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***Neisseria gonorrhoeae* survives within and modulates apoptosis and inflammatory cytokine production of human macrophages**

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Abstract

The human-adapted organism *Neisseria gonorrhoeae* is the causative agent of gonorrhea a sexually transmitted infection. It readily colonizes the genital, rectal, and nasopharyngeal mucosa during infection. While it is well-established that *N. gonorrhoeae* recruits and modulates the functions of polymorphonuclear leukocytes (PMNs) during infection, how *N. gonorrhoeae* interacts with macrophages present in infected tissue is not fully defined. We studied the interactions of *N. gonorrhoeae* with two human monocytic cell lines, THP-1 and U937, and primary monocytes, all differentiated into macrophages. Most engulfed bacteria were killed in the phagolysosome, but a subset of bacteria were able to survive and replicate inside the macrophages suggesting that those cells may be an unexplored cellular reservoir for *N. gonorrhoeae* during infection. *N. gonorrhoeae* was able to modulate macrophage apoptosis, *N. gonorrhoeae* induced apoptosis in THP-1 cells whereas it inhibited induced apoptosis in U937 cells and primary human macrophages. Furthermore, *N. gonorrhoeae* induced expression of inflammatory cytokines in macrophages, suggesting a role for macrophages in recruiting PMNs to the site of infection. These results indicate macrophages may serve as a significant replicative niche for *N. gonorrhoeae* and play an important role in gonorrheal pathogenesis.

Keywords

Neisseria gonorrhoeae; gonorrhea; macrophages; apoptosis; cytokines

Introduction

The Gram-negative bacterium *Neisseria gonorrhoeae* is the causative agent of gonorrhea, a sexually transmitted infection that affects more than 100 million people annually (WHO, 2012). This exclusive human pathogen primarily infects the urogenital tract often giving rise to local inflammation. Dissemination from the local site of infection can also occur and lead to pelvic inflammatory disease, dermatitis, endocarditis and arthritis (reviewed in Hook *et al.*, 1985). As an exclusive human pathogen with no known alternative reservoir, *N. gonorrhoeae* has evolved strategies to promote growth and persistence in the host by modulating the host innate and adaptive immune systems. Symptomatic *N. gonorrhoeae*

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infection triggers an inflammatory response driven by large numbers of polymorphonuclear leukocytes (PMNs) and the role of PMNs in *N. gonorrhoeae* pathogenesis has been extensively investigated. These studies suggest that some *N. gonorrhoeae* survive and replicate within PMNs, notably by modulating their ability to phagocytose and release antimicrobial components (reviewed by Johnson *et al.*, 2011, Criss *et al.*, 2012).

The genitourinary mucosae also contains significant populations of macrophages, supporting the idea that *N. gonorrhoeae* encounter these cells during infection (Givan *et al.*, 1997). Moreover acute gonorrhea is characterized by an intense inflammatory exudate that contains macrophages, exfoliated epithelial cells, and numerous PMNs (Hook, 1999). Therefore, it is likely that macrophages have an important role in the pathogenesis of *N. gonorrhoeae* infections. There is little consensus concerning the survival of *N. gonorrhoeae* inside the macrophages. Ota *et al.*, (Ota *et al.*, 1975) suggested that *N. gonorrhoeae* may be capable of intracellular survival within murine macrophages, whereas Cooper and Floyd (Cooper *et al.*, 1982) showed that *N. gonorrhoeae* phagocytosed by mouse macrophages were completely killed after 30 min of incubation. A more recent study shows that after macrophage internalization the number of *N. gonorrhoeae* rapidly decreases at early time points but, importantly, a portion of bacteria were able to survive intracellularly over a more prolonged period (Leuzzi *et al.*, 2005). This study was consistent with another report that suggested that some intracellular *N. gonorrhoeae* can survive inside macrophages (Zughaier *et al.*, 2014).

One common strategy used by microbial pathogens to establish infection in human hosts is the modulation of apoptosis. Apoptotic cells are characterized by distinct morphological and chemical changes that results from signaling cascades initiated through two distinct pathways, the extrinsic and intrinsic pathways (for a review, Elmore, 2007). Engagement of death receptors on the cell surface leads to activation of the extrinsic apoptotic pathway, whereas the intrinsic pathway is activated by intracellular events that cause mitochondrial release of cytochrome *c* into the cytoplasm. Both pathways result in caspase cleavage and DNA fragmentation. The effect of *N. gonorrhoeae* infection on apoptosis of different host cells has been studied extensively, but there are conflicting reports. *N. gonorrhoeae* has been shown to induce apoptosis in epithelial cells (Muller *et al.*, 1999, Muller *et al.*, 2000, Kepp *et al.*, 2007, Kepp *et al.*, 2009), but other studies have shown *N. gonorrhoeae* to have no effect on apoptosis in the same cell type (Massari *et al.*, 2003). *N. gonorrhoeae* can inhibit apoptosis induced by staurosporine (STS) in epithelial cells and HL-60 cells (Binnicker *et al.*, 2003, Howie *et al.*, 2008, Follows *et al.*, 2009, Chen *et al.*, 2011). *N. gonorrhoeae* also inhibits spontaneous apoptosis in primary human PMNs and HL-60 cells (Simons *et al.*, 2006, Chen *et al.*, 2011). The reasons for the divergence in the observed experimental results are unknown, but may be due to the use of different *N. gonorrhoeae* strains, host cell lines, or infection conditions (Massari *et al.*, 2003). The mechanisms by which *N. gonorrhoeae* induces and/or inhibits apoptosis have been explored mainly in epithelial cells and PMNs, but nothing is known about the mechanisms used within macrophages, and whether *N. gonorrhoeae* utilizes similar mechanisms of apoptosis regulation remains to be determined.

In this study, we used the two most common differentiated cell lines, U937 and THP-1 cells as models to study the interaction of *N. gonorrhoeae* with human macrophages. We

demonstrate that *N. gonorrhoeae* is able to survive in association with these macrophage cell lines and that a subset of intracellular bacteria can survive intracellularly. We also show that during infection *N. gonorrhoeae* modulates apoptosis in macrophages with the outcome differing in the two cell lines used: *N. gonorrhoeae* induced apoptosis in THP-1 cells but protected against STS- and TNF- α -induced apoptosis in U937 cells. The phenotypes observed in U937 cells were replicated in primary human macrophages. *N. gonorrhoeae* also induced the secretion of many cytokines from infected macrophages suggesting that *N. gonorrhoeae* interactions with macrophages could be an instrumental step in the induction of the inflammatory response characteristic of symptomatic gonorrhoea. All of these interactions between *N. gonorrhoeae* and macrophages strongly support the hypothesis that macrophages are an important innate cell type involved in the pathogenesis of gonorrhoea.

Results

***N. gonorrhoeae* survives in presence of U937 and THP-1 macrophages**

The survival of *N. gonorrhoeae* in association with differentiated THP-1 and U937 cells was examined using viable counts (CFUs). *N. gonorrhoeae* grew better when associated with the macrophage cell lines than in RPMI medium alone, a medium in which the bacteria can replicate (Fig. 1A). While *N. gonorrhoeae* viable counts tended to decrease slightly over time in PBSG medium alone since this medium does not support growth, bacteria incubated in the presence of U937 and THP-1 cells in PBSG medium for 7h grew an average of 139- and 40-fold, respectively (Fig. 1A).

To establish whether the U937 and THP-1 cells bind and phagocytose *N. gonorrhoeae*, association of bacteria with challenged cells was monitored by differential immunofluorescence of intra- and extra-cellular bacteria (Fig. 1B). After 1.5h, 3.5h and 21.5h of infection, there were both extra- and intra-cellular bacteria associated with the cells. Phagocytosis occurred rapidly as intra-cellular bacteria could be observed as early as 30 min after infection, and when phagocytosis is blocked by pretreatment of the cells with cytochalasin D, no intra-cellular bacteria are detected (data not shown). Furthermore, an increase of macrophage internalized bacteria was observed over the duration of the experiment in both RPMI and PBSG medium (Fig. 1B). Based on the rapid entrance of the bacteria into the macrophages, we conclude that macrophages can phagocytose *N. gonorrhoeae* without opsonization. We cannot from these analyses determine whether some bacteria can enter using an alternative mechanism from phagocytosis.

To examine bacterial survival within macrophage cell lines, U937 and THP-1 cells were challenged with *N. gonorrhoeae* at a multiplicity of infection (MOI) of 23 in RPMI medium for 1.5h, and extracellular bacteria were killed by gentamicin treatment. After removal of gentamicin and incubation in fresh media, the number of viable bacteria remaining in association with macrophages was determined at various times (Fig. 1C, data not shown for THP-1). 1% (± 0.2) of the total inoculum was internalized in U937 macrophages after 3h of infection. The number of bacteria associated with both cell types decreased initially; however, 3h after the removal of gentamicin, the number of CFUs increased, suggesting that the bacteria were able to replicate in association with the cells. When gentamicin was maintained in the culture medium for the entire duration of the experiment, no viable *N.*

gonorrhoeae were detected after 6h. Two possible explanations for these results are that the macrophages are becoming compromised by the infection to allow gentamicin access to intracellular bacteria, or that *N. gonorrhoeae* are escaping from the macrophages and were killed extracellularly by the antibiotic.

