Kinetics of Neutrophil Recruitment and their NF-κB Response to *Neisseria gonorrhoeae* Uterine Infection

by

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Department of Molecular Genetics
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Abstract

Neisseria gonorrhoeae (*Ngo*) is the bacterial pathogen responsible for the sexually transmitted infection gonorrhea. Aside from the cervicitis typical of gonococcal infection in women, untreated infections may ascend into the upper genital tract, leading to a devastating inflammatory response that can result in pelvic inflammatory disease. In this thesis, I reveal the first leukocyte responders to *Ngo* uterine infection using a transgenic mouse model that expresses green fluorescence protein (GFP) when the immediate-early transcription factor NF-κB is activated. Using flow cytometry, my results demonstrate recruitment of neutrophils into the infected uterus, where only a subset of these neutrophils displays activated NF-κB, and most of which are not associated with gonococci, suggesting that these populations may represent different neutrophil phenotypes. These findings make it enticing to consider that the responding neutrophils are of a phenotype incapable of clearing infection, and may help explain persistent colonization observed in untreated individuals.

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List of Abbreviations

EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FMO	fluorescence minus one
FSC-A	forward scatter area
FSC-H	forward scatter height
GFP	green fluorescence protein
hCEACAM	human carcinoembryonic antigen-related cell adhesion molecule
IL-10	interleukin-10
IL-12	interleukin-12
IL-17	interleukin-17
IL-6	Interleukin-6
LGT	lower genital tract
Ngo	*Neisseria gonorrhoeae*
MIP-2	macrophage inflammatory protein 2
NETs	neutrophil extracellular traps
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NK cell	natural killer cell
PBS	phosphate buffer saline
PID	pelvic inflammatory disease
SD	standard deviation
SSC-A	side scatter area
SSC-H	side scatter height
TGF-β	transforming growth factor beta
TNF	tumor necrosis factor
TRXSE	texas red succinimidyl ester
UGT	upper genital tract
WT	wild type

Chapter 1
Introduction

1 Introduction

1.1 *Neisseria gonorrhoeae* infections

Neisseria gonorrhoeae (*Ngo*) is a human-restricted bacterial pathogen responsible for causing the sexually transmitted infection, gonorrhoea. Following chlamydia, gonorrhoea is the second most commonly reported sexually transmitted infection [1]. In fact, the World Health Organization estimates 78 million cases worldwide (2012), and the infection rates have been rising in Canada since 1997 [2, 3]. The majority of cases involve genital infections, however anorectal, pharyngeal, eye, and systemic infections can also occur.

Genital gonococcal infections present differently in men and women. In men, *Ngo* colonize the urethra, with disease presenting as urethritis that results in painful urination and urethral discharge. The symptomatic nature of gonococcal infections in men usually leads to treatment at onset of symptoms [4]. In contrast, infections in women are commonly asymptomatic and if symptoms develop they are often mild and unspecific [5]. This can include symptoms such cervicitis and vaginal discharge. Taken together, this means that many infected women are left undiagnosed and untreated. When left untreated, infection can persist and ascend into the female upper genital tract (UGT). This can lead to devasting sequalae such as pelvic inflammatory disease (PID), UGT scarring, and infertility.

1.2 Pelvic inflammatory disease (PID)

Women with asymptomatic or untreated gonococcal infections are at risk for developing PID. This can result in acute endometritis, salpingitis, and peritonitis, which all fall under the umbrella term of PID [5]. It is estimated that 10 to 20% of women with untreated gonococcal infections will develop PID [6]. Symptoms of PID can range from mild to severe, and include abdominal and pelvic pain, unusual vaginal discharge, and scarring that can lead to complications such as ectopic pregnancies, infertility, chronic pain, and tubo-ovarian abscesses. Often, these severe symptoms are the first indications that a gonococcal infection is present, leading to diagnosis and treatment of infection. Even after treatment of infection, the sequelae can persist due to

permanent damage of the reproductive tract. Despite these devastating consequences, factors contributing to disease severity remain poorly understood.

1.3 Treatment

Traditionally gonococcal infections were easily treatable with antibiotics, however with the rise of antimicrobial resistance, gonococcal infections are becoming an increasing concern and public health threat globally [7]. As an emerging superbug, *Ngo* has developed resistance to nearly all of the antibiotics used to treat it, including sulfonamides, penicillin, older cephalosporins, tetracyclines, macrolides, and fluoroquinolones [8]. Current treatments include a combination of extended spectrum cephalosporins and azithromycin. There is, however, an increasing number of isolates resistant to both cephalosporins and azithromycin, demonstrating an obvious threat to these last remaining treatment options [9, 10]. This is particularly worrisome, given the serious outcomes of untreated infections in women. Therefore, continuous research efforts are focused on understanding gonococcal virulence, host-pathogen interactions, and the immune response mounted in order to further develop antibiotic or immune-based treatment strategies as well as gonococcal vaccines.

1.4 In vivo models of infection

With the rapid spread of gonococcal infections, the rise in its antimicrobial resistance, and the possible severity of untreated infections, research on gonococcal infections is imperative. However, since infections in men and women are unique in terms of disease presentation and immunological outcome, the independent study of male and female infections is required.

1.4.1 Male infection model

For research concerning infections in men, there exists a urethral challenge model whereby human volunteers are intra-urethrally inoculated with *Ngo* and treated with antibiotics upon onset of symptoms [11]. This model enables the study of the early innate response towards *Ngo* in the male genital tract, as well as the study of virulence factors required for early stages in colonization. No animal model of urethral infection currently exists.

1.4.2 Female infection models

While human-based research is considered ethical in male volunteers, these types of studies are not permitted in females due to the serious consequences of ascending infection. Instead, a mouse model of female gonococcal lower genital tract colonization has been developed, and has proven to be a useful tool to study factors mediating gonococcal immune evasion and host-pathogen interaction between the female host and the gonococci [12, 13].

However, since *Ngo* is a human-restricted pathogen, gonococcal colonization eventually clears the lower genital tract (LGT) without ascending into the mouse UGT. To overcome this challenge, our lab has recently developed a mouse model of female gonococcal UGT infection [14]. In this model, *Ngo* are directly inoculated into the uterine horns of female mice.

Female mice undergo a sex hormone cycle called the estrous cycle. This cycle can be divided into four stages: proestrus, estrus, metestrus, and diestrus. Importantly, the immune response to UGT infections varies depending on the stage of the cycle. Gonococcal transcervical infection during the diestrus stage of the cycle results in a severe inflammatory response and extensive gonococcal penetration into the uterine tissue. In contrast, infection during the estrus stage of the cycle results in a mild inflammatory response [14]. Therefore, to study the severe inflammatory response that would occur during severe PID, progesterone treatment is used to induce the diestrus stage of the cycle [14].

1.5 Comparing Human and Mouse Female Reproductive Tracts

The female reproductive tract is a mucosal compartment that must balance the ability to carry a pregnancy to term with the ability to protect the mother and fetus against microbial challenge. As such, the female reproductive tract undergoes distinct morphological changes throughout the reproductive cycle in both mouse and humans. Although these cycles differ in duration, they are similarly regulated by hormones that ultimately affect the tissue microenvironment and immunological response within the genital tract in a similar manner [14].

Anatomically, the female reproductive tract can be divided into the lower genital tract (LGT), which is comprised of the vagina, which opens through the cervix into the uterus and fallopian tubes of UGT. The UGT in mice differs from that of humans as it is divided into two uterine horns that join the corpus in the middle (Figure 1.1). In both humans and mice, the LGT is

protected by squamous epithelium and the UGT is composed of a monolayer of columnar epithelial cells [15]. The cervix, which is the opening between the vagina and uterus, contains the zone within which the multilayered squamous epithelia transitions into columnar cells. Both of these epithelial cell types provide protection from pathogenic stimuli, with a thick-walled barrier in the LGT and the UGT epithelium forming strong tight junctions [15].

Beneath the epithelial cells lie fibroblasts, which form the stroma and provide structure to the tissue. Within the stroma exists a population of immune cells and, although the distribution varies depending on the stage of the cycle, the primary immune cell types within the female reproductive tract are T cells, macrophages, natural killer (NK) cells, neutrophils, and mast cells [16]. In addition to the abundance of leukocytes, the female reproductive tract of both mice and humans produces antimicrobial secretions (acting as a chemical barrier) and mucus (acting as a physical barrier) against invading pathogens.

Overall, the similarities between the human and mouse female reproductive tract make mice a useful tool for studying mucosal immunology in the female reproductive tract.

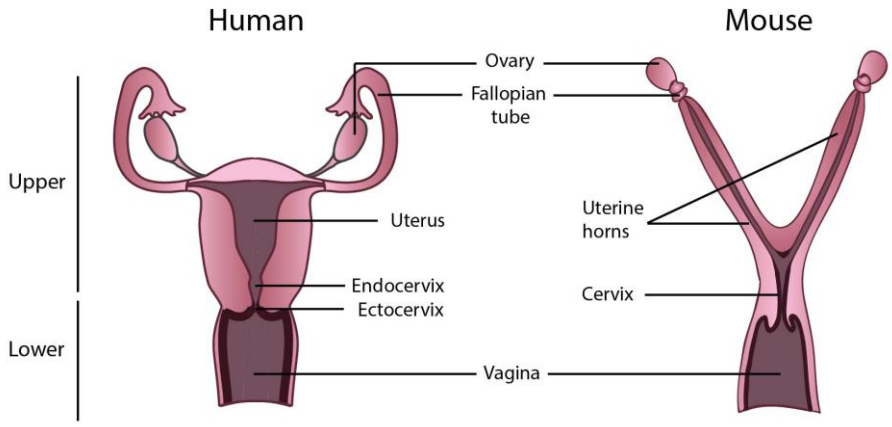


Figure 1.1 [17]: Comparison between the human (left) and mouse (right) female genital tracts. (Figure taken with permission from Islam, E. A, and Gray-Owen, S.D.)

1.6 Immunological Response to *Ngo*

1.6.1 Human response to *Ngo*

Studies on the immune response to *Ngo* in humans can be performed either using naturally occurring infections, or experimental infections in male volunteers. Although limited, human studies have revealed insight into the contribution of several different gonococcal virulence factors contributing to disease pathogenesis.

In both men and women, symptomatic infections are characterized by mucopurulent discharge, consisting mostly of neutrophils. Many of these neutrophils in the purulent discharge are found to be associated with intact gonococci. In spite of this neutrophil response, gonococci can be cultured from the exudates of infected individuals [18] and, further, have been shown to be able to survive and replicate within neutrophils [19, 20, 21, 22, 23]. These findings highlight the fact that the neutrophil response to *Ngo* may be ineffective at clearing *Ngo* infections, and this ineffective neutrophil response may help explain the persistent colonization observed in untreated individuals.

Despite the presence of an innate inflammatory response, humans do not develop protective immunity against *Ngo* infections [24], although there have been reports of the development of transient anti-*Ngo* antibodies in both serum and the genital mucosa[25, 26, 27 28]. In fact, recurrent *Ngo* infections can occur without any sign of disease improvement throughout subsequent infections. This is because *Ngo* have developed an arsenal of mechanisms to evade the host's immune response, including antigenic variation of outer membrane proteins such as pilin and Opa [29, 30], the ability to evade complement-mediated killing [31], and production of IgA1 protease [28].

1.6.2 Murine response to *Ngo*

As with human infection, *Ngo* infection in the mouse LGT results in recruitment of neutrophils [13]. During infection, there is a marked upregulation of pro-inflammatory cytokines including IL-6, TNF, KC, and MIP-2 at 5 days post-infection, although these responses seem to be limited to BALB/C mice [32]. Like humans, mice produce antibodies against *Ngo* after infection in the lower genital tract, but do not develop immunity or secondary antibody responses against recurrent *Ngo* infections [33].

Further, it has been demonstrated that *Ngo* infection elicits a Th17 response in the mouse model of lower genital tract infection [34]. Th17 responses and their IL-17 production occurs early on during infection and is considered the bridge between innate and adaptive immunity. This Th17 response is thought to drive the neutrophil influx during infection but is also responsible for the shift away from a Th1/Th2 adaptive response. In fact, Liu et. al demonstrated that *Ngo* are able to suppress Th1 and Th2 responses by driving the Th17 response in a TGF-β dependent manner [35]. Additionally, *Ngo* infection elicits the production of IL-10 and type 1 regulatory T cells, and blockage of this response led to an enhanced and protective Th1/Th2 response [36]. These findings suggest that *Ngo* elicit an immune response that interferes with the development of an adaptive immune response that would protect against subsequent infections. However, further work is required to define the type of response that is required to confer protection and the effector processes that confer this protection.

The UGT and LGT represent distinct immunological compartments and, as such, respond differently to *Ngo* infection. Inoculation with *Ngo* into the UGT of FVB mice results in a rapid recruitment of neutrophils, and the induction of pro-inflammatory cytokines as early as 6 hours post-infection. These cytokines include interleukin IL-1α, IL-1β, MIP-1α, MCP-1, MIG, CXCL10, and IL-12 [14]. Additionally, both local and systemic antibody production occurs upon re-infection of *Ngo* into the UGT of female mice, although these antibodies do not significantly reduce bacterial burden post-infection, highlighting that they likely do not confer protection against recurrent infections [14].

1.7 Neutrophils and their response to *Neisseria gonorrhoeae*

1.7.1 Neutrophil function

Neutrophils are an integral part of the innate immune system and are the most abundant cell type circulating in human blood. Neutrophils are produced in the bone marrow and circulate in blood. They are one of the first cells recruited to the site of infection and are thus considered to be the first line of defense against invading microorganisms. Upon encounter with an invader, they have the capacity to capture and destroy the invader through a variety of mechanisms including phagocytosis, the release of granules, and the formation of neutrophil extracellular traps (NETs), thereby killing the microorganism and protecting the host from infection [37]. Dead or dying

neutrophils are cleared by macrophages [38]. Until recently, these functions were thought the be the only functions performed by neutrophils.

1.7.2 Neutrophil heterogeneity

Recently, neutrophils have been redefined as a population of innate immune cells able to nuance their response to varying stimuli, displaying more heterogeneity than previously thought [39]. Neutrophils have the capacity for transcriptional activation and cytokine production, and have been shown to play a role in resolution of inflammation [40] and in diseases such as cancer [41].

Considering these recent findings, the study of different neutrophil phenotypes in health and disease has become of interest. An example of such neutrophil diversity was recently described by Fine et. al [42]. They described a population of neutrophils that was 'primed' for more rapid recruitment to sites of acute infection, that differed in surface markers from the bulk neutrophil population. These neutrophil subpopulations were termed 'primed' and 'resting' state neutrophils, respectively. They found that at steady state in the mouse, approximately 10% of neutrophils in the blood and bone marrow are primed. They demonstrated that upon peritoneal infection with *Escherichia coli*, the percentage of primed neutrophils increases as this population gets recruited into the site of infection. Primed neutrophils were characterized by upregulation of specific adhesion markers on mouse neutrophils, including CD66a^{hi}, CD5, CD193, and CD11b^{hi} [42]. These neutrophil populations have yet to be studied in the context of the UGT or in the context of gonococcal infections. In fact, their contribution to the outcome of any infection has not yet been explored.

1.7.3 Neutrophil response during *Neisseria gonorrhoeae* infection

It is well established that neutrophils are the first line of defense against pathogens, and that they get recruited to the genital tract during gonococcal infection. Once neutrophils are recruited into the genital tract, they are able to interact with gonococci. One such mechanism involves opsonins such as antibodies or complement, which ultimately leads to phagocytosis of the bacteria [19]. However, curiously, the interaction between neutrophils and *Ngo* is also facilitated by the expression of several bacterial factors known to promote attachment and phagocytosis by host cells. For example, gonococci associate with neutrophils through opsonin-independent

mechanisms such by their Opa proteins binding to neutrophil-expressed CEACAMs, and via the gonococcal pilin.

Opa proteins are phase variable proteins expressed on the surface of gonococci that can bind CEACAM1, 3, 5, and/or 6. These CEACAMs are expressed on a variety of cell types within the human host (Figure 1.2). Human neutrophils express CEACAM1, 3 and 6 [43]. Although the majority of CEACAM-Opa interactions promote infection by facilitating penetration into host tissues and inhibiting activation of host immune cells, Opa binding to CEACAM3 leads to neutrophil activation, thus promoting phagocytic uptake of the *Ngo* [44]. Interestingly, CEACAM3 lacks the cell adhesion function of other CEACAMs and is expressed exclusively by human neutrophils, while other CEACAMs are expressed by numerous different cell types. For these reasons, CEACAM3 is thought to be a type of decoy receptor as it promotes phagocytosis of Opa-expressing bacteria. In addition to CEACAMs, *Ngo* express pili on their surface, which facilitate bacterial adherence to epithelium and promote bacterial colonization. Pili association with neutrophils may also promote phagocytosis of the bacteria [19], though it remains unclear whether this most benefits the host or the pathogen.

