TNF-α-Independent IL-8 Expression: Alterations in Bacterial Challenge Dose Cause Differential Human Monocytic Cytokine Response[1]

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We examined the effects of different bacterial doses of *Neisseria gonorrhoeae* on the cytokine response of primary human monocytes. The data indicate that a low multiplicity of infection (MOI) challenge (MOI = 0.1) results in substantial production of IL-8 and other chemokines/cytokines, in the absence of significant TNF-α production. Positive control challenges (MOI = 10) induced levels of IL-8 that were comparable to the low MOI challenges, but now induced significant levels of TNF-α. Induction of IL-8 expression in low MOI challenges was not mediated by an autocrine response as pretreatment of monocytes with neutralizing Abs against TNF-α or IL-1β had no effect on IL-8 expression. IL-8 induction resulting from gonococcal challenge was shown to require NF-κB activation, though this activation was limited by the inoculating dose. These data indicate that IL-8 induction results from direct contact between bacteria and monocytes. Analysis of the overall cytokine profile revealed patterns of expression for growth-regulated oncogene, MCP-1, and IL-6 that were similar to IL-8. Analysis of various MAPKs indicated that low MOI challenges were able to efficiently activate both the ERK and p38 pathways, but in contrast to positive control samples, failed to activate the JNK pathway. A lack of phosphorylated JNK leads to decreased production of AP-1 dimers, transcription factors that are critical for efficient transcription of TNF-α. Therefore, we propose a mechanism where a low MOI gonococcal challenge results in diminished AP-1 activity and TNF-α production while IL-8 levels remain constant. ***The Journal of Immunology,*** **2006, 177: 1314–1322.**

Neisseria gonorrhoeae, the causative agent of gonorrhea, classically manifests as an intense inflammatory response. Disease in men is characterized by a massive infiltrate of polymorphonuclear leukocytes (neutrophils) and monocytes to the site of infection, which results in pain and purulent discharge (1, 2). However, almost 80% of infections in women occur without noticeable symptoms (3). The reason for this gender-based discrepancy is unknown and individuals with asymptomatic gonorrhea often harbor infections which persist at subclinical levels. This is presumably advantageous for the bacterium, allowing continued transmission to other hosts. Prolonged gonococcal infections in women can lead to serious sequelae, i.e., pelvic inflammatory disease and disseminated gonococcal disease. Damage resulting from these complications is thought to be caused by the proinflammatory cytokine TNF-α (4), and this damage can predispose women to ectopic pregnancy or fallopian tube scarring and subsequent sterility (for review, see Burnakis and Hildebrandt (5)).

The first stage of gonococcal infection is considered to be the colonization of host epithelial cells. Type IV pili mediate the initial attachment of gonococci to host cells where the bacteria form microcolonies of 10–100 diplococci (6). Eventually gonococci retract their pili and adhere tightly to the host cell plasma membrane (7). Invasion and transcytosis of urogenital tissues then follows, as demonstrated by a number of in vitro studies (8–11). Invasive gonococci have been observed within intracellular vacuoles in secretory epithelial cells (12). Gonococcal transcytosis, however, appears to occur very inefficiently, even when the initial inoculum is quite high, resulting in a very low overall percentage of invading bacteria (11, 13). These surviving bacteria must then encounter immune cells, such as monocytes, within the submucosa. These early cell-cell interactions are followed by the elicitation of neutrophils, the classic diagnostic of symptomatic gonorrhea. The interaction of gonococci with mucosal immune cells is therefore likely to be of significance during the disease process.

Several other key surface molecules contribute to adherence and invasion including gonococcal opacity-associated protein (Opa) and lipo-oligosaccharide (LOS)[3]. Some (Opas) have been shown to bind to host heparin sulfate proteoglycans (14, 15), while others bind members of the carcinoembryonic Ag-related cell adhesion molecules (16, 17). LOS displayed by the gonococcus is antigenically phase variable and mimics mammalian glycosphingolipids. LOS containing a terminal lacto-*N*-neotetraose moiety has been shown to bind the host asialoglycoprotein receptor (18) and increase gonococcal invasion (9).

Although it is known that other Gram-negative pathogens such as *Salmonella spp.* are able to establish infections via an extremely low inoculum (19), the infectious dose for *N. gonorrhoeae* is unknown and likely differs depending upon host gender and the site of colonization. Schneider et al. (20) demonstrated that an initial

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Received for publication October 14, 2005. Accepted for publication April 25, 2006.

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[1] This work was supported by National Institutes of Health Grant AI24452 (to D.C.S.).

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[3] Abbreviations used in this paper: LOS, lipo-oligosaccharide; PVDF, polyvinylidene fluoride; MOI, multiplicity of infection; TPCK, *N*-tosyl-l-phenylalanine-chloromethyl ketone; GRO, growth-regulated oncogene; Opa, opacity-associated protein.

0022-1767/06/$02.00

inoculum of only 250 gonococci was sufficient to establish infection in three of seven healthy male volunteers in a clinical challenge (19, 20). The best estimates of the number of bacteria transferred to women suggest an inoculating dose for women ranging from $\sim 2 \times 10^4$ to 6×10^6 gonococci (21). Though data are limited to male clinical trials and tissue culture models of infection, it is probable that even given a high inoculating dose, only a very small number of gonococci will successfully invade the reproductive tract.

The degree of inflammation usually reflects disease severity and symptoms, and serves to clear the host of infectious microbes. Mucosal inflammation can occur as a result of the direct interaction of a pathogen with surface epithelial cells or with specific immune cells beneath the epithelial layer. The production and regulation of inflammatory cytokines is a critical component of the human innate response to bacterial infections. The cytokines produced as a result of infection can have both local and systemic effects including alteration of vascular permeability, recruitment of immune cells, accumulation of toxins, and tissue damage (22). Inflammation is widely accepted as the classic host response to gonococcal colonization; however, the intricacies of the human immune response to this bacterium are unknown. In this study, we chose to measure the production of several proinflammatory cytokines such as TNF-α and IL-1β, as well as chemokines such as IL-8. These cytokines are associated, respectively, with gonococci-induced tissue damage/inflammation and neutrophil/monocyte influx to the site of infection.

