



HHS Public Access

Author manuscript

Mucosal Immunol. Author manuscript; available in PMC 2012 July 01.

Published in final edited form as:

Mucosal Immunol. 2012 January ; 5(1): 19–29. doi:10.1038/mi.2011.38.

Protective Role of Toll-like Receptor 4 in Experimental Gonococcal Infection of Female Mice

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Abstract

Neisseria gonorrhoeae is a common bacterial sexually transmitted infection. Like all Gram-negative bacteria, the outer membrane of the gonococcus is rich in endotoxin, a known ligand for Toll-like receptor (TLR)-4. However, the role of endotoxin and its cognate receptor TLR4 in the mucosal response to acute gonococcal infection in the genital tract of women is unclear. To test this, we examined the course of infection following vaginal inoculation of *Neisseria gonorrhoeae* in mice carrying the *Lps^d* mutation in *Tlr4*, which renders them unresponsive to endotoxin. While there was no difference in the duration of colonization, the *Lps^d* mice had a significantly higher peak bacterial burden which coincided with a massive polymorphonuclear cell influx and the concomitant upregulation of a subset of inflammatory cytokine and chemokine markers. Notably, infected *Lps^d* mice showed a decrease in IL-17, suggesting Th17 responses are more dependent on TLR4 signaling *in vivo*. Defective PMN-mediated and complement-independent serum killing of gonococci in *Lps^d* mice was also observed and may account for the increased bacterial burden. This is the first *in vivo* evidence that TLR4-regulated factors modulate the early inflammatory response to gonococcal infection in the female reproductive tract and control bacterial replication.

Keywords

Toll-like receptors; TLR4; *Neisseria gonorrhoeae*; cytokines; mouse models

INTRODUCTION

Neisseria gonorrhoeae is the second most commonly reported notifiable disease in the United States, and infections due to gonorrhea are associated with the development of pelvic

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DISCLOSURE: The authors declare no conflict of interest with this work.

inflammatory disease (PID) in women, which can lead to tubal infertility and chronic pelvic pain¹. In addition, gonococcal infections have been reported to enhance HIV transmission in co-infected individuals². Recently released statistics from the Centers for Disease Control (CDC) for 2009 show a record low 301,174 cases of gonorrhea were reported in the United States that year, reflecting about 111 cases per 100,000 people¹. However, while overall rates were decreased from previous years, significant disparities remain with regard to gender and race. In this report the CDC noted 71% of all cases of gonorrhea occurred in African Americans, and African American women age 15 through 19 showed the highest rates of 2,613.8 cases per 100,000 people.

Gonorrhea is almost exclusively transmitted by close sexual contact, and can occur at several mucosal surfaces, including the urethra, cervix, rectum and pharynx. Early events in the establishment of infection involve interactions between gonococci and epithelial cells, which lead to colonization of the mucosal surface, release of local inflammatory mediators, and the recruitment of professional immune cells. The current model for gonococcal pathogenesis in women suggests that *N. gonorrhoeae* is unable to invade the squamous epithelium of the vagina and ectocervix, but rather colonizes and transmigrates across the columnar epithelium of the uterine endocervix³. In women, infection with gonorrhea usually remains localized to the lower reproductive tract, where it can induce an inflammatory cervical exudate containing polymorphonuclear cells (PMN) with intracellular gonococci. However, like many sexually transmitted infections in women, gonococcal cervicitis is often described as subclinical or asymptomatic since patients rarely report subjective evidence of increased vaginal discharge or pelvic pain. This can lead to continued transmission between sexual partners and delayed treatment⁴. The latter is believed to contribute to the development of PID in a subset of women. PID is a syndrome associated with an acute infection of the upper genital tract structures, including the uterus, oviducts, and ovaries, which can then lead to the complications of tubal infertility and ectopic pregnancy^{5,6}.

While *in vitro* studies have implicated the involvement of several innate immune receptors and pathways in the induction of inflammatory signals during gonococcal infection, none have been able to adequately address the complex interaction of various cell types at the mucosal surface during an *in vivo* infection in women. To better address this aspect of gonococcal pathogenesis, we developed a female mouse genital tract infection model that mimics several aspects of human infection⁷. The model, although limited by a number of host restrictions, has been used successfully to study several aspects of host-pathogen interactions during gonococcal infections. In this study, we used our model to evaluate the role of endotoxin, a key virulence factor for all Gram-negative bacteria, in the pathogenesis of lower genital tract infection with *N. gonorrhoeae*. Endotoxin is a potent proinflammatory trigger by virtue of its ability to engage the Toll-like receptor (TLR)-4 receptor complex, which is expressed on the surface of a variety of cells including monocytes, macrophages, dendritic cells and PMNs [reviewed in⁸]. We examined the course of infection and production of proinflammatory cytokines in mice that are sufficient or deficient in TLR4 signaling by comparing BALB/c mice and BALB/c mice carrying the *Lps^d* mutation in *Tlr4*⁹. Although there was no difference in the rate of clearance of infection between wild type

and *Lps^d* mutant mice, the colonization load and a number of acute inflammatory markers were significantly lower in the wild type mice. The TLR4 mutant mice displayed an intense inflammatory cytokine and chemokine response *in vivo*, with a massive PMN influx and a significantly higher peak bacterial burden. The only cytokines that appeared to be down regulated *in vivo* were IL-17 and related family members, including IL-6, IL-21, and IL-22, suggesting that Th17-mediated responses were dependent on TLR4 signaling events. Interestingly, the induction of proinflammatory cytokines *in vitro* was largely dependent on TLR4 function, suggesting a more complex interplay of cell types and endogenous flora occurs *in vivo*. Finally, we observed defective PMN-mediated and complement-independent serum killing of gonococci in the TLR4 mutant mice, suggesting a role for TLR4 in mediating antibacterial responses. These data suggest that a TLR4-regulated factor provides a barrier to gonococcal colonization, and that TLR4 plays a negative role in regulating inflammation during gonococcal cervicitis.

RESULTS

TLR4 mutant mice have a higher colonization load of *N. gonorrhoeae* following vaginal inoculation

Female BALB/c mice exhibit a significant localized PMN influx in response to gonococcal infection in the lower reproductive tract, and support high levels of colonization over the course of infection^{10, 11}. In order to determine the importance of TLR4 signaling during experimental murine infection, we took advantage of the C.C3-*tlr4LPS-d/J* mouse strain, in which C3H/HeJ mice were back bred onto the BALB/c background to create a congenic BALB/c mouse strain that contains a segment of chromosome 4 which includes the *Lps^d* allele of *Tlr4*⁹. Thus, these mice, which we will refer to as BALB/c-*Lps^d*, express the *Lps^d* mutation in *Tlr4*¹² and, like the C3H/HeJ parental strain, are unresponsive to LPS. We found no difference in the duration of gonococcal colonization in BALB/c mice (mean, 7.1 days; range 0–16) versus the BALB/c-*Lps^d* mice (mean, 9.3 days; range 2–16). However, as shown in Fig. 1a, a significant difference in the bacterial burden was observed between days 1–10 of infection, with ~1–2 logs more bacteria recovered from the BALB/c-*Lps^d* mice compared to BALB/c mice. This result suggests that TLR4 is required for control of bacterial replication during infection in the lower reproductive tract of female mice.

We recently reported that, in contrast to BALB/c mice, mice of the C3H/HeN strain are resistant to gonococcal colonization¹⁰. We were therefore curious if loss of TLR4-mediated signaling would have any effect on this strain that, otherwise, appeared unable to support gonococcal infection. To test this possibility, we compared the colonization loads of C3H/HeN mice, which are wild type for TLR4, with C3H/HeJ mice, which express the *Lps^d* mutation in *Tlr4*¹². A group of BALB/c mice was inoculated in parallel. As expected, C3H/HeN mice were resistant to infection with *N. gonorrhoeae* (mean duration of recovery, 0.7 day; range 0–2) compared to BALB/c mice (mean duration of recovery, 7.5 days; range 2–9), confirming our previously published data¹⁰. In contrast, the C3H/HeJ mice exhibited a longer duration of infection (2.3 days; range 0–5) and significantly higher colonization load compared to the C3H/HeN mice, although they did not reach the same level of colonization as the BALB/c mice (Fig. 1b). While the basis of resistance in the C3H/HeN

mouse strain remains unknown, these data suggest that the innate resistance to gonococcal colonization of this inbred mouse strain can, at least partially, be overcome by the loss of TLR4-mediated signaling events.