Through these mechanisms of attachment, *Ngo* associate with neutrophils in the genital tract. Evidence for this interaction is observed in pus exudates from infected individuals, wherein neutrophils can be observed directly interacting with *Ngo*. Surprisingly, however, *Ngo* can be cultured from these samples, suggesting that *Ngo* possess the ability to resist neutrophil killing [5]. Further, in vitro evidence suggests that *Ngo* can survive and replicate within neutrophils by expressing factors that inhibit killing by neutrophils [20]. Since these discoveries, several mechanisms employed by gonococci to inhibit killing by neutrophils have been identified. These mechanisms affect killing by limiting phagocytosis [31, 45], manipulating the phagosome [46], evading extracellular traps [47], suppressing ROS production [48], and protecting themselves from antimicrobial peptides [49].

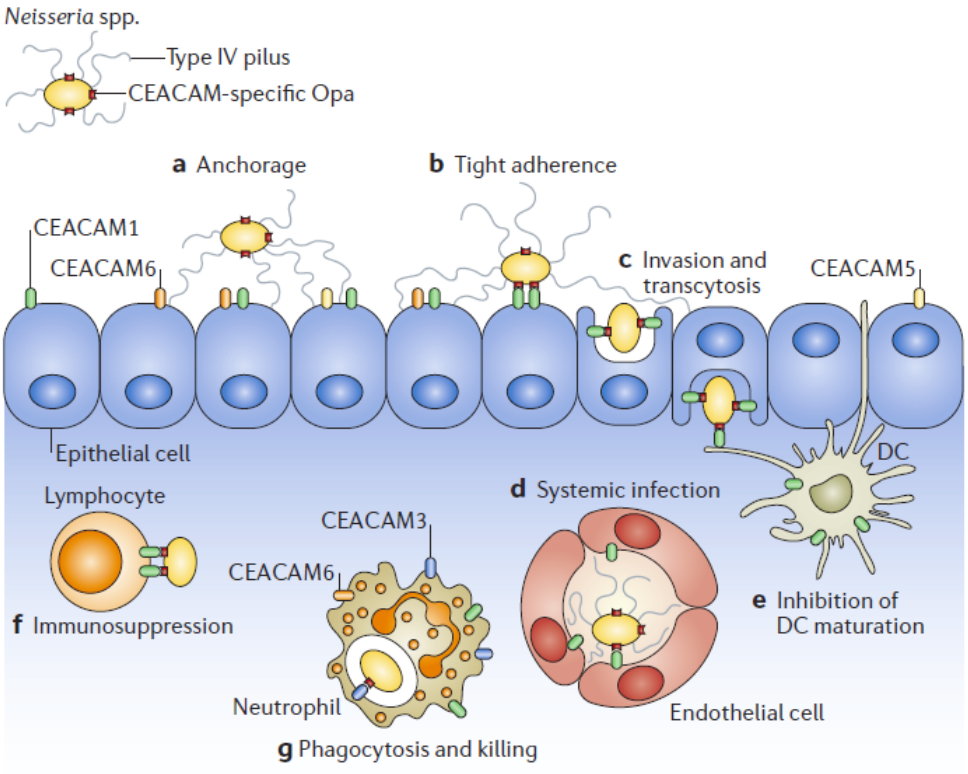


Figure 1.2: Gonococcal association with host cells. Type IV pili enable anchorage to the mucosal epithelium, and interaction with host cells. Opa proteins bind CEACAM 1, 5, and 6 on epithelial cells. Opa proteins can also bind CEACAMs present on leucocytes, including lymphocytes, dendritic cells, and neutrophils. (Figure taken with permission from Gray-Owen, S.D.)

The described evidence suggests that neutrophils play a major role during gonococcal infection, however, their contribution to bacterial clearance versus pathogenic inflammation during *Ngo* infection remain elusive. Work focused on the neutrophil response during PID is imperative to understanding their contribution to the development of the disease. Overall, understanding neutrophil heterogeneity in the context of PID may identify specific target neutrophil populations to prevent PID.

1.8 Thesis Objectives

Due to the challenges associated with studying ascending gonococcal infections in women, many questions remain unanswered about the immune response to *Ngo* in the UGT, as well factors contributing to PID severity and immunopathogenesis. Because the severity of ascending infections is variable, we hypothesized that the earliest immune responders dictate the balance between clearance of infection and immunopathogenesis, and ultimately, disease outcome. For this reason, the overall goal of this thesis was to use the mouse model of UGT infection to identify the first responders to *Ngo* infection, and to characterize the early immune response in the UGT so that we can understand factors contributing to immunopathogenesis.

Previously, it was demonstrated that *Ngo* infection in the UGT of FVB mice leads to a rapid recruitment of neutrophils to the site of infection, in a manner similar to what occurs in humans [14]. Additionally, there was a marked upregulation of pro-inflammatory cytokines in the mouse UGT as early as 6 hours post-infection, however the first responders responsible for this induction of cytokines and the resulting inflammatory response remained unknown. Further, the immunological microenvironment in the UGT during early infection, including the recruited leukocytes and their activation, remain unknown. Finally, the overall kinetics of infection in the mouse have yet to be studied.

For these reasons, I took advantage of a transgenic mouse line that expresses GFP upon the activation of NF-κB, which I term the NF-κB GFP reporter mouse line [50]. NF-κB is a rapidly inducible transcription factor functioning in a variety of both innate and adaptive inflammatory processes. During early infection, including during infections with *Ngo*, NF-κB regulates expression of many pro-inflammatory genes including proinflammatory cytokines [51, 52].

Therefore, I sought out to use NF-κB activation as a readout of early immune activation. The approach used in this thesis was to identify cells with active NF-κB to uncover the first responders to infection in the UGT, and ultimately to uncover cell populations playing an important role in contributing to the overzealous inflammatory response during UGT infections.

Using this model, this work reveals that the majority of immune cells recruited to the UGT post-infection are neutrophils and, unexpectedly, that only a fraction of the recruited neutrophils activates NF-κB. I then work to characterize differences between the NF-κB activated and non-activated neutrophil populations by assessing gonococcal association, neutrophil localization within the UGT, and the differential response of primed and resting state neutrophils. I show that the neutrophils may activate NF-κB regardless of whether or not they are associated with *Ngo*. I also demonstrate that the activated neutrophils are not specifically localized to either the lumen or the tissue of the UGT. Finally, I show that the majority of neutrophils recruited to the UGT are 'primed', which is consistent with their recruitment into the site of infection, but that only a subset of these primed neutrophils activates NF-κB.

Together, this work describes the neutrophil recruitment kinetics to gonococcal UGT infection and provides a clear indication that the infiltrating neutrophils are heterogeneous with respect to their NF-κB-driven response to infection.

Chapter 2
Methods

2 Methods

All animal procedures conducted in this study were approved by the Local Animal Care Committee (LACC) at the University of Toronto (Protocol #20011775), which is in compliance with ethical and legal requirements under Ontario's Animals for Research Act and the federal Canadian Council on Animal Care.

2.1 Mouse strains

6-12 week old female mice in the C57BL6 background containing a GFP transgene expressed upon activation of NF-κB[50] were used for all experiments unless otherwise stated. Transgenic mice in the C57BL6 background expressing human CEACAM1[53] (Tg418) and mice expressing human CEACAM3, 5, 6, and 7[54] (CEABAC10) were independently crossed with the NF-κB GFP reporter mice, which were used for the human CEACAM (hCEACAM) experiments.

2.2 Hormone Administration

All mice used in experiments were induced into the diestrus stage of the estrous cycle unless otherwise stated. To induce the diestrus stage of the cycle, mice were administered 2 mg medroxyprogesterone acetate/mouse (DepoProvera, Pfizer Canada Inc) in PBS subcutaneously 5 days prior to infection. To induce the estrus stage of the cycle, mice were administered 0.5 mg β-estradiol (Sigma-Aldrich) in PBS subcutaneously 2 days prior to infection. On the day of infection, vaginal lavages of the mice were taken, and the diestrus stage of the cycle was confirmed by the presence of neutrophils in the vaginal lavage. The estrus stage of the cycle was confirmed by the presence of cornified epithelial cells in the vaginal lavage.

2.3 Preparation of *Ngo*

A mouse-passaged strain of *Ngo* MS11 was used for all experiments unless otherwise specified. After 16-18 hours of growth on GC agar (BD Biosciences), *Ngo* was swabbed into 1 mL PBS++ (MultiCell), which contains magnesium and calcium, washed once to remove debris, and then diluted to a concentration of approximately $5x10^8$ colony forming units (CFU) per milliliter,

which was calculated by measuring the OD_{550}. For experiments in CEACAM transgenic mice, a recombinant *Ngo* strain MS11 derivative constitutively expressing the CEACAM1, 3, 5 and 6 binding Opa_{57} protein variant (strain N313) was used instead.

2.4 Fluorescent Labelling of *Ngo*

Ngo was prepared as described above. The bacteria were then resuspended in 0.01 mg/mL of Texas red succinimidyl ester (TRXSE)(Invitrogen) in 1 mL PBS++ pH 8.5, and incubated for 30 minutes at room temperature with shaking in the dark. The *Ngo* were washed 5 times in PBS++ ~pH 7.4 prior to a final wash in RPMI (MultiCell) with 10% fetal bovine serum to remove and then quench any remaining reactive substrate. The *Ngo* were then kept in a tube in the dark until infection.

2.5 Transcervical infections

Prior to infection, mice were anesthetized using 3% isoflurane inhalation. Once unconscious, mice were continuously administered 2% isoflurane until the completion of the infection procedure. The vagina was washed 3 times with 30 mL of PBS++ ~pH 7.4. To infect, the mice were placed at a 45-degree angle facing down, upon which a 25G blunt needle was inserted through the vagina into the uterine horn. Insertion into the uterine horn was identified by a decrease in resistance and ease of injection. Then, a suspension of ~10^7 *Ngo* in 20 µl of PBS++ ~pH 7.4 was inoculated into the uterine horn. At the indicated timepoints, the mice were euthanized via CO_2 inhalation followed by cardiac puncture. The genital tract was dissected out, and the cervix, vagina, and ovaries were removed (refer to figure 1.1). The uterine horns were then placed on ice into tubes contained RPMI and prepared for further experimentation.

2.6 Preparation of single cell suspensions from the UGT tissue

To prepare single cell suspensions from the mouse uterine horns, the uterine horns were physically minced into small pieces with scissors, after which they were incubated with 20 ug/mL DNase I (Sigma-Aldrich) and 2 mg/mL collagenase D (Roche) in RPMI with 10% fetal bovine serum. Tissues were digested for 1 hour at 37°C with shaking. Following digestion, tissue was passed through a cell strainer (40 µm pore size) while crushing with a plunger and rinsed thoroughly with RPMI with 10% fetal bovine serum.

2.7 Cell staining and flow cytometry with the general immune cell panel

Cells were stained in 100 µl volumes containing antibodies for the multi-colour immune cell panel (Table 1), 2 µl FC block (BD Biosciences), 50 µl BrilliantViolet buffer (BD Biosciences), and PBS (MultiCell) for 30 minutes on ice in the dark. Aliquots of the same cells were used to prepare the fluorescence minus one controls (FMO) for each antibody to establish appropriate flow cytometry gating. The cells were washed three times in PBS and resuspended in 100 µl volume of PBS containing 0.25 µl of prepared Fixable Live/Dead aqua (ThermoFisher Scientific) for 30 minutes on ice in the dark. The cells were then washed a further three times in PBS and then resuspended in 2% paraformaldehyde (PFA) for 20 minutes on ice in the dark. The cells were then washed in PBS and kept at 4°C until data acquisition. The samples were acquired within 24 hours of staining. Compensation controls were made using UltraComp eBeads Compensation Beads (Invitrogen), as well as with cells from infected tissue for the GFP compensation control.

Marker	Fluorophore	Clone
CD45	BV421	30F11
CD3	PE	17A2
CD19	APC-Cy7	6D5
Ly-6G	Pe-Cy7/BV785	1A8
CD11b	BV605	M1/70
F4/80	APC	BM8
Live/dead aqua	N/A	N/A
GFP	N/A	N/A
Ngo	Texas-red	N/A

Table 1: Flow cytometry panel for identification of immune cells activating NF-κB. All antibodies were supplied by BioLegend.

2.8 Data acquisition and analysis

Data were acquired using a BD LSR Fortessa or a BD LSR Fortessa X20. Data were analyzed using FlowJo version 10, using the basic gating strategies displayed in Figures 2.1 and 2.2. Gates were made based on fluorescence minus one controls. Graphs were generated in Prism (GraphPad).

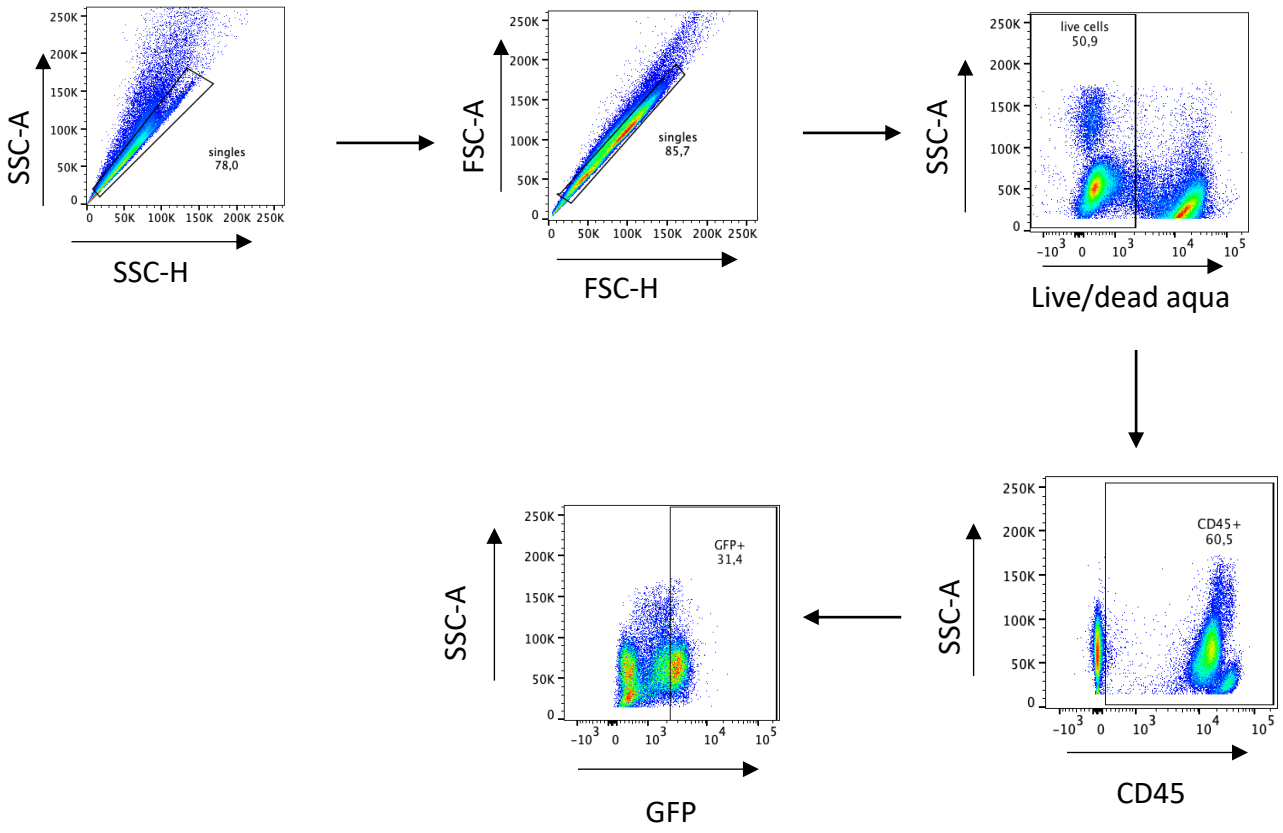


Figure 2.1: Gating strategy used to identify immune cell populations expressing GFP. In each case, gated cell populations (indicated with boxes) were selected for the analysis shown in the subsequent graph.