It was possible that *N. gonorrhoeae* was being killed by the antibiotic because of disruption of the host cell membrane. To test this hypothesis, cytoplasmic lactate dehydrogenase (LDH) release that occurs upon cell lysis was measured (Fig. 1D). The percentage of LDH release was near zero indicating that *N. gonorrhoeae* does not cause extensive lysis of U937 or THP-1 cells. The absence of cell lysis was also confirmed with propidium iodide exclusion staining of challenged U937 cells, which showed that less than 1% of challenged cells had disrupted cell membranes (data not shown). These results suggest that *N. gonorrhoeae* does not induce overt membrane damage during the times studied. Therefore, we reason that the bacteria must be exiting the macrophage cells without lysis. Moreover, treatment of the macrophage cell with the actin polymerization inhibitor cytochalasin D did not inhibit *N. gonorrhoeae* growth indicating that actin polymerization had no role in bacterial exit from macrophages (data not shown). These results show that *N. gonorrhoeae* is phagocytosed by U937 and THP-1 differentiated cells and *N. gonorrhoeae* is able to survive within and potentially escape from these phagocytes.

***N. gonorrhoeae* can survive and replicate inside macrophages**

Although *N. gonorrhoeae* was unable to expand in PBSG medium alone, it was able to grow in presence of macrophages in PBSG, expand greater in RPMI (Fig 1A) and increase intracellularly (Fig 1B). While these results could reflect bacterial replication within the macrophages, it was possible that continual uptake of bacteria from the medium could contribute to the increased internalized bacteria. We therefore examined the number of intracellular bacteria per macrophage over time by fluorescence microscopy, treating the macrophages with cytochalasin D after infection to block additional phagocytosis. The percentage of macrophages containing more than 10 bacteria increased over time whereas the percentage of macrophages with one to five bacteria decreased (Fig. 2A), indicating that the internalized bacteria were able to survive and replicate inside the macrophage.

To better understand the time course of events involved in intracellular survival and replication of *N. gonorrhoeae*, we analyzed the bacterium-containing phagosomes. We first evaluated *N. gonorrhoeae* colocalization with fluorescent microscopy using the acidotropic dye LysoTracker at different time points. After 2h of infection, 58.5% \pm 3.4% of the bacteria colocalized with acidic organelles (Fig 2B), demonstrating that bacterial phagosomes fuse with lysosomes at early stages of infection. However, after 3h of infection, coincident with the increase in the number of bacteria inside the macrophage (Fig 2A), most of the bacteria did not colocalize with the acidotropic dye. Furthermore, the decrease of LysoTracker red colocalization with *N. gonorrhoeae* during the time course of infection suggests that bacteria do not survive within this acidic compartment. In the presence of the acidification inhibitor Bafilomycin A1, the survival of *N. gonorrhoeae* in macrophages increased compared to no inhibitor (Fig 2C). Interestingly, *N. gonorrhoeae* that colocalized with acidotropic dye also colocalized with the late endosome marker LAMP-1, and the bacteria that were not

colocalized with LysoTracker were also not associated with LAMP-1 (Fig. 2D). The *Neisseria* IgA1 protease has been described to cleave LAMP-1 and promote survival of bacteria within epithelial cells (Lin *et al.*, 1997). There was no difference in the survival of an *igA1* mutant and the parental strain in U937 cells (data not shown). Finally, transmission electron microscopy of U937 cells challenged with *N. gonorrhoeae* showed a subset of the internalized bacteria had no obvious surrounding membrane suggesting they can escape either from the phagolysosome or from the vacuole after entering the cell by an active process initiated by the bacterium (Fig. 3A). In contrast, phagolysosome encased *N. gonorrhoeae* were readily observed in Bafilomycin A1 treated cells (Fig 3A). Furthermore, the number of macrophage exhibiting bacteria with no surrounding membrane increased over the time of infection in absence of Bafilomycin A1 (Fig 3B), and the bacteria localized in the cytoplasm retain electron density in contrast to the bacteria localized in vacuole, suggesting that the cytoplasmically localized bacteria retain viability. These results support the hypothesis that a subset of internalized bacteria is able to escape from a phagosome or endosome into the cytoplasm and/or avoid lysosome fusion to prevent killing and it is this population that can replicate within the macrophages.

***N. gonorrhoeae* differentially influences apoptosis in THP-1 and U937 cells**

Since *N. gonorrhoeae* can survive and replicate within macrophages, we wondered whether *N. gonorrhoeae* could modulate caspase-dependent apoptosis in macrophages, as has been reported for PMNs (Simons *et al.*, 2006, Chen *et al.*, 2011). Differentiated U937 and THP-1 cells were challenged with *N. gonorrhoeae* for 3h, and the cells were treated for 3h with either staurosporine (STS) to induce apoptosis or DMSO as a control. Cell lysates were harvested and caspase-3 activity was assessed using the fluorogenic caspase-3 substrate Ac-DEVD-AMC (Fig. 4). Infection of U937 cells with *N. gonorrhoeae* did not induce significant caspase-3 activity compared to the uninfected control, however, infection of THP-1 cells showed an increase of caspase-3 activity (Fig. 4A and B). Treatment of both cells with STS increased caspase-3 activity over that of control DMSO treated cells. Preincubation of U937 cells with *N. gonorrhoeae* at an MOI of 100 significantly decreased STS-induced caspase-3 activity, an effect comparable to that of the general caspase-3 inhibitor z-VAD-fmk (Fig. 4A). In contrast, preincubation of THP-1 cells with *N. gonorrhoeae* did not significantly modify caspase-3 activity levels (Fig. 4B). Studying DNA fragmentation, the last stage of apoptosis, confirmed the absence of apoptosis induction by *N. gonorrhoeae* in U937 cells and the partial induction of apoptosis in THP-1 cells (35%) (Fig 4C). The effect of *N. gonorrhoeae* on STS-induced caspase-3 activation of U937 cells was dose dependent, as the inhibition of caspase-3 activity became more pronounced with increasing MOIs (Fig. 4A). These differential results are reminiscent of the contrasting results about the effect of *N. gonorrhoeae* on apoptosis obtained by different groups using epithelial cell lines and could provide a way to examine the mechanistic basis for these differential responses by comparing the modulation of signal transduction pathways by *N. gonorrhoeae*.

To test whether *N. gonorrhoeae* internalization is necessary to modulate apoptosis, U937 and THP-1 differentiated cells were pre-treated with cytochalasin D to inhibit phagocytosis, then challenged with bacteria and caspase-3 activity was measured. Cytochalasin D treatment did

not alter the effect of *N. gonorrhoeae* on STS induced caspase-3 activation in U937 cells or on the induction of caspase-3 activity in THP-1 (Fig. 4D). Furthermore, apoptosis regulation did not require viable *N. gonorrhoeae*, as heat-killed or paraformaldehyde-killed *N. gonorrhoeae* are still able to fully modulate apoptosis (Fig. 4). These results show that active *N. gonorrhoeae* processes are not required for apoptosis modulation.

***N. gonorrhoeae* inhibits both the intrinsic and extrinsic apoptosis pathways in U937 cells and induces the extrinsic apoptosis pathway in THP-1 cells**

While we established divergent apoptosis responses to *N. gonorrhoeae* in U937 and THP-1 macrophages, the identity of the upstream signaling pathways influenced by *N. gonorrhoeae* remain to be fully defined. In order to define which apoptotic signaling pathways are affected by *N. gonorrhoeae* infection, the loss of mitochondrial membrane potential (MMP) was studied as a marker of the intrinsic apoptotic pathway. STS induced the loss of MMP in over 90% of both U937 and THP-1 cells (Fig 5). In U937 cells differentiated into macrophages, *N. gonorrhoeae* inhibited the loss of MMP induced by STS (around 50%), indicating that *N. gonorrhoeae* is able to inhibit intrinsic apoptosis pathway induced by STS. In THP-1 cells differentiated into macrophages, *N. gonorrhoeae* was unable to inhibit apoptosis induced by STS (Fig. 5), confirming the caspase-3 results. No induced loss of MMP was observed in THP-1 cells challenged by *N. gonorrhoeae* suggesting that *N. gonorrhoeae* does not induce apoptosis via the intrinsic pathway in this cell line. To test whether *N. gonorrhoeae* modulates the extrinsic apoptosis pathway in THP-1 differentiated cells, Bid cleavage was assayed by western-blot analysis. *N. gonorrhoeae* induced Bid cleavage in THP-1 cells (Fig. 6), indicating that *N. gonorrhoeae* induces the extrinsic apoptosis pathway in this cell line. *N. gonorrhoeae* did not induce Bid cleavage in U937 cells, consistent with our prior results. In contrast, *N. gonorrhoeae* inhibited Bid cleavage induced by TNF- α + cyclohexymide (CHX) treatment (Fig. 6). Finally, Caspase-8 cleavage, another step of the extrinsic apoptosis pathway, when induced by TNF- α + CHX in U937 cells, was also inhibited by *N. gonorrhoeae* infection (data not shown). Taken together the results of these assays clearly demonstrate that *N. gonorrhoeae* inhibits both the intrinsic and extrinsic apoptotic pathways in U937 cells, but only induces extrinsic apoptosis in THP-1 cells.

***N. gonorrhoeae* inhibits apoptosis in Human primary macrophages**

The contradictory apoptosis response in two macrophage cell lines, suggested that these standard human monocytic cell lines express distinct phenotypes. In order to determine which macrophage cell line phenotype might be most relevant *in vivo*, apoptosis modulation was examined in macrophages derived from primary human monocytes (MDM). Similarly to the cell lines, *N. gonorrhoeae* was internalized without opsonisation by MDM (Fig. 7A) and the percentage of *N. gonorrhoeae* present within the MDM 2.5h after infection was 1.85% (± 0.25). *N. gonorrhoeae* survived in the presence of MDM, but when gentamicin was present for the entire duration of the assay, no viable *N. gonorrhoeae* were detected after 6 h of infection (Fig. 7B). Furthermore, no LDH release was observed during MDM infection by *N. gonorrhoeae* (Fig. 7C). These results were all consistent with those obtained from the macrophage cell lines. Infection of MDM with *N. gonorrhoeae* alone did not induce significant caspase-3 activity over uninfected controls using cells from six distinct donors

(Fig. 7D). While treatment of MDM with STS increased caspase-3 activity over that of control treated cells, preinfection of MDM with *N. gonorrhoeae* significantly decreased STS-induced caspase-3 activity (Fig 7D). Furthermore, loss of MMP and Bid cleavage are not induced in MDM challenged by *N. gonorrhoeae*, whereas infection with *N. gonorrhoeae* protected against loss of MMP induced by STS (data not shown). Therefore, the effect of *N. gonorrhoeae* on apoptosis of MDM is more similar to the effect in U937 cells than THP-1 cells.

