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AVOIDANCE OF MECHANISMS OF INNATE IMMUNE RESPONSE BY *NEISSERIA GONORRHOEAE*

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Abstract: *Neisseria gonorrhoeae* (gonococcus) is a Gram-negative bacteria and an etiological agent of the sexually transmitted disease – gonorrhea. *N. gonorrhoeae* possesses many mechanism to evade the innate immune response of the human host. Most are related to serum resistance and avoidance of complement killing. However the clinical symptoms of gonorrhea are correlated with a significant presence of neutrophils, whose response is also insufficient and modulated by gonococci.

UNIKANIE MECHANIZMÓW WRODZONEJ ODPOWIEDZI IMMUNOLOGICZNEJ PRZEZ *NEISSERIA GONORRHOEAE*

Streszczenie: *Neisseria gonorrhoeae* (gonokok) to Gram-ujemna dwoinka będąca czynnikiem etiologicznym choroby przenoszonej drogą płciową – rzeżączki. *N. gonorrhoeae* posiada liczne mechanizmy umożliwiające jej unikanie wrodzonej odpowiedzi immunologicznej gospodarza. Większość z nich związana jest ze zdolnością gonokoków do manipulowania układem dopełniacza gospodarza oraz odpornością tej bakterii na surowicę. Jednakże symptomy infekcji *N. gonorrhoeae* wynikają między innymi z obecności licznych neutrofili, których aktywność jest modulowana przez gonokoki.

Key words: bacterial pathogenesis, inflammation, innate immune response
Słowa kluczowe: bakteryjna patogeneza, stan zapalny, wrodzona odpowiedź immunologiczna

1. Introduction

Nowadays, sexually transmitted infections (STIs) still remain a major global health problem with about 1 million of new cases of chlamydia, gonorrhea, trichomoniasis or syphilis daily [55]. However, in 2017 the World Health Organization qualified *Neisseria gonorrhoeae*, as the only etiological agent of STIs, among the "priority pathogens" that pose the greatest threat to human health because of antibiotic resistance [54]. In 2012, an estimated 78 million new cases of gonorrhea caused by an obligatory human pathogen *N. gonorrhoeae* (gonococcus), occurred among 15–45 year-old patients worldwide [16, 55]. Gonococci infect a diverse array of human mucosal surfaces, although the most common place of infection is the genitourinary tract [17]. Infection with *N. gonorrhoeae* leads to acute urethritis in men and cervicitis in women, which in 50–80% cases is asymptomatic [10]. Untreated gonorrhea may result in serious complications in women such as pelvic inflammatory disease (PID), ectopic pregnancy and infertility [16].

The crucial challenge related to gonorrhea treatment is increased multi-drug resistance of *N. gonorrhoeae* [54]. What is more, there are problems with constructing an effective vaccine due to the highly antigenically variable surface of these bacteria and lack of appropriate animal model of this disease, with the exception of chimpanzees [20, 26]. Additionally, infection with *N. gonorrhoeae* does not induce protective immunity [12]. Hence it is crucial to understand the mechanisms underlying the ability of gonococci to avoid the first line defence in the human host, which is the innate immune response [26].

2. Adherence ability

One of the main function of the surface structures of gonococci – Opacity-associated proteins (Opa), as virulence factors, is their involvement in attachment to human cells during mucosa infection which facilitates affective colonization. Different Opa proteins can interact with different hosts receptors, for example, human

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carcinoembryonic antigen-related cell adhesion molecules (CEACAMs): CEACAM1, CEACAM3, CEACAM5 and CEACAM6 as well as heparan sulfate proteoglycans (HSPGs) or integrins [36]. CEACAM1 and CEACAM6 have several functions, for example, in cell adhesion and angiogenesis, and are expressed in different cell types. CEACAM3, on the contrary, is expressed in neutrophils only and functions primarily as a receptor for Gram-negative, human-restricted pathogens like *N. gonorrhoeae* and *Neisseria meningitidis*. Variations in the binding properties of Opa proteins are caused by differences in the sequence of their extracellular loops [2]. Interaction of *N. gonorrhoeae* with CEACAMs proteins expressed on the surface of epithelial cells facilitates bacterial adhesion to host cells and can trigger engulfment and transcytosis through epithelial cells which can lead to further dissemination of the infection [37, 43]. It is commonly known that intracellular bacteria can alter the host's immune response [49]. It has been demonstrated that *N. gonorrhoeae* induces tyrosine phosphorylation of epidermal growth factor receptor (EGFR), what is an essential step *prior* to invasion and is able to escape autophagy-mediated killing in human epithelial cells by activating the autophagy repressor mammalian target of rapamycin complex 1 (mTORC1) [27, 48]. Interestingly, a lack of Opa proteins increases transmigration across epithelial cells [45].