We have hypothesized that in naturally occurring gonococcal infections, colonization and subsequent invasion by a small number of bacteria can result in a dramatic innate immune response by monocytes. We show here that *N. gonorrhoeae* is remarkably efficient at eliciting significant levels of IL-8 in our low dose challenge model, in the absence of detectable TNF-α. Our data indicate that this IL-8 production is likely caused by direct contact of gonococcal surface molecules with monocytes and is NF-κB dependent. In addition, we demonstrate that the MAPK JNK is not activated in our low dose model. Our results demonstrate the importance of chemokines in gonococcal infection and imply that lowering the initial bacterial dose causes a striking per cell increase in the production of specific chemokines/cytokines. We propose a mechanism where a low dose challenge results in diminished AP-1 activity and TNF-α production while overall IL-8 levels remain constant.

Materials and Methods

Bacterial strains and infection

Gonococcal strain F62 was obtained from Dr. P. F. Sparling (University of North Carolina, Chapel Hill, NC). F62ΔlgtD, a derivative of F62 that expresses a nonvarying LOS, lacto-*N*-neotetraose, has been previously described (9). Before each experimental challenge, gonococci were grown on gonococcal growth media (BD Biosciences) containing agar supplemented with Kellogg's solution (23) and piliated, Opa^- organisms were selected. Bacteria were suspended and the concentration of cells was determined spectrophotometrically and verified via viable plate count. Cells were killed by incubation for 3 h with 2 mg/ml gentamicin sulfate. Bacteria were stored at 4°C until challenge of human cells. Before bacterial challenge, gentamicin-killed bacteria were collected by centrifugation (12,000 rpm for 5 min), resuspended in RPMI 1640, and diluted appropriately. Use of killed bacteria prevented growth and phase variation during the incubation period. For the preparation of bacterial lysates, live gonococci were diluted into PBS and sonicated. (Sonications were performed with a Branson Sonifier 250; 20% duty cycle, output control = 4). Sonication was conducted on ice, using a 15 s burst with 30 s of cooling, for a total of 180 s. Bacterial killing was confirmed by the absence of detectable bacteria on viable plate counts. For experiments involving *Escherichia coli*, strain DH5-α was used (New England Biolabs).

Isolation and culture of human monocytes/macrophages

Whole blood was drawn from healthy volunteers and PBMC were prepared by density gradient centrifugation using Ficoll-Paque Plus (Amersham Biosciences). Before bacterial challenges, cells were seeded at 1×10^6 cells/ml in tissue culture-treated plates in RPMI 1640 (Invitrogen Life Technologies) and incubated at 37°C, 5% CO_2 with humidity. After 30 min, the cells were washed to remove the nonadherent population and RPMI 1640 (supplemented with 10% autologous heat-inactivated serum) was added preceding bacterial challenge. Differentiated macrophages were used to represent a resident macrophage phenotype, and provided us a clear model for visualization of NF-κB translocation. For differentiation into macrophages, cells were seeded at $\sim 5 \times 10^5$ cells/ml in untreated petri plates and maintained in RPMI 1640 supplemented with 20% heat-inactivated FCS and 20 mM HEPES for 7 days. On day 7, cells were washed and macrophages were removed from petri plates with Cell Stripper (Cellgro/Mediatech) and reseeded onto glass coverslips for immunofluorescence staining and confocal microscopy. These studies were approved by the University of Maryland Institutional Review Board.

ELISA

Before bacterial challenge, primary human monocytes were isolated and seeded as described. Following challenge with the appropriate dilution (100 μl) of gentamicin-killed gonococci, cells were incubated in RPMI 1640 supplemented with 10% autologous human serum at 37°C 5% CO_2 with humidity for the time specified. Cell supernatants were then collected and assayed for the presence of cytokines by ELISA. Upon collection of supernatants, a protease inhibitor mixture was added and each sample was stored at −80°C until analysis. Ab pairs and recombinant standards for human TNF-α and IL-8 were purchased from BD Pharmingen. Ab pairs, recombinant standards, and neutralizing Ab for human IL-1β were purchased from R&D Systems. In neutralizing experiments, 5 μg/ml TNF-α neutralizing Ab/2 μg/ml IL-1β neutralizing Ab was shown to completely prevent detection of TNF-α/IL-1β in cell supernatants.

Immunofluorescence staining and confocal microscopy

Human macrophages, differentiated from primary blood monocytes, were seeded overnight on sterile, acid-washed coverslips at 2×10^5 macrophages/coverslip. Macrophages were washed once in warm RPMI 1640 before the addition of 100 μl of the appropriate bacterial dilution in RPMI 1640 and 300 μl of RPMI 1640 supplemented with 10% FCS. Cells were incubated at 37°C, 5% CO_2 with humidity. At the specified time point, macrophages were washed once in warm PBS and fixed with cold methanol for 10 min. After incubation, macrophages were washed three times with PBS and blocked with PBS 5% FCS for 15 min at 37°C with humidity. A mouse monoclonal anti-NF-κB p65 (Santa Cruz Biotechnology) was added to coverslips at a concentration of 1 μg/ml in blocking buffer. Coverslips were incubated for 1 h at room temperature with gentle agitation, washed twice in PBS, and incubated with secondary Ab Alexa Fluor 633 goat anti-mouse (Molecular Probes) in blocking buffer at 1 μg/ml, for 1 h at room temperature with gentle agitation. Cells were washed twice in warm PBS and incubated with Alexa Fluor 546 phalloidin (1/50) for 30 min, washed once, and incubated with a 1/5000 dilution of SYTO 13 green fluorescent nucleic acid stain for 20 min. Coverslips were washed three times in PBS and mounted using Gel/Mount antifading mounting medium (Biomeda). Slides were analyzed via laser-scanning confocal microscopy (LSM 510; Zeiss). Fields were randomly chosen and at least 50 macrophages/sample were categorized as positive or negative for the presence of activated NF-κB p65 in the nucleus.

Activation/inhibition of MAPK in monocytes treated with gonococci

Before infection, primary human monocytes were seeded overnight in 6-well tissue culture plates and incubated at 37°C, 5% CO_2. This overnight incubation was necessary to allow monocytes to return to expressing only basal levels of phosphorylated MAPKs before detection by Western blot. Postinfection, the 6-well plates were placed on an ice bath and washed three times with ice-cold PBS. Lysates were collected by adding 100 μl of Nonidet P-40 lysis solution (1% Nonidet P-40, 20 mM Tris-Cl (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM EGTA, 10 μg/ml leupeptin and aprotinin protease inhibitors, 50 mM NaF, 1 mM sodium vanadate) to each well, scraping the cells from the plates, and pipetting the lysate into 1.5-ml microcentrifuge tubes. The lysates were placed on ice for 20–30 min and vortexed briefly every 7 min. All lysates were then centrifuged at 4°C for 30 min and the supernatants were transferred to a new 1.5-ml microcentrifuge tube and stored at −80ºC. Protein concentration of the cellular extracts was determined using the Bio-Rad protein assay. MAPKs were

inhibited by pretreatment with either PD98059, SB203580, or SP600125, 30 min before challenge with gonococci (BioSource International).