TLR4 mutant mice generate an exaggerated inflammatory response to gonococcal infection *in vivo*

BALB/c mice upregulate vaginal proinflammatory cytokines and chemokines in response to infection with *N. gonorrhoeae*, the expression of which peaks on day 5 and coincides with a concomitant PMN influx. We hypothesized that the inability to control early gonococcal replication might reflect inadequate proinflammatory signaling in the absence of TLR4 function, and therefore examined the vaginal milieu for evidence of inflammation by looking for PMNs in vaginal smears and measuring the levels of some classic proinflammatory cytokines and chemokines, specifically IL-1 β , TNF- α , KC and MIP-2, in vaginal washes from infected and uninfected mice of each background. To our surprise, we detected significantly higher percentages of vaginal PMNs on days 4–7 of infection in the BALB/c-*Lps^d* mice compared to wild type BALB/c mice, with as many as 60% PMNs detected in the TLR4 mutant mice that were colonized with high numbers of *N. gonorrhoeae* (Fig. 2a,d). Higher numbers of PMNs were also detected within genital tract tissues and within the vaginal lumen from TLR4-mutant mice using a granulocyte-specific stain (Fig. 2b–c, e–f). Coincident with the PMN influx, we observed upregulation of IL-1 β , TNF- α , KC and MIP-2 in the vaginal washes of infected mice over time, however, the BALB/c-*Lps^d* mice had significantly higher levels compared to the BALB/c control mice (Fig. 3). This difference was most pronounced for the potent neutrophil chemoattractants MIP-2 and KC, which remained elevated relative to control mice even out to 7 days. Of note, we found that at baseline, uninfected control BALB/c-*Lps^d* mice had higher levels of proinflammatory cytokines and chemokines compared to uninfected control BALB/c mice, with levels of IL-1 β and TNF- α ($p = 0.02$, day 3) and KC [days 1 ($p = 0.01$) and 3 ($p = 0.04$)] significantly different between these two groups (data not shown). These data suggest that the TLR4 mutation might have an effect on basal secretion of proinflammatory mediators that could also affect the threshold of activation by bacterial ligands.

Cells from TLR4 mutant mice are hyporesponsive to *N. gonorrhoeae* when stimulated *in vitro*

One possible explanation for the elevated inflammatory markers in the BALB/c-*Lps^d* infected mice was that they were hyperresponsive to the TLR4-independent bacterial ligands encountered during infection, such as bacterial lipoproteins and porin, that activate TLR2. In order to determine if the BALB/c-*Lps^d* mice had altered expression of TLR2 relative to the wild type BALB/c mice, we looked for evidence of TLR2 on the surface of bone marrow derived macrophages (BMDM) by flow cytometry, and tested whether protein expression was altered in response to treatment with *N. gonorrhoeae* or other TLR ligands. As shown in Fig. 4, we found that both strains of mice expressed similar levels of TLR2 on the surface, which were upregulated in response to exposure to TNF- α , the synthetic triacylated gonococcal lipopeptide H.8/Lip¹³ and to lysates of *N. gonorrhoeae* FA1090. Predictably, only the BALB/c strain upregulated TLR2 in response to LPS, while BALB/c-*Lps^d* did not.

These data suggest that TLR2 expression in the BALB/c-*Lps^d* strain is not significantly altered compared to the BALB/c mice.

In order to determine if cells derived from BALB/c and BALB/c-*Lps^d* mice responded similarly to TLR ligands and *N. gonorrhoeae*, we next examined the response of BMDM (Fig. 5a) and splenic mononuclear cells (Fig. 5b) *in vitro*. We found that cells from both mouse strains responded similarly to a TLR2 ligand, the synthetic lipopeptide Pam₂-Cys-SK₄ in terms of upregulation of TNF- α and IL-6, while cells from the BALB/c-*Lps^d* only were unresponsive to the TLR4 ligand, LPS. However, when exposed to live *N. gonorrhoeae* or crude gonococcal lysates, cytokine induction was reduced in the BALB/c-*Lps^d* mice compared to the BALB/c mice, although not to baseline. These results confirm that the response to *N. gonorrhoeae*, at least by mononuclear cells, is primarily driven by TLR4 but that TLR4-independent cell activation can occur. This finding is similar to what we previously reported with the related pathogen *N. meningitidis*¹⁴. We conclude from these *in vitro* studies that cells derived from mice carrying the *Lps^d* mutation in TLR4 are not hyperresponsive to TLR2 ligands or *N. gonorrhoeae in vitro*.

Serum and PMNs from TLR4 mutant mice are defective in killing *N. gonorrhoeae in vitro*

The inability of BALB/c-*Lps^d* mice to control infection in the presence of a marked inflammatory response was intriguing, and we therefore compared the capacity of serum and PMNs from normal BALB/c and BALB/c-*Lps^d* mice to kill *N. gonorrhoeae*. For serum killing assays, we used heat-inactivated (HI) sera in order to examine complement-independent factors. Incubation of FA1090 bacteria with HI-serum from BALB/c mice at a final concentration of 20% resulted in a 1–2 log decrease in recovery after 120 and 150 min incubation, compared to the number of gonococci recovered from HI-serum from BALB/c-*Lps^d* mice (Fig. 6).

To test the relative capacity of TLR4-sufficient and -deficient PMNs to kill bacteria, gonococci were serum-opsonized and incubated with PMNs from BALB/c versus BALB/c-*Lps^d* mice, as described in the Methods. Although the percentage of serum used in these assays (10%) was lower than that used in the serum killing assays (20%) and shown to not significantly kill *N. gonorrhoeae* at 90 min, serum from normal BALB/c mice was used to opsonize gonococci for phagocytic uptake by both normal and TLR4-defective PMNs to control the potential differences in the killing activity of sera from the two mouse strains at this lower concentration. Recovery of *N. gonorrhoeae* following incubation with PMNs from BALB/c mice was 40% reduced relative to the HI-NMS control (Fig. 7). In contrast, PMNs from BALB/c-*Lps^d* mice, which were tested in parallel, showed no evidence of being able to kill *N. gonorrhoeae* under at the same time point. We conclude that both cellular and heat stable soluble factors may contribute to the inability of BALB/c-*Lps^d* mice to clear infection despite the induction of an intense inflammatory response.

Th17 responses are upregulated in wild type BALB/c mice but not in *Lps^d* mutants

We previously reported that IL-17 responses are induced and are protective during experimental murine gonococcal infection¹⁵. We therefore examined cytokines, receptors and transcription factors known to be critical for the induction of a Th17 response to

determine whether IL-17 responses were absent or less pronounced in TLR4 mutant mice. As shown in Fig. 8, transcripts for RORGT, STAT3, IL-6, IL-17A, IL-17E, IL-17F, IL-17RB, IL-21 and IL-22 were highly upregulated in infected wild type BALB/c mice compared to BALB/c-*Lps^d* mice by relative real-time PCR, suggesting that Th17 responses were more dependent on TLR4 signaling *in vivo*. Thus, while many of the classic inflammatory markers were elevated in the TLR4 mutant mouse, those involved in the IL-17 response were higher in wild type mice. This result is consistent with the ability of wild type mice to better control gonococcal replication and is also suggestive that Th17 responses were more dependent on TLR4 signaling.

DISCUSSION

The role of Neisseria endotoxin in the pathogenesis of meningococcal sepsis is generally accepted, and endotoxin levels in the blood stream correlate with clinical presentation, activation of complement, coagulopathy and plasma TNF- α and IL-6 levels^{16–20}. In contrast, the role of endotoxin in the pathogenesis of gonococcal infection, which is most often a localized mucosal infection, is less clear. Several reports with cultured human cells that suggest that the columnar epithelium of the endocervix is deficient in expression of the endotoxin receptors TLR4 and MD2, leading us to hypothesize that epithelial cell responses to gonococci are primarily driven by TLR2, which recognizes bacterial lipoproteins and neisserial porin^{21, 22}. However, there is the caveat that *in vivo* there could be alterations in TLR4 levels as a result of hormonal influences and endogenous flora^{21, 23, 24}. Moreover, TLR4 signaling could play a role in the pathogenesis of gonococcal cervicitis independently of epithelial cells, since professional phagocytic cells, including neutrophils, macrophages, and dendritic cells, are present in the lumen as well as the submucosal space of the lower genital tract, and they express the TLR4 receptor complex and are exquisitely sensitive to endotoxin. Thus, an *in vivo* model is required to test the role of LOS and TLR4 signaling during gonococcal infection in order to examine the cooperation between multiple cell types and the panel of receptors they express.