3. Serum resistance and complement system

The bactericidal function of human serum depends mostly on the interaction of IgM antibodies with pathogenic bacteria and the further complement-mediated killing of sensitive strains [7]. However, complement activation may occur by 3 different pathways: i) classical, due to the presence of an antigen-antibody complex; ii) alternative, related to interaction with the surface of the pathogen; iii) lectin, based on interactions between soluble pattern recognition molecules (Mannose-binding lectin (MBL), collectin-10, collectin-11 and ficolins) and microbial surface [32, 13]. Activation of these pathways leads to C3-convertase activation, which performs an essential effector function in the whole complement cascade and results in, for example, C3b deposition on the bacterial surface, which enables phagocytic cells to internalize the pathogen, inflammation to progress due to C3a and C5a activity and a Membrane Attack Complex (MAC) to form that eradicates the bacteria directly [19, 32]. However, efficient complement deposition on most pathogenic bacteria requires the initiation of complement activation via the classical pathway [4].

It is generally considered that the complement system plays a crucial role in an innate immune response against *N. gonorrhoeae*: patients with a deficiency of complement components are more susceptible to disseminated gonococcal infections (DGI) [30]. *N. gonorrhoeae* mainly activates the classical complement pathway and gonococci isolated from the female genitourinary tract are coated with complement components [18, 29]. However, gonococci possesses an arsenal of mechanisms that enable this pathogenic bacteria to evade complement-mediated killing.

The most common location of gonococcal infection is the mucosa of the female and male genitourinary tract [17]. It has been demonstrated that *N. gonorrhoeae* freshly isolated from the place of infection is mostly characterized by resistance to the normal human serum (NHS), and that this resistance is lost during the cultivation period [53]. On the basis of gonococcal resistance to NHS, *N. gonorrhoeae* in a population are divided into serum-sensitive and serum-resistance bacteria [34]. Serum-sensitive gonococci mainly cause local infections with symptoms of inflammation while the presence of serum-resistance bacteria is related to the disseminated gonococcal infection [31]. The serum-resistance phenotype is maintained by sialylation of lacto-*N*-neotetraose (LNT) of gonococcal lipooligosaccharide (LOS) by 5-cytidinemonophospho-*N*-acetylneuraminic acid (CMP-NANA) acquired by *N. gonorrhoeae* from its human host [28]. This modification decreases antibody binding and prevents complement-dependent killing of the bacteria by human sera. This phenomenon is called "unstable" serum-resistance, because the loss of LOS sialylation leads to a serum-sensitive phenotype [9]. It has been demonstrated that the resistance to NHS featured by gonococci with sialylated LOS is related to the ability of this bacteria to bind to factor H (fH) (Figure 1) [41]. Factor H is a complement regulatory protein, which inhibits the complement alternative pathway by, for example, being a cofactor for the cleavage of C3b to hemolytically inactive iC3b and acceleration of C3-convertase decay [52]. However, the bactericidal potential of some domains of this protein has also been demonstrated *in vitro* [40]. Another analysis revealed that interaction of sialylated LOS with fH is influenced by porin (Por) proteins of *N. gonorrhoeae* [28]. Por1A and Por1B proteins represent up to 60% of all surface proteins of *N. gonorrhoeae* and they function as selective anion channels. These two major allelic isoforms of gonococcal porin proteins undergo an equable antigenic variation [31]. Por1A strains are more likely to cause DGI, without symptoms of local inflammation and those gonococci expressing Por1B protein are more related to local genital tract infection and PID [1]. The reason of such a variation of symptoms between these two isoforms is the difference in the ability of Por1A and Por1B expressing gonococci to bind fH: most Por1A proteins can bind to fH and Por1B proteins bind to fH weakly, unless gonococcal LOS is sialylated [41].

