QUALIFICATION PROPOSAL

Contribution of heptose 1,7-bisphosphate to gonococcal infection and disease

[redacted]

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ABSTRACT

The Gram-negative bacteria *Neisseria gonorrhoeae* (*Ngo*) is the causative agent of gonorrhea, a sexually transmitted infection. In females, gonococcal infection of the endocervix is often asymptomatic and therefore more likely to remain untreated. When untreated, gonococci can ascend from the endocervix to the uterus and fallopian tubes, causing pelvic inflammatory disease. This, combined with increasing antibiotic resistance and a lack of vaccine make gonorrhea a re-emerging health problem. Strikingly, infected individuals do not develop protective immune memory, and thus remain susceptible to reinfections. While partly attributable to the frequent variation of surface antigens, this effect is also attributable to the apparent suppression of Th1 and Th2 responses by *Ngo*. The mechanisms by which *Ngo* achieves this latter effect remain unclear.

Studies undertaken by our lab to characterize gonococcal factors that modulate host immunity have revealed a previously unrecognized pathogen-associated molecular pattern, heptose 1,7-bisphosphate (HBP). HBP drives strong inflammatory responses in a variety of immune and non-immune cells. Although this molecule is required for the synthesis of lipooligosaccharide and is thus conserved in most Gram-negative bacteria, liberation of HBP during growth is unique to *Neisseria* species. Given that *Ngo* is a human-restricted pathogen, and has evolved multiple mechanisms to suppress the host immune response, it is unclear why gonococci have evolved the ability to liberate a factor which activates the immune system. We therefore hypothesize that **liberation of HBP drives inflammation so as to ultimately enhance gonococcal persistence in the population through its avoidance of protective adaptive responses.** This may occur through the induction of Th17 responses, as well as yet-to-be uncharacterized mechanisms. These studies aim to describe the effect of HBP on host immunity during two distinct stages of gonococcal infection in females: lower genital tract colonization and pelvic inflammatory disease.

I. BACKGROUND AND SIGNIFICANCE

Gonococcal pathogenesis: The Gram-negative bacteria *Neisseria gonorrhoeae* (*Ngo*, gonococci) is the etiological agent of the sexually transmitted infection (STI) gonorrhea. Gonorrhea is the second-most prevalent bacterial STI worldwide, with ≥106 million cases occurring per year.[1] Acute infections occur primarily at the urethra in males and the endocervix in females (Figure 1), where they persist within the submucosal space. Gonococci can infect additional sites including the rectum, oropharynx, and conjunctiva. Despite increased inflammation, colonization of the endocervix is most often asymptomatic but carries a persistent risk of progressing to more complicated disease.[2] When untreated, gonococci can ascend from the endocervix to the uterus and fallopian tubes, causing pelvic inflammatory disease (PID).[3] Damage to these tissues as a result of host inflammation can lead to complications including ectopic pregnancy, chronic pain, and infertility.[3] Unfortunately, infected individuals do not develop protective immune memory, and thus remain susceptible to reinfection. These effects, combined with increasing resistance to the last line of available antibiotics[4] and a lack of vaccine, make gonorrhea a significant re-emerging health problem.

Mouse models of gonorrhea: While cell-based studies have suggested both pro- and anti-inflammatory effects of *Ngo* virulence factors, understanding how these combine to direct the host response to *Ngo* necessitates the use of mouse models. Acute urethral challenge experiments can be performed in male volunteers. However, they are prohibitively expensive for basic studies and the risks associated with endocervical infection preclude the use of female volunteers for infection studies. Fortunately, mouse models of upper (UGT) and lower genital tract (LGT) infection in females now exist,[5,6] and our group is working to establish urethral infection in "humanized" male mice.

Female mice undergo a reproductive cycle comparable to the menstrual cycle in humans (Figure 1). Due to fluctuating hormones, immune cell recruitment and tissue architecture in the genital tract differ dramatically between various stages (Figure 1).[7] These differences profoundly affect outcomes of infection[5,6], and vaginal lavages are performed on naturally cycling mice to select

mice at the same stage for inoculation. Alternatively, the administration of 17β-estradiol or progesterone (Depo-Provera) "locks" mice to states reflecting the proestrus/estrus or diestrus stage, respectively. Experimental challenge of mice have focused largely on modelling uncomplicated infections. In this model, administration of exogenous β-estradiol and antibiotics are required for colonization of the LGT post-intravaginal inoculation.[5,8] Mice at the diestrus stage are resistant to infection of the lower genital tract.[5]

Modelling infections in mice is complicated by the fact that *Ngo* is an obligate human pathogen, and exploits several human factors for optimal colonization, pathogenesis, and immune evasion. Murine orthologues of these proteins are absent or not recognized by gonococci.[9] One such interaction is that between Opa adhesins on the surface of *Neisseria* and human carcinoembryonic antigen-related cell adhesion molecules (hCEACAMs1, -3, -5, and -6).[10] These interactions facilitate gonococcal invasion of the submucosal space[11–14], induce host inflammation[15–17], and suppress immune cell activation[12,18–22]. The majority of research to date has been conducted in wild type mice, and do not take into account the contribution of Opa or other human-specific factors.