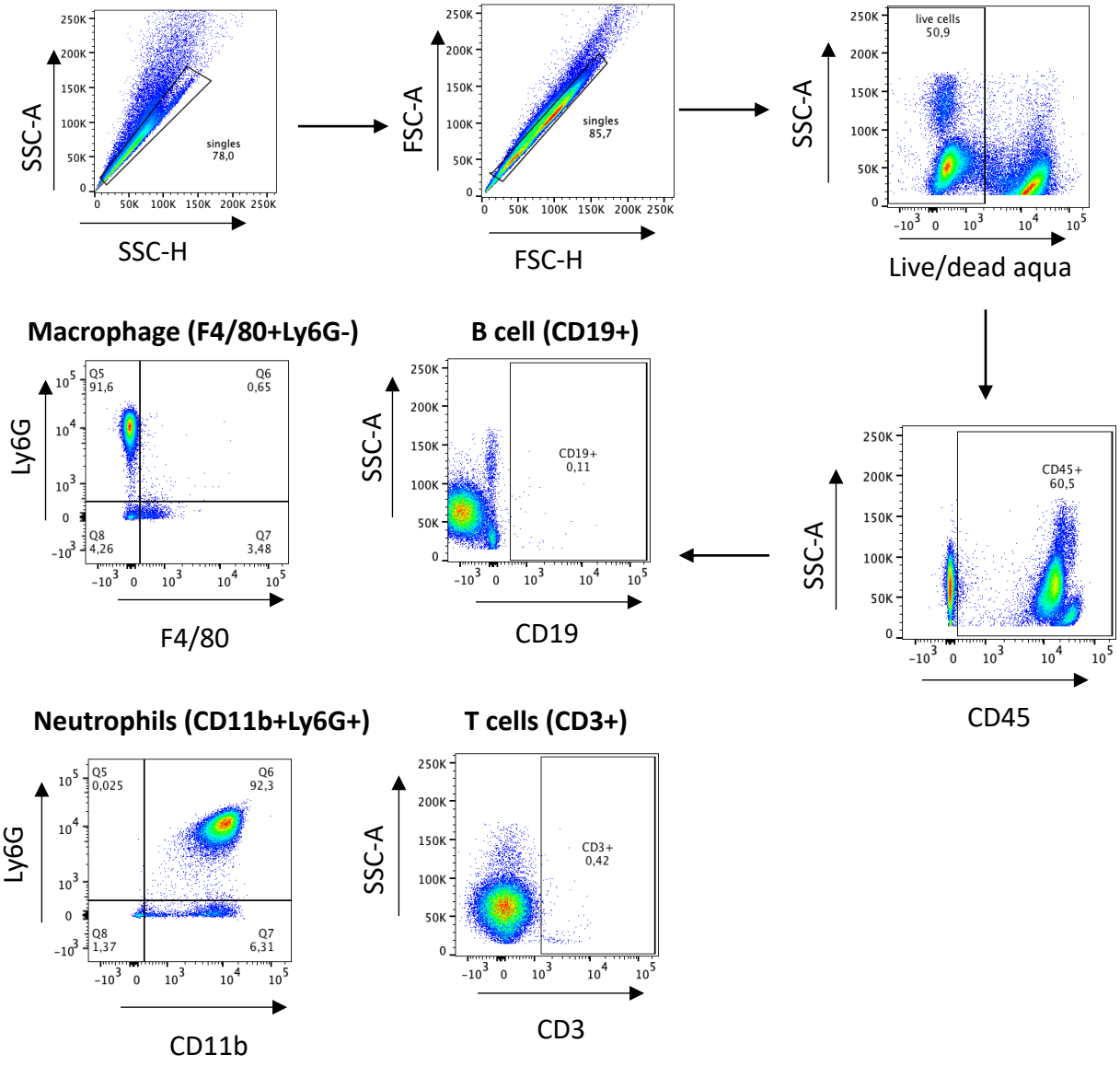


Figure 2.2: Gating strategy used to identify immune cell subsets present in the upper genital tract. In each case, gated cell populations (indicated with boxes) were selected for the analysis shown in the subsequent graph(s), except that specific cell populations were all selected from the SSC-A versus CD45 gate.

2.9 Identification of primed neutrophils

2.9.1 Cell isolation and fixation

Samples were collected as described [55]. Briefly, bone marrow, blood and UGT luminal lavages were collected at various timepoints post-infection. Bone marrow was collected from the hind limb of animals after their humane sacrifice by CO_2 inhalation. For blood collection, 200 µl was collected by cardiac puncture and placed into a tube already containing 22.2 µl EDTA to prevent clotting. 400 µl of PBS was added to each blood sample. For the uterine lavages, the UGT were dissected, distal tip removed to open each uterine horn, and 500 µl of PBS was passed through the lumen. All bone marrow, blood, and UGT lavage samples were fixed for 15 minutes in 1.6% methanol free formaldehyde (Thermo Scientific Pierce), and then washed with PBS. Red blood cells were lysed (Pharmlyse, BD Biosciences) in bone marrow derived cell suspensions and blood samples in a 10 mL volume for 5 minutes, spun down, and washed with PBS. Samples were then prepared for staining.

2.9.2 Staining

FC receptors were blocked using 2 µl FC block (BD Biosciences) in 100 µl PBS for 20 minutes on ice. Cells were then stained in 100 µl volume of FACS buffer (1x $HBSS^{-/-}$ (1litre) with 1% BSA and 2 mM EDTA) using the multicolour panel to identify primed neutrophils (Table 2). The cells were washed in PBS, and kept at 4°C until data acquisition. The samples were acquired within 24 hours of staining. Compensation controls were made using UltraComp eBeads Compensation Beads (Invitrogen), or with cells from infected tissue for the GFP compensation control.

2.9.3 Data acquisition and analysis

Data were acquired using either a BD LSR Fortessa or a BD LSR Fortessa X20. Data were analyzed using FlowJo version 10, according to the gating strategy in Figure 2.3. Gates were made based on fluorescence minus one controls. Graphs were generated in Prism (GraphPad).

Marker	Fluorophore	Clone
CD45	BV421	30F11
Ly-6G	BV785	1A8
CD11b	BV605	M170
CD193	PE	J073E5
CD5	PE-Cy7	53-7.3
GFP	N/A	N/A

Table 2: Flow cytometry panel for identification of primed neutrophils activating NF-κB. All antibodies were supplied by BioLegend.

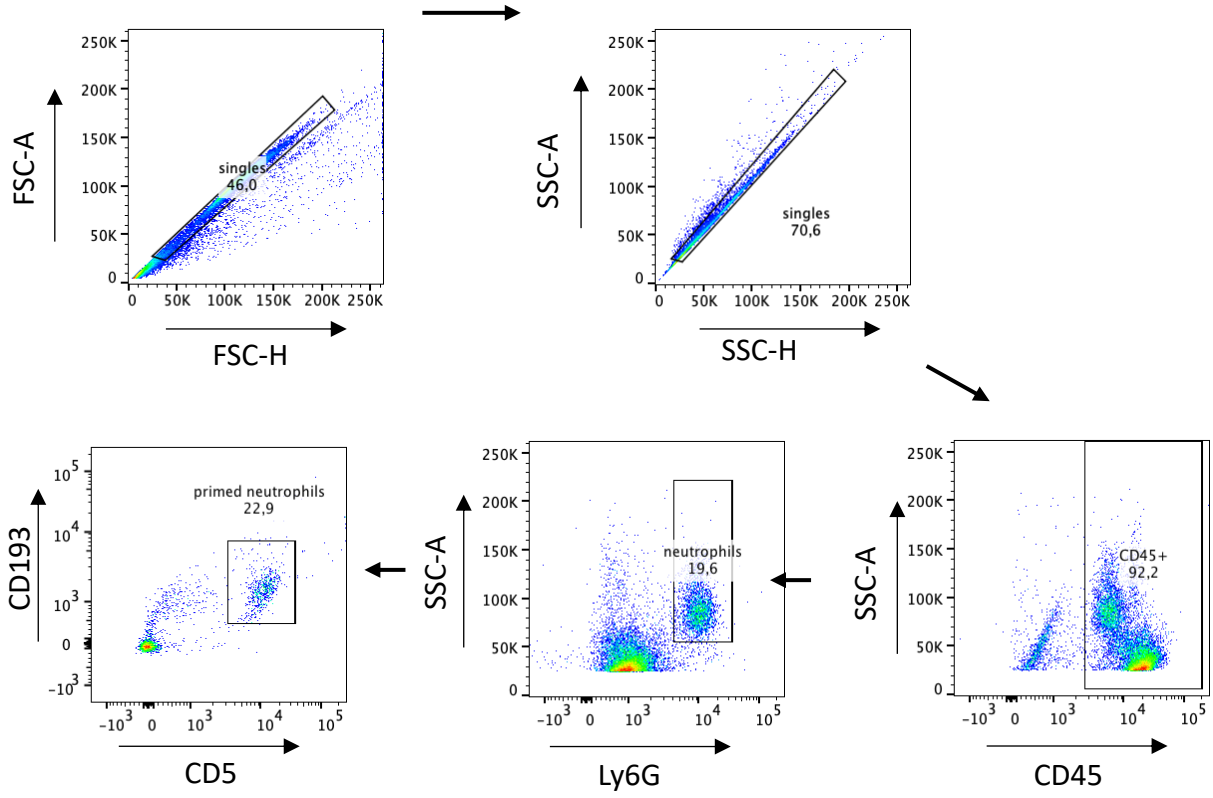


Figure 2.3: Gating strategy used to identify primed neutrophil populations. In each case, gated cell populations (indicated with boxes) were selected for the analysis shown in the subsequent graph.

2.10 Preparation and staining of frozen tissue sections

For tissue sections, the UGT of mice were dissected after infection and placed into 4% PFA for 10-24 hours. The samples were then washed twice in PBS, and placed in 30% sucrose in PBS for 24 hours [50], after which they were frozen using liquid nitrogen and stored at -80°C. Five µm sections were cut on a cryostat and mounted on charged slides. DAPI nuclear stain was then incubated on the sections for 5 minutes prior to washing and mounting in Molwiol mounting media.

2.11 Cytospins

After infection, the UGT of mice were dissected and the lumens were washed in 1 mL of RPMI. These luminal lavages were fixed in 2% PFA for 20 minutes, and then washed and resuspended in 300 µl of PBS. The samples were spun on to slides using Hettich ROTOFIX 32A. Slides were then DAPI stained for 5 minutes prior to washing and mounting in Mowiol mounting media.

2.12 Fluorescence microscopy

Images were taken using an Imager 2 (Zeiss) and analyzed using the Zen2 software.

2.13 Statistical analysis

All *p*-values were determined by unpaired two-tailed *t*-tests. $p \leq 0.05$ was considered statistically significant. All statistical analyses were performed using Prism (GraphPad).

Chapter 3
Results

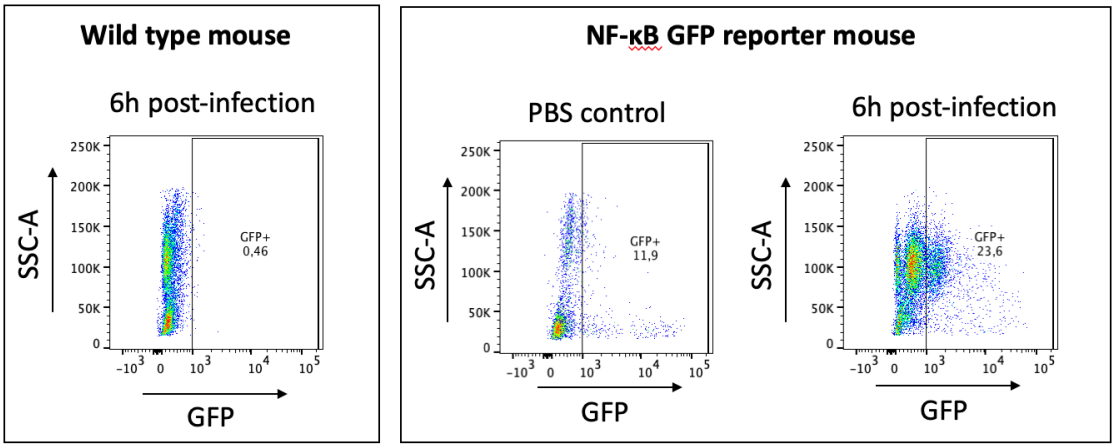
3 Results

3.1 Understanding the early immune response to *Ngo* infection

3.1.1 Validation of the model.

I first validated the use of this model by verifying that cells expressing NF-κB-driven GFP were apparent during uterine infection with *Ngo* when monitored via flow cytometry and microscopy. To do this, I compared wild type C57BL6 mice to the NF-κB GFP reporter mice in the C57BL6 background during *Ngo* infection in the UGT. GFP expression in uterine cells was assessed by flow cytometry at 6 hours post-infection, when bacterial burden is the highest [14]. Flow cytometric analysis revealed that a portion of the cells present in the uterine horns expressed GFP in the transgenic GFP reporter mice, but not the wild type mice (Figure 3.1A). Further, cryosections from infected mouse UGTs reveal a portion of cells expressing GFP in the NF-κB GFP reporter mice (Figure 3.1B). Together, these data validate the potential usefulness of this transgenic mouse as a tool for studying the early inflammatory response during gonococcal infections in the UGT.

A.



B.

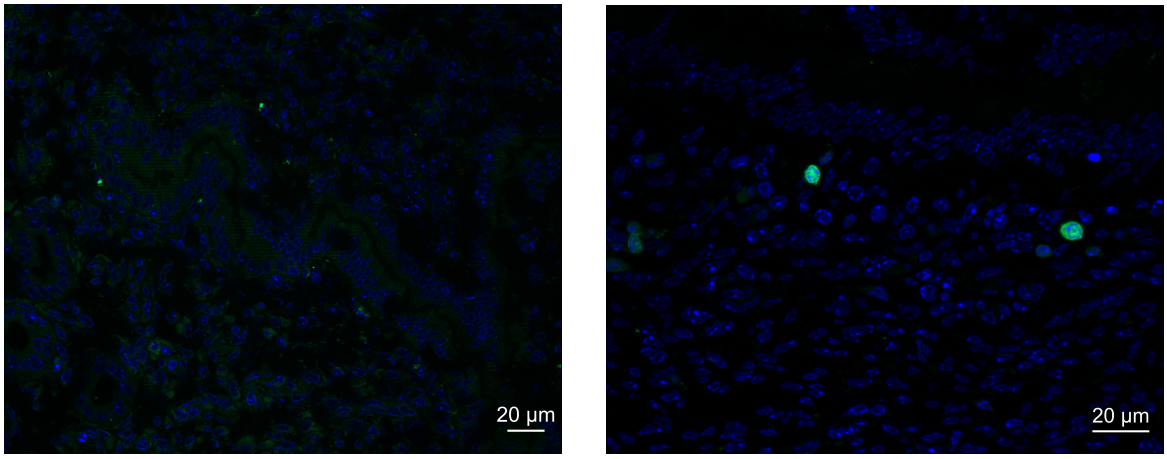


Figure 3.1: GFP expression in NF-κB GFP reporter mice can be observed via flow cytometry and microscopy. Wild type C57/BL6 mice and transgenic mice expressing GFP upon NF-κB activation in the C57BL6 background were transcervically infected with *Neisseria gonorrhoeae* and euthanized at 6 hours post-infection. **(A)** GFP expression in CD45+ cells isolated from the uterine horns of infected mice was analyzed by flow cytometry. Plots representative of 2-3 mice per group. **(B)** Cryosections of an infected upper genital tract 6 hours post-infection from a wild type C57/BL6 mouse (left) and an NF-κB GFP transgenic mouse (right). GFP expression assessed by fluorescence microscopy. GFP signal is in green, DAPI staining is in blue.

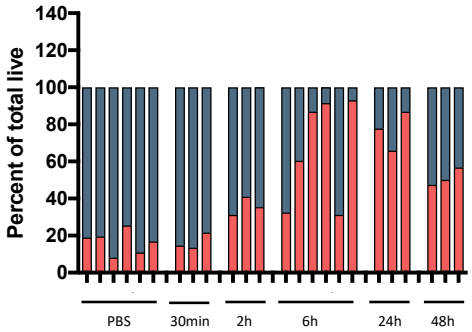
3.1.2 *Ngo* infection in the UGT results in inflammation that peaks at 6 hours post-infection and begins resolving by 48 hours post-infection.

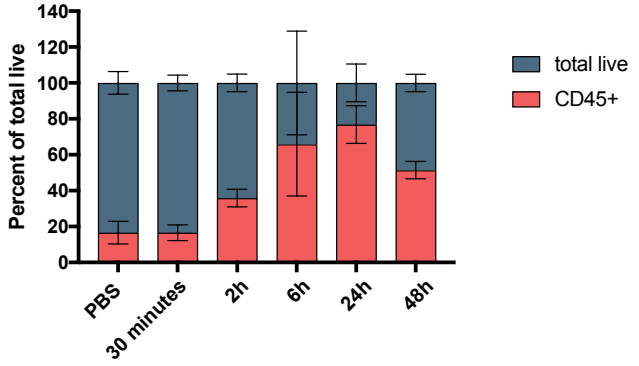
Massive neutrophil recruitment to the site of gonococcal infection has been characterized previously [14], but recruitment of other immune cells has not been explored in depth. To this end, I developed an immune cell-specific antibody panel in order to identify neutrophils, macrophages, T cells, and B cells in the uterine horn cells by flow cytometry. NF-κB GFP reporter mice were transcervically infected with *Ngo* or PBS, after which mice were euthanized at 30 minutes, 2 hours, 6 hours, 24 hours, and 48 hours post-infection to assess the kinetics of immune cell recruitment into the UGT. Using the immune cell panel described in Table 2.1, flow cytometric analysis of CD45, which is present on all immune cells, revealed that immune cell recruitment into the uterine horns occured by 2 hours post-infection, peaks at 6 hours post-infection, and begins resolving by 48 hours post-infection (Figure 3.2A, B).

Further analysis of CD45+ cells revealed that the majority of recruited immune cells were neutrophils (Figure 3.2C, D). The number of macrophages, the most abundant leukocyte recovered from uninfected animals, remained relatively stable throughout the infection. T and B cells were of very low abundance in uninfected animals and this did not change with infection.