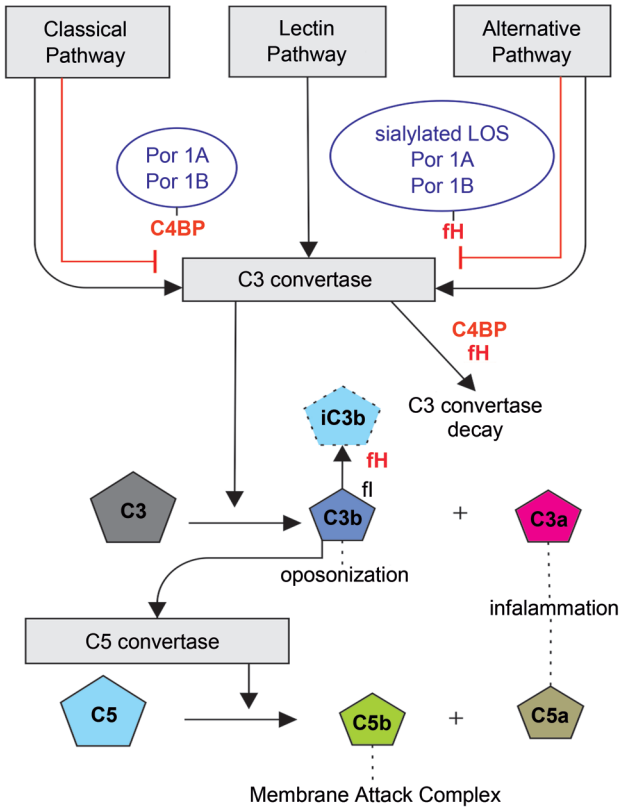

Fig. 1. Diagram of the complement system inhibition by *N. gonorrhoeae* [according to 13, 19, 32, 41, 52]. Por1A – porin 1A; Por1B – porin 1B; fH – factor H; fI – factor I, C4BP – C4b-binding protein; LOS – lipooligosaccharide.

Additionally, despite its function as an alternative pathway inhibitor, fH also enhances the adhesion and invasion of *N. gonorrhoeae* to cervical cells through Complement Receptor 3 (CR3). It has been demonstrated that Por1A-expressing gonococci invade CR3-expressing cells with greater efficiency than Por1B strains and this may be the reason why Por1A strains mostly cause disseminated disease with no signs of inflammation [1].

Another complement regulatory protein that is used by gonococci to evade complement mediated killing is C4b-binding protein (C4BP). This big serum protein can alter all three complement pathways thus inhibiting opsonization and killing by phagocytic cells, and Por1A and certain Por1B strains are able to interact with this protein. Interaction between Por proteins and C4BP protein is strongly correlated with serum resistance phenotype, as it is demonstrated for fH protein [19].

4. Neutrophils

N. gonorrhoeae, during colonization of the urogenital tract mucosa, release pathogen-associated molecular patterns (PAMPs), that activate, for example, toll-like receptor 2 (TLR2), toll-like receptor 4 (TLR4) and nucleotide-binding oligomerization domain 1 (NOD1) [8, 33]. As a consequence, the nuclear factor kappa-lightchain-enhancer of activated B cells (NF-κB) is activated in epithelial cells and immune cells residing in infected tissue, which results in up-expression and further secretion of many proinflammatory cytokines and chemokines including interleukin 8 (IL-8) [33, 46]. This chemokine acts as a chemoattractant to polymorphonuclear leukocytes (PMNs, neutrophils) circulating in the bloodstream and guides them to the place of infection [46]. In infected tissue, the effective response of PMNs toward pathogenic bacteria depends on: phagocytosis, production of neutrophil extracellular traps (NETs) and degranulation of neutrophils, with the involvement of oxygen-dependent and oxygen-independent mechanisms [3].

Clinical symptoms of gonococcal infection are mainly caused by infiltration of these immune cells. However, it has been demonstrated that *N. gonorrhoeae* is able to survive within PMNs isolated from the site of infection and has evolved many mechanisms to evade neutrophil mediated killing [38] (Figure 2).