Gonococcal immunity: The host immune response to *Ngo* is poorly understood. Symptomatic infection is characterized by a massive inflammatory response and the recruitment of neutrophils to the site of infection. Despite this overwhelming innate response, infection in both mice and humans does not lead to the development of protective memory responses. Individuals remain susceptible to reinfections by even the same strain, with no observed reduction in the severity or duration of disease.

A series of studies in wild type mice using a model of lower genital tract infection suggest that *Ngo* has evolved to selectively enhance neutrophil-dominated Th17 responses, but avoids inducing protective Th1- and Th2-mediated memory responses (Figure 3).[23–27] Specifically, gonococcal infection in wild type mice induces the expression of Th17-associated cytokines (TGF-β, IL-6, IL-17A, IL-23) but not the Th1-polarizing cytokine IL-12.[25,27] Mirroring this, cytokines associated with Th17 responses in humans IL-17A, IL-23, and IL-1β are elevated in individuals with gonorrhea.[28–30]

Although Th17 responses aid in gonococcal clearance by recruiting neutrophils and improving mucosal barrier function, they do not result in the development of protective memory responses.[27] In contrast, administration of exogenous cytokines or antibodies to induce T cell polarization toward Th1 and Th2 phenotypes led to enhanced resistance of the mice to reinfection.[24–26] This correlated with an increase in anti-*Ngo* antibodies in the serum and vagina upon reinfection.[26] Moreover, intravaginal administration of microencapsulated IL-12 (Th1-polarizing cytokine, Figure 3) during primary infection enhanced both the development of Th1 cells and anti-*Ngo* antibodies, resulting in faster clearance of secondary challenge.[24] Similar effects were achieved through administration of anti-TGF-β (transforming growth factor β) to block Th17 polarization, and anti-TGF-β in combination with anti-IL-10 to block immunosuppressive type 1 regulatory T cells.[25,26]

Cumulatively, these studies suggest that *Ngo* elicits an innate response that subverts the development of a protective adaptive response. One major mechanism that mediates this process is the recognition of pathogen-associated molecular patterns (PAMPs; *e.g.* lipopolysaccharide, LPS) via germline encoded pattern recognition receptors (PRRs; *e.g.* Toll-like receptors, TLRs).[31] Indeed, host recognition of lipooligosaccharide (LOS, Figure 5) through TLR4 has been shown to contribute to Th17 responses elicited by gonococci. However, it is evident that additional gonococcal factors are required, as gonococcal infection in TLR4 knockout mice still result in the generation of Th17 responses.[25,27,32] The contributions of other gonococcal-derived PAMPs have not been investigated.

II. EXPERIMENTAL PROGRESS

Previous work in the laboratory

Modeling gonococcal infections: In mice, gonococci instilled vaginally rare ascend into the UGT.[5] Our lab has developed and characterized a model of gonococcal PID in which inoculum is transcervically deposited into the uterine horns of mice using a blunted needle (Figure 1). As in the LGT, outcomes of infection largely depend on the stage of the reproductive cycle. Infection at diestrus induces high levels of inflammation, as evidenced by elevated cytokine levels (in the uterus, lower

genital tract, and serum), neutrophil recruitment to the genital tract, and penetration of gonococci into the uterine endometrium (summarized in Figure 4).[6] In comparison, mice infected at estrus show modest levels of inflammation in the uterus and serum, with no bacterial penetration of the uterine endometrium. We have also modeled the effect of hCEACAMs on outcomes of both UGT and LGT infections using transgenic mice expressing human CEACAMs (hCEACAMs) 1, 3, 5, and 6. During estrus, hCEACAM expression results in increased bacterial penetration of the uterine endometrium and inflammation compared to wild type mice (unpublished). Preliminary data suggest that epithelial expression of hCEACAMs prolong gonococcal colonization of the LGT. These studies are ongoing.

HBP as a novel PAMP: Studies undertaken by our lab to characterize gonococcal factors which modulate host immunity have identified a previously unrecognized PAMP, heptose 1,7-bisphosphate (HBP).[33,34] HBP is an intermediate of the LPS biosynthesis pathway, where the heptose from HBP is eventually incorporated into the inner core of LPS (Figure 5).[35] Although this pathway is conserved across most Gram-negatives, *Neisseria* species appear unique in their propensity to liberate HBP during bacterial growth.[33,34] Furthermore, neither heptose nor its phosphorylated derivatives are found in mammals, meaning that their presence in tissues is a sign of infection. HBP drives inflammation in immune and non-immune cells (Figure 6), as well as mice.[34] Signalling downstream of HBP requires the cytosolic protein TIFA (TRAF-interacting protein with forkhead-associated domain) and culminates in NF-κB activation.[34] TIFA appears to be ubiquitously expressed, is well-conserved between mice and humans, and is unique to the detection of HBP (Figure 6).[34] While TIFA is clearly essential for the response to HBP, experiments to determine whether TIFA is the receptor that directly binds HBP are ongoing. It is unclear why *Neisseria* species alone have evolved the ability to liberate HBP, and how host responses to HBP might affect the outcome of gonococcal infections.