Our approach, using the BALB/c mouse strain expressing the *Lps^d* mutation in TLR4, allowed us the opportunity to test the role of gonococcal endotoxin during lower reproductive tract infection in a susceptible surrogate female host. We found a significantly higher colonization load in the TLR4 mutant mice compared to the wild type mice, which correlated with significantly higher PMN influx. As the *in vitro* data demonstrate, *N. gonorrhoeae* is capable of inducing TNF- α and IL-6 even in the absence of TLR4 signaling. This is likely a result of signaling via TLR2 and possibly other innate immune receptors, and may therefore explain the ability of the TLR4 mutant mice to upregulate inflammatory cytokines and chemokines in the lower genital tract. However, while both mouse strains mounted an inflammatory cytokine response to gonococcal infection, the infected BALB/c-*Lps^d* mice developed a markedly exaggerated chemokine and PMN response when compared to the BALB/c control mice. One possible explanation for this observation rests with the increased bacterial burden that we observed within the same time frame. The presence of more bacteria in the genital tract may drive a more exaggerated induction of the potent neutrophil chemoattractants, MIP-2 and KC, leading to a more pronounced PMN influx. In fact, the timing of the PMN peak coincided with the increased bacterial

colonization that we observed, which is consistent with excessive bacterial replication driving the induction of proinflammatory mediators via TLR4-independent pathways. Of note, while IFN- γ has been reported to suppress both MIP-2 and mouse KC secretion^{25, 26}, we found no detectable levels of IFN- γ in either mouse strain following inoculation with *N. gonorrhoeae*. Thus, the explanation for the increased chemoattractants is not a result of differential IFN- γ production in the two genetic backgrounds. Additional factors may have contributed to the massive number of PMNs detected in the genital tract in the TLR4 mutant mice. For example, we cannot exclude an additional role for TLR4 signaling in either the induction of PMN apoptosis or the clearance of apoptotic PMNs by activated macrophages.

The more interesting question then becomes, why did the TLR4 mutant strain fail to control early bacterial replication, especially in light of the massive PMN influx that we observed? The role of PMNs in controlling gonococcal replication and clearance is a topic of debate in the field of gonococcal pathogenesis. The hallmark of gonorrhea is the appearance of Gram-negative diplococci within PMNs in genital secretions²⁷⁻²⁹, but it remains controversial if these intracellular bacteria are replicating or in the process of being killed. One of the earliest reports to promote the idea that gonococci are rapidly killed within PMNs was by Watt and colleagues³⁰, and numerous subsequent studies supported this model³¹⁻³³. However, Casey *et al.* later reported that while the majority of intracellular gonococci within PMNs are killed by O₂-independent antimicrobial systems, a small subset of intracellular bacteria, approximately 2%, are able to survive and replicate³⁴. Similar results were found by Simons³⁵ and Criss³⁶, although the percent of surviving bacteria differs from study to study. Our observation that PMNs from TLR4 mutant mice are less able to kill gonococci over time suggests that TLR4 activation is required for PMNs to limit bacterial replication. Moreover, our data that heat-inactivated serum from TLR4 mutant mice is also defective in bacterial killing suggests that there are additional complement-independent antibacterial factors found in serum that are dependent on TLR4 expression.

Reports on the innate immune responses to other infectious processes have similarly revealed an important role for TLR4 signaling in controlling bacterial burden. For example, Montminy *et al.* demonstrated that the absence of TLR4 activation by the hypoacylated lipid A of *Yersinia pestis* directly contributed to mortality³⁷. When *Y. pestis* was engineered to produce a more biologically active hexaacylated form of lipid A, the pathogen was completely avirulent via the subcutaneous route, demonstrating that evasion of TLR4-dependent innate immune activation by the pathogen was a virulence mechanism. Similarly, TLR4 mutant mice were found to be incapable of controlling or eradicating Gram-negative respiratory infections secondary to *Klebsiella pneumoniae*³⁸, *Haemophilus influenzae*³⁹, and *Pasteurella pneumotropica*^{40, 41}. In the same manner, we would argue that early activation of TLR4-dependent responses during the mucosal challenge of mice with *N. gonorrhoeae* is an important protective mechanism by the host. Evidence that removal of this essential host defense mechanism was able to partially compensate for the innate resistance of the C3H/HeN mouse strain to gonococcal colonization lends further support to this model.

One additional mechanism behind the important role of TLR4 in controlling gonococcal infection in the mouse model rests with the link between TLR4 and IL-17. Our recent report

in Feinen *et al.* implicates a role for IL-17 and the Th17 axis in bacterial clearance during gonococcal infection in the mouse model, as the duration of colonization was almost doubled in the IL-17 receptor deficient mouse strain compared to control mice¹⁵. Furthermore, it was reported that IL-17 induction by spleen cells from TLR4 mutant C3H/HeJ mice was markedly abrogated in response to *N. gonorrhoeae* compared to TLR4 wild type mice. Our current data further support a role for TLR4 in the induction of IL-17, as we again observed a reduction in IL-17 and related cytokines in the absence of TLR4 signaling. This finding is consistent with other reports that suggest TLR4 helps to direct the induction of Th-17 cells during infections⁴²⁻⁴⁴.

While our data suggests a role for TLR4 in controlling the acute bacterial burden and regulating the inflammatory response, the effect was most pronounced during the time period that corresponds with the host inflammatory response (days 5–7) and was not seen at later time points. In wild type mice, a 4–5 day period of reduced recovery began on day 5, which was followed by an increase in the number of gonococci recovered. This pattern is typical of the colonization kinetics of *N. gonorrhoeae* strain FA1090 in this model⁷. Gonococcal colonization of estradiol-treated female mice is cyclical and appears to be hormonally regulated as periods of reduced recovery are not observed in ovariectomized mice⁴⁵. As TLR4 has been shown to be hormonally regulated^{46, 47}, we hypothesize that the protective effect of TLR4-mediated responses may also be cyclical. It is difficult to dissect the reason for clearance of *N. gonorrhoeae* in the model used here since the need for exogenously delivered estradiol to promote susceptibility to long-term *N. gonorrhoeae* infection in mice must also be considered. When water-soluble estradiol is used, as in this study, clearance of infection occurs at an average of 10–12 days post-inoculation and coincides with the resumption of the estrous cycle¹¹, and administration of additional doses of estradiol later in infection sustains colonization (A.E. Jerse, unpublished observation). The reason that estradiol is required for infection is not known, other than it synchronizes mice into a proestrus-like state on the day of bacterial challenge, which is the most susceptible stage for experimental *N. gonorrhoeae* infection in mice⁴⁸ and maintains an estrus-like state based on the presence of predominantly epithelial cells in vaginal smears. Clearance of infection upon resumption of the reproductive cycle may have an immunological basis, however, that involves one or more innate defenses associated with the post-ovulatory stage, which, based on similar rates of clearance of infection in wild type and *Lps^d* mutant mice, we would predict to be TLR4-independent. Effects mediated by TLR2 and other innate immune receptors, and amplification of the immune response by cytokine feedback mechanisms, thus appears to make the mice fully capable of ultimately eradicating the pathogen.

Another limitation of the model that should be considered when interpreting these data is the host restriction for several receptors utilized by *N. gonorrhoeae* to adhere to and invade epithelial cells. These receptors include the carcinoembryonic adherence molecules, which are the major class of opacity protein receptors⁴⁹ and the human CR3 molecule⁵⁰, which while having a high degree of similarity to that of mice, is likely to be host-restricted in that a monoclonal antibody that blocks gonococcal invasion of human cells through this receptor did not stain genital tract tissue from infected mice (unpublished data in collaboration with

Drs. Jennifer Edwards and Michael Apicella). The human membrane cofactor protein (CD46) is also host restricted and hypothesized by some to serve as the neisserial pilus receptor ⁵¹. In spite of these restrictions, gonococci do adhere to murine epithelial cells via unknown ligand/receptor interactions ⁷, and because TLR4 is on the surface of cells, gonococcal invasion of epithelial cells through host-restricted pathways would not be required for TLR4-mediated signaling. Therefore the significance of TLR4 signaling for infection as shown here, is unlikely to be altered by the absence of these receptors.

In conclusion, this study reveals a role for TLR4 signaling in the pathogenesis of gonococcal cervicitis. The induction of proinflammatory cytokines and chemokines, as well as similar rates of clearance of the infection over the same period of time regardless of TLR4 expression, speaks to redundancy in the innate immune system whereby alternate receptors are capable of alerting the host to the danger of an invading pathogen. However, the inability of the host to control early bacterial replication during the first week of infection, and the concomitant massive PMN influx, suggests an important role for TLR4 in controlling gonococcal replication during this period, specifically via the antibacterial activity of PMNs and soluble complement-independent serum factors. It remains to be seen what impact TLR4 signaling will have in the upper reproductive tract, where its expression may be higher, and we hope to examine the role of TLR4 in additional experimental systems in future studies.

MATERIALS AND METHODS

Reagents

Ultrapure LPS purified from *E. coli* serotype O111:B4 was purchased from List Pharmaceuticals (Woburn, MA); the synthetic lipopeptides, *Neisseria* lipoprotein H8/Lip and Pam₂-Cys-Ser-Lys₄, were purchased from EMC Microcollections (Tuebingen, Germany).