Macrophages can be polarized to M1 or M2 phenotypes in response to environmental signals, including bacterial infection. It was possible that the macrophage-like cell lines were more closely replicating one of the polarized phenotypes. To determine whether polarized primary macrophages have a similar apoptotic phenotype than non-differentiated primary macrophages, MDM were treated with TNF- α plus INF- γ or IL-4 to polarize them to subtype M1 or M2a phenotypes, respectively, and caspase 3-activity was measured. *N. gonorrhoeae* did not induce apoptosis in either M1 or M2a MDM (Fig. 7E). *N. gonorrhoeae* inhibited caspase-3 cleavage induced by STS with M2a macrophages, but not with M1 macrophages (Fig. 7E). Therefore with respect to the regulation of apoptosis during infection, U937 cells, MDM and M1 macrophages were the most similar and THP-1 cells are not a good model.

***N. gonorrhoeae* induces cytokine production in MDM, U937 and THP-1 cells**

Upon recognition of invading microorganisms, macrophages secrete inflammatory mediators including cytokines and chemokines to activate immune defenses that are designed to limit or clear infection. However, as host-adapted organisms it is unclear whether immune responses are effective in limiting infection. To determine whether infected macrophages could be a significant source of cytokines and chemokines during gonococcal infection, primary macrophages and differentiated THP-1 and U937 cells were challenged with *N. gonorrhoeae* at an MOI of 35. The cell-free supernatants were collected 10h post-infection and the induction of various inflammatory cytokines in the supernatants was assessed by ELISA (Figure 8). Uninfected U937 cells showed higher levels of many of the measured cytokines than THP-1 cells or MDM. None of the cells produced significant amounts of IL2, IL-4, IL-17A and INF- γ in response to *N. gonorrhoeae* infection. However, *N. gonorrhoeae* infection induced MDM, THP-1 and U937 cells to secrete IL-1 α , IL-1 β , IL-10, GM-CSF, and high level of IL-6, IL-8 and TNF- α . The primary macrophages and THP-1 were induced to secrete higher levels of cytokines/chemokines than U937 cells. *N. gonorrhoeae* infection resulted in the induction of pro-inflammatory cytokines IL1- β , IL-6 and TNF- α from all of the macrophages, and the anti-inflammatory cytokine IL-10 was also induced, but only in large amounts from MDM. IL-12 was only detected from challenged primary MDM. In summary, upon *N. gonorrhoeae* infection, human macrophages are able to upregulate production of the immunoregulatory cytokines IL-1, IL-6, IL-8, IL-10 and TNF- α . These data indicate that when macrophages interact with *N. gonorrhoeae* during colonization, the induced cytokines may stimulate both inflammation and immune suppression.

Discussion

N. gonorrhoeae recruits large number of PMNs during symptomatic infection of both men and women and a proportion of intracellular gonococci survives and multiplies within these cells (Simons *et al.*, 2005). What is less well understood is the results of interaction of *N. gonorrhoeae* with resident or recruited macrophages and whether macrophages could help recruit the large numbers of PMNs to the genital track. The presence of macrophages in genitourinary mucosae and in gonococcal exudates suggests that macrophages could have an important role in gonococcal pathogenesis (Givan *et al.*, 1997, Hook, 1999). Using two standard monocytic cell lines, differentiated into macrophages, and primary human macrophages; we confirmed that *N. gonorrhoeae* are phagocytosed without opsonization and are able to survive and grow in association with macrophages. It was previously described that *N. gonorrhoeae* survives exposure to macrophages but whether survival occurred intracellularly or extracellularly was not established in those studies (Leuzzi *et al.*, 2005, Zughailer *et al.*, 2014). We conclusively show that although many bacteria are killed by macrophages after phagocytosis, a subset of the internalized bacteria are able to survive and replicate.

Macrophages eliminate engulfed microorganisms by delivering them into the lysosomal system. Bacteria not adapted for an intracellular life cycle are effectively digested within the phagolysosome, an acidic environment that contains various activated hydrolytic enzymes, whereas some bacteria are able to survive by escaping into the cytoplasm to avoid lysosomal digestion, or other bacteria inhabit vesicles that do not fuse with lysosomes. Since the subset of *N. gonorrhoeae* that survives with macrophages does not co-localize with the acidic compartment or LAMP-1, it is possible that *N. gonorrhoeae* arrests phagosome maturation to survive inside macrophages. This is consistent with the idea that the Neisserial porin (PorB) inhibits phagosome maturation in human macrophages (Mosleh *et al.*, 1998). This alteration in cellular trafficking may also be related to the ability of *N. gonorrhoeae* containing phagosomes to delay fusion with primary granules inside PMNs (Johnson *et al.*, 2013). The *Neisseria* IgA1 protease cleaves LAMP-1 and promotes survival of bacteria within epithelial cells (Lin *et al.*, 1997), but does not seem to play a role in the survival of *N. gonorrhoeae* macrophages as we did not observe any difference of survival between an *igA1* mutant and the parental strain in U937 cells. Electron microscopy images indicated the presence of *N. gonorrhoeae* in the cytoplasm as there was no observable membrane surrounding viable *N. gonorrhoeae* cells in U937 macrophages (Fig. 3A). To gain entrance to the cytoplasm the bacteria could escape from the vacuole, as has been observed in other pathogens (for a review, Smith *et al.*, 2013), or could use a separate mechanism to enter as they do in epithelial cells (for a review, Sadarangani *et al.*, 2011). In the last hypothesis where *N. gonorrhoeae* could actively invade the macrophage, actin polymerization will be required, as cytochalasin D addition in the medium before challenging the cells do not allow detection of intracellular bacteria. Free *N. gonorrhoeae* in the cytoplasm was already observed in urethral epithelial cell (Apicella *et al.*, 1996). While the mechanisms used by the subset of *N. gonorrhoeae* to avoid macrophage killing, potentially escape the phagolysosome, and survive inside macrophages will need further investigation, it was

already suggested that *N. gonorrhoeae* can subvert the iron-limiting immune defenses to facilitate iron acquisition and intracellular survival (Zughaier *et al.*, 2014).

Many studies have shown both pro- and antiapoptotic effects of both *N. gonorrhoeae* and *Neisseria meningitidis* on epithelial cells (Muller *et al.*, 1999, Muller *et al.*, 2000, Binnicker *et al.*, 2003, Kepp *et al.*, 2007, Howie *et al.*, 2008, Follows *et al.*, 2009, Kepp *et al.*, 2009, Chen *et al.*, 2011) and lymphocytes (Massari *et al.*, 2003, Deghmane *et al.*, 2009). Moreover, *N. meningitidis* has been reported to prevent macrophage apoptosis in U937 macrophages and MDM (Tunbridge *et al.*, 2006), but also has been reported to induce apoptosis in RAW 264.7 cells and THP-1 macrophages (Sjolinder *et al.*, 2012). Our results suggest that these contradictory results could all be correct and show that the effect on apoptosis is not strain or species specific but is most likely due to differences between host cells. While many of the previous studies in different laboratories used the same cell lines, it is well established that immortalized cells can change during propagation and that the characteristics or identity of the cell lines reported are not always correct (Hughes *et al.*, 2007). We found that apoptosis modulation by *N. gonorrhoeae* in U937 and THP-1 cells does not require live bacteria nor internalization, suggesting gonococcal surface components are sufficient for apoptosis modulation. The induction of the extrinsic apoptosis pathway in THP-1 but not in U937 macrophages by live and killed *N. gonorrhoeae* suggests there are surface receptor differences between the two cell lines.

During gonococcal infection, TNF- α induction has been observed during experimental human challenge, in vaginal secretions from infected mice, and after challenge of human fallopian tube explants (Ramsey *et al.*, 1995, Maisey *et al.*, 2003, Packiam *et al.*, 2010). Moreover *N. gonorrhoeae* has been reported to induce TNF- α secretion from epithelial cells and THP-1 monocytes *in vitro* (Christodoulides *et al.*, 2000, Zughaier *et al.*, 2014), and we confirmed TNF- α secretion from all three types of macrophages infected by *N. gonorrhoeae* (Fig. 8). While TNF- α secretion can result in accelerated macrophage apoptosis (for a review, Parameswaran *et al.*, 2010), we report that *N. gonorrhoeae* is able to inhibit apoptosis induced by TNF- α in U937 macrophages. The fact that *N. gonorrhoeae* is able to inhibit caspase 8 and Bid cleavage induced by TNF- α and loss of MMP induced by STS in U937 macrophages suggests that *N. gonorrhoeae* acts by blocking an early event in both apoptosis pathways. These mechanisms are supported by the fact that Bcl-2 family proteins have been described to play roles in the anti- and pro-apoptotic phenotypes observed during gonococcal infection in epithelial cells (Binnicker *et al.*, 2003, Binnicker *et al.*, 2004, Kepp *et al.*, 2007, Howie *et al.*, 2008, Follows *et al.*, 2009, Kepp *et al.*, 2009).

