in inhibiting innate recognition during FT infection by targeting the function of patrolling submucosal dendritic cells.

In summary, our data suggest a modulatory role for gonococcal pili during infection of the human tubal mucosa. Expression of pilus enhances the initial rapid attachment of the gonococcus to the Fallopian tube mucosal epithelium but significantly attenuates the local production of several inflammatory mediators. Although phase variation of pilus and opa expression in the upper reproductive tract is likely to be a dynamic process during interactions with the host, it is likely that the Pil<sup>-</sup> phenotype is probably present at a lower percentage of the gonococcal population. Switching off pilus expression would not be beneficial to the gonococcus in that an excess inflammatory response contributory to LOS-mediated stimulation could result. Thus, a consequence of limiting the innate response of the epithelium is to reduce immune effector cell recruitment to the site of infection. This would add to the mechanisms that could allow repeated gonococcal infections in the same individual without ever generating protective immunity.

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