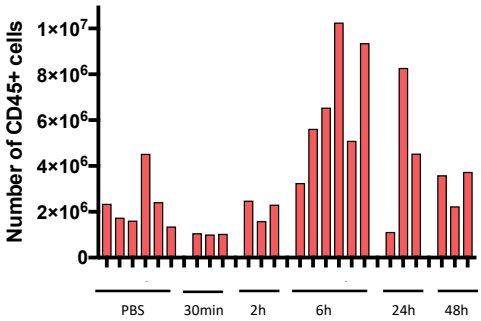
Importantly, I have repeated the 2- and 6-hour timepoints several times. I have been able to obtain consistent results at these timepoints, where, by pooling the data, I observed a significant increase in the proportion of immune cells present in the uterine horns both at 2- and 6- hours post-infection compared to the PBS control. I have also observed a significant increase between the proportion of immune cells present in the uterine horns at 6 hours compared to 2 hours post-infection (Figure 3.2E). Specifically, neutrophils were the only identified immune cell subset whose proportion significantly increased at 2- and 6- hours post infection compared to the PBS control group (Figure 3.2F).

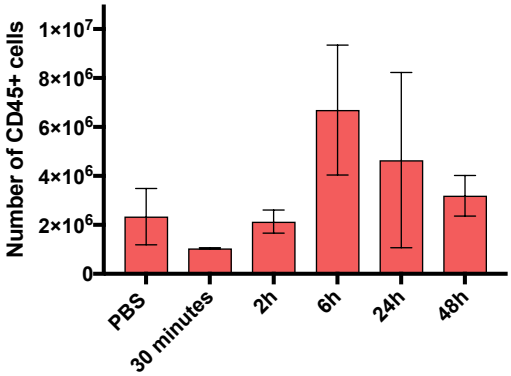
A.


Percent of total live
140
120
100
80
60
40
20
0
PBS
30min
2h
6h
24h
48h

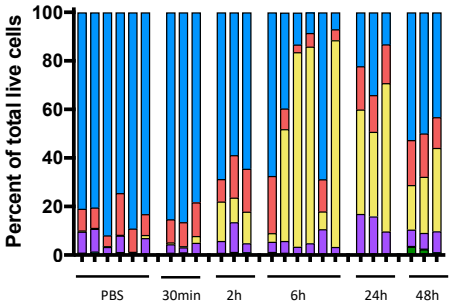

Percent of total live
140
120
100
80
60
40
20
0
PBS
30 minutes
2h
6h
24h
48h
total live
CD45+

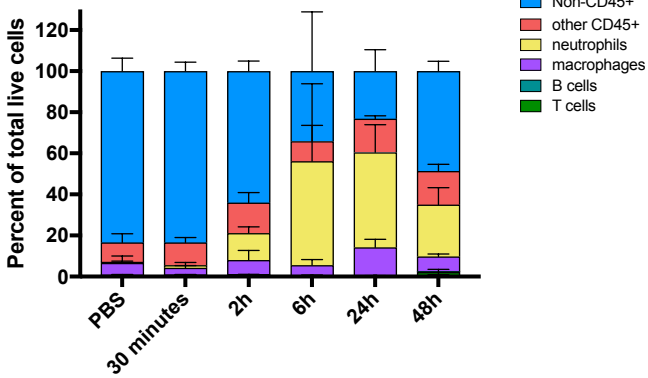
B.


Number of CD45+ cells
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
PBS
30min
2h
6h
24h
48h

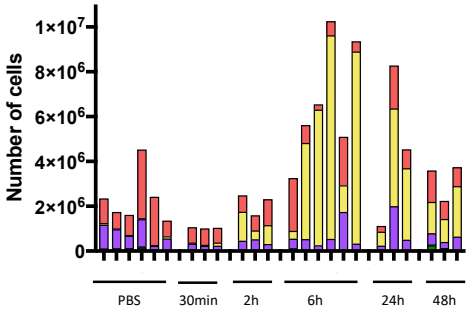

Number of CD45+ cells
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
PBS
30 minutes
2h
6h
24h
48h

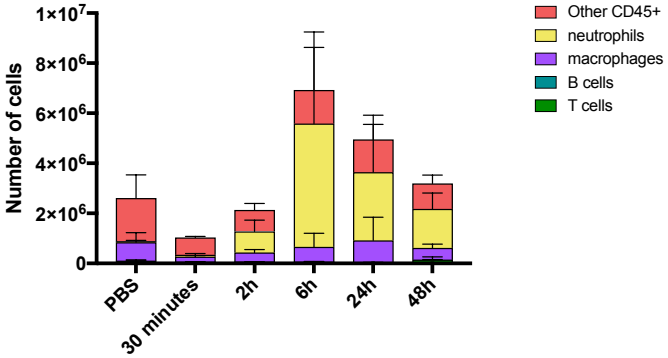
C.


Percent of total live cells
100
80
60
40
20
0
PBS
30min
2h
6h
24h
48h

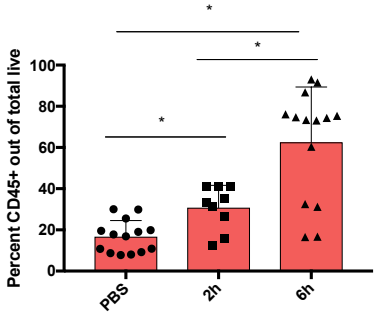

Percent of total live cells
120
100
80
60
40
20
0
PBS
30 minutes
2h
6h
24h
48h
Non-CD45+
other CD45+
neutrophils
macrophages
B cells
T cells

D.


Number of cells
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
PBS
30min
2h
6h
24h
48h


Number of cells
1×10^7
8×10^6
6×10^6
4×10^6
2×10^6
0
PBS
30 minutes
2h
6h
24h
48h
Other CD45+
neutrophils
macrophages
B cells
T cells

E.


Percent CD45+ out of total live
100
80
60
40
20
0
PBS
2h
6h
*
*
*

F.

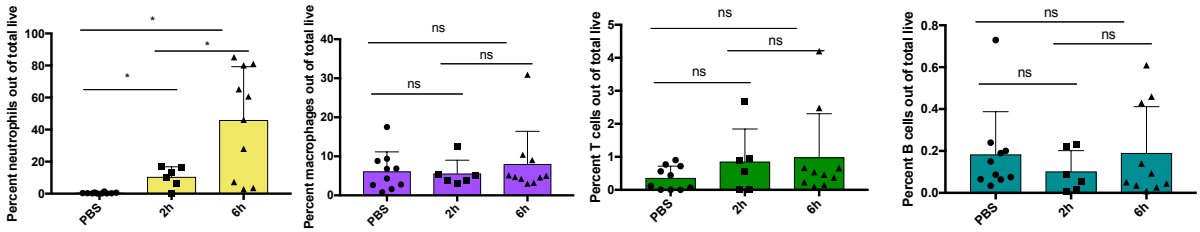


Figure 3.2: *Ngo* infection in the UGT results in inflammation that peaks at 6 hours post-infection and begins resolving by 48 hours post-infection. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Ngo* and euthanized at various timepoints post-infection. Immune cells from the uterine horns were analyzed by flow cytometry. Bars in graphs on the left represent individual mice, and bars in graphs on the right represent mean values of mice in each group, where error bars represent SD. **(A-B)** CD45+ cell recruitment into the uterine horns shown as a percent of total live cells **(A)** or total cell counts **(B)**. **(C-D)** Recruitment of immune cell subsets into the uterine horns post-infection shown as a percent of total live **(C)** and total cell counts **(D)**. **(E-F)** Pooled data from independent experiments showing **(E)** total CD45+ and **(F)** individual immune cell subsets graphed as a percent of total live cells in the uterine horns at 2- and 6- hours post-infection. Each point represents an individual mouse in each group. $p \leq 0.05$ (*t*-test) is indicated by '*'.

3.1.3 NF-κB gets activated in a subset of recruited immune cells in the UGT.

Next I assessed GFP expression in the recruited immune cells to determine which of the recruited cells were activating NF-κB (Figure 3.3A). It was previously discovered that several pro-inflammatory cytokines are upregulated as early as 6 hours post-*Ngo* infection in the UGT, both locally and systemically [14]. Yet, the first responders that produce these cytokines leading to the overall inflammatory response during early infection in the UGT were unidentified.

GFP expression in uterine horn cells was assessed at the various time points post-infection. At all timepoints post-infection, the majority of cells in the uterine horns expressing GFP were CD45+ (Figure 3.3B). Notably, out of the all of the recruited CD45+ cells, only a portion activated NF-κB (Figure 3.3C, D). Although there is variation in the amplitude of response among mice, this general response was consistent among all infected animals, which can be observed in the graphs with bars of individual mice.

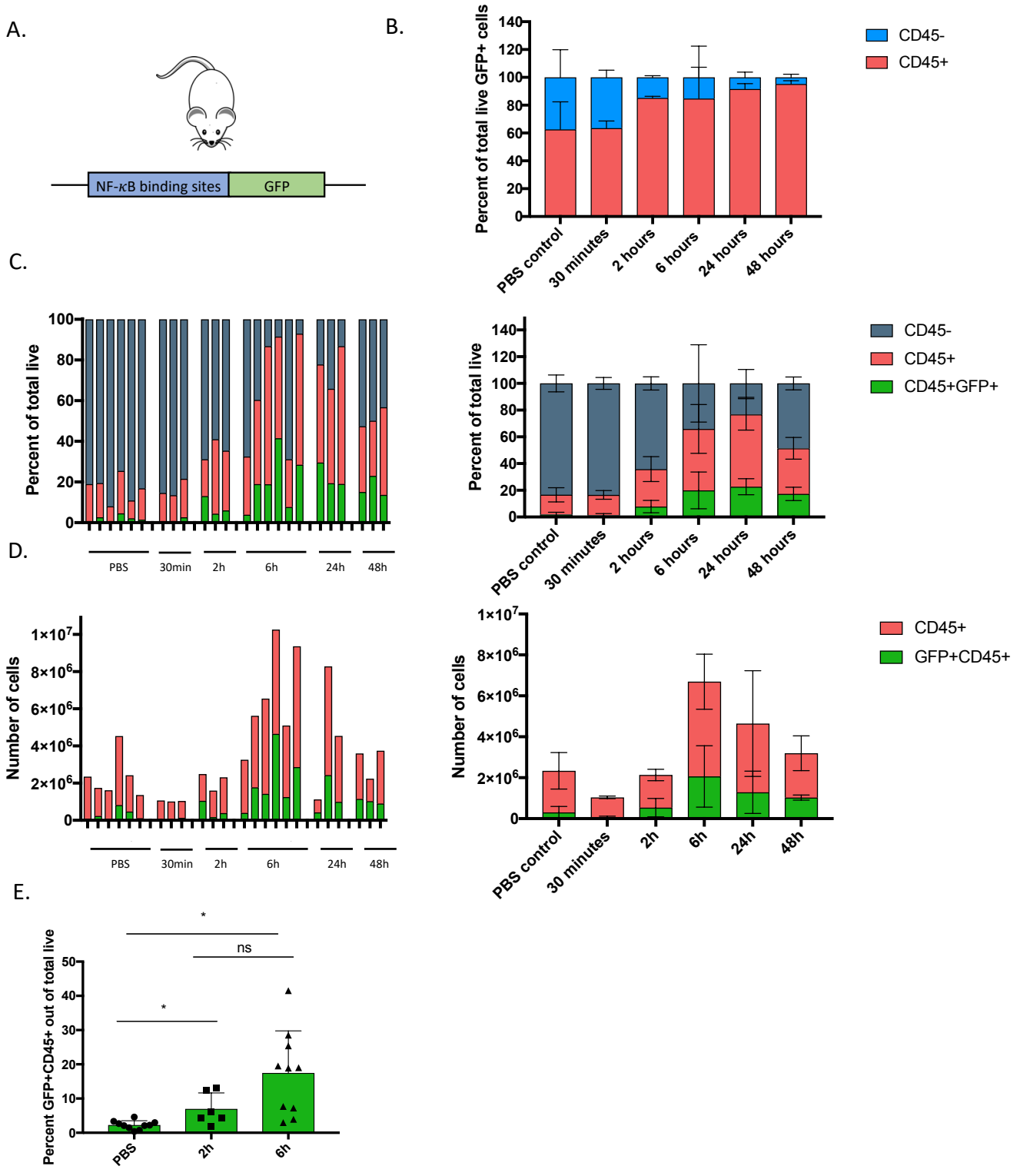


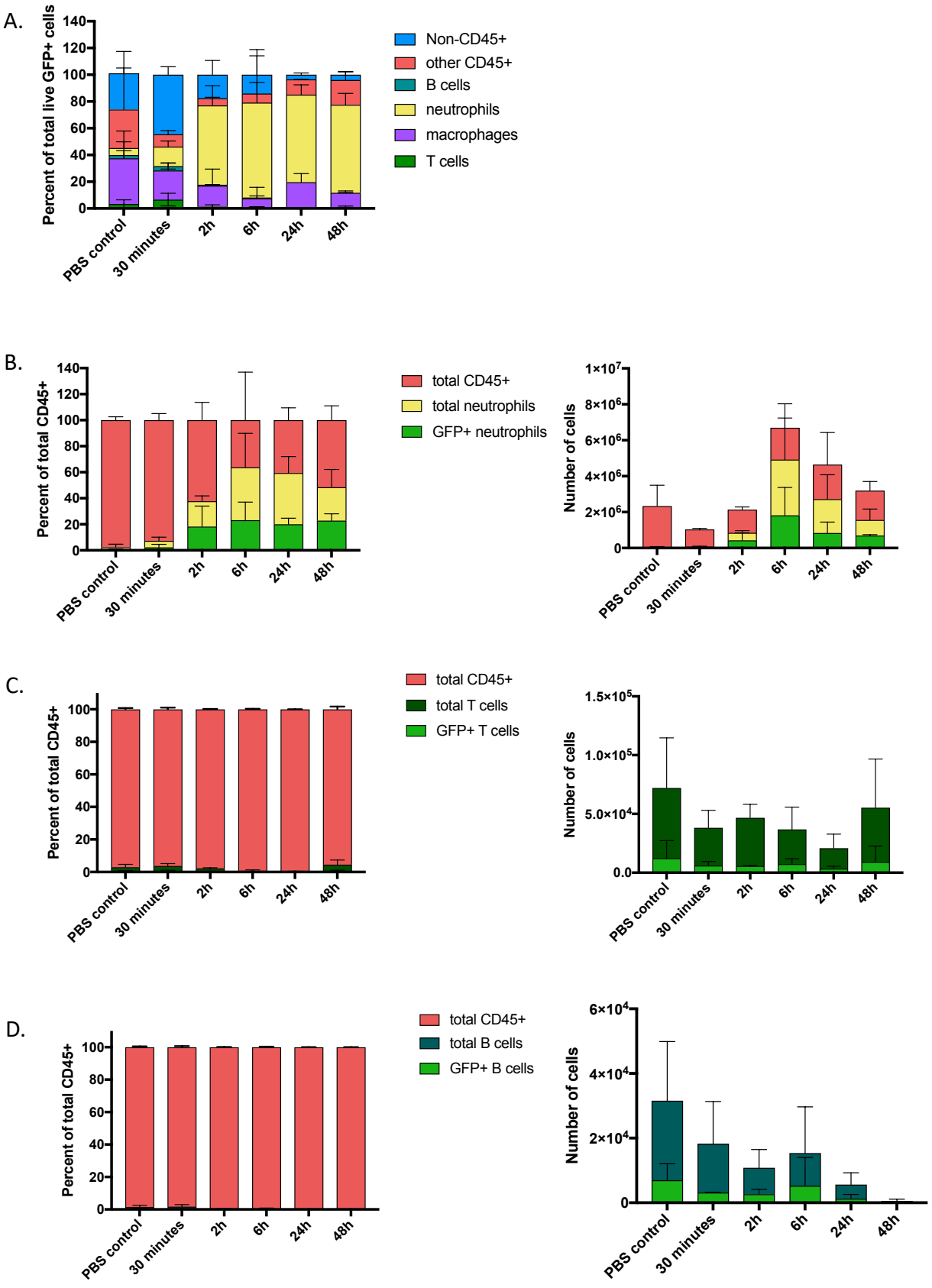
Figure 3.3: NF-κB gets activated in a subset of recruited immune cells in the upper genital tract post-infection. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Ngo* and euthanized at various timepoints post-infection. Uterine horn cell suspensions were analyzed by flow cytometry. **(A)** Graphical representation of the GFP mouse model. **(B)** GFP expressing cells that are CD45+ versus CD45-

at various timepoints post-infection, graphed as a percent of total live GFP+ cells. **(C-D)** CD45+ immune cell recruitment and NF-κB activation in uterine horn cells post-infection. Bars in graphs on the left represent individual mice, and bars in graphs on the right represent mean values of mice in each group, where error bars represent SD. Data graphed as a percent of total live cells **(C)** and total cell counts **(D)**. **(E)** Pooled data from three independent experiments showing GFP expression in CD45+ cells from the uterine horns at 2- and 6- hours post-infection. Data graphed as a percent of GFP+CD45+ out of total live cells. Each point represents an individual mouse in each group. $p \leq 0.05$ (*t*-test) is indicated by '*'.

3.1.4 Neutrophils are the primary cell population with active NF-κB.

To identify which specific cell types were activating NF-κB, I further characterized CD45+GFP+ cells as either neutrophils, macrophages, B cells or T cells, following the gating strategy in Figure 2.2. After transcervical infection with *Ngo*, the majority of cells that activated NF-κB in the uterine horns at all time points were neutrophils. There was very little change in the proportion of T cells or B cells that express GFP over this time period (Figure 3.4C-D), but there was a notable fraction of macrophage that became GFP+ (Figure 3.4E). There was also a population of immune cells expressing GFP that were not T cells, B cells, neutrophils or macrophages. This population was termed 'other CD45+' (Figure 3.4A).