The opposite effects of *N. gonorrhoeae* on apoptosis in U937 and THP-1 macrophages led us to examine these processes in MDM. The difference between U937 and THP-1 cells in our assays may be due to the different origin and maturation stage of cells. In fact, U937 cells are of tissue origin (histocytic lymphoma), thus at a more mature stage, whereas THP-1 cells are of blood leukemic origin, at a less mature stage (Ralph *et al.*, 1976, Tsuchiya *et al.*, 1980). *N. gonorrhoeae* was able to inhibit STS induced apoptosis in MDM, like in U937 macrophages. Therefore, the U937 cell line is a more relevant model to study the modulation of apoptosis by *N. gonorrhoeae*. Since *N. gonorrhoeae* inhibits STS induced apoptosis in M2a polarized macrophages, similarly to undifferentiated MDM and U937 cells, and *N.*

gonorrhoeae induces the secretion of high level of IL-10, but low levels of IL-12 (Fig. 8), we postulate that M2 macrophages may be the more relevant phenotype within the genital tract. Escobar *et al.* had previously suggested that cytokines induced by *N. gonorrhoeae* from RAW 264.7 mouse macrophages correspond to an M2 profile (Escobar *et al.*, 2013), and had recently published that *N. gonorrhoeae* can polarize human macrophages to a M2 profile (Ortiz *et al.*, 2015). The phenotype of macrophages in the genital tract of patients will have to be investigated to determine whether there is polarization during natural gonococcal infection.

Secretion of IL-6 and TNF- α by MDM and U937 macrophages in response to *N. gonorrhoeae* or purified *N. gonorrhoeae* LOS was already described (Ellis *et al.*, 2001, Makepeace *et al.*, 2001), and in this study we showed MDM and two human macrophage cells line respond robustly to the presence of *N. gonorrhoeae* by secreting different cytokines (Fig. 8). The induction of pro-inflammatory cytokines, especially IL-8, by macrophages, in addition to epithelial cells, could contribute to the recruitment of PMN during gonococcal infection. The secretion of TNF- α and GM-CSF can also contribute to prolong the life of PMNs. The secretion of the regulatory cytokine IL-10 can have a potent T-cell suppressive function (Escobar *et al.*, 2013) and it has been previously shown that *N. gonorrhoeae* suppresses T cell function (Boulton *et al.*, 2002, Lee *et al.*, 2008, Zhu *et al.*, 2012) and may inducing a localized immune suppression (Liu *et al.*, 2014). IL-6 and TNF- α may trigger the influx of lymphocytes. This suggests that macrophages are at least in part responsible for the increased level of TNF- α and IL-6 observed in the urine of men and vaginal secretion of women infected with *N. gonorrhoeae* (Ramsey *et al.*, 1995, Hedges *et al.*, 1998). Interestingly, IL-1 β was also secreted by macrophages in response to *N. gonorrhoeae* infection, suggesting that the inflammasome would be activated. Inflammasome activation by *N. gonorrhoeae* was already described in monocytes (Zhou *et al.*, 2014). Gonococcal stimulation of macrophages could have a profound influence on the continued expression of a pro-inflammatory, often damaging, response during natural infection.

Our results suggest that macrophages could be an unexplored cellular reservoir for *N. gonorrhoeae* during infection. In fact, macrophages may serve as a significant replicative niche for a subset of *N. gonorrhoeae* bacteria which are able to resist phagocytic killing and the inhibition of apoptosis in MDM may play an important role in facilitating *N. gonorrhoeae* replication and contribute to gonorrhea pathogenesis. Determining the mechanisms used by *N. gonorrhoeae* to differentially manipulate macrophage apoptosis and the other relevant host cells will be important to understanding the pathogenesis of this disease.

Experimental procedures

Bacterial strains and culture conditions

N. gonorrhoeae isolate FA1090 piliated and Opa positive was used in this study and was cultured on gonococcal medium base agar (GCB, Difco) plus Kellogg's supplements (Kellogg *et al.*, 1963). The strain was typically grown at 37°C and 5% CO₂ for approximately 15 hours. Prior to cell infections, bacteria were suspended in gonococcal liquid medium containing Kellogg's supplements and 0.042% Na₂HCO₃ at an OD₅₅₀ of

0.16, and grown at 37°C to mid-logarithmic phase. A target number of CFUs was used for each experiment but due to the variability of *N. gonorrhoeae* growth, for each experiment the measured CFU are reported. For some experiments FA1090 strain harboring a plasmid encoding a green fluorescent protein, pCmGFP, was used (Srikhanta *et al.*, 2009). For experiments with killed bacteria, *N. gonorrhoeae* was heat-killed at 65°C or treated with 4% paraformaldehyde for 30 minutes.

Cell culture and differentiation

The human monocytic cell lines U937 and THP-1 were obtained from ATCC and maintained at 2.5×10^5 in RPMI 1640 medium supplemented with 10% FBS at 37°C and 5% CO₂. U937 and THP-1 cells at 1.10^6 cells/ml were differentiated to macrophages using 80 nM phorbol 1-myristate 13-acetate (PMA, Sigma-Aldrich) for 2 days in 24 well (1ml/well) or 48 well (0.5ml/well) tissue culture plates, and when necessary on coverslips for microscopy.

Human peripheral blood mononuclear cells (PBMC) were isolated by Ficoll Paque (GE healthcare) density centrifugation from human buffy coats provided by the National Red Cross (PA). Monocytes were negatively isolated from freshly isolated PBMC using Dynabeads untouched human monocytes (Life technologies). Cells at 1.1×10^6 /ml were plated in RPMI, after 1h non-adherent cells were removed, and adherent cells were cultured in RPMI with 20% heat-inactivated FBS (Atlanta) and 10ng/ml M-CSF (Peprotech) during 7 days to allow differentiation of monocytes into monocyte-derived macrophages (MDM), medium was exchanged at 3 days intervals. For macrophage polarization experiments, these MDM were then stimulated for 24h with either 2ng/ml TNF- α and 20ng/ml INF- γ or 20ng/ml IL-4 to obtain M1 or M2a phenotypes, respectively (Peprotech). Flow cytometry on surface markers CD80 and CD163 was performed to confirm macrophages polarization in M1 or M2a phenotypes, respectively.

Bacterial survival assays

Host cells (5×10^5 cells/well in 48 well plates) were challenged with FA1090 in RPMI + 10% FBS or PBSG (Chen *et al.*, 2011) medium and incubated at 37°C in the presence of 5% CO₂ after centrifugation for 4 min at 1500 rpm. At different times after infection (as indicated in each Figure legend), the number of bacteria in the supernatant and cells lysed by 1% saponin was determined by serial dilution and plating. In some assay, 1.5 h after infection, non-adherent bacteria were removed by washing three times with PBS, and cells were incubated at 37°C in 5%CO₂ in fresh medium containing gentamicin 200 μ g/ml (time 0). After 1h, gentamicin was maintained or cells were washed 3 times with PBS and incubated in fresh medium without gentamicin. To study the influence of acidification on bacterial survival, bafilomycin A1 0.5 μ M (Sigma) was added in the macrophage well 1h before infection and maintained during each experiment.

Immunofluorescence microscopy

Host cells (1×10^6 cells/well in 24 well plates, on coverslips) were challenged with FA1090 at 37°C in the presence of 5% CO₂. At different times after infection (as indicated in each Figure legend), the cells were washed in PBS and fixed with 4% paraformaldehyde in PBS.

In some experiments, one hour after infection cells were washed in PBS and fresh medium with 10 µg/ml cytochalasin D (sigma) was added. When necessary, 100 nM of LysoTracker red (life technologies) were added in the medium 15 min before fixation. After fixation, extracellular bacteria were detected with a polyclonal anti-*N. gonorrhoeae* antibody (Bioss), and stained with an Alexa Fluor 647 or 350-conjugated goat anti-rabbit secondary antibody (Jackson and Molecular Probes). Cells were permeabilized with 0.2% saponin, and intracellular bacteria were detected by incubation with the same anti-*N. gonorrhoeae* antibody and stained with a fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody, except when GFP expressing bacteria were used. LAMP-1 was detected after permeabilization of the cells with 0.2% saponin using a rabbit-LAMP-1 antibody (ab24170, Abcam) and Alexa Fluor 350-conjugated goat anti-rabbit secondary antibody. The coverslips were mounted on slides with ProLong gold antifade reagent with or without DAPI staining (Life technologies), and the cells were visualized with a Nikon Eclipse 90i microscope. The number of intracellular bacteria and the percentage of bacteria that colocalized with LysoTracker was calculated by examination of at least 100 macrophages.

Lactate dehydrogenase release assay

Primary human macrophages, U937 and THP-1 differentiated cells (2×10^5 cells/well in 96-well plates) were challenged with FA1090 and centrifuged at 1500 rpm for 4 min to synchronize the infection. At different times after infection, supernatants of the infected macrophages were collected and assayed for lactate dehydrogenase activity (LDH) using the CytoTox 96 kit (Promega) according to the manufacturer's instructions. The percent cell death was calculated as: $100 \times (\text{experimental release} - \text{spontaneous release}) / (\text{total release} - \text{spontaneous release})$, where spontaneous release is the amount of LDH activity in supernatants of cells incubated in medium alone, and total release is the activity from macrophages lysed with 1% Triton X-100. A control with medium alone (without cells) was also performed.

DNA fragmentation assay

U937 and THP-1 differentiated cells (1×10^6 cells/well in 24-well plates) were challenged with FA1090 at an MOI of 22 to 107 and the plates were centrifuged at 1500 rpm for 4 min. After 13h of infection, the cells were harvested by trypsinisation, washed with PBS, incubated in ice-cold 70% ethanol for 10 min at 4°C, washed twice more with PBS, and stained with 0.1 ml PBS containing 50 µg/ml propidium iodide (Sigma) and 0.5 mg/ml RNase A at room temperature for 30 min in the dark. Genomic DNA content was then assessed by flow cytometry (BD LSRII, BD Biosciences).

