Depo Provera downregulates the expression of genes involved in maintaining the mucosal immune barrier and protecting against *Chlamydia* infection in the murine female genital tract

by

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ABSTRACT

Depo Provera (depot medroxyprogesterone acetate, DMPA) is a standard pre-treatment in *Chlamydia* animal models to ensure reliable female genital tract infection. DMPA also increases infection with simian-human immunodeficiency virus, herpes simplex virus, and *Mycobacterium tuberculosis* in mice and non-human primates. In humans, DMPA has been associated with an increased risk of sexually-transmitted infection, prompting the World Health Organization to call for more research on how DMPA impacts mucosal immunity. Here, we report a transcriptomics analysis of how DMPA modulates the expression of genes related to the immune barrier in the murine female genital tract. C56Bl/6 female mice were treated subcutaneously with 2.5 mg DMPA at day -7 or days -10 and -3, corresponding to different protocols in the *Chlamydia* field. The cervix, uterus, and ovaries were excised, total RNA was extracted and applied to the NanoString PanCancer Immune Profiling and Host Response Panels, representing 1114 genes. DMPA treatment resulted in the differential expression of over 300 genes, including the significant downregulation of many genes involved in mucosal and cell-autonomous immunity (e.g. lipocalin 2, mucin 1, CCR3, STING, and caspase 8). Studies investigating the relative contributions of key genes and their relationship to *Chlamydia* infection are ongoing. These data support a model wherein DMPA weakens immune barrier functions to allow reliable *Chlamydia* infection in the murine female genital tract, and may also explain why DMPA use in humans is associated with increased sexually-transmitted infection.

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INTRODUCTION

I. Background

Depo Provera (depot medroxyprogesterone acetate, DMPA) is a synthetic form of progesterone. DMPA suppresses ovulation and is used clinically as a hormonal contraceptive that is highly effective and discreetly delivered by injection once every three months (1). Worldwide, thirty-five million people use DMPA, and it accounts for half of all contraceptives used in sub-Saharan Africa (2). It is included on the World Health Organization's Model List of Essential Medicines (1).

DMPA is a standard pre-treatment in *Chlamydia* animal models to ensure reliable female genital tract infection (3). DMPA also increases infection with herpes simplex virus (4-5), simian-human immunodeficiency virus (6), Zika virus (7), and *Mycobacterium tuberculosis* (8) in mice and non-human primates. In humans, multiple epidemiological studies have linked DMPA use with an increased risk of human immunodeficiency virus (HIV) infection (9). This has prompted the World Health Organization to call for more research on how DMPA impacts mucosal immunity (9). The cell and molecular mechanism(s) for how DMPA alters susceptibility to pathogens is unknown.

There are likely direct and indirect mechanisms by which DMPA changes host susceptibility to pathogens. Such factors may include 1.) the modulations to barrier morphology in the genital tract, 2.) widespread transcriptomic changes affecting the immune barrier and secreted defense molecules such as cytokines, chemokines, antibodies, and mucus, 3.) effects on the movement and receptors of incoming pathogens. By measuring the differential gene expression and morphological changes in the murine cervix, uterus, and ovaries we can aid the interpretation of epidemiological and pathogenic data and provide insight to these fields.

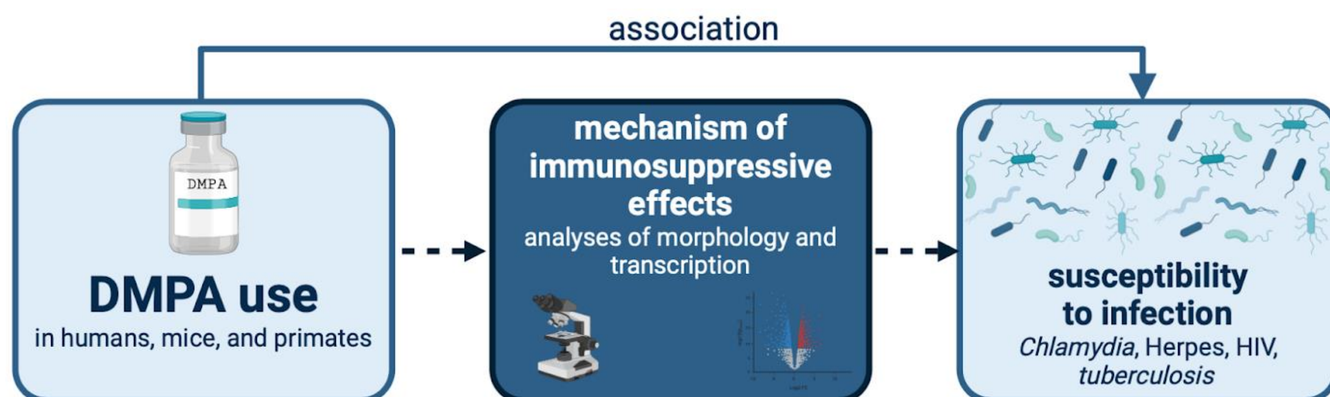

Figure 1. Model of the project rationale and approach. Multiple studies have shown that DMPA use in humans, mice, and non-human primates is associated with an increase in susceptibility to mucosal infection, but the cellular and molecular mechanism(s) for DMPA's immunosuppressive effects are currently unknown. We hypothesize that this is based on a reduction in the immune barrier, which we will measure by analyzing transcriptomic and morphological changes.

Critical evaluation is needed to understand the biological mechanisms involved in the susceptibility to sexually transmitted infection, and how this may be modulated by DMPA. This study is focused on analyzing changes in the morphology and transcription profile of the female genital tract of mice treated with DMPA. These results will allow us to evaluate how DMPA alters immunity in these tissues to increase susceptibility to infections and facilitate reliable *Chlamydia* infection.