Despite a massive influx of neutrophils into the uterine horns, many of the recruited neutrophils did not activate NF-κB (Figure 3.4B). This does not seem to be due to the need for an extended period before the neutrophils become activated after entering the tissues, since the proportion of neutrophils expressing GFP, which was between 20-30%, remained consistent at all the time points post-infection (Figure 3.4B). Considering recent evidence demonstrating the heterogeneity of neutrophil populations [56], these data make it enticing to consider that 'NF-κB active' and 'NF-κB non-active' neutrophils may represent different neutrophil phenotypes responding to the infection.


A.
Percent of total live GFP+ cells
Non-CD45+
other CD45+
B cells
neutrophils
macrophages
T cells
PBS control
30 minutes
2h
6h
24h
48h
B.
Percent of total CD45+
total CD45+
total neutrophils
GFP+ neutrophils
Number of cells
C.
Percent of total CD45+
total CD45+
total T cells
GFP+ T cells
Number of cells
D.
Percent of total CD45+
total CD45+
total B cells
GFP+ B cells
Number of cells

E.

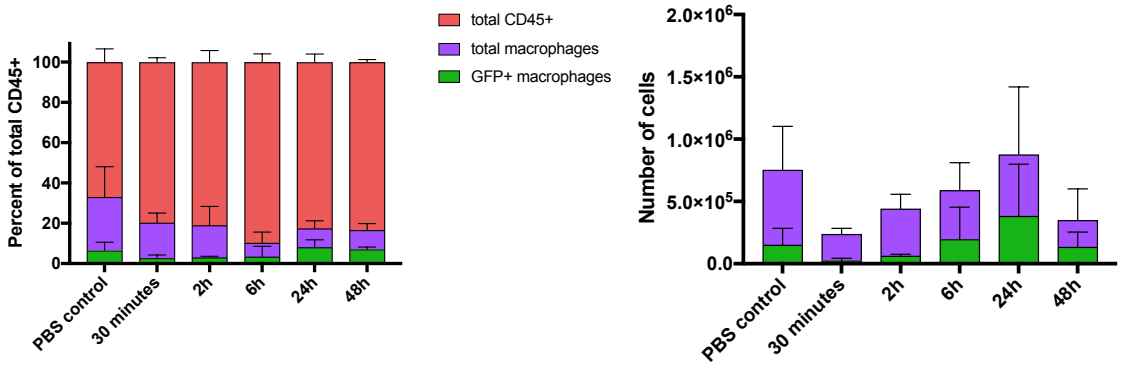


Figure 3.4: Neutrophils are the primary cell population with active NF-κB. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Neisseria gonorrhoeae* and euthanized at various time points post-infection. Uterine horn cell suspensions were analyzed by flow cytometry. **(A)** Immune cell subsets expressing GFP graphed as a percent of total live GFP+ cells. **(B-E)** GFP expression in immune cell subsets as a percent of total CD45+ (left) or total cell counts (right) in **(B)** neutrophils, **(C)** T cells, **(D)** B cells, and **(E)** macrophages. Note the different y axis scales for different cell types.

3.2 Characterizing the neutrophil response during *Ngo* infection.

3.2.1 GFP expressing neutrophils are present in both the uterine lumen and tissue.

Since neutrophils appeared to play a significant role as first responders during *Ngo* infection in the UGT, I wanted to further study this neutrophil response to assess what may be differentiating the 'NF-κB activating' and 'NF-κB non-activating' neutrophils.

Ngo is inoculated into the lumen of the uterine horns during UGT infection. I proposed that neutrophils in the uterine lumen, and not tissue associated neutrophils, may be more likely to come in direct contact with the gonococci, and therefore be more likely to activate NF-κB. This would explain why only a fraction of neutrophils present in the infected uterine horns were activating NF-κB and would help explain the heterogeneous neutrophil response during infection.

To assess this, I compared immune cell recruitment and activation in the uterine tissue compared to the uterine lumen. NF-κB GFP reporter mice were transcervically infected with *Ngo*, and at 6 hours post-infection, I separately recovered uterine luminal lavages and uterine tissue for analysis. Flow cytometric analysis revealed very few cells in uninfected uterine luminal lavages compared to that in the uninfected uterine tissue, which included a small proportion of various types of immune cells including macrophages, neutrophils, and 'other CD45+' cells (Figure 3.5A, PBS samples). At 6 hours post-infection with *Ngo*, there was a dramatic increase in neutrophils present in both the uterine tissue and the uterine lumen, consistent with their rapid recruitment to the infected tissue and passage across the epithelia. Notably, the immune cells present in the lumen were almost exclusively neutrophils, while the uterine tissues showed that the increase in neutrophils occurred without a concomitant increase in other leukocyte populations (Figure 3.5A, Infected). The rapid movement of neutrophils directly into the lumen reflects the purulent discharge in patients with gonorrhea, and may be a contributing factor to immunopathogenesis.

To address whether the neutrophils displaying an active NF-κB response are specifically localized to the lumen, I assessed GFP expression in the tissue versus the uterine lavages. While there were more CD45+ cells in the tissue of infected animals, I observed that there were

actually similar numbers of GFP-expressing immune cells in both the uterine horn tissue and lumen (Figure 3.5B). Consistent with my prior observations, the majority of these NF-κB activated immune cells were neutrophils. However, perhaps surprisingly, the same proportion of neutrophils were GFP+ regardless of whether they were recovered from the lumen or the tissues (Figure 3.5C). The presence of NF-κB activated neutrophils in both the uterine lumen and tissue suggests that NF-κB activation and subsequent cytokine production is not necessarily restricted to neutrophils in the lumen during *Ngo* infection.

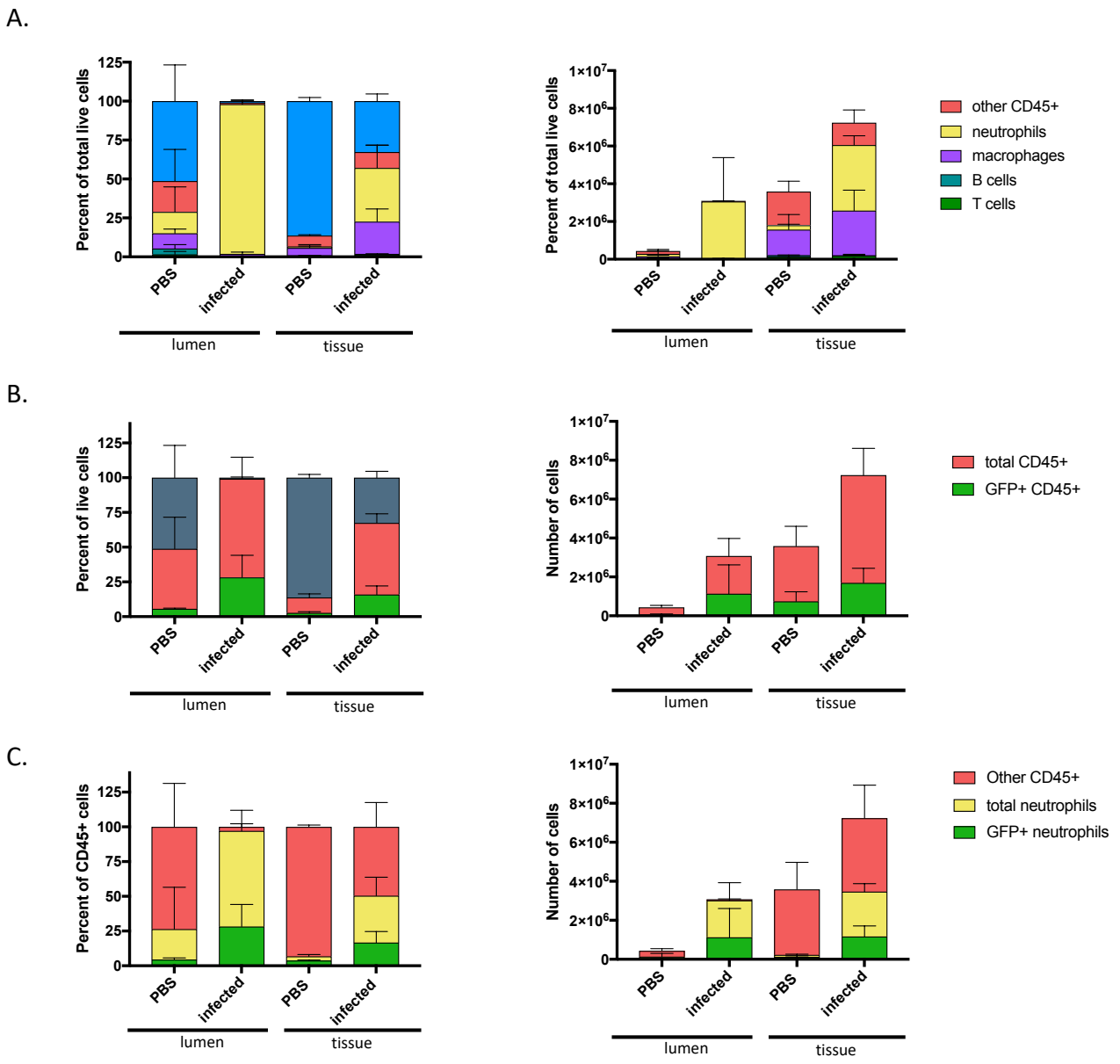


Figure 3.5: GFP expressing neutrophils are present in both the uterine lumen and tissue. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Ngo* and euthanized at 6 hours post-infection. Uterine horns were washed with PBS to dissociated luminal cells from tissue cells. Data were analyzed using flow cytometry. **(A)** Immune cell subset recruitment into the lumen and tissue, plotted as a percent of total live (left) and total cell count (right). **(B)** CD45+ cell recruitment and NF-κB activation into the upper genital tract at 6 hours post-infection. Plotted as a percent of total live cells (left) and total cell counts (right). **(C)** Neutrophil recruitment and NF-κB activation in the upper genital tract 6 hours post-infection, plotted as percent of total CD45+ cells (left) and total cell counts (right). Bars represent mean of mice in each group. Error bars represent SD.

3.2.2 The majority of neutrophils do not associate with *Ngo*, and those that do are not necessarily activating NF-κB.

Gram stains of pus exudates from *Ngo*-infected individuals reveal a large number of neutrophils present at the site of infection, some of which have engulfed multiple gonococci, however the majority of neutrophils in these exudates are surprisingly not associated with any bacteria [19]. While it has often been considered that the neutrophils without bacteria had simply not encountered them, my observation that only certain neutrophils display an active NF-κB-driven transcriptional response at the infection site prompted me to consider that these neutrophils may actually be of a distinct phenotype that does not respond to the gonococci.

To test whether the NF-κB activating neutrophils may be those that are associating with *Ngo*, I utilized an amine reactive dye, termed TRXSE, to label *Ngo* prior to infection. Mice were euthanized at 2- and 6- hours after transcervical infection with the pre-labeled gonococci into the NF-κB GFP reporter mice. Following tissue processing, I stained the uterine horn cells with the described immune cell panel and acquired data by flow cytometry. Although there was some background autofluorescence leading to a small percentage of cells in the red fluorescent channel in the PBS control groups, there was an increase in the percentage of TRXSE+ neutrophils in the infected group, and these cells tended to be brighter than those apparent from the uninfected animals (Figure 3.6A, C). At both 2- and 6-hours post-infection, the majority of immune cells in the uterine horns associated with *Ngo* were neutrophils (Figure 3.6B). However, despite a large influx of neutrophils into the genital tract by 6 hours post-infection with a dense inoculum (~10^7 bacteria in 20 µl per mouse), most of these neutrophils were not associated with *Ngo* (Figure 3.6C), which was consistent with what is observed during human infections. Notably, most of the neutrophils associated with *Ngo* did not display activated NF-κB and, conversely, most of the neutrophils with active NF-κB did not have associated gonococci (Figure 3.6D). These findings suggest that the initiation of an NF-κB response occurs independent of gonococcal binding.

To validate these findings, and to confirm that the TRXSE signal was indeed due to gonococcal association and not background fluorescence, I performed microscopy on cytospins of cells taken from the uterine horns of infected mice at 2 hours post-infection. The images revealed GFP+ cells, likely neutrophils, however consistent with the flow cytometry data, these cells were not associated with gonococci (Figure 3.7E). I also observed many gonococcal associated cells that were not GFP+ (figure 3.7E).

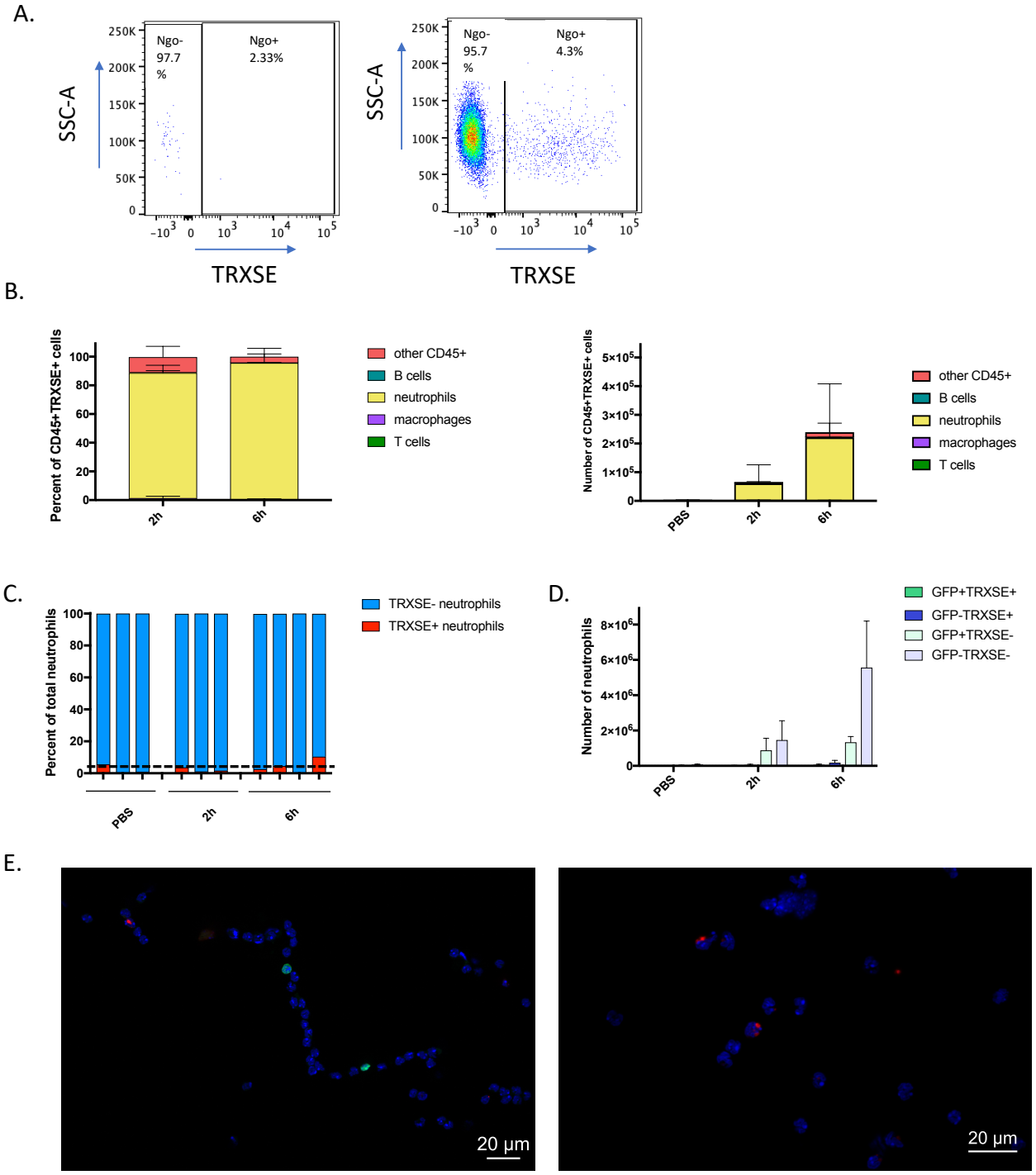


Figure 3.6: The majority of neutrophils do not associate with *Neisseria gonorrhoeae*, and those that do are not necessarily activating NF-κB. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with Texas Red X (TRXSE) pre-labelled *Ngo* and then euthanized at 2- and 6-hours post-infection. **(A-D)** Uterine horn cells were analyzed by flow cytometry. 3-4 mice per group. Error bars represent SD. **(A)** Representative dot plots for PBS control (left) and 6 hours post-infection (right) gated on

neutrophils as outlined in Figure 2.2, with gonococcal association reflected on the x axis. **(B)** Immune cell subsets associating with *Ngo,* graphed as a percent of total TRXSE+ cells (left) and total cell counts (right). **(C)** Neutrophil association with *Ngo* (TRXSE+) as a percent of total neutrophils in the upper genital tract. Dotted line represents the highest percentage of TRXSE+ cells in the PBS control group. **(D)** Comparison of GFP expression and gonococcal association (TRXSE+) in neutrophils from the upper genital tract graphed as absolute cell counts. **(E)** Cytospin of luminal lavages from infected mice at 2 hours post-infection. TRXSE labelled *Ngo* in red, GFP-expressing cells in green, and DAPI nuclear stain in blue.