Caspase-3 assay

Differentiated U937 cells, THP-1 cells or primary human macrophages were challenged with FA1090 at various MOIs and the plates were centrifuged at 1500 rpm for 4 min. After 3h of infection, the cells were treated with dimethyl sulfoxide (DMSO) or 1 µM STS (Sigma) in DMSO, and in some cases with 20 µM z-VAD-fmk (BD pharmingen) a general caspase inhibitor, for a further 3h for U937 and THP-1 cells and 6h for MDM. When cytochalasin D was used, 5 µg/ml of cytochalasin D was added to cells 1h before infection and cells were lysed with 70 µl cell lysis buffer (BD Pharmingen). The cells lysates were

stored at -80°C before being used for caspase-3 assays. Caspase-3 activity was measured by incubating 25 μl cell lysate with 5 μl reconstituted caspase-3 substrate at 1 mg/ml (Ac-DEVD-AMC, BD Pharmingen) and with 200 μl HEPES buffer (20 mM HEPES (pH 7.5), 10% glycerol, 2 mM dithiothreitol) for 1h at 37°C . 7-amino-4-methylcoumarin fluorescence was then measured using an excitation wavelength of 380 nm and an emission wavelength of 440 nm, using a plate reader (SpectraMax M5, Molecular Devices). Fluorescence levels were normalized to total cellular protein level measured by bicinchoninic acid (BCA) protein assay (Pierce).

Mitochondrial Membrane Potential assay

Differentiated U937 and THP-1 cells (1×10^6 cells/well in 24-well plates) were challenged with FA1090 at MOIs from 22 to 107 and plates were centrifuged at 1500 rpm for 4 min. After 3h of infection the cells were treated with DMSO or 1 μM STS (Sigma) for an additional 13h. Cells were then harvested by trypsin treatment, washed with fresh medium, stained with 200 μM Mito PT TMRE (TMRE Mitochondrial Membrane Potential Assessment kit, ImmunoChemistry Technologies) for 15 min at 37°C and 5% CO_2 , and washed one time before staining was assessed by flow cytometry (BD LSRII, BD Biosciences).

Bid Western blotting

Differentiated U937 and THP-1 cells were challenged with FA1090 at an MOI of ~ 100 and plates were centrifuged at 1500 rpm for 4 min. After 3–4h of infection, the cells were treated with DMSO or TNF- α 10 ng/ml and cycloheximide 200ng/ml for an additional 3h. Cells were washed with PBS and lysed with RIPA buffer for 30 min at 4°C under rotation. Lysates were centrifuged at 12,000 rpm for 10 min at 4°C . The supernatants were transferred to fresh tubes and stored at -80°C . 7 to 23 μg of cellular extracts in SDS sample buffer containing β – mercaptoethanol were separated on 15% SDS-polyacrylamide gel and proteins were transferred to a polyvinylidene fluoride membrane in 10 mM CAPS (N-cyclohexo-3-amino-propanesulfonic acid) buffer with 10% methanol at pH 11 for 1h. After saturation in Tris buffered saline containing 0.1% Tween 20 and 5% dry milk, membranes were incubated overnight with anti-Bid antibody (1:1000, #2002, Cell Signaling) or anti- α - β -tubulin antibody (1:1000, 9F3, Cell Signaling). The membranes were then washed and incubated with secondary goat anti-rabbit antibody conjugated to horseradish peroxidase (1:1000, Jackson ImmunoResearch) for 1h. After washing, blots were developed with ECL Plus Western Blotting detection reagent (Amersham, GE Healthcare) and visualized using a ChemiDox XXRS molecular imager (Bio-Rad).

ELISA for Cytokine Production

Differentiated U937, THP-1 cells and primary human macrophages were either left uninfected or infected with FA1090 strain at an MOI of 35, the plates were centrifuged at 1500 rpm for 4 min and infection were carried out at 37°C with 5% CO_2 . After 10 h of infection the supernatant were harvested, centrifuged and the resulting supernatants were stored at -80°C before being used. The culture supernatants were examined for 12 cytokines using the Multi-Analyte ELISArray kit for detection of human inflammatory cytokines (Qiagen). IL1-A, IL2, IL4, IL10, IL12, IL17, INF- γ , and GM-CSF were read undiluted.

IL1B and IL6 using a 1:10 dilution, and IL8 and TNF- α with a 1:100 dilution. The analyses were performed according to the manufacturer's instructions.

Thin-section transmission electron microscopy

U937 cells (7×10^5 cells/well on coverslip) were challenged with FA1090 at a MOI of 19. In some wells, bafilomycin A1 0.5 μ M (Sigma) was added 1h before infection and maintained during the experiment. 1.5h after infection, non-adherent bacteria were removed by washing three times with PBS, and cells were incubated at 37°C in 5% CO₂ in fresh medium containing 200 μ g/ml gentamicin for 1h before new washing and incubation with fresh medium without gentamicin. At 1.5h, 3h and 6h after infection, the cells were washed and fixed at 4°C in 0.1 M sodium cacodylate buffer (pH 7.3) containing 2% paraformaldehyde and 2.5% glutaraldehyde. Samples were treated with 2% osmium tetroxide in 0.1M sodium cacodylate buffer, rinsed with distilled water, dehydrated in ascending grades of ethanol, embedded in resin mixture of Embed 812 kit, and cured in a 60°C oven. Sample blocks were thin sectioned on a Leica Ultracut UC6 ultramicrotome with 70nm sections collected on 200 mesh Cu grids and then were stained with 3% uranyl acetate and Reynolds lead citrate. Images were captured on a FEI Tecnai Spirit G2 120-kV transmission electron microscope (TEM). The localization of bacteria inside the macrophage was calculated by examination of at least 70 macrophages.

Acknowledgments

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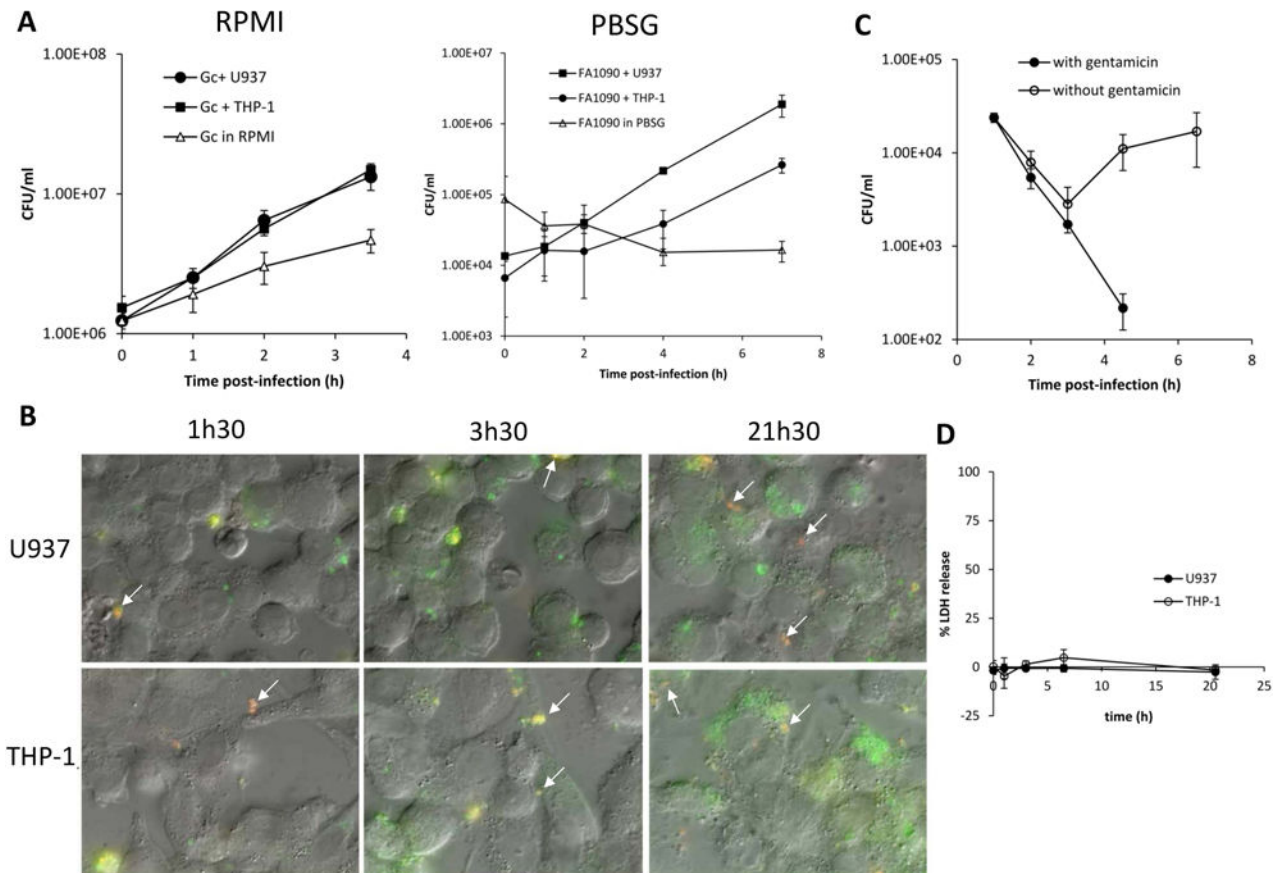


Figure 1. *N. gonorrhoeae* is able to survive and replicate in association with U937 and THP-1 macrophages

(A) *N. gonorrhoeae* survives and replicates in association with U937 and THP-1 macrophages. Differentiated U937 and THP-1 cells were challenged with FA1090 at an MOI of 1 and viable CFU/ml was enumerated at various times post-infection. Data are represented as the mean \pm SD for 3 independent replicates and are representative of 3 (RPMI) and 2 (PBSG) independent experiments. (B) *N. gonorrhoeae* associated with U937 and THP-1 cells are both intra- and extracellular. Differentiated U937 and THP-1 cells were challenged at an MOI of 1 and intra- and extracellular bacteria were differentiated using fluorescent microscopy. Extracellular bacteria appear red and yellow (some are indicated with arrows), and intracellular bacteria appear green. (C) *N. gonorrhoeae* survival in U937 cells. Differentiated U937 cells were challenged for 1.5h with FA1090 at an MOI of 23. After washing, challenged cells were incubated in fresh medium with gentamicin 200 μ g/ml for the entire duration of the experiment (with gentamicin, black circle) or 1h before new washing and incubation with fresh medium without gentamicin (open circle). Viable CFU/ml were enumerated at various times post-infection, and the data are represented as the means \pm SD for three independent replicates and are representative of two independent experiments. (D) *N. gonorrhoeae* does not induce LDH release in U937 and THP-1 macrophages. U937 and THP-1 differentiated cell were challenged with FA1090 at a MOI of two and host cell lysis was assessed by measuring release of cytosolic LDH into the

supernatant at different times post infection. Data shown are mean \pm SD calculated from three replicates and are representative of three independent experiments.