SDS-PAGE and immunoblotting

Lysates were prepared for SDS-PAGE by combining them with 2× loading buffer (50% glycerol, 5% SDS, 0.25 M Tris-Cl (pH 6.8), 0.04% 2-ME, and 25 μg/ml bromphenol blue) in a 1:1 ratio, boiling the samples for 5 min, and centrifuging them for 2 min at 12000 rpm. Equal volumes of each lysate were loaded into a gradient SDS-PAGE minigel and run at 0.01 Amp/gel until the dye front just ran off the bottom of the gel (~110 min). Gels were equilibrated in 1× transfer buffer (25 mM Tris, 192 mM glycine, 15% methanol (pH 8.2)) for 20 min while the polyvinylidene fluoride (PVDF) transfer membranes (GE Osmonics) were prepared (10 s in methanol, 5 min in HPLC grade water, 15 min in 1× transfer buffer). Proteins were transferred onto the PVDF membrane via wet, horizontal transfer at 100 V for 60 min at 4°C. The PVDF membranes were blocked overnight at room temperature in 1× PBS/0.05% Tween 20%/1% fish skin gelatin (Sigma-Aldrich). Membranes were washed twice in wash buffer (PBS/0.05% Tween 20) and incubated in primary Ab solution (wash buffer, 1% gelatin, 1/1,000 rabbit anti-phospho-p38 (Cell Signaling Technology), ERK, or JNK) for 90 min at room temperature. After 5–15 min washes in wash buffer, membranes were incubated in secondary Ab solution (1× PBS/0.5% gelatin, 1/2000 goat anti-rabbit IgG-HRP conjugate) (Cell Signaling Technology) for 60 min at room temperature followed by 4- to 15-min washes in wash buffer. Membranes were developed with luminal/peroxide chemiluminescence reagents (PerkinElmer Western Lightning) and exposed to blue autoradiography film (Marsh Bioproducts). To verify that equal amounts of lysate were loaded on the gel, the amount of β-actin in each lane was determined by incubating the membrane in stripping buffer (62.5 mM Trizma base, 2% SDS, and 100 mM 2-ME (pH 6.7)) for 45 min at 50°C, washed five times with wash buffer and reprobed for actin protein detection following the same procedure as listed above with the following exceptions. The primary Ab was rabbit anti-β-actin (Sigma-Aldrich) at 1/2,000 and the secondary Ab was goat anti-rabbit (Sigma-Aldrich) diluted 1/10,000.

Cytokine array

Primary human monocytes were isolated as described above and seeded into 24-well tissue culture plates at 1×10^6 cells/ml. The appropriate dilution (100 μl) of gentamicin-killed gonococci was added to each sample monolayer, and cells were incubated in RPMI 1640 supplemented with 10% autologous human serum at 37°C, 5% CO_2 with humidity for 18 h. Supernatants from quadruplicate samples were pooled before addition to the array membrane. The RayBio Human Cytokine Array III was purchased from Raybiotech and used according to manufacturer's instructions. Results from two separate experiments were averaged after spots were quantified with Quantity One software (Bio-Rad) and equilibrated according to internal positive controls.

Results

Challenge dose of N. gonorrhoeae *affects cytokine production by monocytes*

Primary human monocytes were challenged with a range of gonococcal doses and the production of several cytokines predicted to be important in gonococcal infections was measured. After several preliminary studies, we determined that the use of gentamicin-killed bacteria was most appropriate for analyzing the primary response of the monocytes, because it removed the problems associated with gonococcal multiplication in the invasion medium, and prevented phase variation of the challenge inoculum. Gentamicin is an aminoglycoside antibiotic and binds directly to ribosomal RNA, thereby inhibiting protein synthesis (24). Use of this antibiotic does not cause lysis of the bacteria. Upon staining and visualization of killed bacteria, the cells appeared as normal diplococci. In our positive control samples (multiplicity of infection (MOI) = 10), TNF-α, IL-1β, and IL-8 were all measurable in large quantities at 18 h postinfection (Fig. 1*A*). In our low dose challenge (MOI = 0.1), ~20 ng/ml IL-8 was measurable, while the other cytokines tested were produced at very low levels or were immeasurable (Fig. 1*B*). The difference in IL-8 production between the positive control and the low dose challenge was statistically insignificant, while TNF-α and IL-1β respectively resulted in a 20- and 10-fold reduction subsequent to low dose challenge (Fig. 1*C*). These data indicate that during a low inoculum gonococcal challenge, large amounts of IL-8 are produced by human monocytes. This occurs in the absence of significant TNF-α, demonstrating that the initial infectious dose can greatly impact the overall host cytokine profile. We next examined whether a similar dose-dependent differential for TNF-α would result from monocytes challenged with either killed *E. coli* or killed *Salmonella typhimurium*. The average fold reduction in TNF-α between doses was shown to be significant for *E. coli* samples, yet insignificant for *S. typhimurium* samples (Fig. 1*D*). As in the case of our gonococcal challenge, no significant differences in IL-8 production were observed between doses for either species (data not shown). These data suggest that that the cytokine differential we observed for *N. gonorrhoeae* is species specific, with *S. typhimurium* failing to elicit this does-dependent difference. This may suggest that the differences seen are due to structural differences in lipid A of the three organisms. Experiments using live gonococci, where gentamicin (100 μg/ml) was added at 30 min postchallenge, resulted in a slight increase in production of TNF-α when compared with challenge by killed gonococci (data not shown). We attribute this difference to growth of the bacteria, as complete killing of the gonococci under these conditions did not occur in <2 h. These experiments revealed no significant difference in the production of IL-8 (data not shown). Because the concentration of IL-8 in these samples was quite high at both doses, it is logical that bacterial growth during gentamicin treatment did not result in significantly different IL-8 levels during live bacterial challenge. In addition, when monocytes were challenged with sonicated bacteria (MOI = 10 and MOI = 0.1), no significant differences in TNF-α or IL-8 production were observed when compared with killed bacterial challenge samples (data not shown). We attribute this observation to the fact that gonococci routinely shed membrane blebs (containing DNA) and may not elicit alternate signaling patterns when lysed (25).