3.2.3 The majority of neutrophils associate with *Ngo* in the uterine lumen

To determine where the neutrophils capture gonococci and whether this location correlates with a difference in NF-κB response, I compared gonococcal association with immune cells in the luminal lavage compared to that occurring within the uterine tissue 6 hours after transcervical infection with *Ngo*. Again, I saw that there was some background fluorescence leading to a small percentage of cells falling in the 'TRXSE+' gate in the PBS control group, but overall there was an increase in the percentage of TRXSE+ cells in the infected group as compared to the PBS control group (Figure 3.7A). The majority of gonococcal-associated immune cells in both the tissue and lumen were neutrophils (Figure 3.7B). However, there were, on average, more gonococcal-associated neutrophils in the lumen compared to the tissue (Figure 3.7C). This suggests that most gonococci encounter the neutrophils within the luminal environment. Importantly, most of the neutrophils that were associated with *Ngo* in both compartments did not display activated NF-κB, and most of the GFP+ neutrophils were not associated with *Ngo* in both the lumen and the tissue (Figure 3.7D).

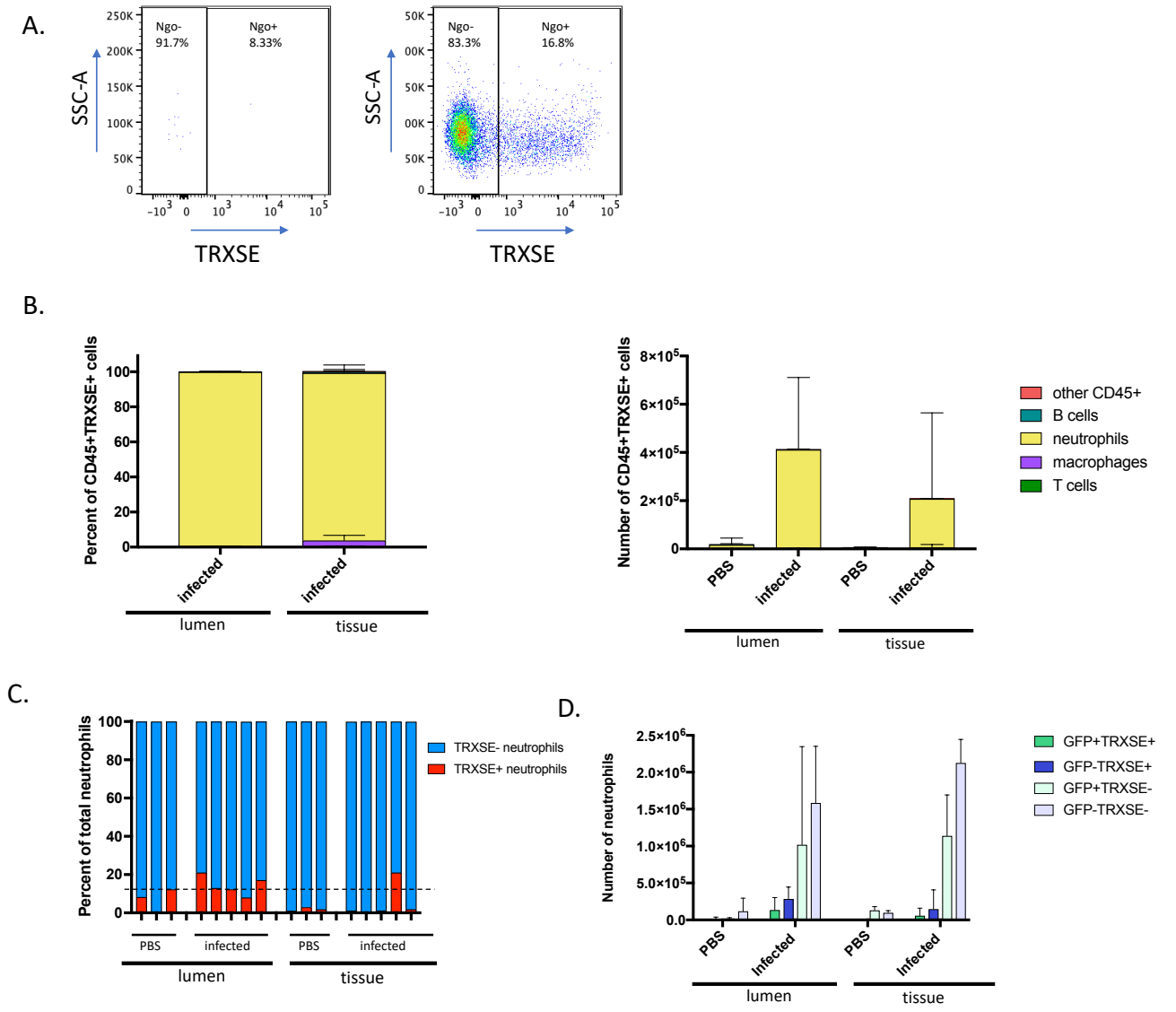


Figure 3.7: Most gonococcal-associated neutrophils are present in the uterine lumen. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with pre-labelled (TRXSE) *Neisseria gonorrhoeae (Ngo)* and euthanized at 6 hours post-infection. Uterine horns were washed with PBS to obtain luminal cells, and uterine tissue was processed separately. **(A-D)** Data analyzed by flow cytometry. 3-5 mice per group. Error bars represent SD. **(A)** Representative dot plots for PBS control (left) and luminal lavage 6 hours post-infection (right) gated on neutrophils, with TRXSE on the x axis. **(B)** Immune cell subsets associating with *Ngo*, graphed as a percent of total CD45+TRXSE+ cells (left) and total CD45+TRXSE+ cell counts (right). **(C)** Neutrophils association with *Ngo* as a percent of total neutrophils in the upper genital tract. Dotted line represents the highest percentage of TRXSE+ cells within the PBS control group. **(D)** Comparison of GFP expression and gonococcal association in neutrophils from the upper genital tract graphed as total cell counts.

3.2.4 Assessing primed neutrophil populations during *Ngo* infection.

A recent report by Fine et. al [42] suggests that neutrophils become 'primed' when danger signals emanate from damaged or infected tissues, presumably allowing them to more effectively extravasate and respond to the threat. They distinguish this 'primed' neutrophil subset from the resting state subset by expression of CD66a^{hi}, CD5, CD193, and CD11b^{hi}. This prompted me to assess whether the differential GFP response was reflective of the primed state of neutrophils recruited to the *Ngo*-infected uterus. Given that the priming event can happen prior to developing neutrophils exiting the bone marrow to enter the circulation, I aimed to determine whether *Ngo* infection in the UGT would influence the proportion of neutrophils in a resting versus primed state in the blood, bone marrow, and genital tract, and to determine whether the primed neutrophils within the genital tract were the subset with an active NF-κB response.

To answer these questions, I examined primed neutrophil recruitment and NF-κB activation in the UGT at various time points post-infection. The NF-κB reporter mice were transcervically infected with *Ngo*, euthanized at 30 minutes, 2 hours, and 6 hours post-infection, and their bone marrow, blood, and UGT luminal washes were stained for markers of primed neutrophils (Table 2). Flow cytometric analysis revealed a small population of primed neutrophils in the bone marrow and blood in the uninfected controls, and this remained relatively consistent throughout the early stages of infection (Figure 3.8A, B). In contrast to the blood and bone marrow, the majority of neutrophils present in the lumen of the UGT were of the primed phenotype, even in the uninfected control mice (Figure 3.8C). Further, there was a small increase in the percentage of primed neutrophils in the uterine lumen at the 30 minutes post-infection.

Next, I assessed GFP expression in the primed versus resting state neutrophils. Flow cytometric analysis of luminal washes revealed that similarly sized populations of resting and primed neutrophils had active NF-κB (Figure 3.8D). This suggests that NF-κB activation is not restricted to the primed neutrophil subset.

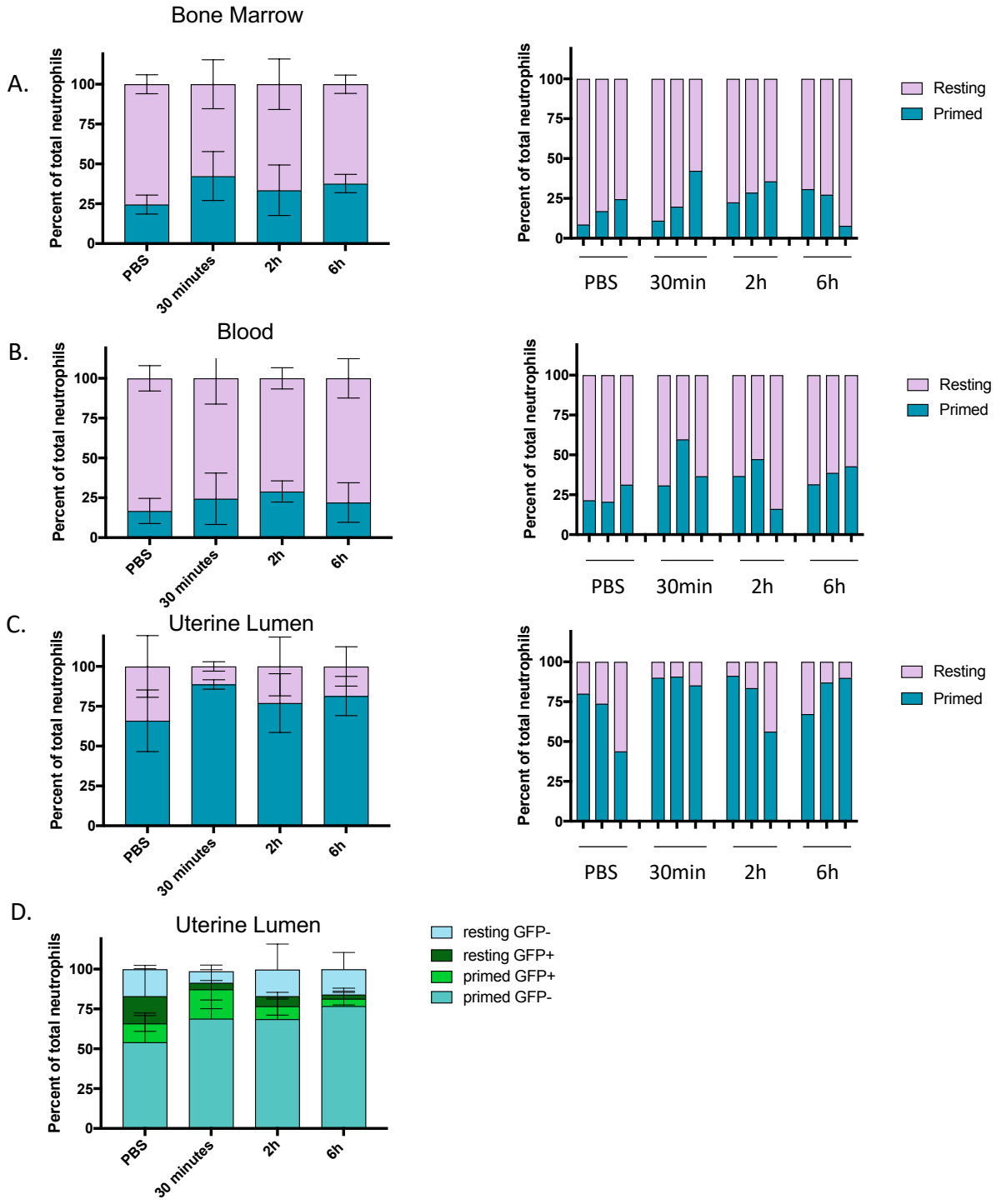


Figure 3.8: Primed neutrophils are recruited to uterine horns post-infection with *Neisseria gonorrhoeae*. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Ngo* and euthanized at 30 minutes, 2 hours, and 6 hours post-infection. Data analyzed by flow cytometry. 3 mice per group. **(A-C)** Primed and resting state neutrophil populations are shown as a mean of percent of total neutrophils in **(A)** bone marrow, **(B)** blood, and **(C)** upper genital tract luminal wash. Bars in graphs on the left represent mean values of mice in each group, where error bars represent SD. Bars in graphs on the right represent individual mice. **(D)** Comparison of resting and primed neutrophil subtypes with GFP expression graphed as a percent of total neutrophils.

Chapter 4
Discussion and Future Directions

4 Discussion and Future Directions

4.1 Discussion

4.1.1 Summary of findings.

Infection of the UGT with *Ngo* can cause a severe inflammatory response resulting in PID. Despite the global burden of *Ngo*-induced PID, the factors contributing to disease pathogenesis remain poorly understood. I hypothesized that the early immune responders dictate the outcome and severity of infection. Therefore, in this work, I took advantage of the mouse model of UGT infection in order to characterize the early immune response to *Ngo* in an attempt to identify factors that may be contributing to PID pathogenesis.

My data revealed the kinetics of the inflammatory response in the mouse model of infection, showing that peak inflammation in the UGT occurs by 6 hours post-infection, and resolution of inflammation by 48 hours post-infection. I showed that the majority of immune cells recruited into the UGT post-infection are neutrophils, and that neutrophils are also the major cell type displaying an active NF-κB response. Interestingly, only a fraction of the recruited neutrophils activated NF-κB. This led me to hypothesize that the NF-κB activated and non-activated neutrophils may represent different neutrophil subsets.

To further investigate this, I assessed neutrophil localization within the UGT and their association with *Ngo*. My data revealed that NF-κB activated neutrophils were not specifically localized to the uterine tissue, but rather were distributed throughout the uterine tissue and lumen. I showed that not all *Ngo*-associated neutrophils had activated NF-κB, and that not all neutrophils activating NF-κB associated directly with *Ngo*.

Finally, I assessed primed versus resting neutrophil populations in the bone marrow, blood, and UGT lumen at various timepoints post-infection. I saw that there was a small population of primed neutrophils in the bone marrow and blood, while the majority of neutrophils in the UGT were of the primed phenotype. Throughout infection, I did not see any large changes in the percentage of primed versus resting neutrophils in either the bone marrow or blood, and only a

modest increase was apparent within the UGT. A similar percentage of GFP-expressing cells were of the resting and primed phenotype. This indicates that priming is not a prerequisite for neutrophil induction of their NF-κB response.

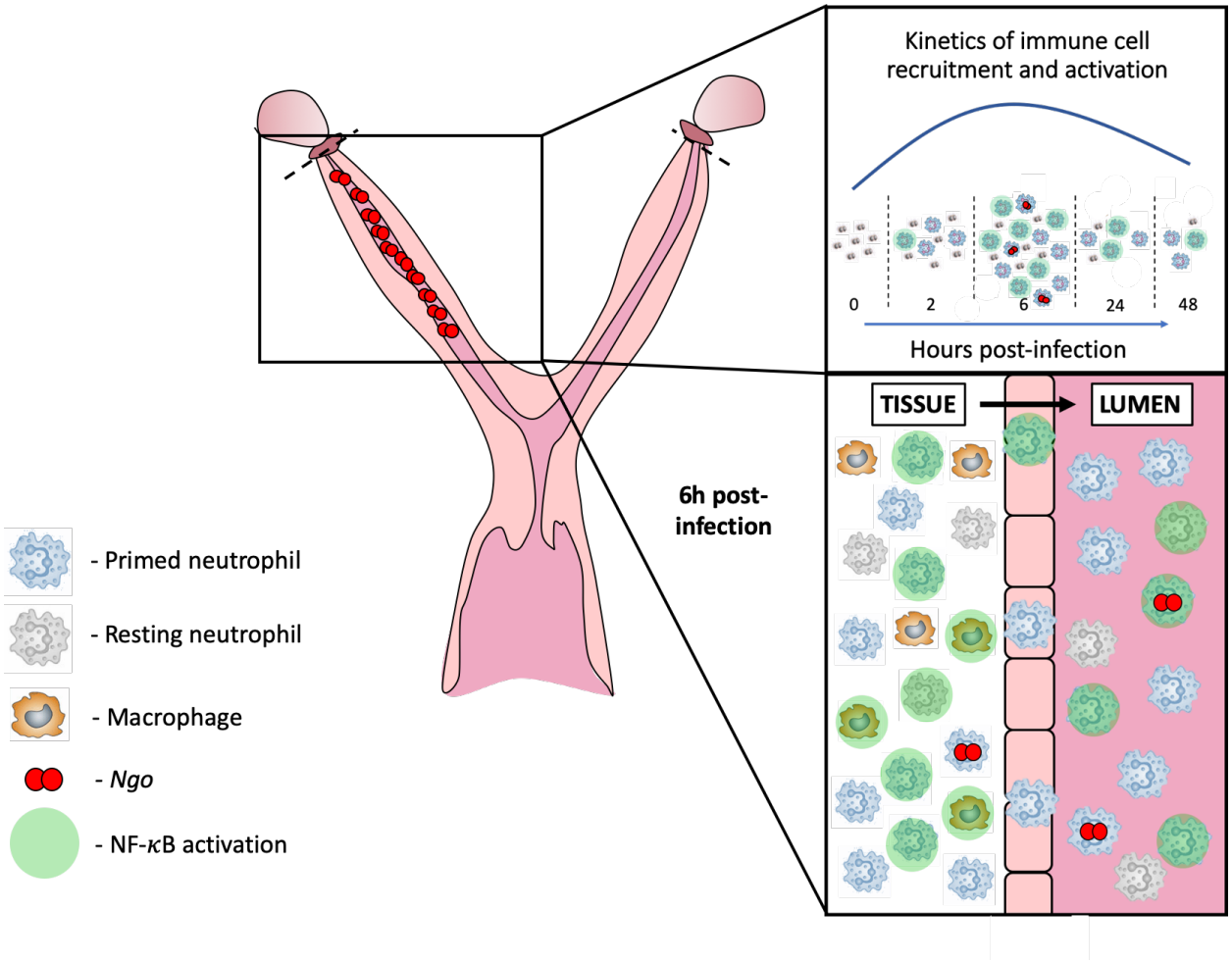


Figure 4.1: Schematic summary of immune cell recruitment and NF-κB activation in the mouse upper genital tract after transcervical gonococcal infection.