Bacterial strains and culture conditions

N. gonorrhoeae FA1090 (streptomycin-resistant, serum resistant) is a well characterized serum resistant *porB1B* strain ⁵². Bacteria were cultured on GC agar with Kellogg's supplements and FeNO₃ at 37°C under 7% CO₂ as described ⁷. GC agar with vancomycin, colistin, nystatin, trimethoprim sulfate, and streptomycin (GC-VCNTS agar) was used for mouse infection experiments ⁷. For *in vitro* stimulation assays, crude whole cell gonococcal lysates were made by subjecting the bacterial suspensions to a freeze-thaw cycle at -80°C, followed by vigorous vortexing.

Experimental murine infection

Two sets of mouse strains that are congenic except for the *Trl4* gene were tested: BALB/cJ (TLR4 wt) and C.C3-*tlr4LPS-d/J* (TLR4 mutant), from Jackson Laboratories; and C3H/HeN (TLR4 wt) and C3H/HeJ (TLR4 mutant), from National Cancer Institute. For simplicity, the C.C3-*tlr4LPS-d/J* mouse strain is abbreviated BALB/c-*Lps^d*. For all experiments, female mice were purchased at 4–6 weeks of age and allowed to acclimate to the animal facility for 10 days. Mice were then treated with water-soluble 17β-estradiol (Sigma) subcutaneously on

day -2, day 0 and day 2 and antibiotics (2.4 mg streptomycin sulfate and 0.4 mg vancomycin twice daily via intraperitoneal injection and 0.04 g trimethoprim sulfate per 100 ml drinking water) were administered starting on day -2 through day 10 to promote long-term colonization with *N. gonorrhoeae* as described¹¹. For experiments with BALB/c and BALB/c-*Lps*^d mice, mice were inoculated intravaginally with 1×10^6 colony forming units (CFU) of *N. gonorrhoeae* strain FA1090, which is a dose that results in infection of 80% of BALB/c mice, or PBS. For experiments with BALB/c, C3H/HeN, and C3H/HeJ mice, a dose of 10^7 CFU was used. Following inoculation, the number of gonococci recovered from vaginal mucus was quantitatively cultured daily for 10 or 16 days. Vaginal smears were prepared at each time point and stained with a modified Wright stain⁷ and the percentage of PMNs per 100 vaginal cells was determined by cytological differentiation under a light microscope. For immunohistochemical staining of tissue for granulocytes, whole genital tracts were harvested from three mice per group on day 7 post-inoculation with *N. gonorrhoeae* or PBS. Tissues were fixed, sectioned and stained for immunohistochemical analysis by Histoserv, Inc. (Germantown, MD) as described⁵³ using Anti-Gr1 (1:100) (BD Pharmagen, catalog number 550291) and anti-rat IgG-horse radish peroxidase as the primary and secondary antibodies, respectively. Sections were viewed under a light microscopy and images were scanned using Nanozoomer Digital Pathology (NDP) software (Olympus, USA). All mouse infection experiments were performed at least twice. All protocols were approved by the USUHS and the BUMC Institutional Animal Care and Use Committees.

Chemokine and cytokine measurements

Vaginal washes from wild type and TLR4 mutant mice used in the infection experiments were collected on days 1, 3, 5, and 7 by gently pipetting 50 μ l of PBS in and out of the vagina 20 times as described⁵⁴. The lavage fluid was then centrifuged at $13,000 \times g$ for 3 min. The supernatant was frozen immediately and stored at -70°C for further analysis. Levels of IL-1 β , TNF- α , KC and MIP-2 proteins were measured using a custom Milliplex multiplex assay (Millipore, Billerica, MA). Relative real-time PCR method was used to assess gene expression levels between infected wild type and TLR4-deficient mice as described¹⁰. Briefly, total RNA was extracted from pooled vaginal samples using Qiagen mini RNAeasy isolation kits and 100–500 ng of total extracted RNA were then treated with genomic elimination mixture (SABiosciences, Frederick, MD). Complementary cDNA was synthesized by adding reverse transcriptase cocktail (SABiosciences) to the DNase-treated RNA and incubated at 42°C for exactly 15 min, after which the reaction was stopped by heating at 95°C for 5 min. Oligonucleotide primers used to measure Th17 cytokines groups were purchased from SABiosciences. A relative change of 2-fold or greater between infected BALB/c vs. infected BALB/c-*Lps*^d mice was considered significant.

Preparation of bone marrow derived macrophages and splenic mononuclear cells

Bone marrow derived macrophages (BMDM) from BALB/c and BALB/c-*Lps*^d mice were prepared as follows. Briefly, femurs and tibiae were dissected from female mice aged 6–8 weeks, bone marrows were flushed, and after lysis of red blood cells, cells were cultured in RPMI 1640 supplemented with 10% FBS, 20 μ g/ml of gentamicin, 10 μ g/ml of ciprofloxacin, and 20% (v/v) of L929 condition medium (containing M-CSF). The cells were incubated at 37°C , 5% CO_2 incubator for 7–9 days to allow macrophage

differentiation, and removed from ciprofloxacin at least three days prior to infection with *N. gonorrhoeae*. Differentiated BMDM were plated in 96 well plates (5×10^4 cells/well) and subsequently stimulated. Supernatants were collected after 24 hours and analyzed for cytokines using commercial ELISA kits for TNF- α (eBioscience; San Diego, CA) and IL-6 (R&D Systems; Minneapolis, MN). Splenic mononuclear cells were prepared as follows. Spleens were removed from mice and spleen cells were prepared. After red blood cell lysis, splenocytes were plated in 24 well plates (1×10^6 cells/well) in RPMI 1640 supplemented with 10% FBS, 20 $\mu\text{g/ml}$ of gentamicin, 10 $\mu\text{g/ml}$ of ciprofloxacin. Supernatants were collected 3 and 5 days after stimulation and analyzed for cytokines by ELISA as described above.

Serum killing assay

Venous blood was obtained from wild type BALB/c and BALB/c-*Lps^d* mice by retro-orbital bleeding and the serum separated by centrifugation. Sera were heat-inactivated at 56°C for 30 min. Strain FA1090 was suspended in PBSGCM and passed through 1.2 micron filters to remove bacterial aggregates and adjusted to an optical density of 0.07 at 600 nm and diluted 1:10. Twenty μl (10^5 CFU) of the bacterial suspension were added to heat-inactivated serum (HI-serum) from BALB/c and BALB/c-*Lps^d* mice or buffer (no serum control) in complete Hanks balanced salt solution (HBSS buffer with 10 mM glucose, 0.1% gelatin, 1mM CaCl₂ and 1mM MgCl₂). Final serum concentration was 20% in a final assay volume of 300 μl . After 30, 90, 120 and 150 min of incubation at 37°C, 10 μl of samples were serially diluted in GC broth with 0.05% saponin and cultured on GC agar. The number of colonies from triplicate wells was counted after overnight incubation and the data are expressed as the average log₁₀ CFU/ml.

PMN killing assay

PMNs were elicited from wild type BALB/c and BALB/c-*Lps^d* mice via peritoneal lavage and the capacity of PMNs to kill *N. gonorrhoeae* was measured using a modification of the tumbling tube assay as described previously⁵⁵. Serum (10%) from normal BALB/c mice (NMS) was used to opsonize the bacteria to control for differences in the killing activity of sera from these two mouse strains. Bacteria that were pre-incubated with HI-NMS, which we previously found does not opsonize gonococcal for PMN uptake^{55, 56} were incubated with PMNs in parallel to provide a baseline for loss of bacterial viability during the assay. The number of viable gonococci recovered from each test condition was determined after 0, 45 and 90 min incubation by quantitative culture. The averages of triplicate cultures were calculated and results are expressed as the percent survival [$100 \times (\text{number of NMS-opsonized CFU recovered at 90 min divided by number of HI-NMS opsonized CFU})$].

Flow cytometric analysis

Differentiated BMDM from BALB/c and BALB/c-*Lps^d* mice were prepared as above. After stimulation under different conditions for 24 hrs, cells were collected and stained with a phycoerythrin (PE) conjugated anti-TLR2 antibody (eBioscience, Inc., San Diego, CA; cat# 12-9021-82) and a biotinylated antibody for the macrophage marker F4/80 (BioLegend, San Diego, CA; cat#122603) plus streptavidin-PE-Cy5 (BD Biosciences, San Jose, CA;

cat#554062). Cells were analyzed by flow cytometry using a FACScan microfluorimeter (Becton Dickinson, San Jose, CA). A total of 10,000 events were counted for each condition.