To determine whether the low inoculum was simply resulting in slower kinetics of cytokine production, we measured cytokine levels produced over time (Fig. 2). In positive control samples, all three cytokines tested were detectable at 2 h postchallenge. TNF-α and IL-1β both increased over time until reaching a maximal level at ~6–12 h postchallenge. IL-8 expression continued to increase throughout the entire time course. During the low dose challenge (MOI = 0.1), TNF-α and IL-1β were detected much later than IL-8 (12 and 6 h, respectively) and when measurable, peaked at very low levels. TNF-α and IL-1β measured after a longer time period (24 h) never approached levels seen in the positive control (data not shown), demonstrating that the low inoculum challenge did not affect cytokine kinetics. As in the positive control, IL-8 continued to increase during the time course at levels far above the other cytokines measured. In addition, because the low dose challenge results in the production of IL-8 in the absence of measurable TNF-α, it suggests that IL-8 is not produced as a result of primary production of TNF-α.

IL-8 production is independent of TNF-α and IL-1β

Because IL-8 production is often attributed to prior production of TNF-α and IL-1β during bacterial infections, we tested whether small amounts of these cytokines in culture supernatants were driving production of IL-8 during low dose gonococcal infections. Primary cultures of human monocytes were pretreated with neutralizing Abs against TNF-α and IL-1β (the Ab concentration was experimentally shown to neutralize all of the TNF-α or IL-1β in culture supernatants), and challenged with either an MOI of 10 (positive control) or an MOI of 0.1. Resulting IL-8 production was

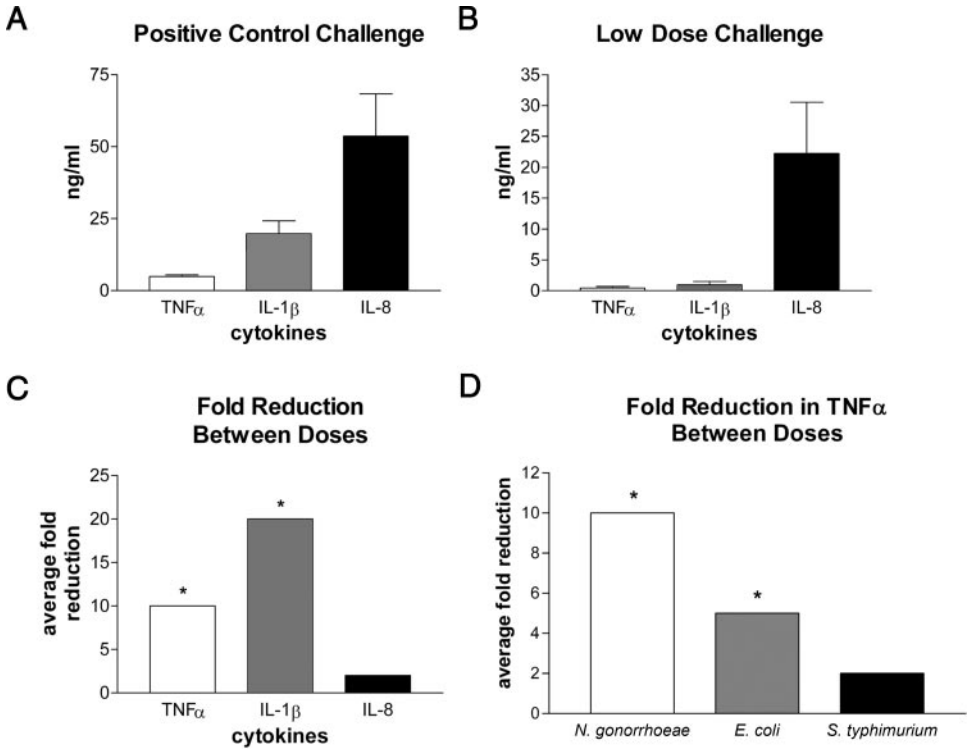


FIGURE 1. Production of cytokines by primary human monocytes after in vitro challenge. Cytokine profile of monocytes (10^6/sample) after an 18-h challenge with killed *N. gonorrhoeae*, strain F62ΔlgtD. Shown are TNF-α, IL-1β, and IL-8 levels as measured by ELISA. Data are representative of at least three independent experiments, each performed in triplicate. Error bars represent the SEM. *A*, MOI = 10; positive control samples, (*B*) MOI = 0.1; low dose challenge samples. *C*, Statistical analysis of positive control and low dose samples using a paired, two-tailed, *t* test with a 95% confidence interval indicates that low dose inoculations resulted in significantly lower levels of TNF-α (10-fold) and IL-1β (20-fold), but not IL-8 (2-fold). *, A statistically significant difference between doses: $p < 0.05$. *D*, Statistical analysis of positive control and low dose challenges using *E. coli* or *S. typhimurium* in comparison to *N. gonorrhoeae*. Results of a paired, two-tailed, *t* test with a 95% confidence interval demonstrate that low dose inoculations resulted in significantly lower levels of TNF-α for *E. coli* (as for *N. gonorrhoeae*) but not *S. typhimurium*. *, A statistically significant difference between doses: $p < 0.05$.

measured by ELISA (Fig. 3). Pretreatment of monocytes with the two Abs had no effect on the amount of IL-8 expressed at each dose. These data indicate that IL-8 production by monocytes challenged with *N. gonorrhoeae* is likely a result of direct stimulation by a bacterial product and not due to autocrine stimulation by TNF-α or IL-1β.

NF-κB activation is proportional to the bacterial challenge dose

The *TNF-α*, *IL-1β*, and *IL-8* genes can all be transcriptionally regulated via NF-κB binding to specific DNA sequences within their promoters (26–28). However, the importance of NF-κB in IL-8 expression is controversial (28–32). We hypothesized that NF-κB may not be activated during the low dose challenge. To test this hypothesis, primary human monocytes were differentiated into macrophages and challenged with different doses of gonococci (MOI = 10 and MOI = 0.1). Immunofluorescence staining and laser scanning confocal microscopy were used to visualize the degree of NF-κBp65 translocation into macrophage nuclei. Random fields at each time point and dose were examined. At least 50 cells from each sample were analyzed and categorized as positive or negative for activated NF-κB staining in their nuclei (Fig. 4*A*). The positive control challenge (MOI = 10) resulted in NF-κB activation in nearly 100% of the macrophages by 90 min postchallenge. In the low dose challenge (MOI = 0.1) at 90 min, NF-κB was translocated to the nucleus in ~12% of the macrophage population. At 120 min postinfection, this percentage did not change significantly (Fig. 4*B*). The percentage of activated cells in the low dose is reflective of the 10% of the macrophage population expected to come into direct contact with bacteria. Therefore, NF-κB activation appears to result from direct contact between macrophages and gonococci. These data demonstrate that NF-κB is translocated into macrophage nuclei in low dose challenges and that the activated cell population is limited by the initial dose of infection. Based on these findings, it is likely that NF-κB is driving at least a portion of the IL-8 response. These data as well as the previous cytokine measurements imply that the low dose challenge results in a marked increase in IL-8 production on a per cell basis.