My work to date

Response to HBP at diestrus: My work to date has focused on examining the role of HBP in pelvic inflammatory disease. Because *Neisseria* release HBP during growth, culture supernatants from wild

type gonococci or HBP-deficient gonococci can be processed to obtain preps with or without HBP, respectively. Both preps lack contaminating PAMPs (including CpG DNA, peptidoglycan, and LOS), which is evident from the low immune stimulatory potential of preps from HBP-deficient bacteria.[34] To determine whether HBP alone can drive inflammation in the uterus, I transcervically inoculated naturally cycling mice at diestrus with PBS (vehicle control), preps from WT gonococci (containing HBP), or preps from HBP-deficient (*hldA*::Tn5) gonococci. Expression of cytokines in the serum and genital tract tissues were assayed as a readout for inflammation. A range of proinflammatory cytokines (KC, MIP-1α, MIP-2, TNF) were significantly upregulated by 1 hour post-inoculation (P.I.) in the genital tract compartments and serum (Figure 7), indicating that local administration of HBP is potent enough to induce a systemic response. These patterns of induction are highly consistent with responses seen in murine gonococcal infections.[6,27,30,29]

To assess whether HBP can contribute to the Th17-biased response elicited by gonococci, I analyzed genital tract tissues for the expression of Th1-, Th2-, or 17-associated cytokines by qRT-PCR. HBP induced the expression of cytokines associated with Th17 responses (IL-6, IL-1β, IL-23A, IL-17A; Figure 9), but did not induce cytokines associated with Th1 (IL-12, IFN-γ) and Th2 (IL-4, IL-2) responses (Figure 8), suggesting HBP can potentially drive the induction of Th17 subsets during infection. Further studies will be required to implicate HBP in this process.

The broad range of cytokines induced at the transcriptional level may not be surprising, as signalling downstream of HBP culminates in NF-κB activation.[33,34] In general, the kinetics and pattern of induction were similar between all three tissues. Peak induction for most upregulated cytokines occurred at 1 hour P.I. and returned to baseline levels as early as 3 hours P.I. Interestingly, the only cytokine to show a delayed response was IL-17A, whose expression was restricted to the LGT and peaked at 6 hours P.I. Together, these data demonstrate that HBP can drive rapid and potent inflammatory responses in a physiologically relevant setting, and contribute to the body of evidence supporting HBP as a novel PAMP.

Response to HBP during estrus: I repeated the experiment described above to determine whether HBP induces similar patterns of inflammation in mice at estrus. An initial experiment conducted in naturally cycling mice yielded inconclusive results; expression of proinflammatory cytokines in the serum peaked at 1 hour P.I. in 2/4 mice, but remained at baseline in the remainder of mice (not shown). This experiment was difficult to repeat in naturally cycling mice at the time due to the very large cohort size required to capture mice in the same stage. I used β-estradiol-treated mice instead, with *E. coli*-derived LPS added as a positive PAMP (Figure 10, 11). In stark contrast to mice at diestrus, HBP induced low levels of inflammation in mice at estrus. LPS induced high levels of inflammation at all three sites, with patterns of expression similar to that of HBP during diestrus. This data suggests that the receptor required for signalling may not be expressed during estrus, or that HBP cannot access the endometrium due to increased mucus production in the uterus during this stage[6]. Additional studies will be required to differentiate between these possibilities.

Responses to HBP in the context of live gonococci: To determine the contribution of HBP to inflammation in the context of live bacteria, I inoculated mice at diestrus (Depo-Provera-induced) with PBS^{++} (vehicle) or 10^7 colony forming units (CFUs) of WT or HBP-deficient gonococci (*hldA*::Tn5). Gonococci lacking HBP induced less inflammation (Figure 12). As expected, this effect was more pronounced in the serum than the genital tract compartments. Interestingly, this does not correlate with differences in the number of neutrophils recruited, but correlates with decreased bacterial burden in mice infected with *hldA*::Tn5 mutants. Overall, these results support the hypothesis that liberation HBP contributes to inflammation-induced pathology in the UGT. However, while exciting, the *hldA*::Tn5 mutant has a truncated LOS phenotype which likely to renders it more sensitive to killing by antimicrobial peptides. Therefore, a Δ*gmhB* mutant which expresses HBP but has the same LOS surface carbohydrate truncation has been generated (Figure 6). This mutant will also control for any nonspecific effects the LOS might have on the host immune response (*e.g.* bacterial aggregation), and will be used in future experiments.