Statistical analysis

The average duration of recovery and colonization load over time were compared between groups by repeated measures analysis of variance (ANOVA) with Bonferroni correction using SPSS software. The influx of PMNs in the infected group on any day was compared to its uninfected group by unpaired t-test. Levels of vaginal cytokines and chemokines as measured by multiplex assay were analyzed using a Mann-Whitney U-test with 95% confidence intervals. For the *in vitro* stimulation assays, each data point was assayed in triplicate, and graphed as the mean \pm standard error, and compared using an unpaired t-test. GraphPad software was used for all other statistical tests.

Acknowledgments

This work was supported by NIH/NIAID grants R01 AI42053 and U19 AI31496 (to A.E.J.), and R01 AI46613 and U19 AI084048 (to R.R.I.).

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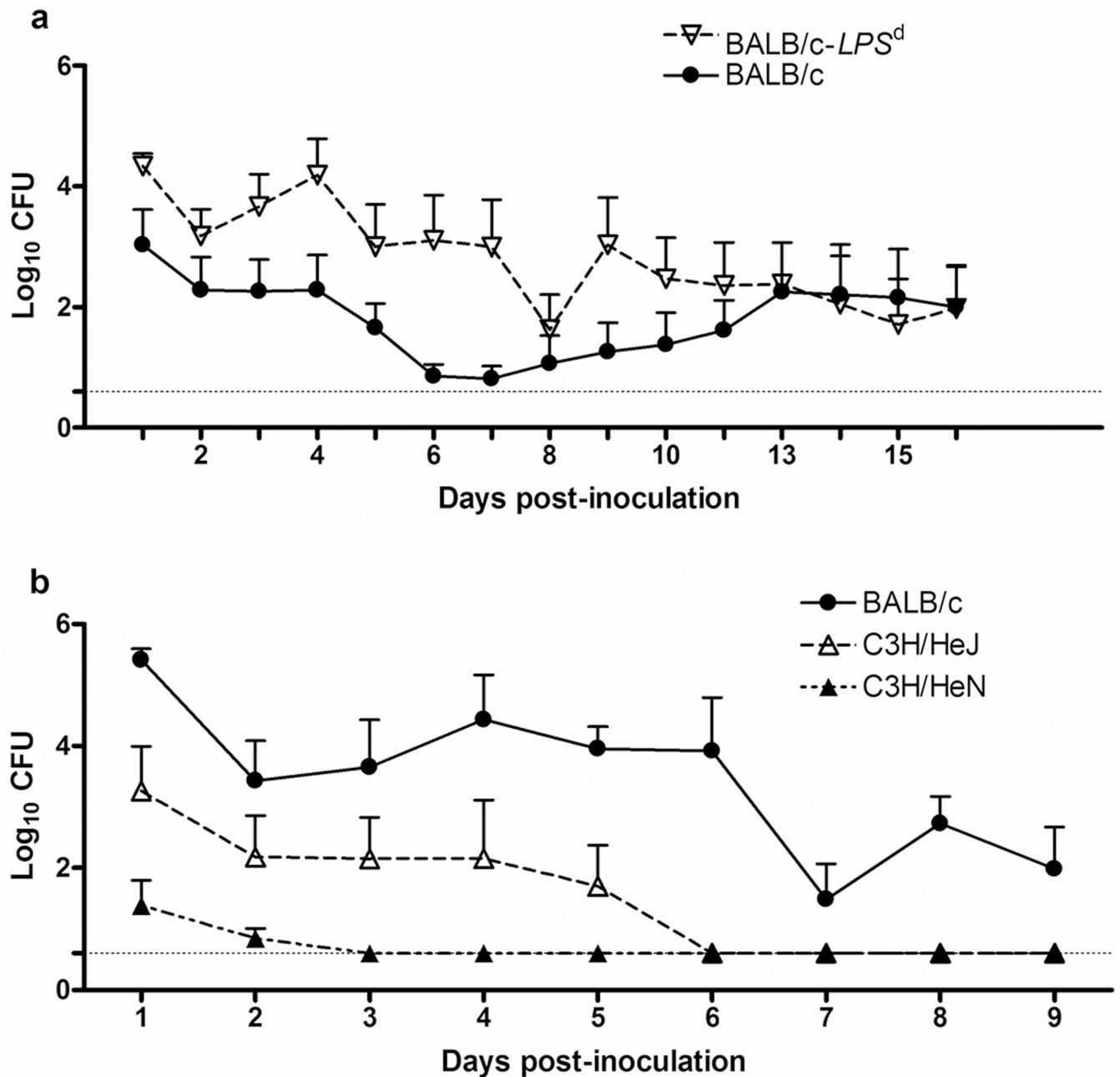


FIG. 1. TLR4 controls gonococcal colonization load in both susceptible BALB/c mice and resistant C3H mice

Mice were inoculated intravaginally with strain FA1090 or PBS as described in the text. The recovery of gonococci from wild type or congenic mutant mice that are homozygous for the *tlr4LPS-d/J* gene (BALB/c-*Lps^d*) was determined and is expressed as log₁₀ CFU per 100 μ l of vaginal swab suspension (\pm Standard Error). **(a)** BALB/c (TLR4 wt; n=8) and BALB/c-*Lps^d* (TLR4 mutant; n=8); **(b)** C3H/HeN (TLR4 wt; n=7), C3H/HeJ (TLR4 mutant; n=5), and BALB/c mice (n=6). In each experiment, mice were inoculated intravaginally with 1×10^6 (Fig. 1A) or 1×10^7 CFU (Fig. 1B) of *N. gonorrhoeae* strain FA1090 or PBS, and

bacterial recovery was determined at the indicated times. Limit of detection for the assay is Log_{10} 0.6 CFU, and is indicated by the dashed line. *, $p = 0.007$ (panel A) and $p = 0.026$ (panel B), as determined by a repeated measures ANOVA. These results are representative of two independent experiments with 4–6 mice per group in repeat experiments.

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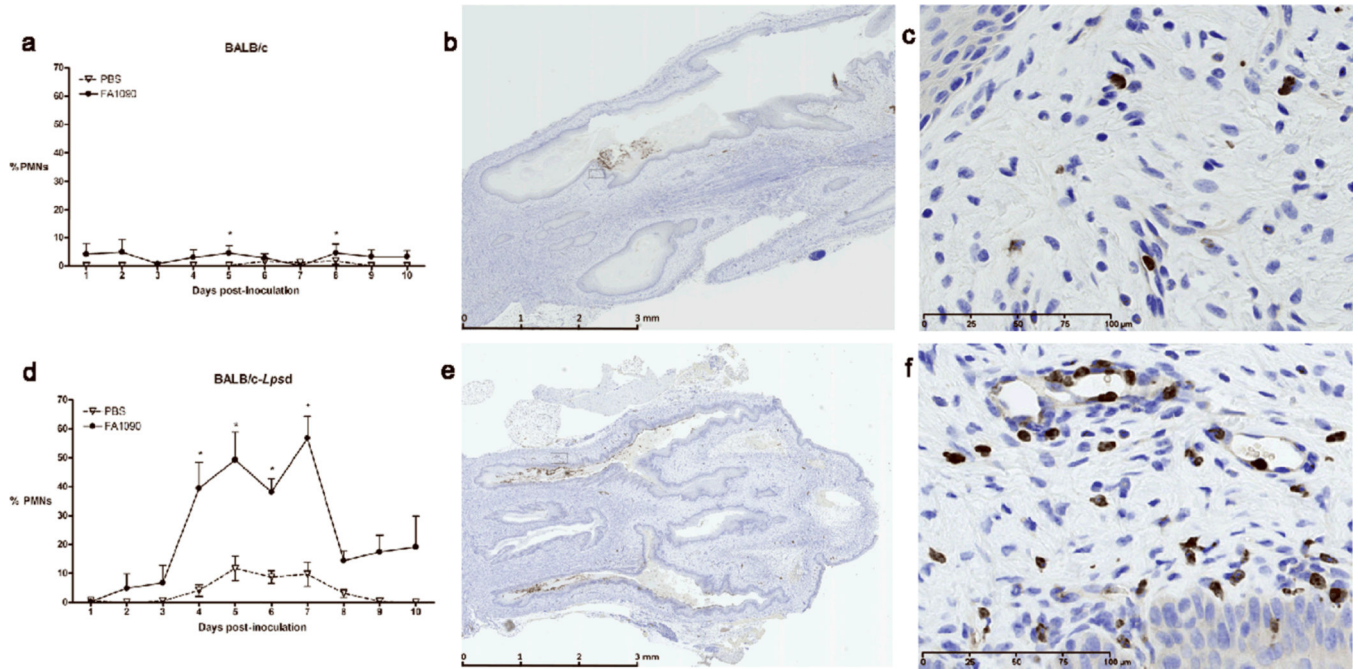


FIG. 2. TLR4 mutant mice have an exaggerated PMN influx in response to gonococcal infection Mice were inoculated intravaginally with strain FA1090 or PBS as described in the text, and the vaginal PMN influx was measured by determining the percent of PMNs among 100 vaginal cells in stained vaginal smears. **(a,d)** PMN count in vaginal smears from infected and uninfected mice. PMN influx in vaginal smears is shown for infected vs. uninfected mice over time for **(a)** BALB/c and **(d)** BALB/c-*Lps^d* mice. Significance was calculated using an unpaired t-test. *, There was a significant difference between infected vs. uninfected strains, with $p < 0.05$. These results are representative of two independent experiments. **(b–c, e–f)** Immunohistochemical analysis of PMN influx into the lower genital tract. Genital tract tissue was extracted from infected BALB/c and BALB/c-*Lps^d* mice mutant mice on day 7 of infection and stained with Gr-1-specific antibodies to detect PMNs, as described in the text. Shown above are representative images for infected **(b,c)** BALB/c and **(e,f)** BALB/c-*Lps^d* mice. Original magnification: $100\times$ (b, e) and $400\times$ (c, f).