II. Relevant immunological concepts

The immune system protects from outside invaders, it is made up of two parts: the innate and adaptive systems (14). The innate immune system is the first line of defense against pathogens, which are microorganisms that cause or can cause disease. Innate immunity is composed of physical barriers as well as effector cells, antimicrobial peptides, and molecular complexes. The innate immune system works quickly - within minutes to hours - and requires no external training or memory to function, though the response is non-specific. The role of the innate immune system is to recognize pieces of non-self called "antigens," prevent infection,

eliminate pathogens, and stimulate the adaptive immune response. In contrast, the adaptive immune system, which is made up of B and T cells, is highly specialized and can develop immunological memory to respond more robustly to a second infection.

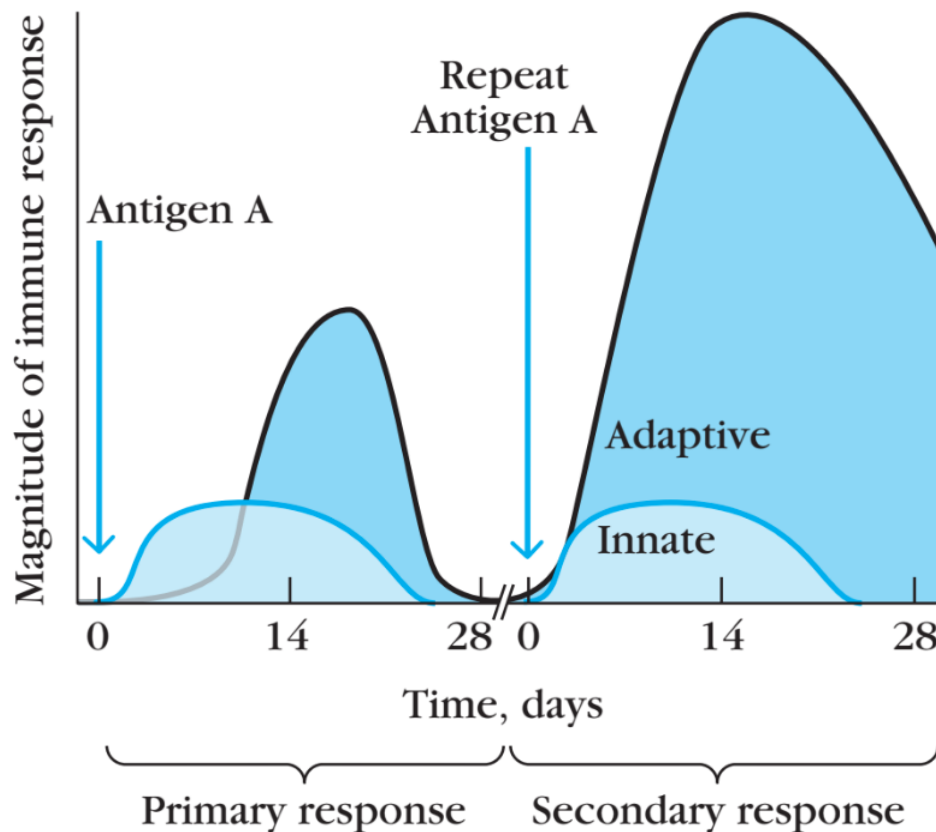

Figure 2. Model of the differences between the innate and adaptive responses to antigen exposure. The innate response is low magnitude and does not differ between the primary and secondary response to the same antigen. The adaptive immune response comes later, but it is more robust in magnitude and is antigen-specific for a greater, faster response upon repeat antigen exposure (adapted from 14).

Within the innate immune system, there are a multitude of components that work together to protect the host against infectious insults. Some important components for this project include the complement system, barrier function, nutritional immunity, cell-autonomous immunity, and the inflammasome.

A. Barrier function

Mucosal barriers are physical protective surfaces standing between the internal milieu and the non-sterile external environment, providing both physical and immune defense against infection (15). Anatomical barriers are also able to completely avoid interaction with microbes to prevent the need for an immune response. They are selectively permeable to allow the influx of water and nutrients and the efflux of bacteria and toxins. It needs to maintain tight junctions while allowing for directive transport across the epithelium. These tight junctions between neighboring cells further reinforce this barrier, preventing pathogens from passing through the gaps between cells as breaches can serve as an entry site.

Additionally, cells often secrete mucus, enzymes, and antimicrobial peptides onto their surfaces, which creates an inhospitable environment for pathogens and hinders their ability to adhere to or penetrate the cell membrane (16). The immune system supports barrier repair and maintenance. The effectiveness of these tissues in thwarting infection, regulating pathogen entry, and controlling dissemination is heavily influenced by their composition and functionality (17).

B. Cell-autonomous immunity

In addition to serving as a structural barrier, epithelial cells exhibit cell-autonomous immunity (18, 19). This process is ubiquitous and well-developed, operating independently within each cell. Each cell can defend itself through various mechanisms that are in place to detect and neutralize invading pathogens. These mechanisms can include the production of antimicrobial proteins or the activation of cell death pathways to kill infected cells and the pathogen along with them (19). Cells first recognize the presence of pathogens through various sensors such as pattern recognition receptors (PRRs) which detect foreign components such as viral nucleic acids or bacterial cell wall components (15).

Inflammation is a vital protective measure against infection (16). One way that cells can exhibit autonomous immunity is through inflammasome activation. Inflammasomes are multimeric protein complexes that assemble when pattern recognition receptors detect the presence of a pathogen in the cell (20). Upon recognition, inflammasomes undergo assembly, triggering the activation of inflammatory caspases. These caspases, in turn, orchestrate the production and release of key cytokines while also initiating a form of inflammatory cell death called pyroptosis. This form of cell death contributes to immune defense against infectious diseases as it restricts infection, and the inflammatory molecules can alert the immune system of the ongoing threat (20, 15).