III. RATIONALE

In both mice and humans, gonococcal infection does not lead to the generation of protective memory responses. Experimental data from mice have shown that this effect is in part due to the unexpected paucity of Th1 and Th2 responses induced by *Ngo* during colonization of the lower genital tract. *Ngo* instead elicits a potent Th17-mediated response that aids in the clearance of infection, but does not lead to the generation of protective memory. The gonococcal factors that drive this response are largely unknown. Our lab has discovered HBP, a previously unrecognized PAMP that drives strong inflammation in a variety of immune and non-immune cells. Although this molecule is conserved in most Gram-negatives, liberation of HBP during bacterial growth is specific to *Neisseria* species. Given that *Ngo* is an obligate human pathogen and has evolved multiple mechanisms to subvert the host immune response, it remains unclear why gonococci have evolved to liberate a factor that activates the immune system. I therefore hypothesize that **liberation of HBP drives inflammation to ultimately enhance gonococcal persistence through subversion of protective adaptive responses.** To test this hypothesis, I will be taking advantage of two established mouse models of gonococcal infection to examine how HBP modulates the host immune response, and how this affects outcomes of infection.

Specific Aims

1. **Describe the role of HBP on outcomes of lower genital tract colonization.**
2. **Determine the effects of HBP on pelvic inflammatory disease.**
3. **Generation of a TIFA-knockout mouse with conditional potential.**

IV. REASEARCH DESIGN AND METHODS

Unless otherwise stated, the experiments outlined in Aims 1 and 2 will be conducted using our recently generated Δ*gmhB* and Δ*hldA* mutants in mice expressing hCEACAMs -1, -3, -5, and -6. Although naturally cycling mice are preferred, the number of mice required and the complexity of experiments necessitate use of exogenous hormones. Routinely, experiments will also be conducted in naturally cycling mice to confirm that outcomes reflect that for mice given exogenous hormones.

However, previous experiments by myself and others in our lab suggest that overall trends are highly similar in naturally cycling mice and hormone-synchronized mice.[6]

AIM 1: Describe the role of HBP in lower genital tract colonization.

Rationale and Strategy: An abundance of evidence *in vitro* and *in vivo* suggest that liberation of HBP modulates host immunity. In gonococcal infections, HBP may hinder the development of protective adaptive immunity by subverting Th1 and Th2 responses and/or enhancing Th17 responses to allow rapid clearance of the infection in a manner that does not elicit an adaptive response. My preliminary experiments in mice suggest that purified HBP might enhance Th17 polarization (Figures 8, 9). I will characterize how HBP moderates the generation of adaptive immunity against the bacteria in a model of lower genital tract infection (see Figure 13 for proposed design). My strategy is to identify broad correlates of protection first, which I will then dissect in greater detail in future studies.

Experimental Plan:

1.1 Effect on primary infection. Using the protocol outlined in Figure 13, I will inoculate mice with Δ*gmhB* or Δ*hldA* gonococci and monitor the rate of clearance of each strain. I expect that Δ*gmhB* mutants will induce more inflammation, and will therefore be cleared more rapidly from the lower genital tract. I will monitor gonococcal clearance by plating vaginal washes obtained daily from mice throughout the duration of the experiment. We have shown in our lab that the levels of KC, a proinflammatory cytokine in mice similar to human IL-8, is a reliable indicator of general inflammation. Therefore, I will monitor KC (proinflammatory cytokine in mice) levels in both the serum and vaginal lavages as a readout for inflammation. To characterize the adaptive response, I will monitor the generation of anti-*Ngo* antibodies (IgM, IgG; IgG subclasses IgG1, IgG2a, IgG2b, IgG3) in the serum and vaginal washes by whole bacterial ELISAs.

1.2 Protection against secondary challenge. I will use the same strategies described in Section 1.1 to characterize the how the presence of HBP during primary challenge affects outcomes of secondary challenge (measured by inflammation, antibody response, and bacterial clearance). Secondary

challenge will be performed with Δ*hldA* gonococci. If my initial hypothesis is correct, I predict that mice initially challenged with Δ*gmhB* gonococci will not be protected against secondary challenge, whereas mice initially challenged with Δ*hldA* gonococci may be equally or less susceptible to re-infection. This experiment allows me to assess whether protection against secondary challenge correlates with the levels and classes of anti-*Ngo* antibodies produced. Protective adaptive immunity against gonococci may occur independently of anti-*Ngo* antibodies, and may instead be primarily cell-mediated. To explore this, I will sacrifice mice at 5 days and 14 days after primary challenge and analyze the draining lymph nodes, uterus, and lower genital tract for relative proportions of Th1, Th2, and Th17 cells (previously implicated) by flow cytometry.

Feasibility and Future Directions: The combined results obtained from these experiments will enable me to formulate and test more specific hypotheses as to how HBP modulates the outcome of infection in the LGT. For instance, if protection against re-infection is mediated by the production of anti-gonococcal antibodies, passive immunization of naive mice with these antibodies should protect mice against subsequent gonococcal challenge.[36,37] If protection is cell-mediated, I expect adoptive transfer of the candidate subset will protect naive mice against subsequent challenge. Alternatively, protection against secondary challenge should be abrogated when I deplete of these candidates from mice after primary challenge. These studies will indicate whether HBP modulates host immunity through Th1, Th2, or Th17 responses as previously described, or through alternative means.

AIM 2: Determine the effects of HBP on outcomes of pelvic inflammatory disease.