Effects of the NF-κB inhibitor TPCK on IL-8 production

To directly test the effects of NF-κB translocation on production of IL-8, we pretreated monocytes with *N*-tosyl-l-phenylalanine-chloromethyl ketone (TPCK), a chemical that blocks the activation of NF-κB by blocking phosphorylation and therefore subsequent degradation of IκB. Primary human monocytes were pretreated with TPCK over a dose range for 45 min before challenge with gonococci. A positive control challenge (MOI = 10) was compared with the low dose challenge (MOI = 0.1) and supernatants were collected for cytokine ELISA analysis (Fig. 5). This concentration of inhibitor was able to inhibit ~80% of the IL-8 produced in both low dose and positive control samples. TNF-α production was completely inhibited by TPCK pretreatment (data not shown), as has been demonstrated previously (33, 34). These experiments demonstrate that inhibition of NF-κB almost completely inhibits the IL-8 production resulting from gonococcal challenge. Therefore, NF-κB is critical for the production of IL-8 during a gonococcal infection.

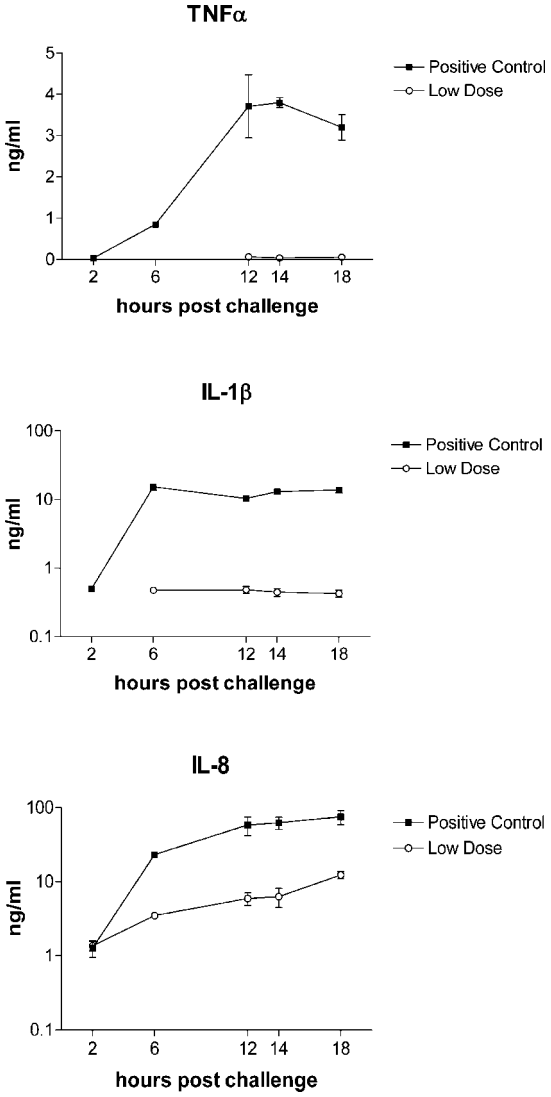


FIGURE 2. Cytokine production by challenged primary human monocytes over a time course. Monocytes (10^6/sample) were challenged with killed *N. gonorrhoeae*, strain F62ΔlgtD. In the positive control challenge time course, an MOI of 10 was used. All three cytokines tested were measurable during the entire time course. In the low dose time course, an MOI of 0.1 was used. These experiments were performed twice in triplicate. Error bars represent the SD of the mean.

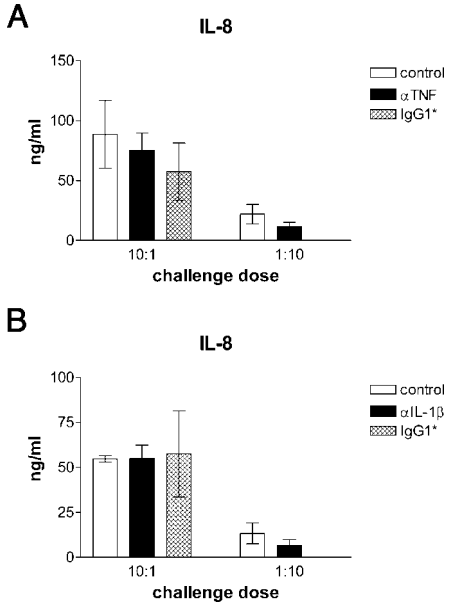


FIGURE 3. Cytokine production by challenged primary human monocytes after pretreatment with neutralizing Abs. Monocytes (10^6/sample) were pretreated with neutralizing Abs for 30 min before challenge with killed *N. gonorrhoeae*, strain F62ΔlgtD. Monocytes were challenged with an MOI equal to either 10 or 0.1. *A*, IL-8 measurement at 18 h postchallenge (in the presence of Ab able to neutralize all available TNF-α). *B*, IL-8 measurement at 18 h postchallenge (in the presence of Ab able to neutralize all available IL-1β). Data represent the average of at least two independent experiments, each performed in triplicate. Error bars represent the SD of the mean. ∗, A total of 3 μg/ml isotype control (mouse IgG1)

Phosphorylated JNK is undetectable in low dose infections

AP-1 is a collective term which refers to a family of transcription factors. AP-1 exists as a dimer and is comprised of proteins from the Jun, Fos, Maf, and ATF subfamilies These molecules contribute to the regulation of many cytokine genes, including *IL-8* and *TNF-α* (35–37). Activation of this transcription factor family is regulated by the MAPKs (for review, see Karin et al. (37, 38)). Because *N. gonorrhoeae* has been shown to activate AP-1 in an epithelial cell tissue culture model through activation of JNK (39), we chose to examine the roles of this and other MAPKs in our low dose monocyte model of infection. We hypothesized that differential MAPK regulation may be responsible for our observed dose-dependent cytokine profile. We challenged monocytes with both low dose and positive control doses of gonococci for various lengths of time, collected lysates and analyzed the level of phosphorylation of several kinases by SDS-PAGE/Western blotting experiments, using Abs specific for phosphorylated p38, JNK, and ERK (Fig. 6*A*). These data provide evidence that, unlike p38 and ERK, JNK is not phosphorylated in low dose challenge samples. (Overexposure of the JNK blot did not result in significant signal when compared with negative controls (data not shown)). This indicates a dose-dependent difference in the MAPK-signaling pathway.