C. Complement

The complement system is part of the early induced immune response. It is a collection of soluble and membrane-associated proteins circulating in the blood that are involved in a catalytic cascade to fight off pathogens and foreign materials in the body (14). The cascade is activated by the detection of a pathogen's presence, or the recognition of antibodies bound to the pathogen. Upon activation, a series of proteins undergo a sequential cascade of conformational changes and enzymatic cleavage of downstream complement proteins. Fragments are released that have different functions: binding to the pathogen's cell membrane to flag it for phagocytosis in a process called opsonization, attracting other immune cells to the site of inflammation, and forming pores in the pathogen's plasma membrane to damage its structure (21).

D. Nutritional Immunity

Iron is essential for life, but its concentration and metabolism require tight regulation (22). Pathogens also require nutrients to survive. Host cells and tissues actively sequester or withhold critical nutrients such as iron, zinc, manganese, and other micronutrients from

pathogens, thereby impairing their ability to thrive within the host environment. The detection of infection-induced cytokines or the activation of transcription factors that respond to iron or microbial sensing can lead to the induction of genes involved in nutritional immunity.

Iron is a crucial micronutrient required for microbial growth and virulence (22). Host cells employ various strategies to sequester iron, making it less accessible to pathogens. For example, iron-binding proteins such as lactoferrin and transferrin sequester extracellular iron, while intracellular iron is stored in ferritin, reducing its availability to intracellular pathogens.

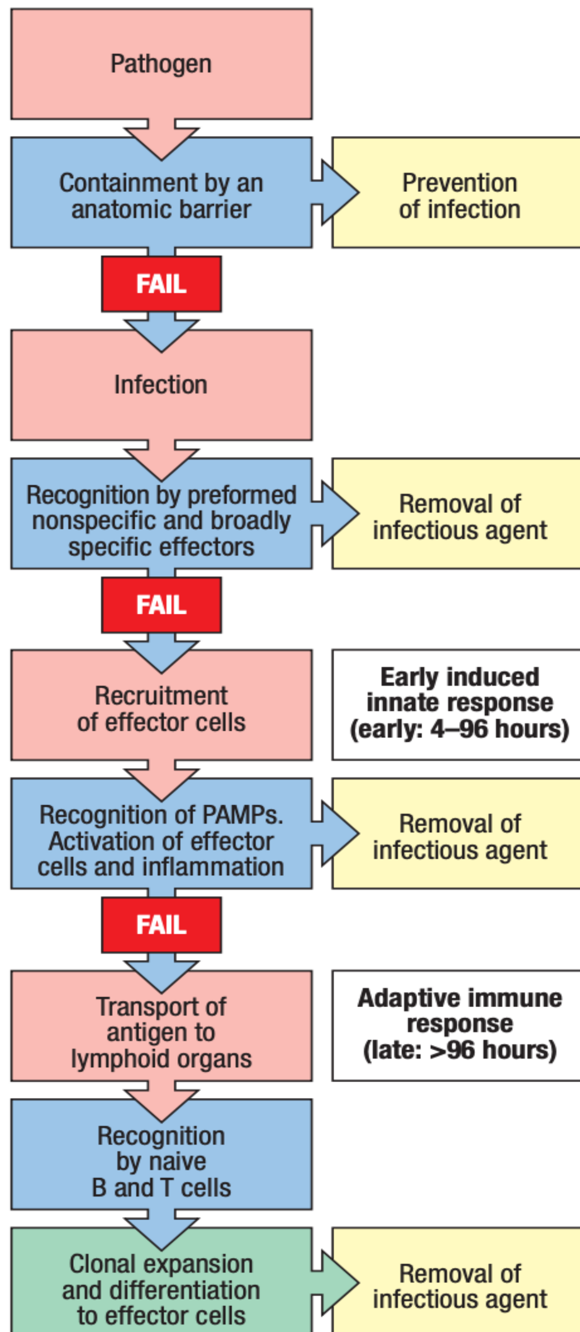

Figure 3. A schematic showing the initial response to primary infection. The innate response includes barrier breech, the induced innate response with the recruitment of effector cells, and the adaptive response. A breach or failure at any step can result in infection (adapted from 15).

III. Host-pathogen interactions in the female genital tract

The biology of the female genital tract (FGT) is composed of a series of connected organs, each with distinct cellular composition and architecture (23). These tissues are relatively understudied, and much of our understanding is inferred from the gastrointestinal and respiratory tracts. Yet it is a crucial immune site with multiple roles and is the site of initiation and dissemination of sexually transmitted infections (STIs). Sexually transmitted pathogens must cross a complex series of mucosal defenses to infect host cells. This includes the physical barrier of a wall of tightly adjoined epithelial cells and sentinel immune cells lining the FGT. Weakening of this barrier could increase susceptibility to infection.

Epithelial cells, fibroblasts, and immune cells respond directly to estrogen and progesterone in the FGT while responding indirectly to the cytokines and chemokines that signal to other cells of the innate and adaptive immune systems (11). Epithelial cells provide barrier protection, while also transporting immune molecules such as IgA and IgG into secretions and producing bactericidal and virucidal antimicrobials to combat pathogens (12). If the tight junctions in the UGT are damaged, it can lead to infection, potentially resulting in secondary effects such as infertility. Uterine epithelial cells secrete antimicrobials that have broad-spectrum activity and are effective in inhibiting fungi, viruses, and Gram-positive and Gram-negative bacteria including *Chlamydia trachomatis*, Herpes Simplex Virus-2, HIV-1, *Neisseria gonorrhoeae*, and *Candida albicans* (12).