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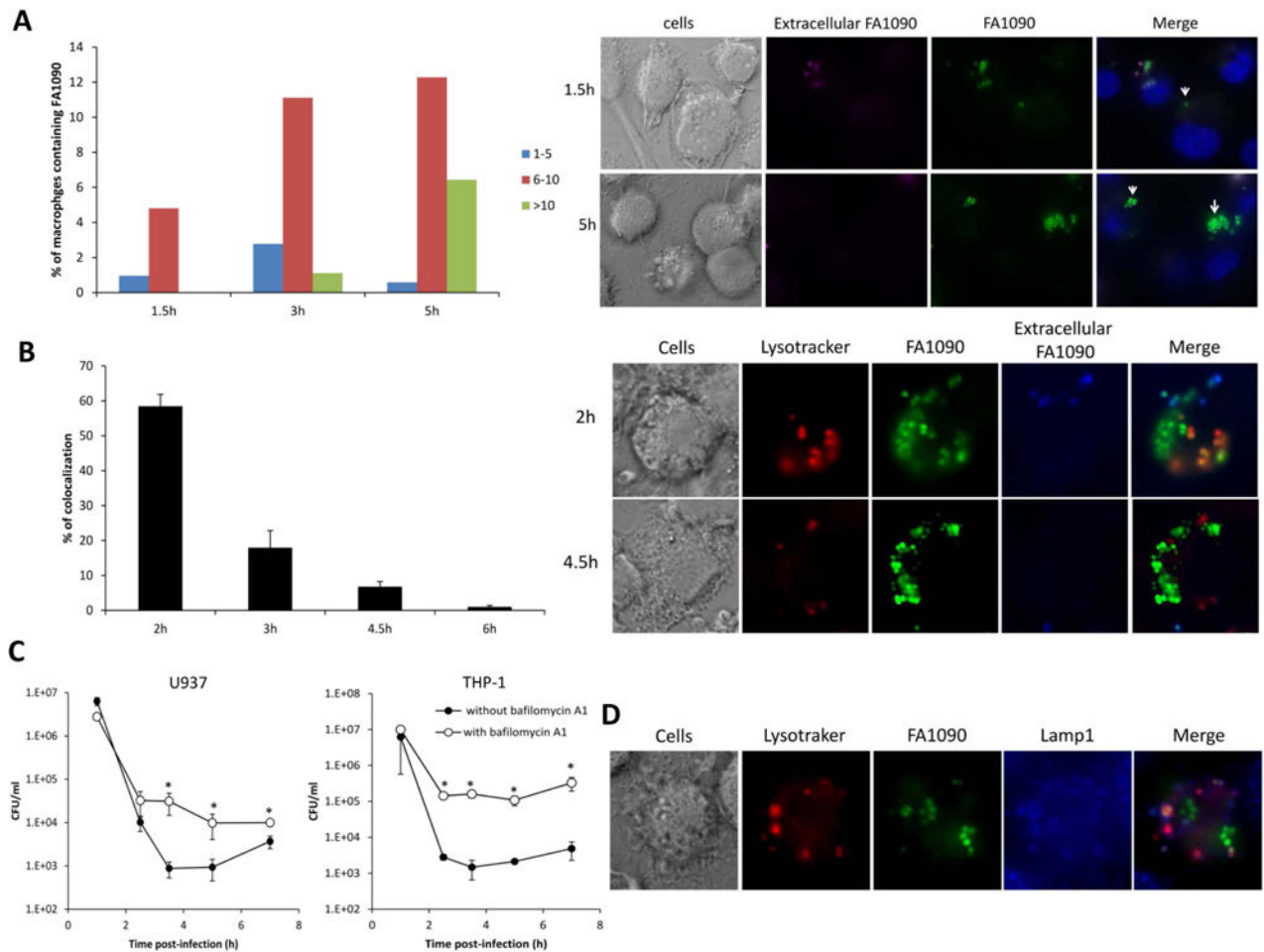


Figure 2. A portion of *N. gonorrhoeae* is able to survive and replicate inside macrophages in nonacidic compartment

(A) Quantification of bacterial loads in U937 macrophages over time. U937 macrophages incubated with GFP-expressing FA1090 (MOI 5) for 1.5h were washed and further incubated with cytochalasin D to block new phagocytosis of extracellular bacteria. At 1.5h, 3 and 5 h after infection, cells were fixed, extracellular bacteria stained with Alexa Fluor 647-coupled soybean agglutinin, and the number of bacteria per macrophage was analyzed by fluorescence microscopy. Data shown on the left panel are representative of 3 independent experiments. Representative images at time 1.5h and 5h after infection are shown on the right panel, extracellular bacteria appear green and purple, and intracellular bacteria appear green. (B) Time course of *N. gonorrhoeae* colocalization with the acidotropic dye Lysotracker. GFP-expressing FA1090 were incubated with U937 macrophages (MOI 5 to 15) for 1h. After being washed, challenged macrophages were incubated with cytochalasin D before Lysotracker staining at 2h, 3h, 4.5h and 6h. Data shown on the left panel are means \pm SD calculated from 3 independent experiments. Representative images at time 2h and 4.5h after infection are shown on the right panel, cells are stained with Lysotracker red, extracellular bacteria appear green and blue, and intracellular bacteria appear green. (C) *N. gonorrhoeae* survival in U937 and THP-1 macrophages in presence of bafilomycin A1. Differentiated U937 and THP-1 cells,

pretreated with bafilomycin A1 or not, incubated with FA1090 (MOI 20) for 1h were washed and further incubated with cytochalasin D and gentamicin for 1.5h. After those 1.5h, the cells were washed and incubated in fresh medium with cytochalasin D. Viable CFU/ml were enumerated at that times shown. Data shown are means \pm SD calculated from three replicates and are representative of three independent experiments. Values that are significantly different ($P < 0.05$) as determined by the Two-tailed, unpaired Student *t* tests are indicated with an asterisk. (D) Characterization of *N. gonorrhoeae* containing phagosome. Differentiated U937 cells incubated with GFP-expressing FA1090 (MOI 38) for 1h were washed and further incubated with cyochalasin D before Lysotraker red and LAMP-1 (blue) staining 4h after infection.

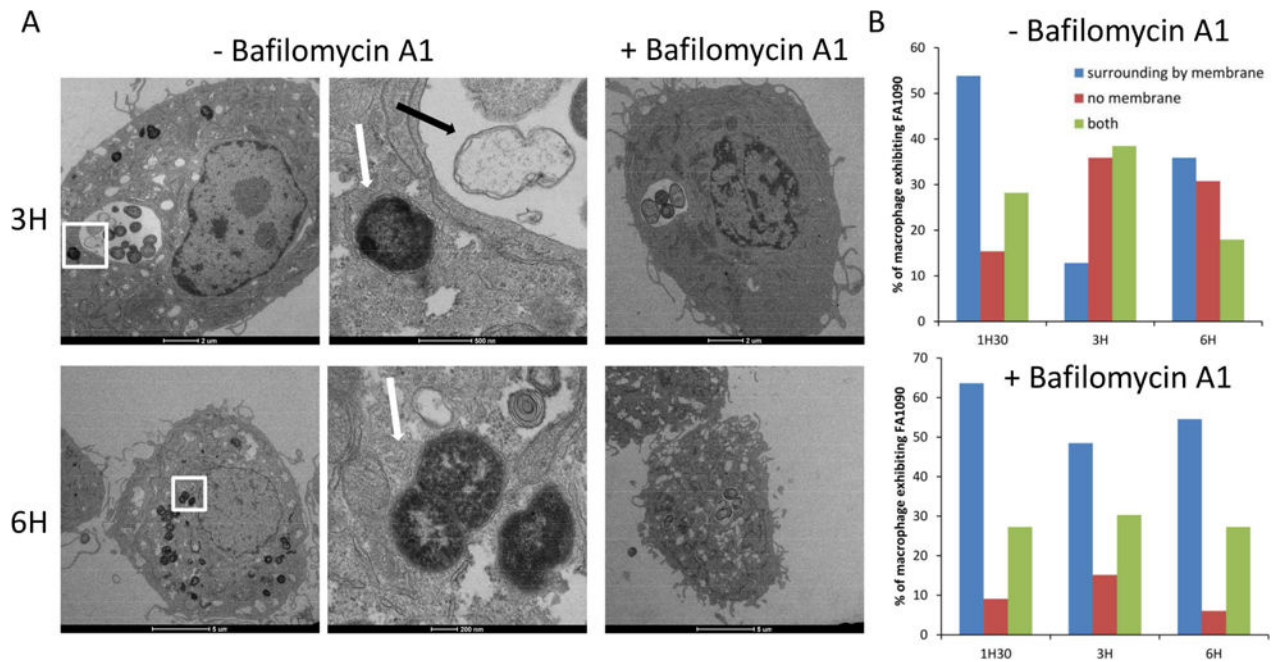


Figure 3. Localization of *N gonorrhoeae* within macrophage cell line

Differentiated U937 cells, were challenged with FA1090 (MOI 19) for 1h30, after washing, challenged cells were incubated in fresh medium with gentamicin 200 µg/ml for 1h before new washing and incubation with fresh medium without gentamicin. At 1.5h, 3h and 6h after infection, cells were fixed and processed for thin section TEM. (A) Left panels show cells without Bafilomycin A1 treatment and the right panels show cells treated to inhibit acidification of the phagolysosome, at 3 and 6h after challenging. The second column of micrograph corresponds of a magnified view of the boxed region of the first micrograph, showing the absence of membrane surrounding the bacteria. Black arrow indicates non-viable intracellular bacteria, while white arrows denote intracellular bacteria that retain electron density, suggesting that they have retained viability. (B) Quantification of U937 macrophages containing FA1090 surrounding by membrane or not, over the time. The localization of bacteria per macrophage was analyzed by TEM.