Three MAPK inhibitors were used to examine the roles of p38, ERK, and JNK in low dose challenge IL-8 production. The specificity of these inhibitors has been previously demonstrated (40–42). The data in Fig. 6*B* show the percent inhibition of IL-8 after pretreatment with the inhibitors and subsequent bacterial challenge. The p38 inhibitor SB203580 inhibited most of the IL-8 production, while the ERK inhibitor PD98059 inhibited ~30%. The JNK inhibitor SP600125 had almost no effect on IL-8 production. These data further indicate that the activation of the JNK pathway (and thus AP-1 production/activation) is not contributing significantly to IL-8 production resulting from our low dose challenge.

Overall cytokine profile is altered depending upon the initial challenge dose

To gain a greater understanding of overall gonococcal-induced dose-dependent cytokine expression, we used a cytokine expression array. Monocytes were challenged with a specific dose of gonococci, incubated for 18 h, and supernatants were collected for array analysis (Fig. 7). Several cytokines demonstrated dose-dependent differences in expression (2-fold change or greater). Included in this group were TNF-α, IL-1β, and IL-10. This correlates with previous ELISA data where TNF-α, IL-1β, and IL-10 (data not shown) were all up-regulated as a result of the higher challenge dose. Similar to IL-8, the cytokines IL-6, growth-related oncogene (GRO), and MCP-1 were also substantially up-regulated compared with negative control samples, yet did not differ significantly between doses. These data indicate that the lower bacterial challenge elicits a dramatic immune response and that significant

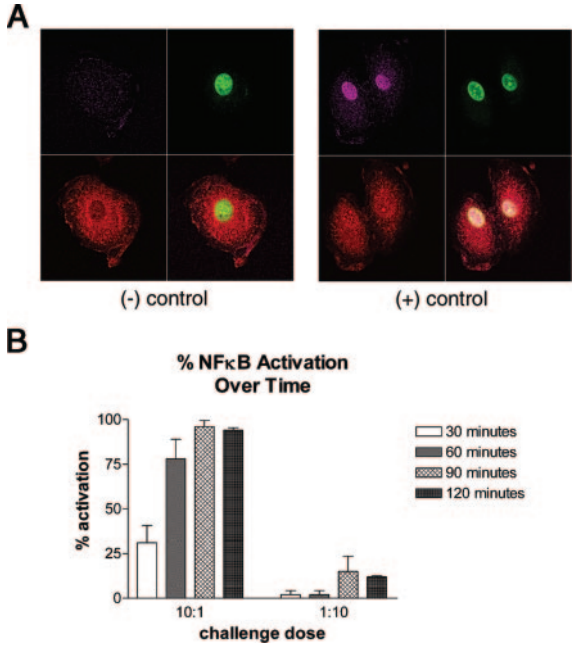


FIGURE 4. Analysis of challenged primary human macrophages by confocal microscopy. Differentiated macrophages were challenged with killed *N. gonorrhoeae*, strain F62ΔlgtD, over time. Macrophages were challenged with an MOI of either 10 or 0.1, stained, and analyzed via confocal microscopy. Staining included phalloidin (red), SYTO-13 nucleic acid stain, and anti-NF-κBp65 (purple). Red shows cellular actin (*lower left*), green shows nuclear material (*upper right*), and purple shows activated NF-κBp65 (*upper left*). *Lower right panel*, An overlay of the three colors. At least 50 cells/time point were analyzed and placed into one of two categories: those with NF-κBp65 in the nucleus and those without. *A*, Confocal images showing unstimulated (− control) and activated (+ control) macrophage controls. *B*, The percentage of cells with NF-κBp65 in the nucleus at 30, 60, 90, and 120 min postchallenge for each MOI. Data shown represent the average of at least two independent experiments. Error bars indicate the SD of the mean.

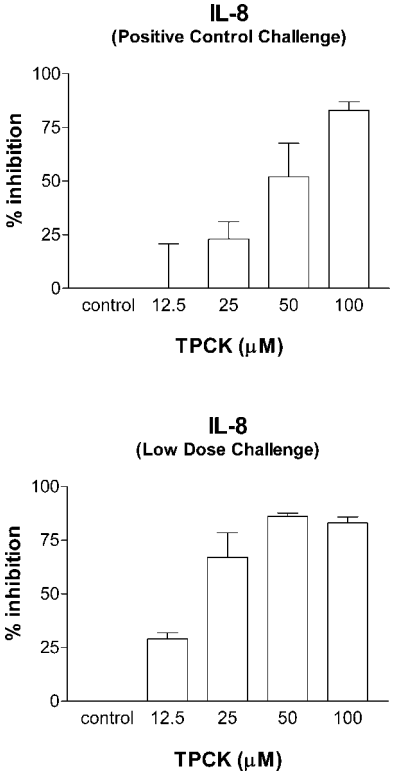


FIGURE 5. Inhibition of cytokine expression by TPCK. IL-8 measured after TPCK pretreatment of primary human monocytes challenged for 18 h with killed *N. gonorrhoeae*, strain F62ΔlgtD. TPCK is a serine protease inhibitor that has been shown to block LPS- or cytokine-mediated activation of NF-κB. Positive control samples were challenged with an MOI of 10, while low dose samples were challenged with an MOI of 0.1. Data shown are from one experiment performed in triplicate and are representative of three independent experiments. Error bars represent the SD of the mean.

differences in the overall cytokine expression profile can result from two different inoculating doses of bacteria.

Discussion

Bacterial infections typically result in the production of specific proinflammatory cytokines and chemokines, leading to clearance of the invading microbes. In this study, we have shown that when confronted with a low challenge dose of *N. gonorrhoeae*, human monocytes are extremely efficient producers of IL-8 as well as several other chemokines/cytokines. In this scenario, host cells do not produce significant levels of TNF-α. This overall cytokine profile could result in an influx of neutrophils and monocytes to the site of colonization, without causing the pain associated with symptomatic infection. Therefore, we propose that a low percentage of invasive bacteria can elicit a dramatic innate immune response without causing substantial cytokine-mediated tissue damage.