Rationale and Strategy: PID is estimated to occur in 10-25% of patients with untreated gonococcal infection of the endocervix.[38–40] Several studies suggest that gonococcal PID presents more rapidly and with more severe symptoms compared to PID caused by other microorganisms.[3,38,40] The onset of symptoms in gonococcal PID is more likely to occur during the first ten days of the menstrual cycle when the human endometrium is structurally similar to that of mice during diestrus (Figure 2).[40] The

degree and speed with which HBP drives inflammation in the uterus during diestrus (Figures 7-9), but not estrus (Figures 10, 11), suggests that it might contribute to these pathogenic outcomes. This hypothesis is further supported by the observation that HBP appears to be uniquely liberated by *Neisseria* species.[33,34] I will use the transcervical inoculation model developed in our lab to determine whether HBP can enhance the pathology of gonococcal PID, and how these effects might be modulated by the female reproductive cycle.

Experimental Plan:

2.1 Innate inflammation. My work to date provides strong evidence that production of HBP by gonococci enhances inflammation in the uterus. My first priority is to repeat the experiment shown in Figure 12 using hCEACAM mice and Δ*gmhB* mutants in addition to wild type and Δ*hldA* gonococci. I will sacrifice mice at several time points P.I. (3, 6, 12, 24 hours) to determine whether the innate response reaches different levels and/or peaks at different times for each mutant. The serum, uterus and lower genital tract will be collected and analyzed for bacterial burden, cytokine production (multiplex analysis of a broad range of cytokines), and myeloperoxidase level as readouts for inflammation. This will indicate which time point should be used as a fair comparison going forward, and which cytokines best correlate with infection outcomes. My future experiments will also include HBP and LOS administered alone (at amount equivalent to the number of inoculated gonococci) as additional controls. Once these conditions are optimized, I will perform histological analysis of the genital tract to assess the cell recruitment and pathology induced by each treatment. I will also perform flow cytometry studies to define the immune cell recruitment by each treatment, starting with broad classes of leukocytes (T cells, B cells, granulocytes, macrophage/monocytes). Overall, I expect Δ*gmhB* mutants to elicit more inflammation than Δ*hldA* mutants.

2.2 Effects of the estrous cycle. The blunted response to purified HBP administered during estrus (Figure 10, 11) may be due to non-specific protection mediated by increased mucus production[6], the absence of cells that drive the response during diestrus, or a downregulation of TIFA expression in

cells always present in the genital tract. To distinguish between these possibilities, I will characterize TIFA expression in the genital tract during various stages of the reproductive cycle at the mRNA level by qPCR and at the protein level by immunohistochemistry and flow cytometry. This will be performed in naturally cycling mice, as well as mice treated with exogenous hormones to compare our models. An additional possibility is that the response to HBP is simply delayed in estrus, and so was not observed at 1 hour P.I. I will therefore sacrifice mice at 3 hours P.I. with the HBP-containing or –lacking preps, using LOS purified from *Ngo* as a positive control. Previous experiments in our lab have shown that hCEACAM expression is necessary for penetration of gonococci into the endometrium at estrus (unpublished). Thus, corresponding experiments involving inoculation with live gonococci at estrus will be performed using hCEACAM-expressing mice. I expect Δ*gmhB* mutants to elicit more inflammation than Δ*hldA* mutants.

Feasibility and Future Directions: These studies are particularly valuable as causal relationships between gonococcal factors and the progression of PID are impossible to study in humans. Once I identify immune responses that correlate with increased pathology (*i.e.* specific cell subsets, cytokines), I will attempt to manipulate these factors to verify their role in outcomes of PID (*e.g.* cell depletion, antibodies against cytokines). Lastly, we have shown that transcervical gonococcal challenge performed during diestrus significantly increases the levels of anti-gonococcal antibodies produced.[6] My future studies will investigate whether this can be enhanced by the presence or absence of HBP.

AIM 3: Generation of a TIFA knockout mouse with conditional potential.

Rationale and Strategy: The protein TIFA is essential for detection of HBP (Figure 6D).[34] TIFA is highly conserved in vertebrates,[34] with 76.6% sequence identity between mice and humans. In both these species, the residues required for TIFA oligomerization and binding to its downstream target TRAF6[34] are conserved. In collaboration with the Toronto Centre for Phenogenomics (TCP), I am currently working to generate a TIFA knockout mouse with conditional potential using a vector purchased from the International Mouse Phenotyping Consortium.[41] I will interbreed these mice with

various Cre-expressing mice to derive progeny that harbour either lineage-specific or whole-organism TIFA deletions[41], which can then be crossed with hCEACAM-expressing mice. Experiments performed using these mice will serve to both validate and expand upon those under Aims 1 and 2.

Experimental Plan: Preparation of the linearized targeting vector is being performed by me; all subsequent steps up to and including the generation of chimeras will be performed as a service by TCP. Before beginning infections in TIFA knockout mice, I will verify knockout efficiency by analyzing various tissues for abrogated TIFA expression using a combination of protein- and gene-based (qRT-PCR) approaches. In parallel, I will verify *in vitro* that TIFA knockout in cells does not significantly impact signalling of other PAMPs (LPS, muramyldipeptide, dsRNA, c-di-GMP, CpG DNA). I expect that HBP will not drive inflammation in mice without TIFA. If knockout efficiency is high, and my expectations prove true, I will proceed with transcervical inoculation of mice with live gonococci. I expect TIFA knockout mice to respond similarly to Δ*gmhB* and Δ*hldA* mutants.