4.1.2 NF-κB activation in immune cells.

In this thesis, I established an antibody-based panel to monitor distinct immune cell subsets within the UGT by flow cytometry. Specifically, I included markers for neutrophils, macrophages, B cells, and T cells. Using these markers, I identified neutrophils as the most

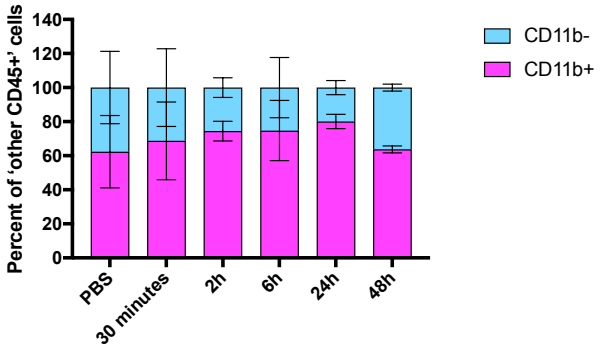
abundant cell type recruited into the UGT post-infection, and neutrophils were also the most abundant activators of NF-κB. Despite mouse to mouse variation, these responses were quite consistent. Although there were small populations of T cells and B cells with activated NF-κB, neither the number of each cell type nor the proportion of them expressing GFP increased in the infected group compared to the PBS control group. There was a small increase in the number of macrophages at the later timepoints post-infection, and a subset of these tended to be GFP+. Together, these data indicated that neutrophils are among the earliest responders to gonococcal infection, and they make up the vast majority of recruited leukocytes.

While neutrophils represent the dominant cell type responding, there appeared to be a small but reproducible percentage cells in the UGT that could not be distinguished using my antibody panel but that displayed an active NF-κB response post-infection. Although it is uncertain what these other immune cell subsets activating NF-κB might be, my data does demonstrate that the majority of these cells appear to express high levels of CD11b (Figure 4.1A). This suggests that these cells are likely monocytes, granulocytes, or uterine natural killer (uNK) cells [57]. My analysis of forward and side scatter revealed a large population of these GFP+ CD11b+ cells falling into the lymphocyte gate, with a smaller percentage falling into the granulocyte and monocyte gates (Figure 4.1B). This suggests that this population of NF-κB activating cells likely represents a heterogeneous population of these different cell types, but mostly consisting of uNK cells.

Apart from these immune cell subsets, there was a small percentage of NF-κB activating cells that were CD45-. This was interesting, given evidence that suggests epithelial cells play a role in the early immune response during in vitro infection with *Ngo* [51, 58]. Although a small percentage relative to other cell types, it is possible that epithelial cells, or other CD45- cells, are playing a role early on during infection. Future studies should work to identify these CD45- populations, with a specific effort being spent on determining whether epithelial cells are effectively recovered by my tissue digestion protocols.

Taken together, my data demonstrate the major role of neutrophils during UGT infections with *Ngo*, but also reveals that other cell types respond with an active NF-κB response, thus providing a tractable model to explore the contribution of these to the inflammatory response observed during pelvic inflammatory disease.

A.



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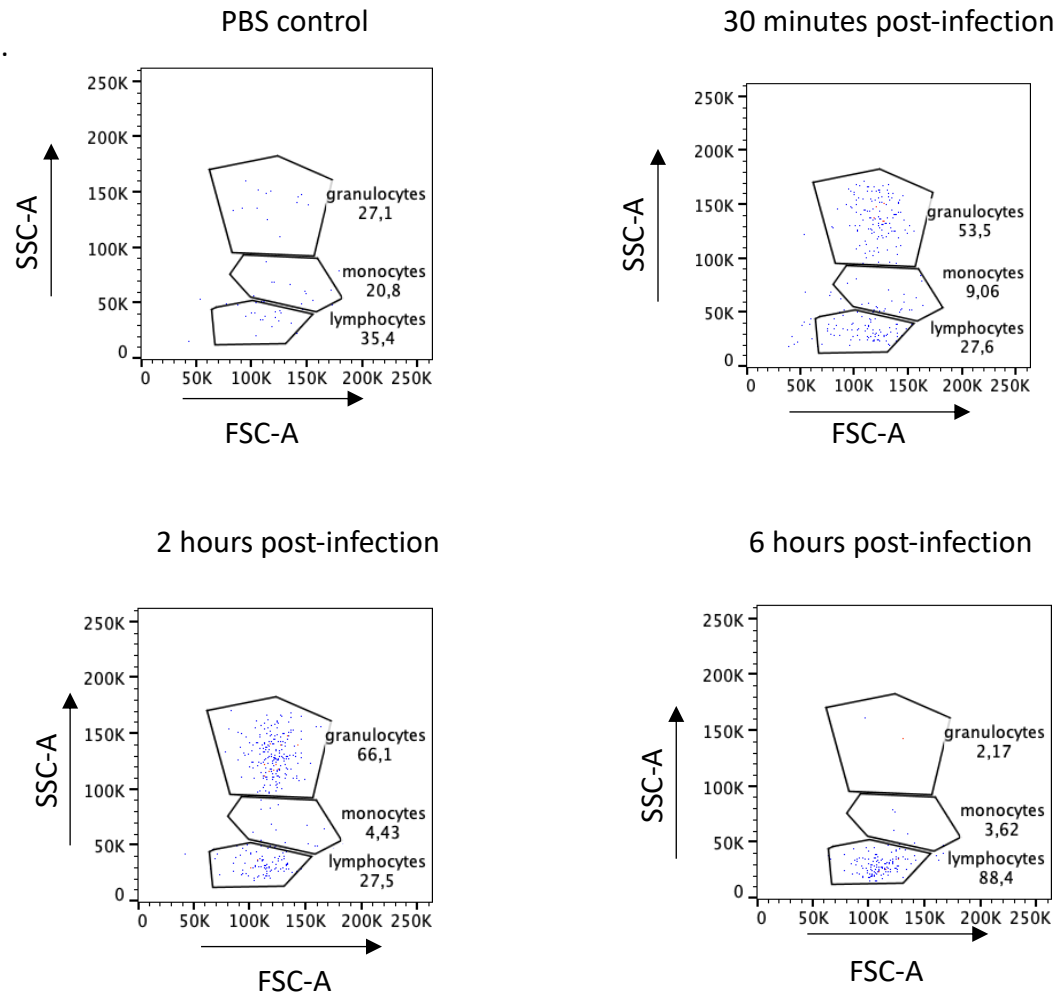

Figure 4.2: Other immune cell populations activating NF-κB are likely a heterogeneous population of CD11b$^+$ granulocytes, monocytes, and lymphocytes. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Ngo* and euthanized at 30 minutes, 2 hours, and 6 hours post-infection. Data analyzed by flow cytometry. 3 mice per group. Error bars represent SD. **(A)** Immune cells that are not B cells, T cells, neutrophils or macrophages (termed 'other CD45+') that express GFP, and that either did or did not express CD11b$^+$. Data graphed as a percent of 'other CD45+ cells' expressing GFP. Data graphed as a percent of 'other CD45+'. **(B)** Forward and side scatter analysis of 'other CD45+ cells' expressing GFP and CD11b. Plots are taken from 1 mouse at each different timepoints.

4.1.3 Heterogeneous activation of NF-κB in recruited neutrophils.

I observed a massive recruitment of neutrophils in the UGT after transcervical infection with *Ngo*. I also noticed that only a portion of these recruited neutrophils had NF-κB activation and that this was consistent at every time point tested. This was intriguing, as emerging work is demonstrating the heterogeneous nature of neutrophils, and the diverse roles that they play in health and disease [60, 61]. For these reasons, it was enticing to consider that the heterogeneous activation of NF-κB could reflect different neutrophil subsets playing distinct roles during infection in the UGT. In an attempt to tease apart the possible differences between the NF-κB activated and non-activated neutrophils, I looked at localization within the uterine horns, and neutrophil association with *Ngo*. I also hypothesized that the neutrophils activating NF-κB would likely be located within the uterine lumen, as this is where they would first encounter *Ngo*. I also hypothesized the NF-κB activated neutrophils would be associated with *Ngo* and would be of the primed phenotype. To my surprise, however, I noticed NF-κB activated neutrophils throughout the uterine horns, and no correlation between gonococcal association and NF-κB activation in the neutrophils. I also noticed that many of the NF-κB activated neutrophils were of the primed phenotype, but that there was also a substantial population of resting state neutrophils that displayed an active NF-κB response.

Although I had hypothesized that the NF-κB activated neutrophils would be specifically localized to the lumen, it was not completely surprising that this was not the case. It is possible that neutrophils activate NF-κB in the tissue prior to migrating into the lumen where they may then directly interact with *Ngo*. Alternatively, neutrophil activation may be independent of tissue localization, and may be influenced more based on the inflammatory cytokines they are exposed to.

Remarkably, there did not seem to be any connection between gonococcal association and GFP expression in the recruited neutrophils. I observed many neutrophils activating NF-κB that were not associated with *Ngo* and many neutrophils associated with *Ngo* but without active NF-κB. I had expected that the close proximity of the gonococci with neutrophils would result in signalling through pattern recognition receptors within the neutrophils, ultimately resulting in activation of NF-κB within that neutrophil. Although it is tempting to consider that *Ngo* may possess a mechanism to inhibit activation NF-κB in associated neutrophils, or that *Ngo*

preferentially associate with a phenotype of neutrophils that does not activate NF-κB, I am mindful that it is also possible that these findings are a result of the relative kinetics of NF-κB activation and subsequent GFP expression versus gonococcal destruction in this model. It is, for example, possible that the gonococci get taken up by the neutrophils and degraded before GFP expression occurs, leading to the apparent disconnect between GFP expression and gonococcal association. This issue will be discussed in the future directions section.

4.1.4 Neutrophils and their role in gonococcal induced PID

Neutrophils are a heterogeneous population of innate immune cells playing roles in microbial killing, tissue repair, and in a multitude of diseases such as cancer [61, 62, 63]. For this reason, there is a growing interest in characterizing different neutrophil populations in health and disease states to understand how we may be able to translate this knowledge into methods to diagnose and/or treat diseases.

Fine et al. recently described the 'primed' neutrophil subset, which are primed for early recruitment to the site of infection [42]. During peritoneal infection with *E. coli*, they found that primed neutrophils in circulation are immediately depleted as they get recruited into the site of infection, after which they become the dominant subtype formed in the bone marrow and released into the blood until resolution of inflammation. From these observations, Fine et al propose that monitoring levels of primed neutrophils may be a useful biomarker for disease susceptibility, because they observed that higher levels of primed neutrophils correlated with improved disease outcome [42]. I therefore aimed to characterize the primed neutrophil levels in the bone marrow, blood, and UGT after transcervical gonococcal infection in the mouse, and questioned whether these primed neutrophils were those activating NF-κB so that I could understand their role in the UGT and during gonococcal infection.

In contrast to Fine et al., I found that the percentages of primed neutrophils in the bone marrow, blood, and UGT did not increase substantially post-infection. This may result from different mucosal surfaces being infected since, for example, the peritoneal cavity is sterile while the genital tract is not. However, I did see that the majority of neutrophils located in the uterine horn lumen were of the primed phenotype, and a subset of these neutrophils displayed an active NF-κB state. However, NF-κB was not exclusively activated in the primed neutrophils in the uterine lumen, and so there did not appear to be any specific association with whether the neutrophil was

primed or resting state. This was surprising, since I expected that as the suspected first responders, the primed neutrophils would rapidly activate their NF-κB-driven inflammatory response. My data may reflect different findings than those described by Fine et al. [42] either because *Ngo* elicits a unique response, or simply due to the differences between peritoneal versus mucosal infection. Understanding this neutrophil response will require further work to decipher their exact role during infection.

4.1.5 Limitations of the model.

Although the mouse model of gonococcal infection in the NF-κB GFP reporter mice provides a useful tool for studying the immune response to *Ngo*, it is important to consider that there may be other pathways leading to cellular immune activation upon gonococcal infection. A number of other rapidly inducible transcription factors have been identified that are involved in inflammatory pathways, such as AP-1, CREB, and SRF [59].

Additionally, it is important to note that mice lack many human specific receptors which bind gonococcal virulence factors. This means that by studying immune responses in wild type mice, key processes that would occur during human infection may be missed.

Specifically, human CEACAMs are differentially expressed on a broad array of human cells, including epithelial cells and immune cells. Phase-variable Opa proteins on the surface of *Ngo* are able to differentially bind to hCEACAMs. Though they are known to play an important role in mucosal association, bacterial clearance, and inflammation, the exact role that they play at the cellular level in the UGT during gonococcal infection remains unclear [64]. To help understand their roles, mouse models expressing human CEACAMs have been developed.

Islam et al. reported that expression of hCEACAM1 in the mouse model of infection enhanced gonococcal penetration into the uterine tissue after transcervical infection during the estrus stage of the cycle [64]. Additionally, an upregulation of pro-inflammatory cytokines was observed in mice expressing hCEACAM1 and in mice expressing hCEACAM 3, 5, and 6, as compared to wild type littermates [64]. Together, these data suggest a role for hCEACAM expression in altering the early immune response during *Ngo* infection in the UGT. For these reasons, it is important to consider the potential impact that hCEACAM expression would have on the kinetics of infection,

NF-κB activation, and immune cell recruitment during UGT infection with *Ngo* in the mice. This will be discussed further in the next section.

4.2 Future Directions

4.2.1 Defining the contributions of human CEACAM expression on the early immune response.

As mentioned above, transgenic expression of human CEACAMs has been shown to alter the early immune response to *Ngo* infection in the upper genital tract of mice [64]. It is unknown how their expression would impact the response in the context of immune cell recruitment and activation into the UGT post-*Ngo* infection. It may be beneficial for future studies to determine how they may impact this response in order to establish an increasingly relevant model for this human-restricted pathogen.

Preliminary experiments, in which transgenic mice expressing hCEACAMs were crossed with the NF-κB GFP reporter mice, reveal that there does not seem to be any dramatic changes to the early response in terms of immune cell recruitment and NF-κB activation when the hCEACAM-expressing mice were compared to their wild type littermates.

In these preliminary experiments, I used one mouse line that expresses hCEACAM1 (Tg418 mice) and another line expresses hCEACAM 3, 5, and 6 (CEABAC mice). I transcervically infected each of these mouse lines and analyzed immune cell recruitment and GFP expression post-infection by flow cytometry. In the mouse line expressing hCEACAM1, I saw little difference between the wild type mice and the transgenic hCEACAM1 mice in terms of immune cell recruitment and neutrophil activation at 6 hours post-infection (Figure 4.2). Similarly, I saw little difference in terms of NF-κB activation and immune cell recruitment into the UGT in the CEABAC mice compared to their wild type littermates (Figure 4.3). However, the CEABAC mice appeared to associate with *Ngo* more quickly than did the wild type mice, likely due to the expression of these hCEACAMs (Figure 4.3E). Taken together, it appears as though hCEACAM expression likely plays a role in gonococcal association at these early time points, but expression does not largely alter the early inflammatory response in the context of immune cell recruitment and activation. These preliminary data suggest that modeling this response using wild type mice may be sufficient, however, further studies should be conducted to validate these findings and

understand their impact during early infection. In particular, the effect of hCEACAMs may not become apparent until other human-derived factors that gonococci use to facilitate their persistence in the tissues, such as the serum complement-regulatory proteins factor H and/or C4b-binding protein are introduced along with the CEACAMs [31, 65].