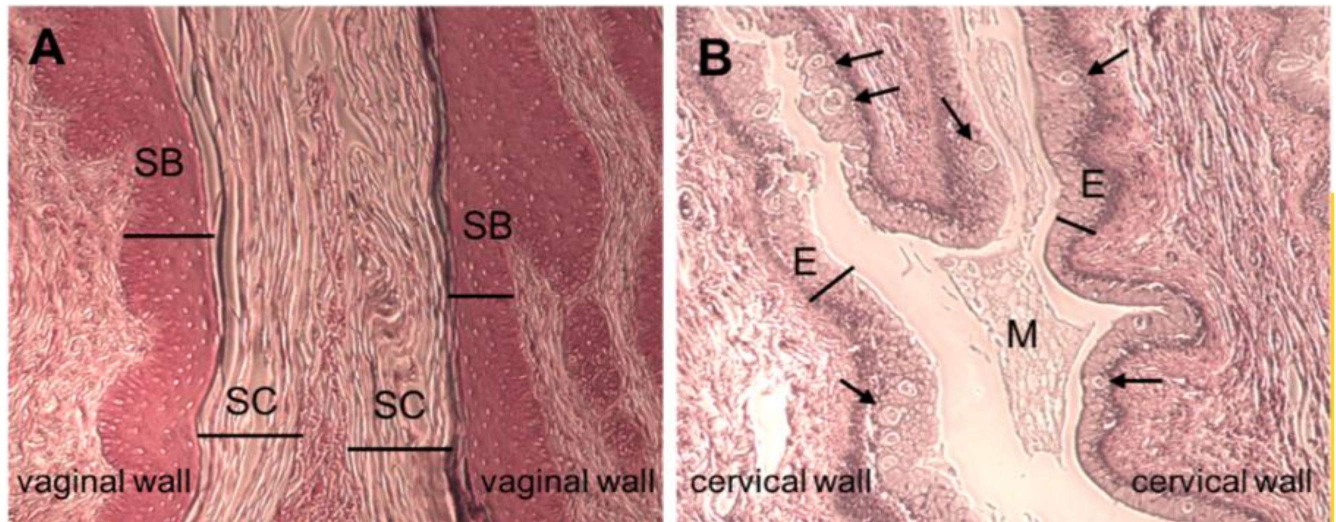

Figure 4. H&E-stained epithelium of the murine vagina (A) and cervix (B) imaged at 10x magnification. SB: stratum basalis. SC: stratum corneum. E: epithelium. M: mucus. Arrows denote intraepithelial immune cells (adapted from 43).

The murine female genital tract is made up of the vaginal vault, cervix, two uterine horns, oviducts, and ovaries. The epithelial layers of the cervix and uterine horns are distinct from the vaginal vault which consists of different strata (24). Simple columnar epithelial cells line the cervical and uterine walls. In contrast, the vaginal wall has a nucleated *stratum basalis* layer and layers of flattened, cornified cells called the *stratum corneum*. The *stratum corneum* consists of loosely connected flattened cells lacking nuclei and internal organelles, with minimal intercellular adhesions. Importantly, it lacks a dense lipid envelope found in the epidermis, allowing for moisture flow. Due to its weaker cell connections, absence of complete keratinization, and lipid envelope, the vaginal stratum corneum layer is permeable to both microbes and immune system components. The epithelium separates the sterile body from the non-sterile outside world. We see this as the empty, white space referred to as the lumen which is the area surrounded by the epithelium.

In the uterus, columnar epithelial cells can act as a physical barrier against microbial infections (25). Tight junctions between these cells ensure the integrity of the mucosal layer; any

disruption to these junctions or damage to the epithelial layer can have consequences such as infection or infertility. These columnar epithelial cells have toll-like receptors that detect pathogens (26). Additionally, they secrete peptides that can destroy harmful microbes and release chemokines and cytokines that attract and activate immune cells, linking the innate and adaptive immune systems. The epithelial cells also facilitate the entry of secretory antibodies into the uterus and can present antigens to T cells. These protective measures maintain a typically sterile uterine environment, unlike the lower genital tract (27).

The mucus serves as another protective component throughout the female genital tract (28). There are also round, motile immune cells (e.g., T cells, macrophages, Figure 4, arrows) that patrol the epithelium to detect invasion by pathogenic microbes.

IV. Summary of past studies evaluating the effects of DMPA on infection

Multiple epidemiological studies have associated DMPA with an increased risk for HIV acquisition, which is a leading cause of morbidity and mortality in sub-Saharan Africa, particularly among women (9, 29-32, 42). Interpreting and comparing data from epidemiological studies can be challenging due to inconsistencies in their design and analysis methods (3, 40). This can lead to conflicting conclusions. However, even after taking confounding variables such as age, number of sexual partners, and sexual behaviors into account, there is a significant increase in sexually transmitted infections following the use of DMPA (40). For example, DMPA use in humans is associated with a 3-fold increase in the risk of cervical *Chlamydia* infection acquisition (41). This increase in susceptibility to sexually transmitted infection is not found with other forms of hormonal contraception (40, 41).

In fact, mice must be treated with DMPA before *Chlamydia* can reproducibly infect the female genital tract (3, 33). DMPA treatment is briefly mentioned in the Methods section of

every *Chlamydia* research paper and has been used in the preclinical trials for all *Chlamydia* vaccines (3). The common rationale within the field for pre-treating before infection with *Chlamydia trachomatis* for research purposes is that DMPA suppresses the murine estrus cycle and makes mice more susceptible to infection. In a genital tract infection with *C. trachomatis*, the hormonal cycle can affect susceptibility in mice. DMPA is intended to create a more consistent and controlled environment for studying the infection's effects. The first paper to describe pre-treating mice with DMPA before infection with *C. trachomatis* was published in 1981 (33). Before this, a mouse model of *C. trachomatis* infection was not possible.

Past DMPA gene expression research has shown the downregulation of genes related to mucosal barrier integrity. Ectocervical biopsies and cervicovaginal lavage specimens collected from HIV-seronegative Kenyan sex workers either regularly cycling or using DMPA assessed with RNA-Seq found DMPA-associated downregulation of genes encoding keratins, small proline-rich proteins, and cell-cell adhesion proteins (34). Interestingly, the same study found DMPA-associated upregulation of some genes that are important for immune-activating pathways including NF-κB-mediated transcriptional regulation. Microarray gene expression analyses using samples from the human ectocervix found significant downregulation in genes critical for the epidermal differentiation complex, stratum corneum development, and the coding of cell-cell junction proteins (13).