Feasibility and Future Directions: TCP has extensive technical expertise in this area, and have successfully targeted genes using this strategy. The estimated time to completion for the generation of chimeras is between 8 months to a year. My preliminary cell-based experiments using TIFA knockout mice will begin shortly after. Because TIFA-mediated signalling has not been thoroughly examined, it is possible that whole-organism TIFA deletion will be embryonic lethal. However, the flexibility of the Cre-loxP system can be exploited to overcome this.

V. SUMMARY

I propose to characterize effects of HBP on the host immune response during two distinct stages of gonococcal pathogenesis, lower genital tract colonization and pelvic inflammatory disease, with an appreciation for the effect of the reproductive cycle on the outcome of infection. Cumulatively, these studies will advance our currently limited understanding of gonococcal infection and immunity, and inform the development of novel strategies to combat gonorrhea. Furthermore, this project will serve as a foundation for future studies on the role of HBP in other Gram-negative infections.

VI. FIGURES

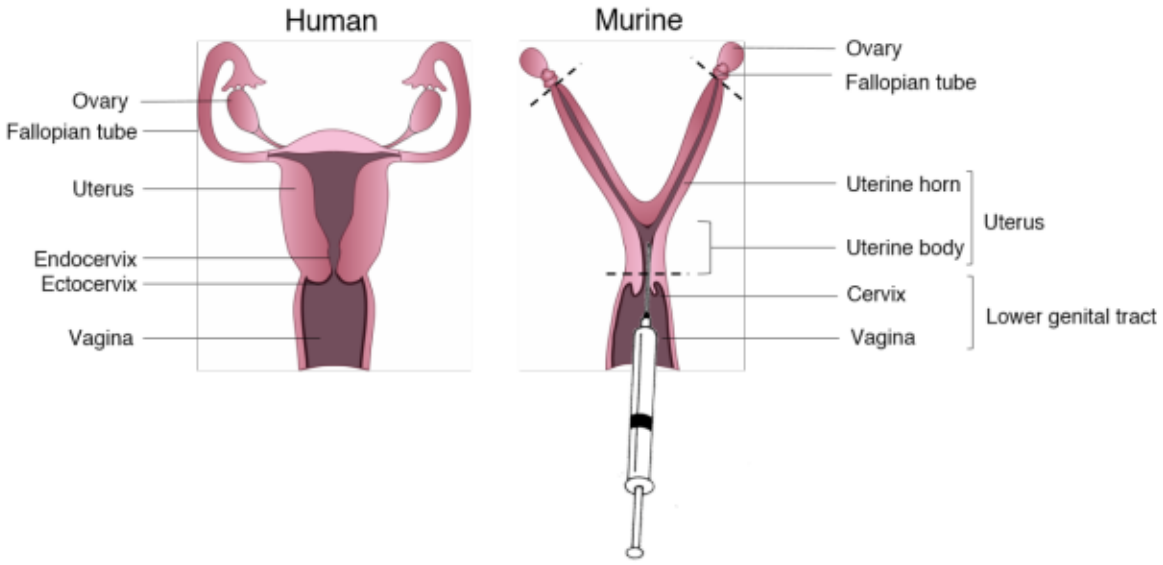

Figure 1. Gross anatomy of the female genital tract. In models of ascending disease, inoculum is deposited transcervically into the upper genital tract using a blunted needle. Dashed lines indicate excision points used when collecting tissues for analysis. Modified from graphic courtesy of Epshita Islam.