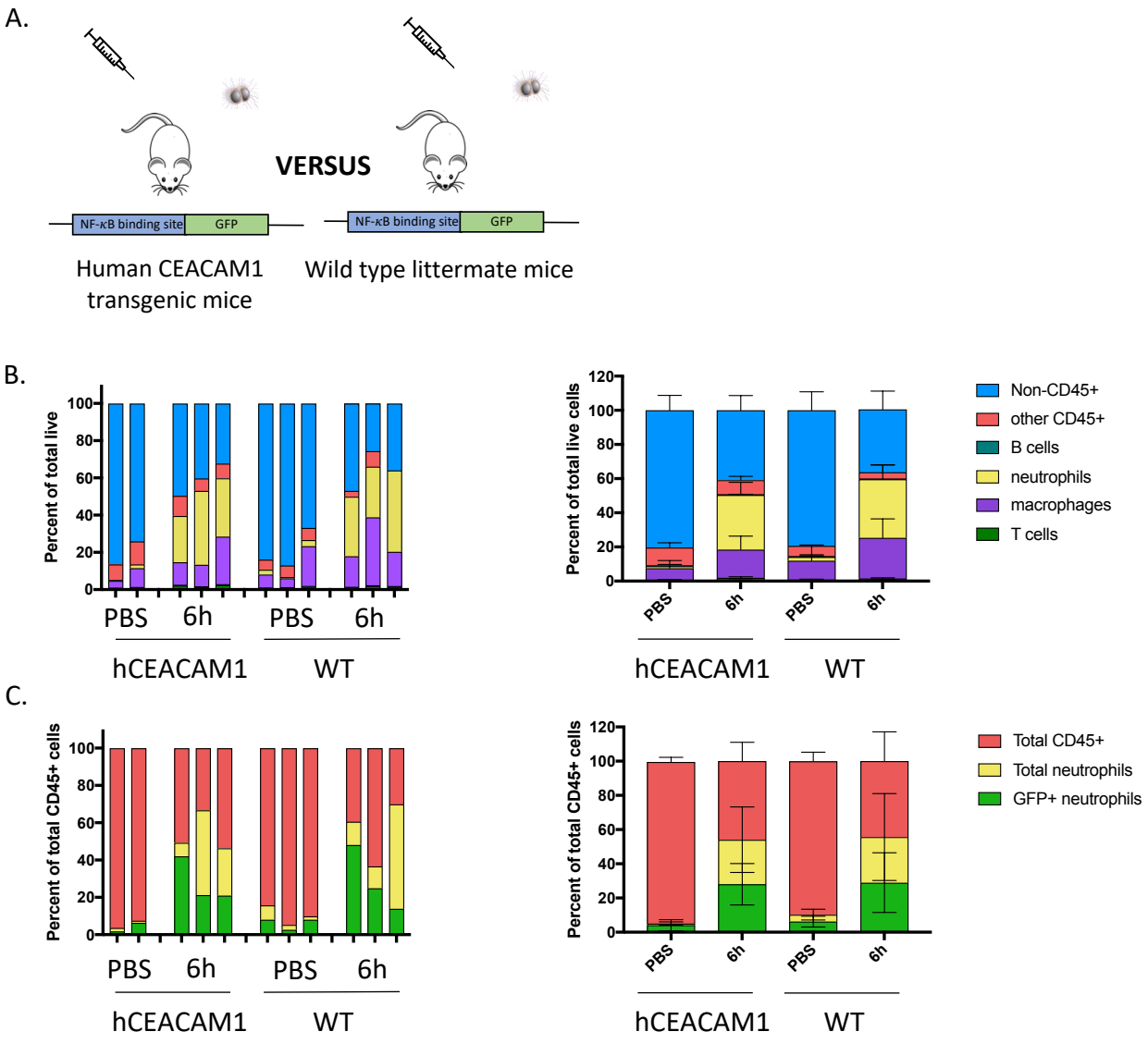

Figure 4.3: Expression of human CEACAM1 does not seem to largely alter NF-κB activation in neutrophils or immune cell recruitment at 6 hours post-infection as compared to wild type littermates. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Ngo* and euthanized at 6 hours post-infection. Mice expressing hCEACAM1 were compared to their wild type littermates. Data analyzed by flow cytometry. 2-3 mice per group. Error bars represent SD. **(A)** Graphical representation of experiment**. (B-C)** Each bar represents individual mice (left) or the mean of mice in each group (right). **(B)** Immune cell subset recruitment into the upper genital tract in hCEACAM1 mice versus wild type mice graphed as a percent of total live. **(C)** Neutrophil recruitment and GFP expression graphed as a percent of total CD45+.

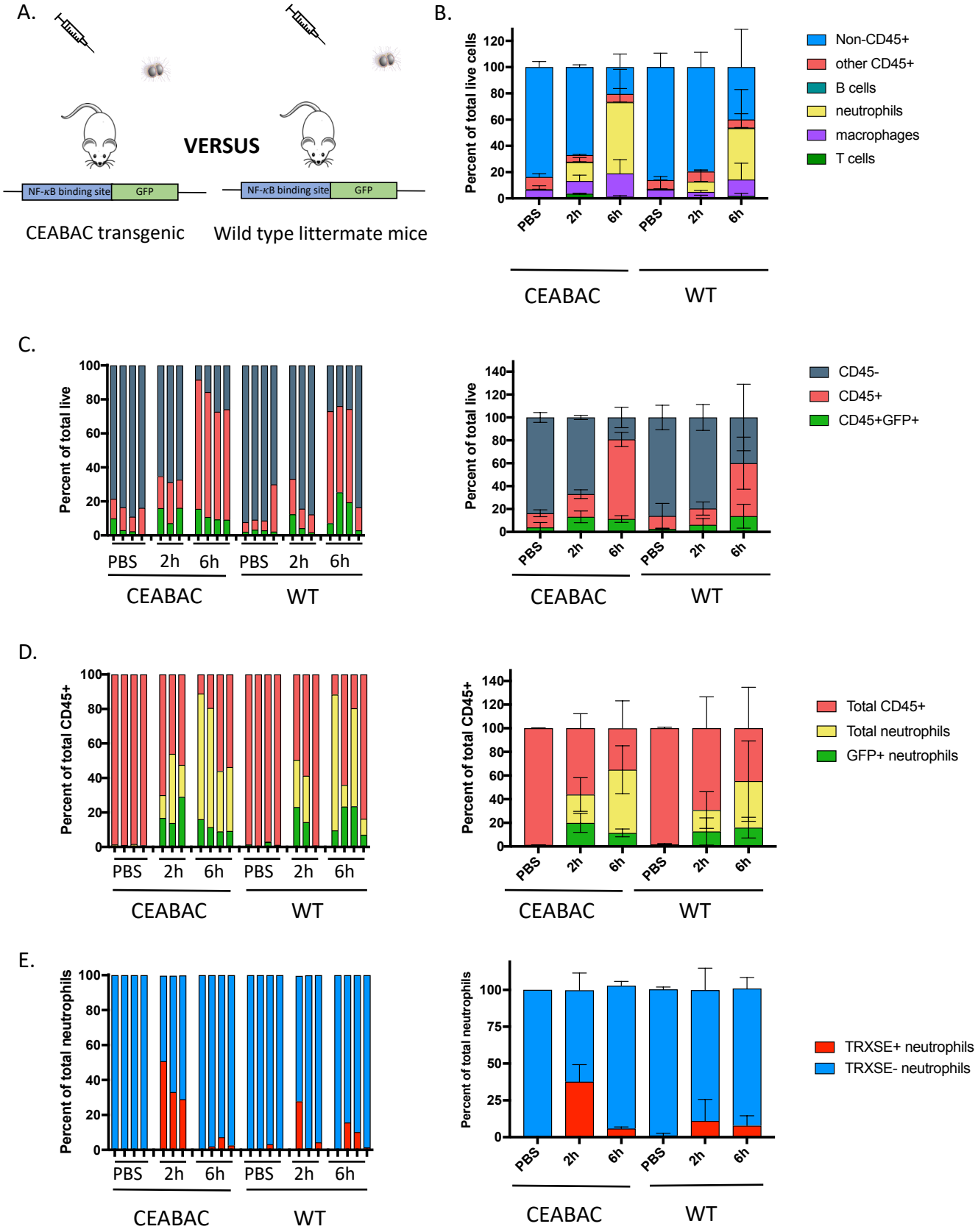
A.
VERSUS
NF-κB binding site
GFP
NF-κB binding site
GFP
CEABAC transgenic
Wild type littermate mice
B.
Percent of total live cells
Non-CD45+
other CD45+
B cells
neutrophils
macrophages
T cells
PBS
2h
6h
PBS
2h
6h
CEABAC
WT
C.
Percent of total live
CD45-
CD45+
CD45+GFP+
PBS
2h
6h
PBS
2h
6h
CEABAC
WT
D.
Percent of total CD45+
Total CD45+
Total neutrophils
GFP+ neutrophils
PBS
2h
6h
PBS
2h
6h
CEABAC
WT
E.
Percent of total neutrophils
TRXSE+ neutrophils
TRXSE- neutrophils
PBS
2h
6h
PBS
2h
6h
CEABAC
WT

Figure 4.4: Expression of human CEACAM 3, 5, 6 does not seem to largely alter NF-κB activation or immune cell recruitment at 2 or 6 hours post-infection as compared to wild type littermates. Transgenic mice encoding a reporter that expresses GFP upon activation of NF-κB were transcervically infected with *Ngo* and euthanized at 2 hours and 6 hours post-infection. Mice expressing human CEACAM 3, 5, 6 (CEABAC) were compared to their wild type littermates. Data analyzed by flow cytometry. 3-4 mice per group. Error bars represent SD. **(A)** Graphical representation of experiment. **(B-E)** Each bar represents individual mice (left) or the mean of mice in each group (right). **(B)** Immune cell subset recruitment into the upper genital tract in CEABAC versus wild type mice graphed as a percent of total live. **(C)** Immune cell recruitment and GFP expression in CEABAC versus wild type graphed as a percent of total CD45+. **(D)** Neutrophil recruitment and GFP expression graphed as a percent of total CD45+. **(E)** Gonococcal association with neutrophils graphed as a percent of total neutrophils.

4.2.2 Identifying neutrophil populations during gonococcal infections through RNA sequencing.

The heterogeneous activation of NF-κB in neutrophils during gonococcal infection makes it enticing to consider that the 'activated' and 'non-activated' neutrophils may represent different neutrophil phenotypes with different roles during infection. An interesting avenue to explore would be to look into the transcriptome of these neutrophil populations. This could be done by FACs sorting the GFP+ and GFP- neutrophils, followed by RNA sequencing of these cell populations. Alternatively, single cell RNA sequencing could be used to understand the broader neutrophil heterogeneity during gonococcal infections. These findings would help to elucidate whether the activated and non-activated neutrophil populations represent different phenotypes and would provide insight into potential functional differences between these neutrophil populations during gonococcal infection.

4.2.3 Further characterizing the neutrophil response during *Ngo* infections in the UGT.

It is clear that neutrophils play an important role during gonococcal infections in both humans and in mice, however the role of NF-κB activation in this neutrophil response remains unclear. An interesting approach to further understand this response would be to use an inhibitor of NF-κB activation, such as a TAK1 inhibitor previously used to study this neutrophil response in vitro [39], to confirm whether NF-κB activation is responsible for the massive recruitment of neutrophils, and to understand how it may impact the early inflammatory response during gonococcal infections.

Another interesting avenue would be to inhibit neutrophil recruitment to the site of infection in order to compare the contributions of possible tissue resident neutrophils to the recruited neutrophils in terms of NF-κB activation, immune cell recruitment, and bacterial clearance. To tease apart these questions, it would be interesting to take advantage of genetic knockout mice to inhibit neutrophil recruitment. For example, CXCR2 knock mice [66] have been studied in the context of microbial infections, and display defective neutrophil migration although their killing functions remain intact [67, 68, 69, 70, 71]. This would provide a useful tool to further understand this neutrophil response.

4.2.4 Understanding why NF-κB activated neutrophils do not appear to be associated with gonococci and gonococcal associated neutrophils do not appear to be activating NF-κB.

When considering GFP expression and gonococcal association, I saw that many neutrophils activating NF-κB were not associated with gonococci, and many of the gonococcal associated neutrophils were not activating NF-κB. Although it appears as though gonococcal association does not necessarily lead to NF-κB, it remains possible that these findings are a result of a loss of fluorescence signal once the *Ngo* is taken up by the neutrophil and destroyed. In this case, my findings might result from a matter of kinetics such that by the time GFP is expressed, the gonococci have already been destroyed and therefore the *Ngo* fluorescence signal might be lost. Future work is required to tease apart these possibilities. One way this response could be followed is by performing an in vitro gonococcal infection on purified neutrophils from the NF-κB GFP reporter mice, and following this infection using live cell imaging or flow cytometry over time.

4.2.5 Comparing the inflammatory response at different stages of the cycle.

Previously, the role of the hormonal cycle has been shown to drastically affect the immune response during *Ngo* infection in the UGT of mice [14]. For the purposes of my work, mice were induced into the progesterone dominant stage of the cycle, diestrus (Figure 4.4A). This was because this stage results in a more damaging inflammatory response as compared to the estrogen dominant stage of the cycle, estrus, and therefore provides a model more reflective of the severe outcomes of PID [14]. In contrast, infections during the estrus stage of the cycle elicit a milder inflammatory response, with less gonococci penetrating into the tissue, and less neutrophil recruitment than seen during the diestrus stage [14]. Consistent with this, I performed a preliminary experiment in which NF-κB GFP reporter mice were transcervically infected and their UGT microenvironment was assessed by flow cytometry. These data also revealed an altered response compared to mice at diestrus, with less neutrophil recruitment (Figure 4.4B-D). Interestingly, studies observing PID in humans show that patients most often present with intense PID within 10 days after menses, suggesting that hormones likely play a role during human disease pathogenesis as well [72]. With evidence to suggest an altered inflammatory response at different stages of the cycle in both humans and mice, it would perhaps be interesting to consider the effects of hormones on immune cell recruitment and NF-κB activation in the NF-κB GFP

reporter mouse model. Studies looking at the inflammatory response at different stages of the cycle would be interesting to compare to what I observed during the diestrus stage of the cycle. These findings would provide insight into how hormones may affect immunological outcome, and how this may impact disease pathogenesis in humans.

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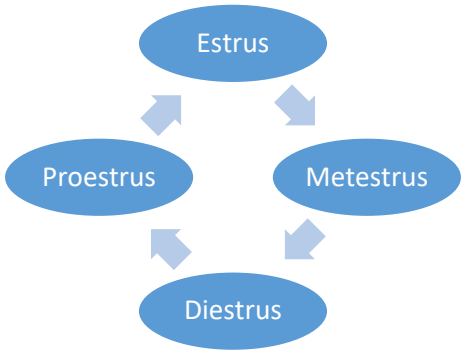

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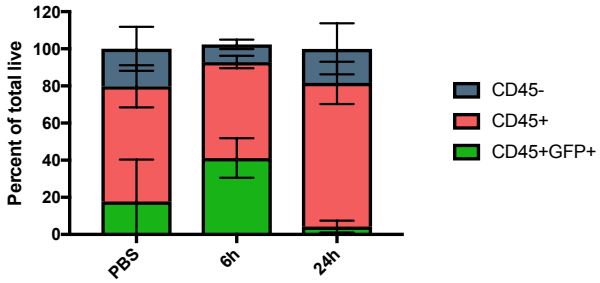

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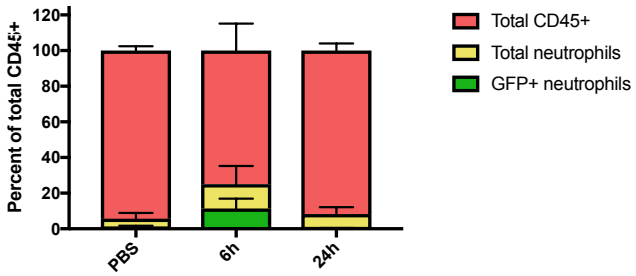

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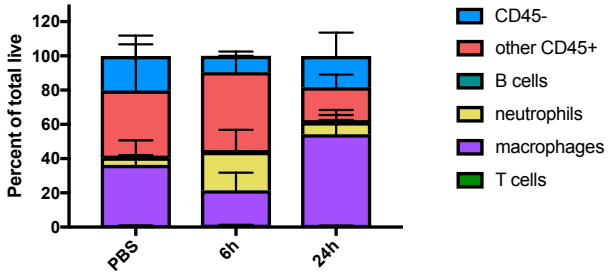

Figure 4.5: Mice in the estrus stage of the cycle display a different immune microenvironment compared to mice at the diestrus stage in control and *Neisseria gonorrhoeae* infected mice. Reporter mice expressing GFP upon activation of NF-κB, that were induced into the estrus stage of the cycle, were transcervically infected with *Ngo* and euthanized at 6- and 24- hours post-infection. Data analyzed by flow cytometry. 3-6 mice per group. Error bars represent SD. **(A)** Graphical representation of the estrus cycle, that lasts approximately four days. **(B)** Immune cell recruitment and GFP expression graphed as a percent of total live. **(C)** Neutrophil recruitment and GFP expression graphed as a percent of total CD45+ cells. **(D)** Immune cell subset recruitment graphed as a percent of total live.

4.3 Conclusion

This work utilized the mouse UGT infection model to understand the early inflammatory response to *Ngo*. I showed that this infection results in a massive recruitment of neutrophils, whereby only a portion of the recruited neutrophils appear to associate with gonococci, and only a portion of them activate NF-κB. This work establishes a model by which to study this differential response of neutrophils to *Ngo* infection. Further work in this model will hopefully provide new insight into protective versus pathogenic neutrophil responses during *Ngo* infection, with the overall goal of understanding how we may be able to target specific neutrophil populations to treat infection.

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