Outside of susceptibility, DMPA may also alter host response and pathogenesis following infection. In a mouse model, it has been shown that DMPA-treated mice have reduced dendritic cell expression of costimulatory molecules, whose role is to activate the immune response, during acute infection with HSV-1 (35). Additionally, sex hormone receptors and the hormonal environment have been shown to alter bacterial shedding, cytokine production, and T cell

recruitment during *Chlamydia* infection *in vivo* (36). The uncertainty surrounding its effects on immune responses and disease progression underscores the need for further research. Addressing these knowledge gaps is essential for developing targeted public health strategies that account for the potential impact of DMPA on infectious diseases.

V. Research question and hypothesis

Our research aims to characterize how DMPA alters global gene expression and tissue morphology in the vagina, cervix, and uterus. We hypothesize that DMPA is weakening the immune barrier by reducing the expression of genes involved in the host response and that it reduces the thickness of the physical barrier of the female genital tract.

A. Use of a murine model to study DMPA-associated host-pathogen interactions in the female genital tract

Prior studies have attempted to characterize this increase in susceptibility to mucosal pathogens, including epidemiological studies and evaluation of the barrier system in the genital tract. Uniquely, this research is targeted at evaluating gene expression in the murine female genital tract. Transcriptomic studies examining DMPA-induced alterations in murine models have not been conducted. Working with a model organism reduces variability in dosage, age, and lifestyle while allowing us to sample the female genital tract including the uterus and ovaries.

Additionally, mouse models are the most common animal model used to simulate *Chlamydia* infection of the female genital tract. This research and the mouse model are of direct relevance to the Chlamydia field. We also utilized *Chlamydia trachomatis* to study infectious acquisition in mice treated with DMPA. For that study, mice were challenged with *Chlamydia trachomatis* serovar L2 transcervically, to mimic sexual transmission in humans and the progression of the infection to the upper genital tract, as performed in previous studies (37).

In the following experiments, comparisons will be made between mice treated with 2.5 mg DMPA or sterile saline (phosphate-buffered saline, PBS) subcutaneously. This dosage is used by the *Chlamydia* field and is scaled from the human dose for contraceptive use. All mouse procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee (IACUC). The transcriptomic work was performed with the assistance of the Boston Children's Hospital Intellectual and Developmental Disabilities Research Center Molecular Genetics Core Facility. Measurement of the infectious acquisition was assessed by evaluating bacterial burden via qPCR, as performed in previous studies (37).

B. Utilization of NanoString technologies to measure differential gene expression

NanoString nCounter technology is a technique similar to RNA sequencing which uses next-generation sequencing to measure the quantity of RNA in a biological sample as a representation of the transcriptome at that time (38). The prime difference is that NanoString does not measure the entire transcriptome, instead it is a targeted approach to evaluating a panel of genes. This targeted approach makes data processing more user-friendly, and we can select panels specific to our phenotype. Here, we are focusing on immune-related genes.

Using NanoString, RNA is extracted and tagged with probes specific to our targets of interest (38). NanoString uses molecular barcodes for genes identified by fluorescently labeled RNA containing a unique color sequence for each gene of interest. Next, these complexes are annealed to a complementary backbone ligated to a target-specific sequence. The fluorescent RNA-DNA hybrid molecule constitutes one reporter half of a probe pair. The second half is the capture probe, which consists of a gene-specific sequence adjacent to the reporter probe and a series of 3' repeats. To quantify these probes and the RNA sequences in the sample, microscopic

imaging is employed in a hybridization. These complexes are then quantified, and we can analyze differential gene expression.

The panels chosen were the PanCancer Immune Profiling Panel and the Host Response Panel. Together they accounted for 1113 unique genes; the two panels had a 55% overlap which also served as an internal control. These panels are pathogen-agnostic and measure the expression of genes relating to many features of the immune response, including both the innate and adaptive immune systems.