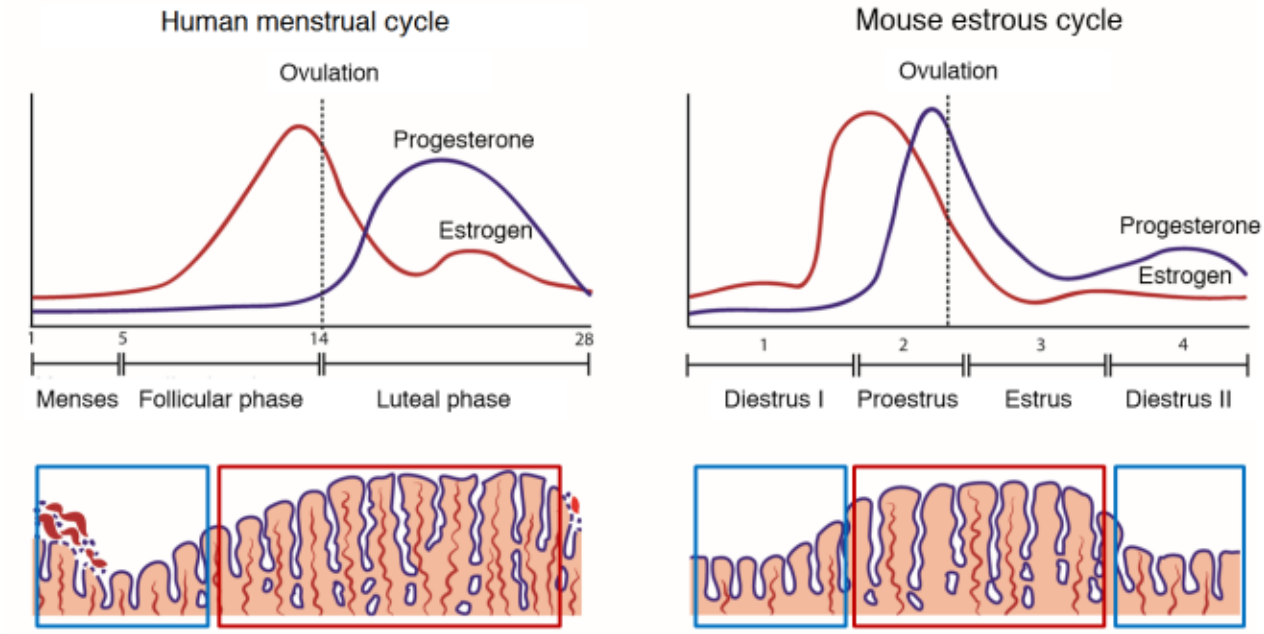

Figure 2. Hormonal and structural differences during the female reproductive cycle. Upper row shows changes in sex hormones throughout the reproductive cycle. Lower row indicates associated structural changes to the uterine endometrium. The estrus and diestrus stages can be hormonally induced using exogenous β-estradiol and Depo-Provera (progesterone), respectively. Figure courtesy of Epshita Islam.

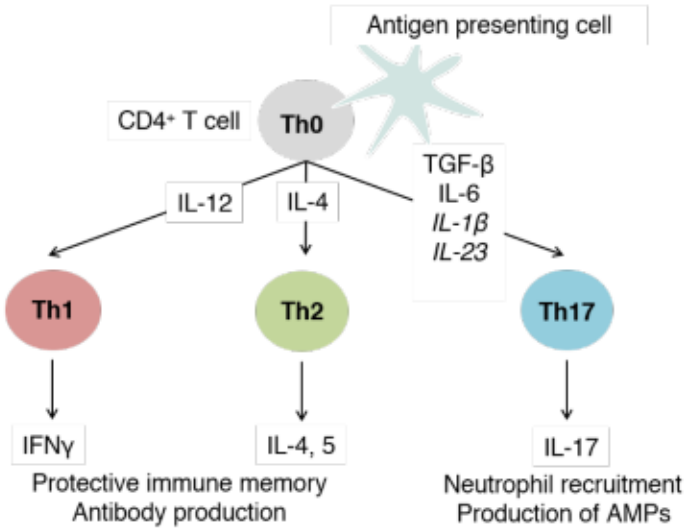

Figure 3. Simplified schematic of T helper (Th) cell polarization and effect on outcomes of gonococcal infection in mice. Activation of naive $CD4^+$ cells by antigen presenting cells secreting distinct cytokines result in the polarization of distinct Th lineages. These subsets differ from one another in the expression of surface markers, transcription factors, and effector cytokines. Differentiation of Th17 cells in mice require TGF-β and IL-6. IL-1β, and IL-23 induce the proliferation of Th17 cells. A series of studies in mice using a model of lower genital tract infection suggests that *Neisseria gonorrhoeae* has evolved to selectively enhance neutrophil-dominated Th17 responses, but avoids inducing protective Th1- and Th2-mediated memory responses. Although Th17 responses aid in gonococcal clearance by recruiting neutrophils and improving mucosal barrier function, they do not result in the development of protective memory responses.[23–27] IL, interleukin; TGF, transforming growth factor; IFN, interferon; AMP, antimicrobial peptides.