Cytokine response to *N. gonorrhoeae* has been investigated by several groups, but to our knowledge, never in the context of a low dose infection. The monocytic inflammatory response to a low dose gonococcal challenge has not been documented, though this dose is almost certainly physiologically relevant. Subsequent to this low dose challenge, we measured levels of IL-8 that were statistically similar to those of high dose challenge samples, yet were produced in the absence of significant amounts of TNF-α. One male challenge study using gonococcal strain MS11 found an increase IL-8, IL-6, and TNF-α levels in the urine samples of volunteers before the onset of symptoms. These cytokines were shown to increase concomitantly with the number of leukocytes found in urine sediment. At the onset of symptoms, IL-1β was detected (43). Fichorova et al. (44) measured IL-6, IL-8, and IL-1β production from challenged immortalized human cervical and vaginal epithelial cells using an MOI of 100, and also found that IL-1β was produced later than IL-6 and IL-8. However, TNF-α was not measurable in these samples. Using a cervical epithelial cell line (ME180) as a model of infection, and an MOI of 5, Naumann et al. (45) demonstrated production of TNF-α and IL-8 by 6 h postinfection. With the exception of a lack of measurable TNF-α from the immortalized epithelial cell model, all of these studies are consistent with our high inoculum challenge samples. In addition, this work supports our conclusion that IL-8 production does not result from autocrine action by IL-1β. Makepeace et al. (46) inoculated primary monocyte-derived human macrophages with *N. gonorrhoeae* at an MOI of 400 and measured the resultant cytokine response. In contrast to our findings, no increase in IL-8 was observed when compared with controls (though negative control samples produced 10 ng/ml IL-8). Additionally, no IL-1β was detected from challenged samples within these experiments. Though these findings seem to be at odds with our results, the challenge dose that they used greatly exceeded our high dose challenge. Therefore, these results further support our observation of dose-dependent alterations in cytokine gene expression.

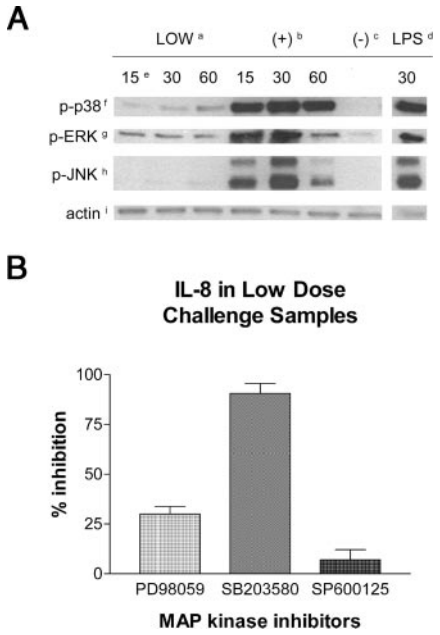
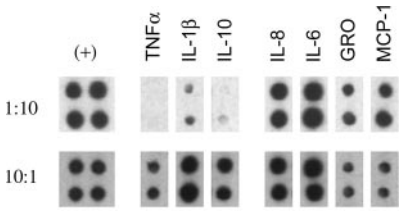


FIGURE 6. Phosphorylation levels of MAPKs from challenged primary human monocytes. Monocytes were challenged for the indicated time period with killed *N. gonorrhoeae*, strain F62ΔlgtD. *A*, Resting monocytes (2×10^6/sample) were challenged with an MOI of 10 or 0.1 over time. Monocyte whole cell lysates were collected and analyzed by immunoblot to detect the phosphorylation levels of p38 (*f*) ERK 1/2 (*g*), and JNK 1/2/3 (*h*). *a*, MOI = 0.1; *b*, MOI = 10 (positive control); *c*, untreated primary human monocytes (negative control); *d*, LPS-treated monocytes (100 ng/ml); *e*, minutes, postchallenge (*f*) probing monocyte lysates with phospho-p38 polyclonal Ab (*g* and *h*) same as *f*, except phospho-ERK and phospho-JNK, respectively. *i*, β-actin levels (loading control). Data are representative of three independent experiments. *B*, Percent inhibition of IL-8 in low dose challenge samples after pretreatment with three specific MAPK inhibitors (PD98059/ERK, SB203580/p38, and SP600125/JNK) and subsequent 18 h challenge. These inhibitors were tested over a dose range and data shown are the result of the pretreatment of monocytes with 5 μM SB203580, 50 μM SP600125, and 50 μM PD98059, 30 min before bacterial challenge. Levels of inhibitors used were experimentally determined to have reached maximal inhibition (data not shown).



	Average fold change between doses
TNFα	5.3
IL-1β	8.6
IL-10	17.5
IL-8	1.8
IL-6	1.0
GRO	1.0
MCP-1	1.8

FIGURE 7. Comparison of cytokine levels from both low dose and positive control inoculations during one experiment. Primary human monocytes were challenged for 18 h with killed *N. gonorrhoeae*, strain F62ΔlgtD. Monocytes were challenged with an MOI of either 10 or 0.1. Densitometric analysis was conducted using a Gel Doc 2000 camera and Quantity One Software (Bio-Rad). Internal positive controls were compared among array exposures and the means were found to be statistically similar ($p = 0.7548$ according to a paired, two-tailed *t* test) before cross-comparison of sample cytokine levels. A fold change greater than or equal to 2-fold was determined to be significant. Table values represent an average of two independent array experiments.

Through the use of neutralizing Abs against TNF-α and IL-1β (two of the strongest activators of IL-8) (30), we verified that the IL-8 measured was not being produced in response to either of these cytokines. We hypothesize that IL-8 production results from a direct interaction between a bacterial structure or product with host monocytes. Lorenzen et al. (47) have shown that neisserial Ig A1-specific serine protease plays a role in eliciting IL-8 from PBMC. In addition, recombinant neisserial PorA has been shown to elicit several proinflammatory cytokines including IL-8. The neisserial surface lipoprotein Lip has also been shown to elicit IL-8 and IL-6 in an immortalized epithelial model (48). Though LPS/LOS activation is known to cause IL-8 production from human monocytes (49), Pridmore et al. (50) demonstrated that both wild-type and lpxA mutant (LOS-deficient) meningococci were similarly able to induce IL-8 promoter activity. Similarly, pili, LOS, and gonococcal outer membrane vesicles were shown to have no effect on IL-8 production in one study using a human macrophage model (46). A conflicting study showed that piliated gonococci were associated with increased production of IL-8 in an ex vivo human endometrial cell model (51). Recently, peptidoglycan has been shown to interact with mannose-binding lectin, ultimately resulting in an increase in chemokine production and a decrease in TNF-α production by macrophages (52). Specific gonococcal LOS molecules have also been shown to avidly bind mannose-binding lectin in vitro (53). It is possible that one or a combination of several gonococcal surface components may be responsible for the cytokine differential we have observed in low dose challenge samples.