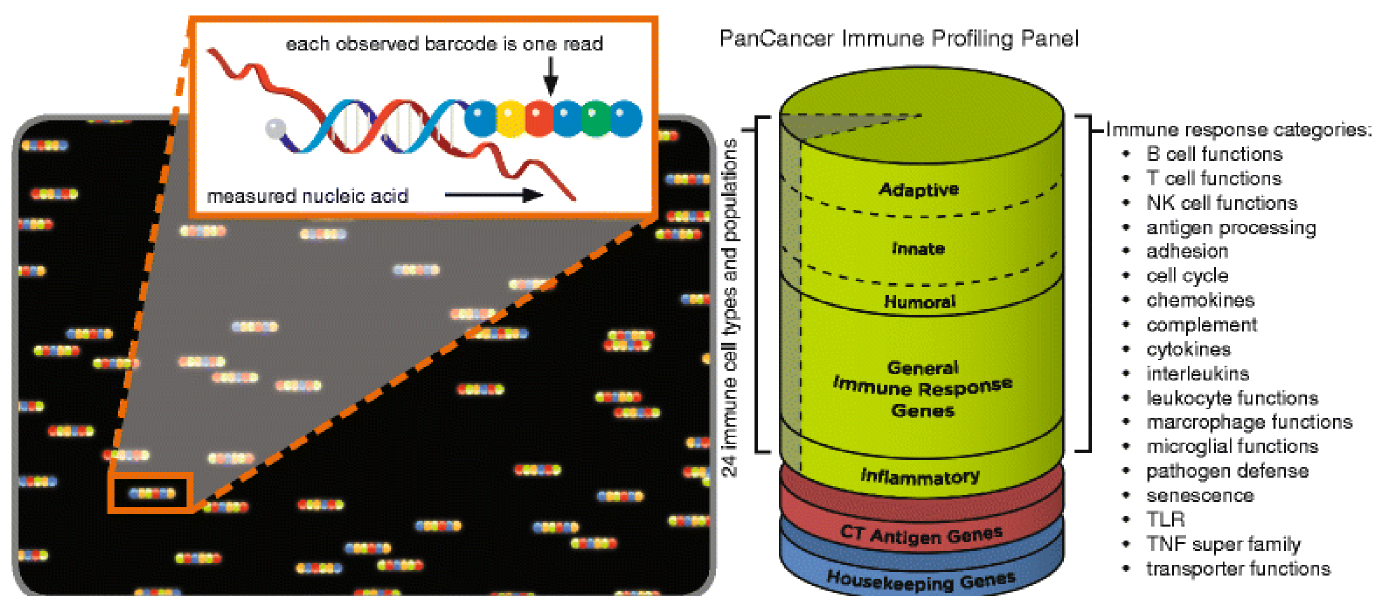

Figure 5. An example of the molecular barcoding that NanoString technology deploys with the capture and reporter probes specific to our targets of interest. The mRNA from the sample of interest is hybridized with the complementary nucleic acid attached to an optical barcode pattern that represents that particular gene (38). The schematic below illustrates the PanCancer Immune Profiling Panel. This panel allows us to study various immune cell types and immune responses by analyzing the expression of RNA in 770 genes. (Image retrieved from 86)

VI. Importance

The association between DMPA use *in vivo* and increased susceptibility to a variety of mucosal pathogens is concerning and underexplored. This research holds significant public health implications, particularly in the context of DMPA usage and its association with a heightened risk of HIV acquisition (29-32). With approximately thirty-five million individuals

globally relying on DMPA as an injectable form of hormonal contraception, understanding its impact on infection risk is paramount. Access to safe contraceptives offers direct benefits, allowing individuals to exert control over their reproductive health. This, in turn, leads to a reduction in unintended pregnancies, a decrease in maternal and infant morbidity and mortality, and a lower risk of vertical disease transmission (2).

The obligate intracellular pathogen *Chlamydia trachomatis* is the most common reportable infection in the United States with an estimated incidence of three million cases per year (3). Although acute infection is asymptomatic, mucosal damage can develop following infection, untreated *Chlamydia* infections increase the patient's risk of severe disease outcomes: blindness caused by ocular trachoma infection; pelvic inflammatory disease (PID), ectopic pregnancy, and infertility caused by female upper genital tract infection (3, 37). Exposure to constant synthetic hormone levels, which is the case with DMPA use, alters the pattern of *Chlamydia* infection compared to that in hormonal contraceptive non-users (41).

A critical public health concern is understanding the mechanism by which DMPA modifies host susceptibility. The World Health Organization has urged further research to gain a more comprehensive understanding of how DMPA may elevate the risk of infection by sexually transmitted pathogens (10).

Furthermore, in mice and non-human primates, DMPA has been found to increase susceptibility to a variety of mucosal pathogens such as *Chlamydia*, herpes simplex virus, simian-human immunodeficiency virus, Zika virus, and *Mycobacterium tuberculosis* (3-9). Therefore, it is crucial to gain a deeper understanding of how DMPA affects animal models and research in the pathogenesis of these infections. These fields will benefit from having a deeper understanding of how DMPA impacts their animal models and research interpretations. Our

insights will not only improve our understanding of infection susceptibility but also guide the development of preventive and therapeutic strategies, ultimately leading to better health outcomes for individuals worldwide.

RESULTS

I. DMPA is required for reliable *Chlamydia* infection in the murine female genital tract

The *Chlamydia* field has historically utilized DMPA to establish a mouse model of female genital tract infection (3, 33). This approach is often justified as a means of minimizing variables, as DMPA induces a progesterone-high diestrus state in mice. For our investigation of the effects of DMPA on infectious susceptibility, it is important to establish whether DMPA treatment is experimentally necessary for *Chlamydia* infection in mice.

We investigated whether DMPA was required for genital tract *Chlamydia* infection in mice. The mice were administered either 2.5 mg DMPA or 100 µL PBS subcutaneously and challenged with 5 x 10^6 IFU *Chlamydia trachomatis* seven days after treatment. Bacterial burden in uterine homogenates was quantified by qPCR three days following the challenge.

Treatment with DMPA resulted in a statistically significant 100-fold increase in *C. trachomatis* burden compared to PBS treatment. Bacterial burden quantifies the amount of *Chlamydia* DNA found in the uterus. This initial result suggests that DMPA is required for reliable *Chlamydia* infection in the murine female genital tract.