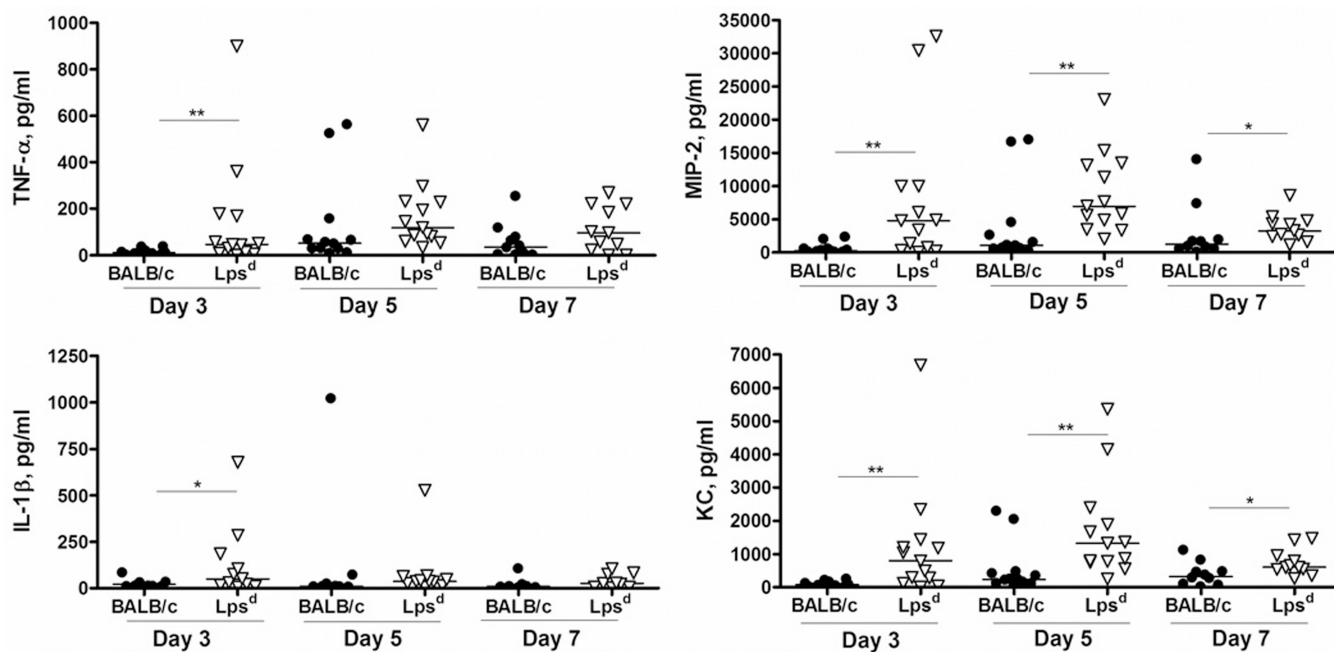


FIG. 3. TLR4 mutant mice upregulate inflammatory cytokines and chemokines in response to gonococcal infection

Mice were infected as described in the text, and vaginal washes were obtained over time for assay of IL-1 β , TNF- α , MIP-2 and KC by custom multiplex assay. Shown above are data pooled from three independent experiments. Each data point represents an individual mouse; n=10–12 for BALB/c, n=11–13 for BALB/c-*Lps^d*. The horizontal line represents the median value for each time point. Statistics were calculated using a Mann-Whitney U-test, with p values as follows: *, p < 0.05; **, p < 0.01.

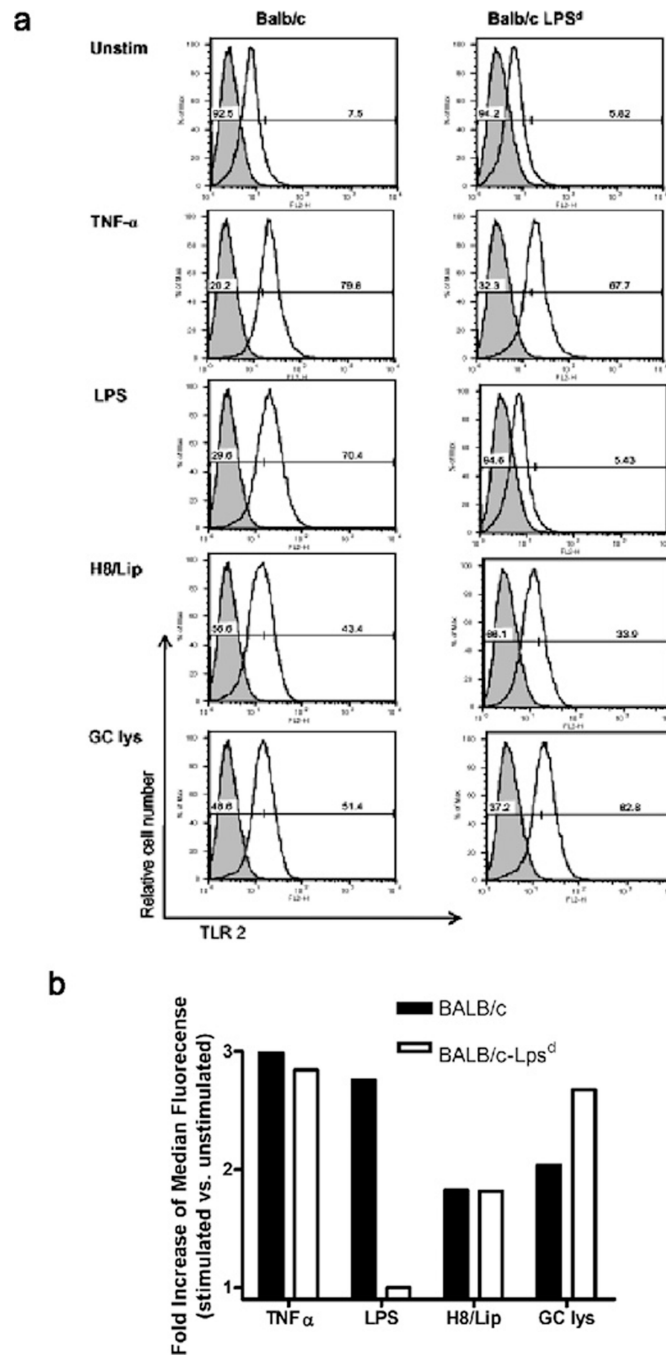


FIG. 4. Expression of surface TLR2 does not differ between BALB/c and TLR4 mutant mouse strains

BMDM were derived from BALB/c and BALB/c-*Lps^d* mice as described in the text. Cells were stained for surface expression of TLR2 either at rest or after 24 hours treatment with the agents noted above. (a) Shown above are FACS histograms for demonstrating staining using either the specific anti-TLR2 mAb (black line) or a matched isotype control (gray shading). The vertical axis represents the relative cell number, while the horizontal axis represents the intensity of fluorescence in the FL2 channel. The percentage of cells that are

either negative or positive for TLR2 is shown for each histogram. **(b)** The bottom graph shows the median fluorescence as calculated from the histograms for each condition. Treatments were as follows: unstim, unstimulated cells; TNF- α , 40 ng/ml; LPS, 100 ng/ml; H8/Lip, 1 μ g/ml; GC lys (*N. gonorrhoeae* FA1090 crude lysates) MOI 10:1. These histograms are representative of two independent experiments.