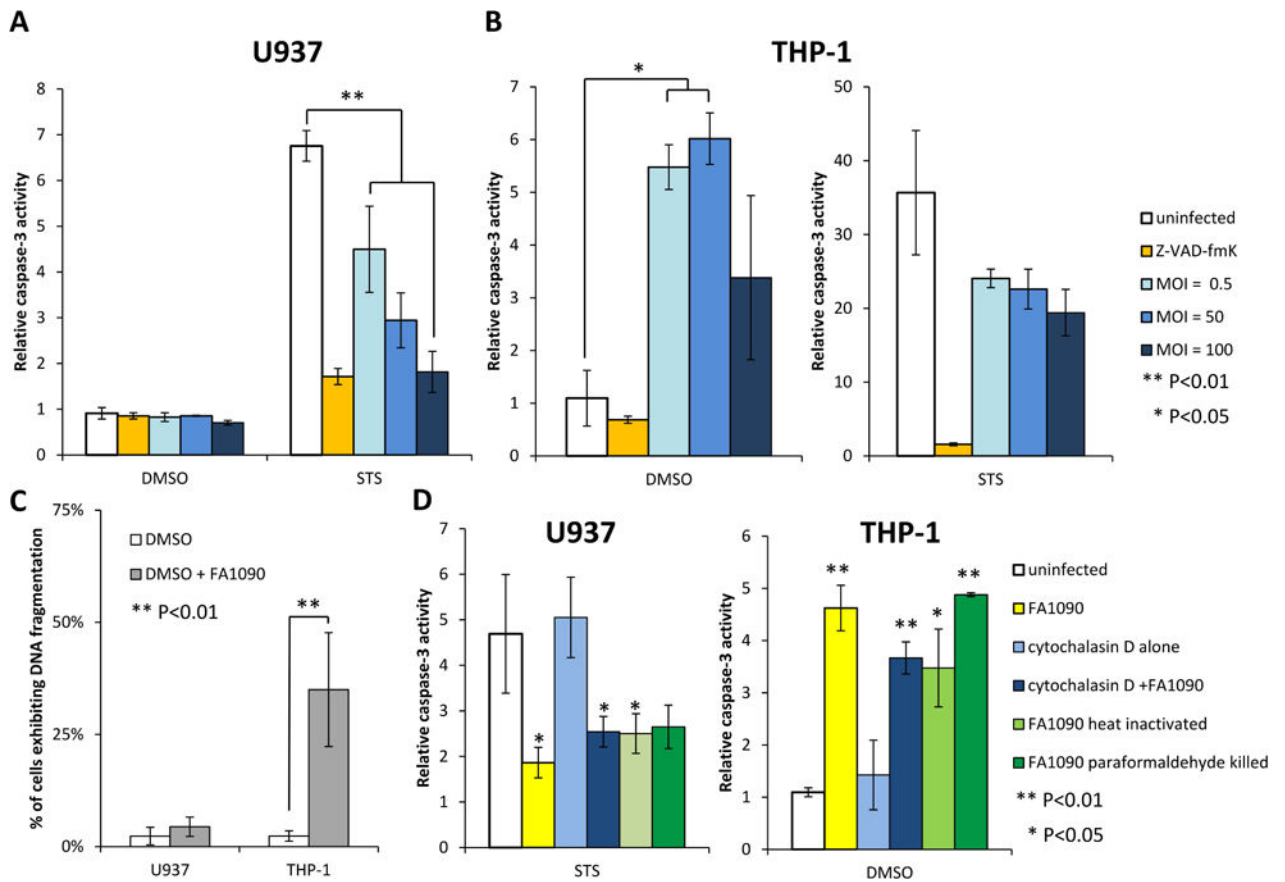


Figure 4. *N. gonorrhoeae* inhibits apoptosis induced by STS in U937 cells and induces apoptosis in THP-1 cells

(A) *N. gonorrhoeae* inhibits caspase-3 activity induced by STS in U937 cells. (B) *N. gonorrhoeae* induces caspase-3 activity in THP-1 cells. (A, B) Differentiated U937 or THP-1 cells were challenged with FA1090 at different MOI as indicated and then treated with STS or DMSO. Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AMC and data are presented as the caspase-3 activation relative to that for uninfected DMSO-treated controls. Data shown are mean \pm SD calculated from three replicates and are representative of three independent experiments. (C) *N. gonorrhoeae* induces DNA fragmentation in THP-1 cells, not in U937 cells. Differentiated U937 and THP-1 cells were challenged or not with FA1090 during 13h, hypodiploid DNA content was then evaluated by flow cytometry and the average percentage of cells exhibiting DNA fragmentation is indicated. The data are averages \pm SD for 5 independent experiments. (D) Neither internalization nor viable *N. gonorrhoeae* are required to modulate caspase-3 activity. Differentiated U937 and THP-1 cells \pm cytochalasin D treated were challenged with live, heat-killed or paraformaldehyde-killed FA1090 at MOI 5 and then treated with STS or DMSO, and caspase 3 activity was measured as described in (A,B). Data shown are mean \pm SD calculated from three replicates and are representative of three independent experiments. Values that are significantly different ($P < 0.05$) and ($P < 0.01$) as determined by the Two-tailed, unpaired Student *t* tests are indicated with one or two asterisks, respectively.

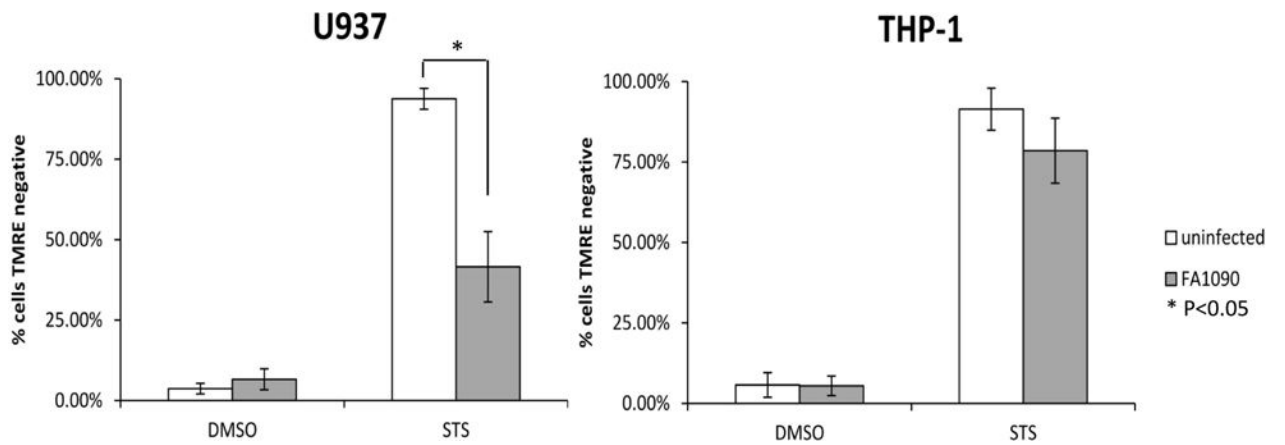


Figure 5. *N. gonorrhoeae* inhibits loss of mitochondrial membrane potential (MMP) induced by STS in U937 cells, not in THP-1 cells

Differentiated U937 and THP-1 cells were challenged or not with FA1090 and then treated with STS or DMSO, the loss of MMP was assessed using the lipophilic cationic dye TMRE and evaluated by flow cytometry and the average percentage of cells TMRE negative is indicated. The data are averages \pm SD for three independent experiments. Values that are significantly different ($P < 0.05$) as determined by the Two-tailed, unpaired Student *t* tests are indicated with an asterisk.