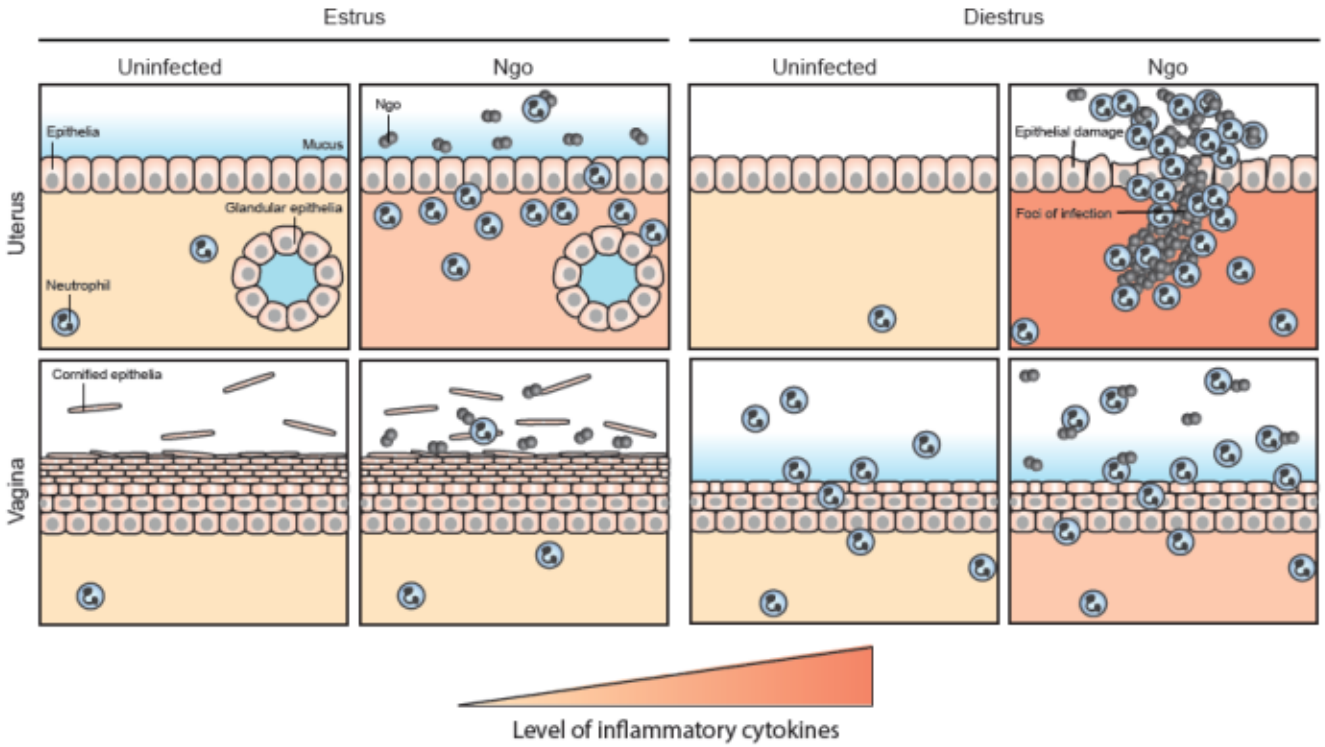

Figure 4. Schematic showing the inflammatory response in the upper and lower genital tracts during different stages of the estrous cycle upon infection with *N. gonorrhoeae*. Early pathological outcome upon gonococcal infection is summarized diagrammatically. The colour of the stroma indicates the level of tissue inflammation, where beige is not inflamed and red denotes presence of inflammatory cytokines. Ngo, *Neisseria gonorrhoeae*. Adapted from Islam *et al.,* (2015)[6].

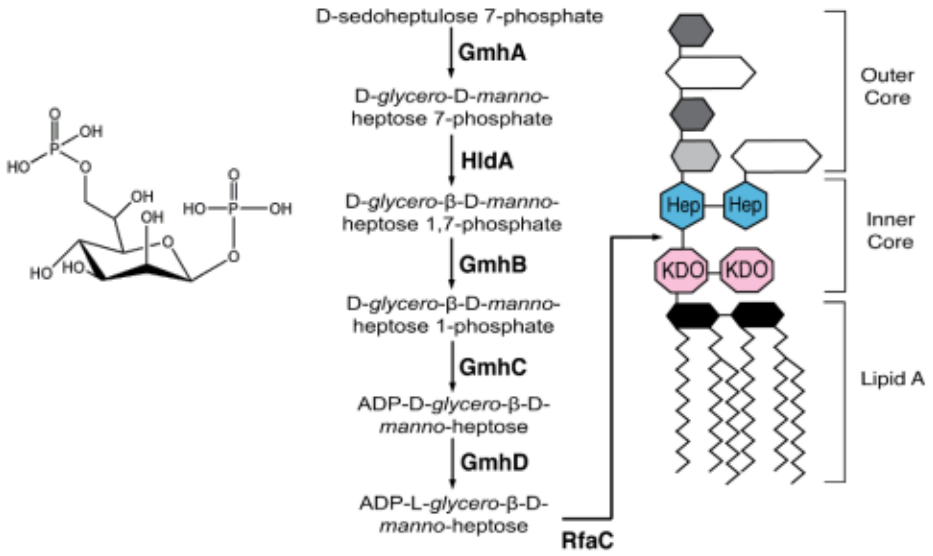

Figure 5. *Neisseria gonorrhoeae* lipooligosaccharide biosynthesis and structure. The seven-carbon phosphorylated sugar, heptose 1,7-bisphosphate (D-*glycero*-β-D-*manno*-heptose 1,7-phosphate; HBP; left), is an intermediate of the lipooligosaccharide (LOS) biosynthesis pathway (right) and is eventually incorporated into the inner core of LOS. In *Neisseria* species, HBP is synthesized solely by the HldA enzyme. LOS is a low molecular weight form of lipopolysaccharide (LPS) that is used by a subset of gram-negatives, including *Neisseria* species. LOS lacks the O-antigen sugars found in LPS, but is otherwise analogous to LPS. In mammals, LOS/LPS is a pathogen-associated molecular pattern, and signals via engagement of the pattern recognition receptor Toll-like receptor 4 (TLR4).