4.1. Phagocytosis

Interaction of particular Opa proteins with PMNs CEACAMs (CEACAM1, CEACAM3 and CEACAM6) leads to more efficient phagocytosis of this bacteria by neutrophils in comparison to Opaless gonococci, even without opsonization by complement or antibodies [2]. Furthermore, engulfment of *N. gonorrhoeae via* CEACAM1 and CEACAM6 results in weak activation of PMNs in comparison to CEACAM3-related internalization, which also activates proinflammatory response in these cells. As previously mentioned, gonococci have a major advantage when interacting with CEACAMs on epithelial cells, but Opa expressing *N. gonorrhoeae* are threatened by CEACAM3-expressing neutrophil killing [37]. PMNs activation results in degranulation and oxidative burst, ending in tissue damage, which can help *N. gonorrhoeae* to colonize the deeper layer of epithelia [22, 38]. The same effect is observed during the infiltration of new PMNs that are attracted from the bloodstream by neutrophils that has already internalized *N. gonorrhoeae* and activated their proinflammatory response [43]. Therefore, it is postulated that a balance between Opaless and Opa-positive gonococci is a strategy that enable these bacteria to sequentially and effectively undergo different stages of infection [22].

4.1.1. Oxygen-dependent intracellular killing

During phagocytosis, PMNs are able to eradicate bacteria by oxygen-dependent and oxygen-independent mechanisms. During the process called oxidative burst, activation of nicotinamide adenine dinucleotide

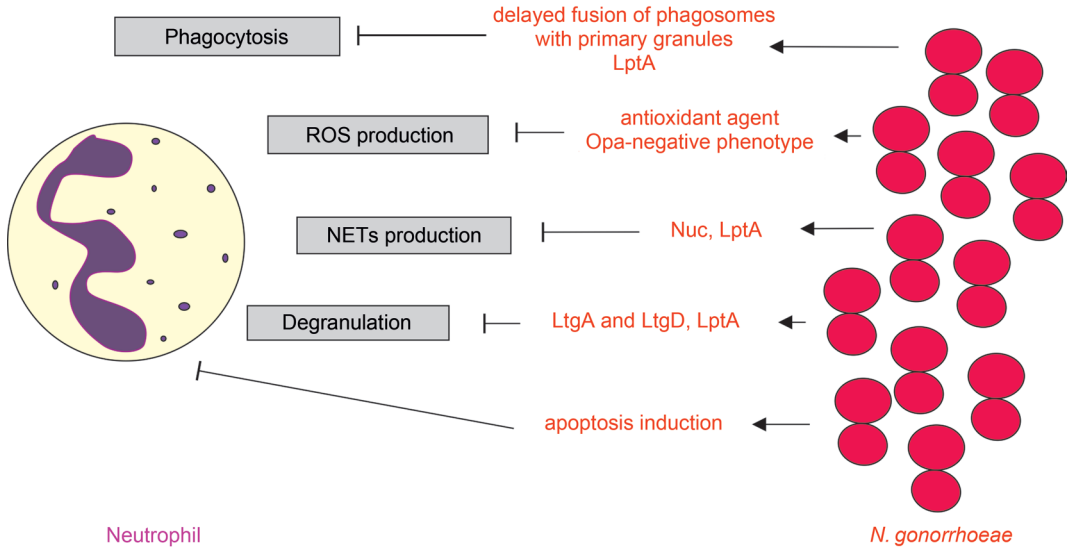

Fig. 2. Evading of neutrophil-mediated killing by *N. gonorrhoeae* [according to 3, 6, 15, 25, 33, 44]. LptA – LOS phosphoethanolamine transferase A; Nuc – thermonuclease homolog; LtgA – lytic transglycosylase A; LtgD – lytic transglycosylase D.

phosphate (NADPH) oxidase in neutrophils sequentially leads to the generation of numerous reactive oxygen species (ROS) such as hydrogen peroxide or hydrochloric acid [38]. *N. gonorrhoeae* features multiple mechanisms that protect this bacterium from oxidative stress. It has been demonstrated that incubation of *N. gonorrhoeae* with sub-lethal concentrations of hydrogen peroxide results in changes of expression of more than 150 genes, including those encoding superoxide dismutase and cytochrome c peroxidase, known antioxidant agents [47]. However, it has also been demonstrated that strains deficient in oxidative stress defence molecules are sensitive to killing by PMNs at the same level as wild type strains [38]. Furthermore, neutrophils from patients with chronic granulomatous disease (CGD), who are deficient in NADPH oxidase, still possesses the ability to kill *N. gonorrhoeae* [42].