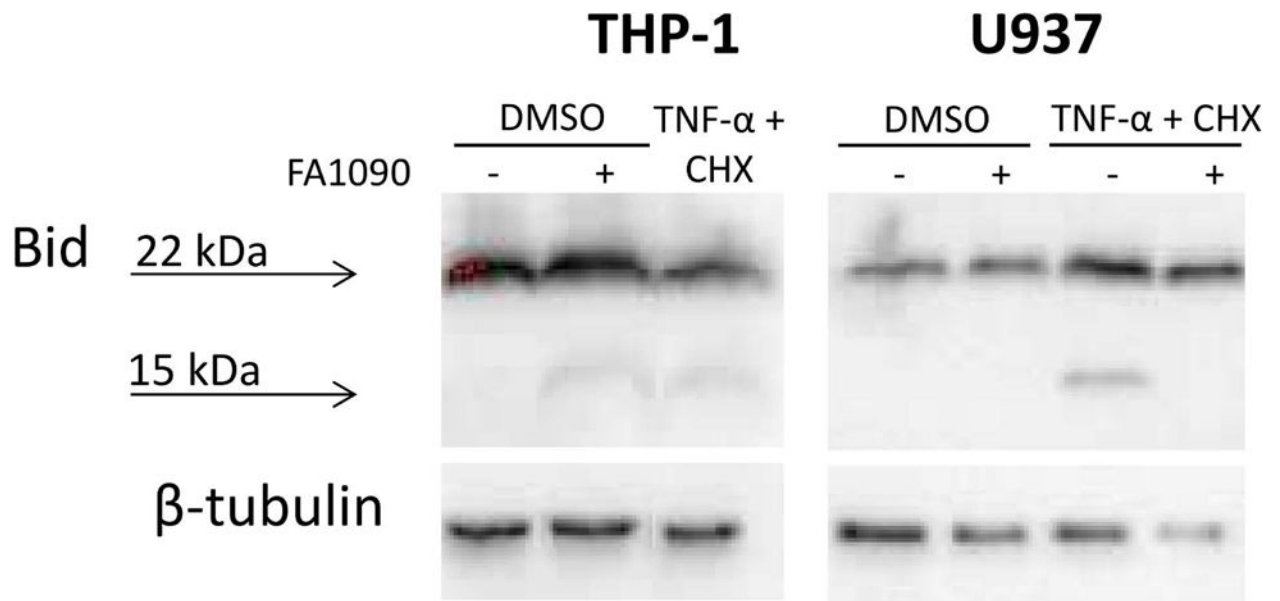


Figure 6. *N. gonorrhoeae* induces Bid cleavage in THP-1 cells and inhibits Bid cleavage induced by TNF- α in U937 cells

Differentiated U937 and THP-1 cells were challenged or not with FA1090 and then treated with DMSO or TNF- α + CHX. Lysates were harvested and subjected to Western blotting using a Bid antibody. The 22 kDa and 15 kDa products correspond to the full length and cleaved Bid, respectively. β -tubulin was used as a loading control. Data are representative of two independent experiments.

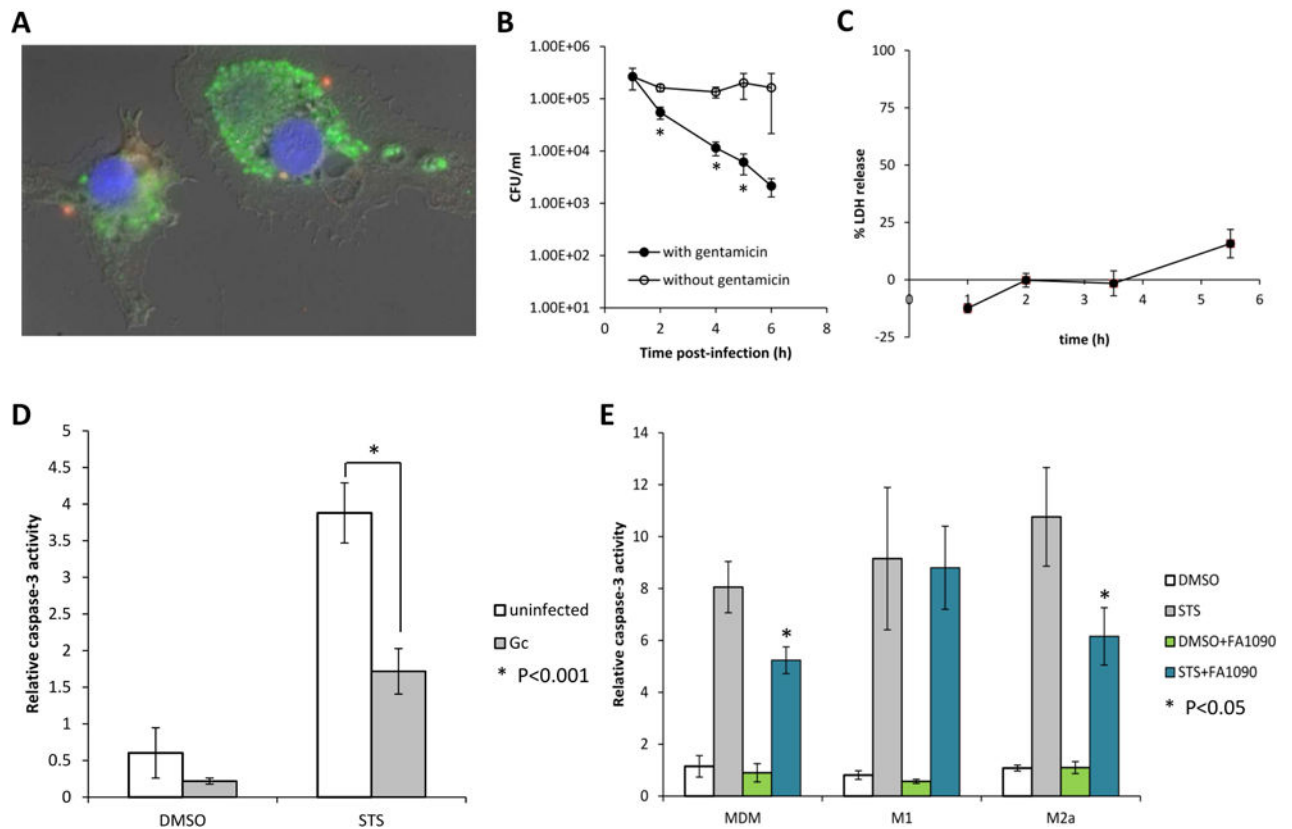


Figure 7. *N. gonorrhoeae* is able to survive and replicate in association with primary human macrophages (MDM) and inhibits STS induced caspase 3 activity

(A) *N. gonorrhoeae* associated with MDM are both intra- and extracellular. MDM were challenged at an MOI of two for 1.5h, after which the cells were fixed and intra- and extracellular bacteria were differentiated: extracellular bacteria appear red and yellow, and intracellular bacteria appear green. Data are representative of three experiments with MDM from three different donors. (B) MDM were challenged for 1.5h with FA1090 at an MOI of 11. After washing, challenged cells were incubated in fresh medium with gentamicin 200 $\mu\text{g}/\text{ml}$ for the entire duration of the experiment (with gentamicin, black circles) or 1h before new washing and incubation with fresh medium without gentamicin (open circles). Viable CFU/ml were enumerated at various times post-infection, and the data are represented as the means \pm SD for three independent replicates and are representative of two independent experiments with two different donors. Values that are significantly different ($P < 0.05$) as determined by the Two-tailed, unpaired Student *t* tests are indicated with an asterisk. (C) *N. gonorrhoeae* does not induce LDH release in MDM. MDM were challenged with FA1090 at a MOI of 15, host cell lysis was assessed by measuring release of cytosolic LDH into the supernatant at different times postinfection. Data shown are means \pm SD calculated from three replicates and are representative of two independent experiments with two different donors. (D) *N. gonorrhoeae* inhibits STS induced caspase-3 activity in MDM. MDM were challenged with FA1090 at a MOI of 38 and then treated with STS or DMSO. Caspase-3 activity was measured using the fluorogenic substrate Ac-DEVD-AMC and data are presented as the caspase-3 activation relative to that for uninfected DMSO-treated controls. Data shown are means \pm SD calculated from three replicates and are representative of six

independent experiments with six different donors. (E) *N. gonorrhoeae* inhibits STS induced caspase-3 activity in MDM polarized in M2a but not in M1 subtype. MDM and MDM polarized in M1 or M2a subtype were challenged with FA1090 at a MOI of 74 and then treated with STS or DMSO and caspase 3 activity was measured. Data shown are means \pm SD calculated from three replicates and are representative of three independent experiments with three different donors. Values that are significantly different ($P < 0.05$) from the value for the uninfected sample as determined by the Two-tailed, unpaired Student *t* tests are indicated with an asterisk.

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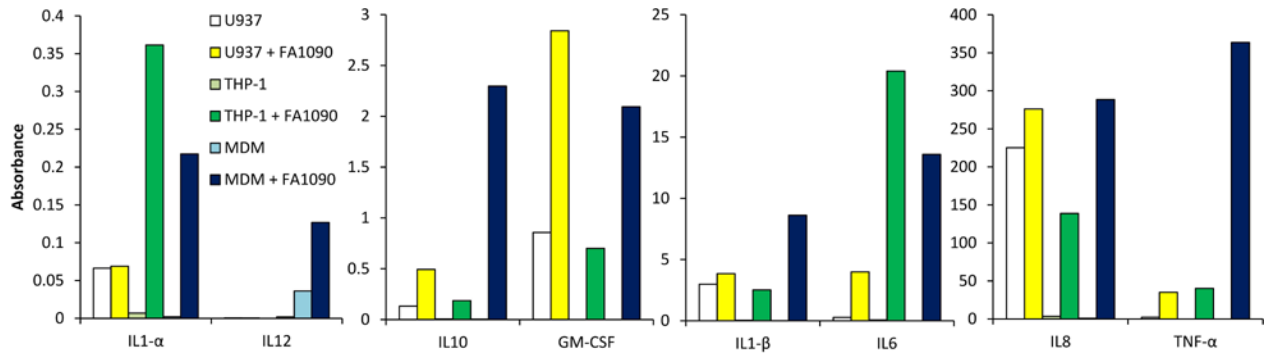


Figure 8. *N. gonorrhoeae* induces cytokine and chemokine production in macrophages

The levels of cytokines and chemokines in U937, THP-1 cells and MDM supernatants were determined by ELISA using the Multi-Analyte ELISArray kit (Qiagen). Data are the corrected absorbance at 450 nm minus 570 nm (correcting for optical background) and the dilution factor.