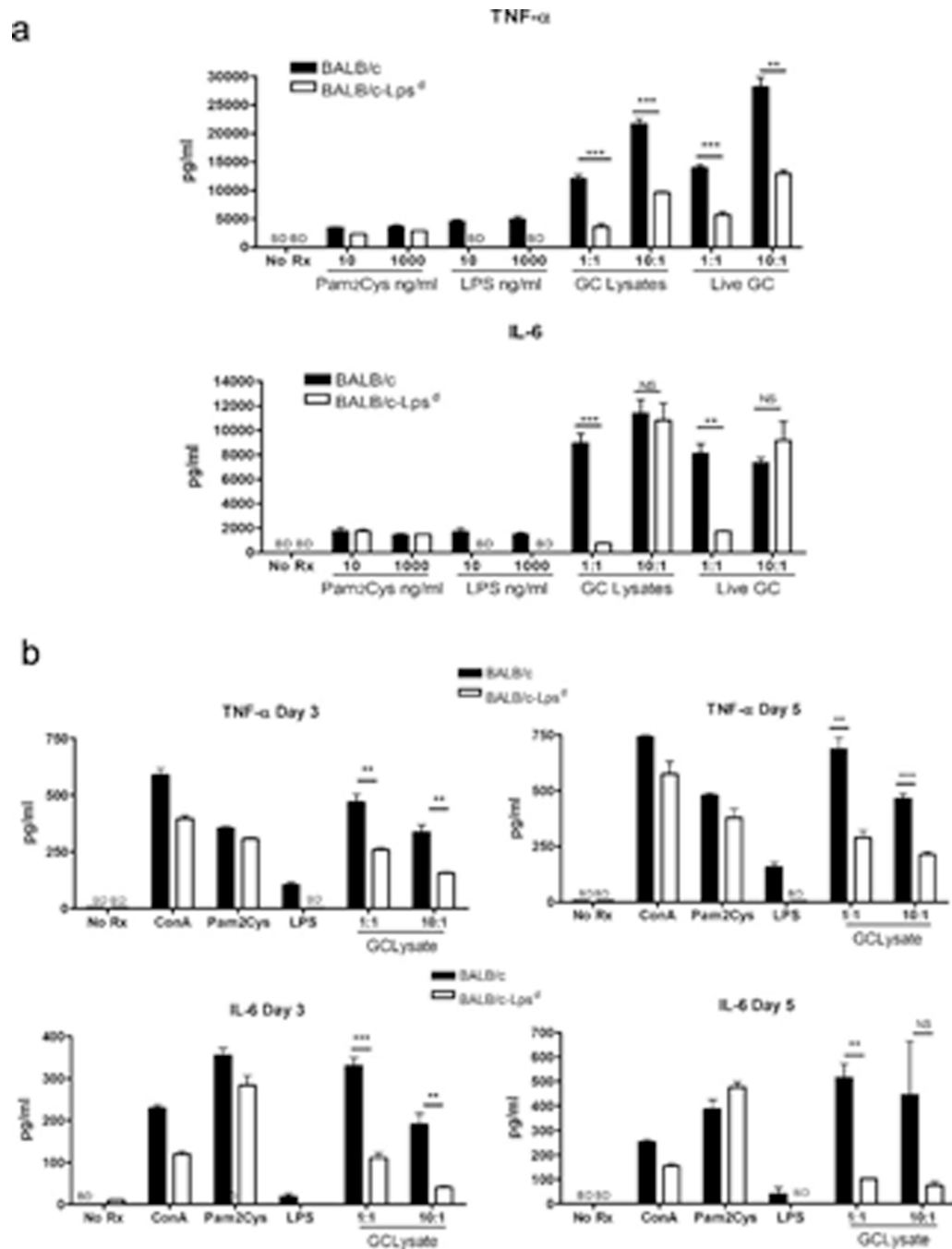


FIG. 5. Cells derived from TLR4 mutant mice are hyporesponsive to stimulation with *N. gonorrhoeae*

(a) BMDM or (b) splenic mononuclear cells were derived from BALB/c and BALB/c-Lps^d mice as described in the text, and treated with the indicated agents. Supernatant was collected at 24 hours for (a) and at the indicated time points for (b), and assayed for TNF- α or IL-6 by ELISA. Pam2Cys, lipopeptide Pam₂-Cys-Ser-Lys₄; GC lys, *N. gonorrhoeae* FA1090 crude lysates. Significance was calculated using an unpaired t-test, with p values as

follows: **, $p < 0.01$; ***, $p < 0.001$; NS, not significant; BD, below detection. These data are representative of two independent experiments.

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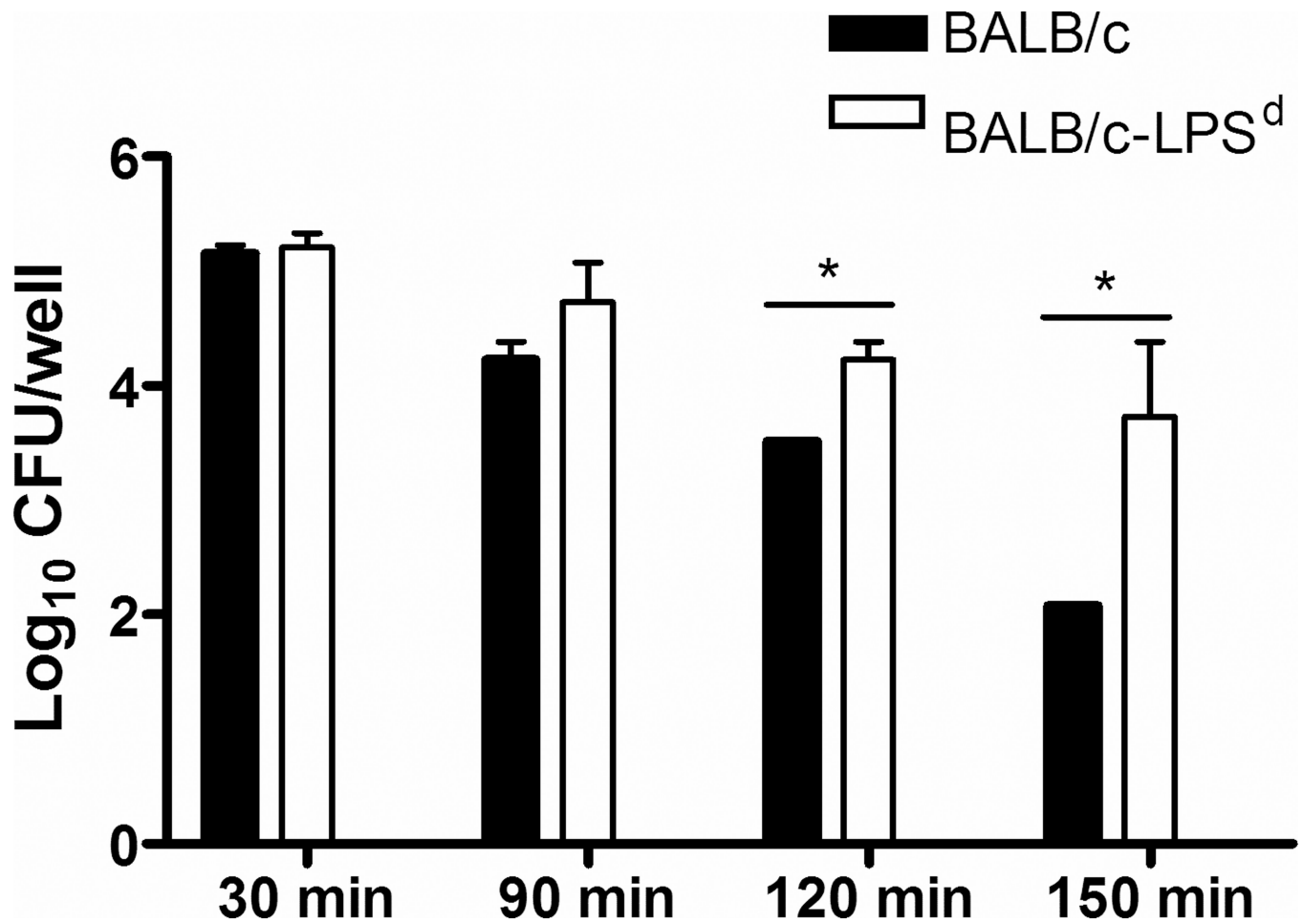


FIG. 6. Heat-stable killing activity of mouse serum is greater in wild type mice compared to *Lps^d* mutant mice

The number of viable bacteria was determined following incubation with HI-serum from BALB/c versus BALB/c-*Lps^d* mice at 30, 90, 120 and 150 min post inoculation. HI-serum from BALB/c mice killed FA1090 bacteria more efficiently when compared to HI-serum from BALB/c-*Lps^d*. Both sera had killing activity when compared to the buffer control. Log₁₀ CFU recovered from no serum controls at any time point ranged from 5.07–5.54 (data not shown). * $p < 0.05$, unpaired t-test. These data are representative of four independent experiments.