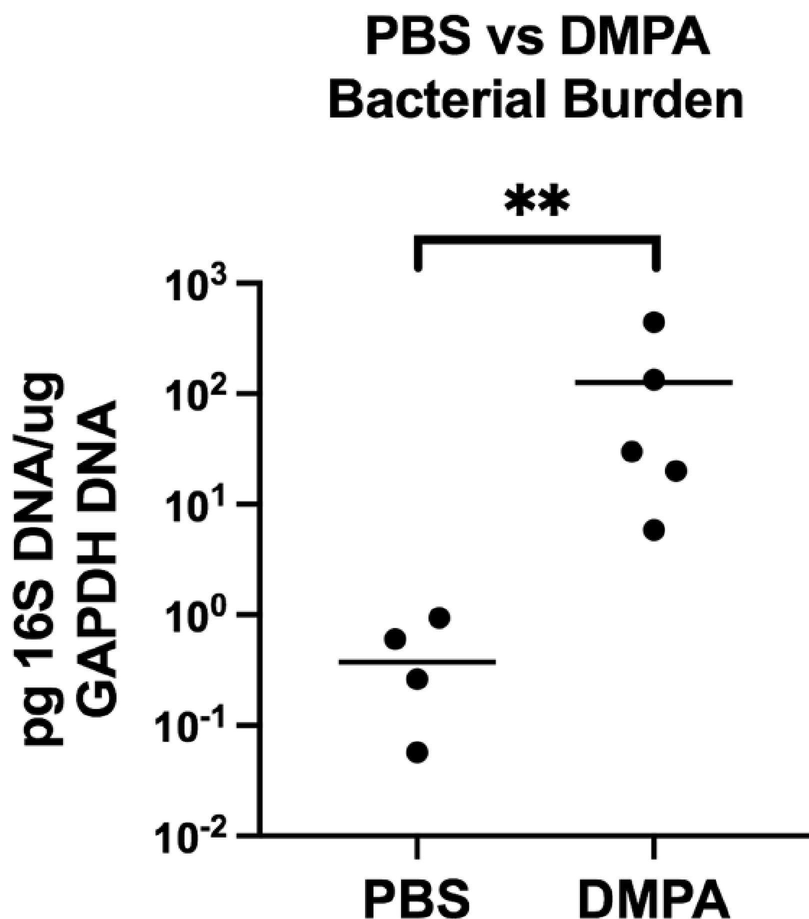

Figure 6. At 5 weeks old, C57BL/6J mice were given 2.5 mg DMPA or sterile PBS subcutaneously. One week later, they were infected transcervically with *Chlamydia trachomatis* serovar L2. *C. trachomatis* bacterial burden in uterine homogenates was quantified by qPCR three days post-infection. Significance was determined using a t-test; ** p = 0.0079

II. DMPA causes epithelial thinning in the murine female genital tract

Various studies have examined how DMPA modulates epithelial barrier function in the reproductive tract (26, 27, 34, 39). This could decrease barrier function to allow for an increase in susceptibility to mucosal infection. A known mechanism of action by which DMPA prevents pregnancy in humans is the thinning of the endometrium (Pfizer). This finding has been corroborated in non-human primates and could enable the entry of pathogens into uterine tissue (39). Recent studies demonstrated increased permeability of the lower genital tract in both non-human primates and mice treated with DMPA (5, 39).

Again, the mice were administered either 2.5 mg DMPA or 100 µL PBS subcutaneously. Seven days following treatment, the entire female genital tract was excised and stained with H&E to assess for histopathological changes. Analysis of the vaginal vault revealed significant morphological changes in mice treated with DMPA. Notably, DMPA-treated mice exhibited a complete absence of the *stratum corneum* layer. These mice also displayed a notable thinning of the mitotically active *stratum basalis* epithelium. Interestingly, there is now an additional epithelial layer that is morphologically distinct from the stratum basalis and stratum corneum layers. It appears to be composed of nucleated cells and is exposed to the lumen.

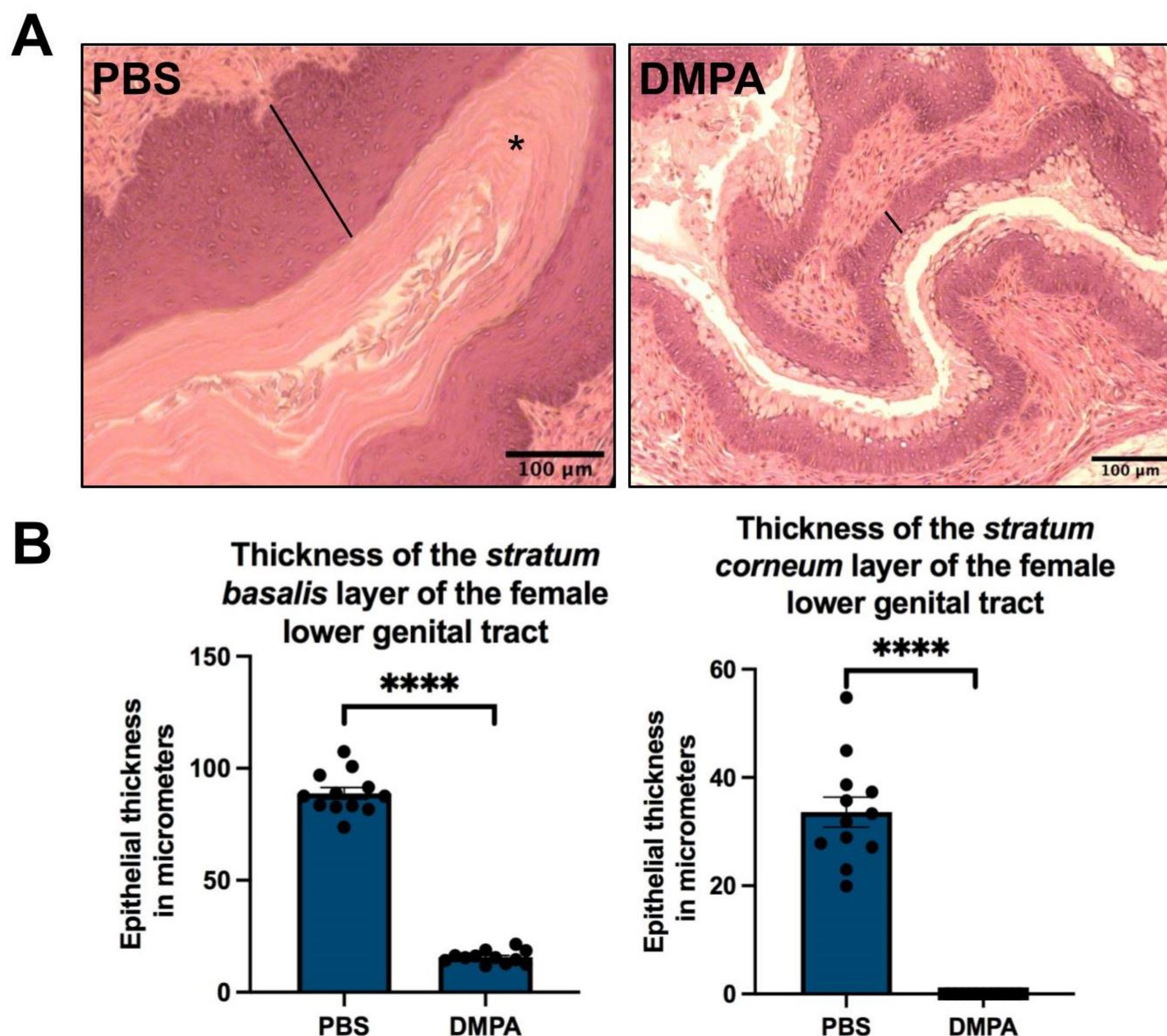

Figure 7. (A) H&E-stained epithelium of the murine vagina treated with either PBS or DMPA imaged at 10x magnification. The line represents an example of the stratum basalis, while the asterisk provides a visual of the stratum corneum. (B) Samples were anonymized and the thickness of the stratum basalis and stratum corneum epithelial strata were measured using ImageJ. Significance was determined using a Mann-Whitney test; **** $p < 0.0001$.