It is worth mentioning that the other aspect of ROS production is related to *N. gonorrhoeae* phenotype. One of the surface structures of gonococci that modulates the interaction with human cells are Opa [37]. *N. gonorrhoeae* possesses at least 12 *opa* loci and encoding 11 Opa proteins [36]. Expression of Opa undergoes frequent phase variation, also during experimental male infection, so the gonococci in a population differ in Opa protein phenotype. Opa-negative (Opaless) gonococci are unable to induce ROS production in PMNs due to the ability to limit assembly of NADPH oxidase in the neutrophil membrane, in contrast to Opa-positive strains that stimulate the formation of this enzyme [44]. Furthermore, they also suppress oxidative burst caused by other stimuli such as serum-opsonized *Staphylococcus aureus* [2]. Suppression of oxidative burst may be a huge survival advantage for this bacterium and may also be connected to differences in the course of infection between men and women. Opaless phenotype is more predominant in women and can contribute to asymptomatic infection [44]. Thus, the Opa phenotype strictly facilitates the evasion of oxygen-dependent mechanisms by *N. gonorrhoeae*, although this protein also contributes to the protection to oxygen-independent mechanisms.

4.1.2. Oxygen-independent intracellular killing

It is therefore postulated that the oxygen-independent mechanisms are more significantly related to destroying the subset of *N. gonorrhoeae* that are sensitive to PMNs killing and that the survival advantage of some of gonococci results from an ability to evade these mechanisms [38]. While evading non-oxidative mechanisms, gonococci are able to delay the fusion of phagosomes with primary granules within PMNs, while maintaining fusion with tertiary and secondary granules [23]. The delayed fusion between phagosome and primary granules results in the delayed contact of gonococci with the antimicrobial content of these granules (BPI) [5, 39]. However, it has been demonstrated that the *N. gonorrhoeae* LptA mutant is more susceptible to killing by non-oxidative components of primary granules including cathepsin G than the wild-type strain, so LptA contributes to the protection of gonococci against this protein. Interestingly, *N. gonorrhoeae* that expresses LptA also decreases the maturation of phagosomes, while LptA deficient gonococci are more frequently found in primary granule-positive phagolysosomes [15]. Thus it is postulated that LptA is an important virulence factor that contributes to the adaptation to mechanisms of the host immune response and ensures the survival advantage of *N. gonorrhoeae* [15, 23].

4.2. Neutrophil extracellular traps

One of the mechanisms that is utilized by neutrophils to destroy pathogenic bacteria is the formation of NETs, which are mesh-like structures composed of chromatin and antimicrobial granule components of PMNs, and can be produced by dying neutrophils, during the cell death process called NETosis, or by live cells [50, 25]. The extrusion of NETs to an extracellular matrix, in order to trap microbes, prevents pathogen dissemination as well as being able to activate other immune cells [3]. NETs production by neutrophils is thought to depend on oxidative burst and require myeloperoxidase (MPO) [14]. However, it has been demonstrated that *N. gonoorhoeae* has the ability to stimulate PMNs to produce NETs through oxygen-independent mechanisms too. Interestingly, common surface antigens of gonococci – OpaD protein and pili – reinforce the stimulation of NETs production in neutrophils but are not required for this process. Nevertheless, NETs produced by neutrophils are unable to kill *N. gonorrhoeae in vitro*, unless the gonococci have a OpaD-positive and nonpiliated phenotype and are internalized by PMNs. The authors suggest that the elimination of this bacteria is related to oxidative burst, as the presence of an inhibitor of the NADPH oxidase in PMNs with internalized OpaD-positive and nonpiliated *N. gonorrhoeae* prevent the killing of this bacteria. As NETs are capable of killing *Lactobacillus crispatus*, which is one of the most predominant commensal bacteria of the women's genitourinary tract, it is possible that reduced competition facilitates the colonization of infected niche by *N. gonorrhoeae* [14, 51].

A thermonuclease homolog (Nuc) encoded by *N. gonorrhoeae* is a protein involved in biofilm reorganization by the degradation of DNA. Furthermore, it also enhances the survival of gonococci in the presence of NETs. It has been demonstrated that recombinant Nuc is able to degrade DNA present in NETs, therefore facilitating the weakening of these structures' integrity. Thus, this protein is considering as a virulence factor of gonococci that can defend these bacteria against extracellular killing by neutrophils [25].