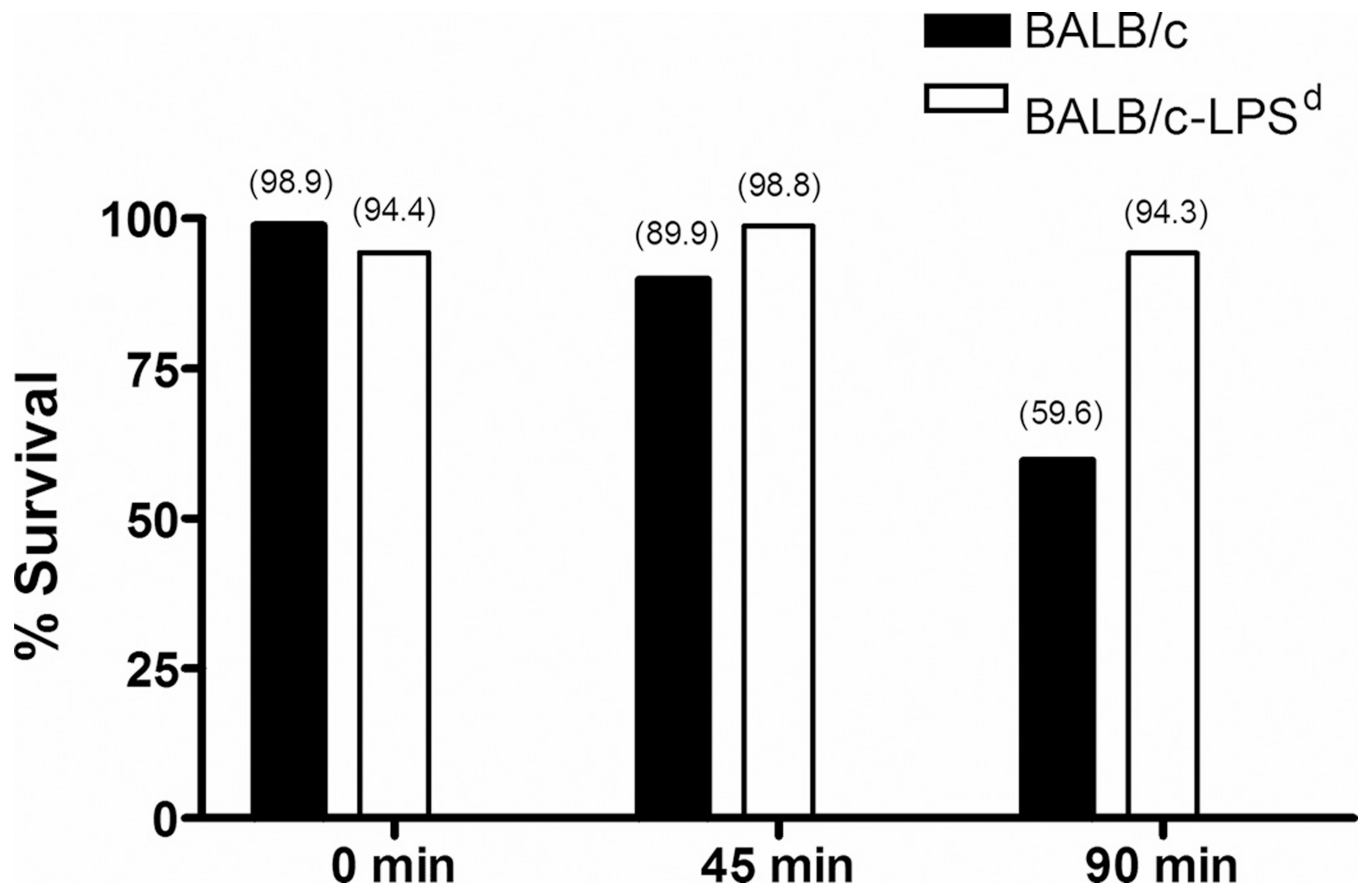


FIG. 7. PMNs from BALB/c-*Lps*^d mice are defective for killing *N. gonorrhoeae*
 Gonococci were opsonized with 10% serum from BALB/c mice and then incubated with PMNs from normal and BALB/c-*Lps*^d mice. The number of gonococci recovered following 0, 45 and 90 min incubation is expressed as the percentage of bacteria recovered from PMNs incubated with bacteria that were pre-incubated in HI-serum. Absolute values are provided in parentheses above the bars. These data are representative of two independent experiments, where reduced recovery of *N. gonorrhoeae* occurred after 90 min incubation with BALB/c PMNs only (59.6% and 52.9% for each experiment).

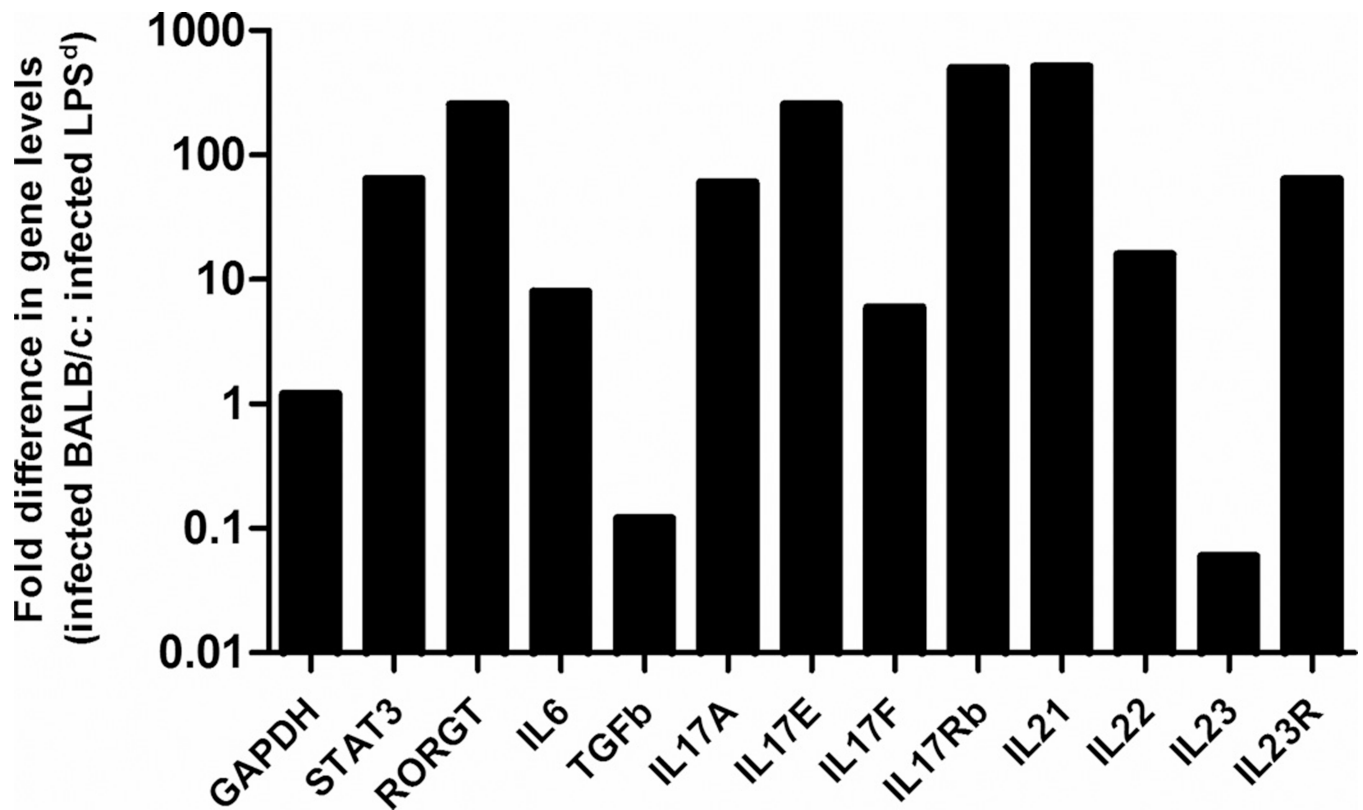


FIG. 8. Th17 type cytokine production is impaired in the TLR4 mutant mice

Relative expression of GAPDH, RORGT, STAT3, IL-6, IL-17A, IL-17E, IL-17F, IL-17 RB, IL-21, IL-22, IL-23A and IL-23R following intravaginal inoculation with FA1090. Total RNA was extracted from vaginal washes and subjected to relative real-time PCR, as described in the text. A relative change of 2-fold or greater between infected BALB/c vs. infected BALB/c-*Lps*^d mice was considered significant. These results are representative of two independent experiments.