It is known that *IL-8* gene expression is affected by the binding of NF-κB, AP-1, and C/EBP (NF-IL-6) to regions of its promoter (for review, see Roebuck (32)). The role that each of these transcription factors plays in the overall expression of IL-8 remains unclear. *TNF-α* gene expression is even more complex and varies among different cell types. In human monocytes, transcription factor binding sites for NF-κB, AP-1 (Jun-ATF-2), Sp1, Egr-1, NFAT, and Ets/Elk have been identified in the TNF-α promoter (36, 54–56). Interestingly, distinct stimuli (viral Ag, a calcium ionophore, *Mycobacterium tuberculosis*, and LPS) have been shown to result in the binding of different sets of transcriptional activators to shared binding sites in the TNF-α promoter, in a cell-type specific manner (55–57). Specific combinations of these regulatory elements are required for recruitment of the coactivator proteins CBP/p300 and subsequent enhanceosome formation (54–56). Though NF-κB-binding motifs have also been identified in the upstream regions flanking the *TNF-α* gene (26), the role of these sites in TNF-α transcription is controversial. Deletion of these sequences has been shown to produce little effect on TNF-α induction by several distinct stimuli (55, 56). However, simultaneous mutations in each of the NF-κB sites of the mouse TNF promoter resulted in a 90% reduction in promoter response to LPS (58). These differences may be stimulus-specific or reflective of differences among cellular models.

Although NF-κB was historically thought to be essential for IL-8 expression in many cell types (28), NF-κB-independent IL-8 expression has been reported (29–32). Our confocal microscopy images (Fig. 4*A*) clearly show that NF-κBp65 is translocated into the nucleus in low inoculum samples, though activation is limited by the initial dose. Though differentiated cells certainly have some altered sensitivity in comparison with monocytes, the NF-κB data

generated from use of this model was reflective of the percentage of macrophages expected to come into direct contact with bacteria. These percentages correlate well with the cytokine differential observed in Fig. 1, where monocyte cytokine production was measured. Therefore, these data support the hypothesis that NF-κB activation and IL-8 production in the low dose are the result of direct contact between bacteria and macrophages. In addition, inhibition of NF-κB translocation through use of the serine protease inhibitor TPCK significantly inhibited IL-8 expression. This indicates the importance of NF-κB in our model of gonococcal disease.

AP-1 has clearly been shown to contribute to both TNF-α and IL-8 expression. Because AP-1 abundance, binding, and activity are impacted by the activity of various MAPKs, we examined the roles of ERK, JNK, and p38 in our low dose challenge model. Our data demonstrate that under these experimental conditions, the JNK pathway is not activated. Therefore, available c-Jun cannot be phosphorylated, which leads to a failure in further c-*jun* induction (for review, see Karin (38)). Because c-Jun is a central component of all AP-1 complexes (37), the data suggests that in our model, AP-1 dimers are not available to bind to the DNA. The protein ATF2 is also phosphorylated in part by JNK (59). Thus, in our model fewer phosphorylated ATF2 proteins are available. Because AP-1 dimers (Jun-ATF-2) are important for enhanceosome formation and efficient transcription of the *TNF-α* gene, it is not surprising that in the absence of phosphorylated c-Jun, and with little available phosphorylated ATF-2, TNF-α production is limited. We hypothesize that the IL-8 produced as result of the low dose gonococcal infection is mediated by NF-κB binding, independent of AP-1. In support of this, the AP-1-binding site has been shown to be dispensable for IL-8 production in some studies (60, 61). Similarly, differential transcription of cytokine genes has been demonstrated in the IL-1β promoter, where fibronectin was shown to induce AP-1 binding, but not NF-κB (62). Additionally, different TLR agonists have been shown to elicit distinct cytokine responses (i.e., induction of TNF-α but not IL-1β) though both AP-1 and NF-κB were activated (63). We believe that in response to a low dose challenge, the TNF-α promoter is not efficiently transcribed. Though the NF-κB inhibitor TPCK resulted in complete inhibition of TNF-α production, we feel that this is due to TPCK-induced inhibition of TNF-α mRNA expression. This effect by TPCK has been previously documented in a model of LPS-stimulated macrophages (33, 34).

The results of our cytokine array experiment provide further insight into the global cytokine response of human monocytes in response to gonococcal infection. Several cytokines and chemokines were up-regulated (in both low and high dose challenge samples) when compared with untreated controls, and the dose differential was apparent for TNF-α, IL-1β, and IL-10 (Fig. 7). Both the chemokines GRO and MCP-1 were shown to be produced in large quantities when compared with negative controls. Interestingly, their production, just as that of IL-8, was not significantly altered in the low dose challenge. Because both GRO and MCP-1 are also transcriptionally regulated in part by NF-κB it is possible that these genes are still efficiently transcribed, as is IL-8, without the presence of AP-1 (64–66). Because chemokine regulation is critical for appropriate regulation of leukocyte infiltration, it is also essential for regulation of the intensity of the inflammatory response. In the context of the low dose, one could imagine an asymptomatic gonococcal infection where a significant chemokine response is generated in the absence of substantial TNF-α production. This would result in continued bacterial transmission and gradual microbial clearance without the host experiencing noticeable symptoms.

We further propose that gonococci in different doses can appear as distinct stimuli to host monocytes. When gonococci exist in sufficient numbers, multicellular aggregates form. These aggregates may physically alter the scope of the interaction between gonococcal pathogen-associated molecular patterns such as Por, Lip, and LOS, and their corresponding pattern recognition receptors. In turn, distinct recognition and/or phagocytosis by host cells may occur, in comparison with that of individual diplococci. In the future, we hope to determine which gonococcal surface molecules contribute to the observed signaling differential.

Taken together, the results of our study indicate that *N. gonorrhoeae* is remarkably efficient at inducing the production of several cytokines/chemokines from host monocytes. This response can occur in the absence of damaging cytokines (TNF-α) and appears to result from direct gonococcal/monocyte surface contact in an NF-κB-dependent manner. This observation may help to explain gonococcal disease in women, which is so often asymptomatic. Although these studies are helpful in understanding gonococcal pathogenesis, we also feel that the implications are broadly applicable to our understanding of the development of immune responses to any infectious disease.

Acknowledgments

We thank Drs. Ken Frauwirth and David Mosser for their critical reviews of the manuscript.

Disclosures

The authors have no financial conflict of interest.

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