Another gonococcal protein that facilitates bacterial survival in the presence of NETs is LOS phosphoethanolamine transferase A (LptA), which is a phase-variable enzyme that catalyzes the addition of phosphoethanolamine (PEA) to 4' phosphate on lipid A. This enzymatic modification of the LOS component by LptA results in less killing of *N. gonorrhoeae* by NETs produced by PMNs, in comparison to gonococci without this modification. This process may occur due to the fact that *N. gonorrhoeae* mutant in the gene encoding LptA protein is more sensitive to the serine protease cathepsin G, which is also a component of NETs, compared to the wild-type strain [15, 11]. Interestingly, it has also been demonstrated that a lack of 4' PEA modification in lipid A in most commensal *Neisseria* species decreases inflammatory response towards these bacteria in human monocytes, thus contributing to the immune privilege of these strains [21]. Surprisingly, it is postulated that the activation of the proinflammatory response by gonococci may be favourable to them, because *N. gonorrhoeae* is able to avoid most of the mechanisms of the innate immune response and may use damaged tissue as a source of nutrients and a way to colonize deeper layers of the epithelia [15].

4.3. Degranulation

Production of NETs is not the only mechanism by which neutrophils combat *N. gonorrhoeae* extracellularly. During the process called degranulation, neutrophil's granules fuse with the cytoplasmic membrane and release antimicrobial molecules including, for example, cathepsin G [3, 11]. Despite the protective role of LptA against extracellular killing by neutrophils, gonococci also possess other enzymes that are involved in the protection against antimicrobial proteins released by neutrophils [15, 33]. Double mutant in genes encoding two lytic transglycosylases (LTs): LtgA and LtgD, which are involved in peptidoglycan release, is more sensitive to lysozyme and neutrophil elastase than double complement *N. gonorrhoeae*. The reason is decreased envelope integrity in the analysed mutant. Additionally, infection of the human primary neutrophils with *N. gonorrhoeae* expressing LtgA and LtgD results in decreased release of the neutrophil's antimicrobial molecules compared to the mutant in genes encoding these two proteins [33]. In conclusion, *N. gonorrhoeae,* as a highly adapted human pathogen, has many factors that contributes to its response against the extracellular antimicrobial mechanism of neutrophils.

4.4. Apoptosis

Another mechanism that *N. gonorrhoeae* uses to its own advantage is the election of an anti-apoptotic effect in neutrophils. This process occurs through inhibition of caspase-3 and suppression of effect of proapoptotic agents like staurosporine by *N. gonorrhoeae*. Due to the fact that infection with gonococci results in NF-κB activation and further neutrophil migration toward the site of infection, it is postulated that the inhibition of apoptosis in these cells provides a notable niche for the replication of gonococci in these normally short-lived cells [6].

5. Summary

Innate immune response is a first line of defence against pathogenic bacteria including *N. gonorrhoeae* [26]. However asymptomatic infections in women, a lack of protective immunity observed in many patients and long-term complications like disseminated gonococcal infections indicate that this pathogen possesses an arsenal of mechanisms to avoid this response [1, 10, 12].

As demonstrated, *N. gonorrhoeae* has many virulence factors that undergo phase and antigenic-variation and play a crucial role in adapting to the hostile environment of infected human tissues, which includes elements of the host's innate immune response [31, 44, 51]. The ability of gonococci to evade complement-mediated killing through, for example, C4BP protein is considered to be the reason why this bacteria can only establish infection in humans and experimental infection in chimpanzees, which indicates that *N. gonorrhoeae* is a highly adapted human pathogen majorly through the ability to avoid the human innate immune response [31].

Furthermore, *N. gonorrhoeae,* in contrast with many pathogenic microorganisms, possesses mechanisms (like the modification of lipid A) that boost the detection of this bacteria by the human host, thus enhancing inflammation [15]. However, due to the ability to evade both oxygen-dependent and oxygen-independent antibacterial activities of PMNs, gonococci take advantage of the tissue degradation caused by inflammation [15]. Consequently, persistent gonococcal infection in the presence of neutrophils leads to an effective colonization of infected tissues and further dissemination of bacteria [23]. Thus, phase and antigenic-variation of many virulence factors that are used by gonococci to evade complement and neutrophil-mediated killing is involved in the overall modulation of the innate immune response in order to take advantage of subsequently induced stages of this response by *N. gonorrhoeae* [15, 23].

As *N. gonorrhoeae* is a strictly human pathogen and many virulence factors of this bacteria undergo phase and antigenic-variation, there has still been limited progress in making a gonorrhea vaccine [35]. Taking into account the increasing antibiotic resistance of *N. gonorrhoeae* and the absence of an effective vaccine, a full understanding of the mechanism underlying the modulation of the host's immune response by this pathogen seems to be imperative [17].

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