The columnar epithelium was significantly thinned in the uteri of DMPA-treated mice as compared to PBS-treated mice.

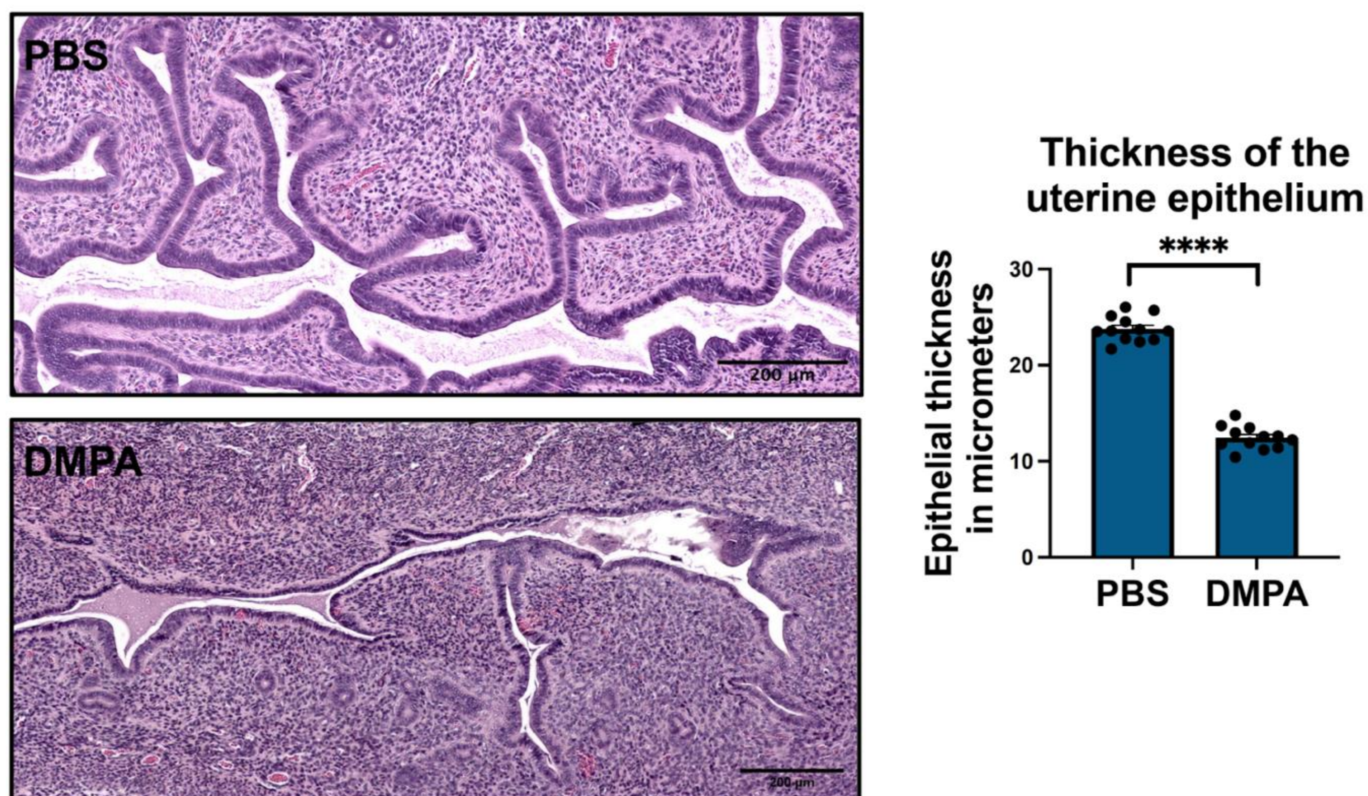

Figure 8. H&E-stained uteri from PBS or DMPA-treated mice imaged at 10x magnification. Samples were anonymized and the thickness of the columnar epithelium was measured using ImageJ. Significance was determined using a Mann-Whitney test; **** $p < 0.0001$.

III. DMPA causes significant differential gene expression in the female genital tract

We hypothesized that DMPA reduces the expression of genes involved in maintaining the immune barrier. Based on the documented increase in susceptibility to a variety of pathogens as

well as the widespread morphological changes, we decided to take a broad approach to identify how DMPA modulates gene expression by using NanoString.

Mice were subcutaneously administered either 100 μL PBS as a negative control or 2.5 mg DMPA in either a single at day -7 or a double dose at both days -10 and -3. Subsequently, the upper genital tract, comprising the endocervix, uterine horns, oviducts, and ovaries, was excised and processed at the Boston Children's Hospital Molecular Genetics Core Facility. RNA from these samples was applied to the two selected NanoString chips: the PanCancer Immune Profiling Panel and the Host Response Panel.

To help assess the similarity of gene expression changes between treatment groups, we used Principal Component Analysis (PCA). Our analysis demonstrated that the primary differentiating factor among the samples was the experimental condition, indicating that the observed changes in gene expression were associated with DMPA treatment. Notably, the gene expression profiles of mice treated on day -7 exhibited similarities to those treated on both day -10 and -3. This observation was further supported by the clustering of these treatment groups within the PCA plot, suggesting a lack of substantial differences between these conditions. Additionally, the specific genes affected by treatment did not vary significantly, and statistical significance was minimal or absent, further corroborating the consistency of gene expression changes across the different treatment time points. This encouraged us to bin the two DMPA dosage schedule treatment groups together for the rest of our gene expression analyses.

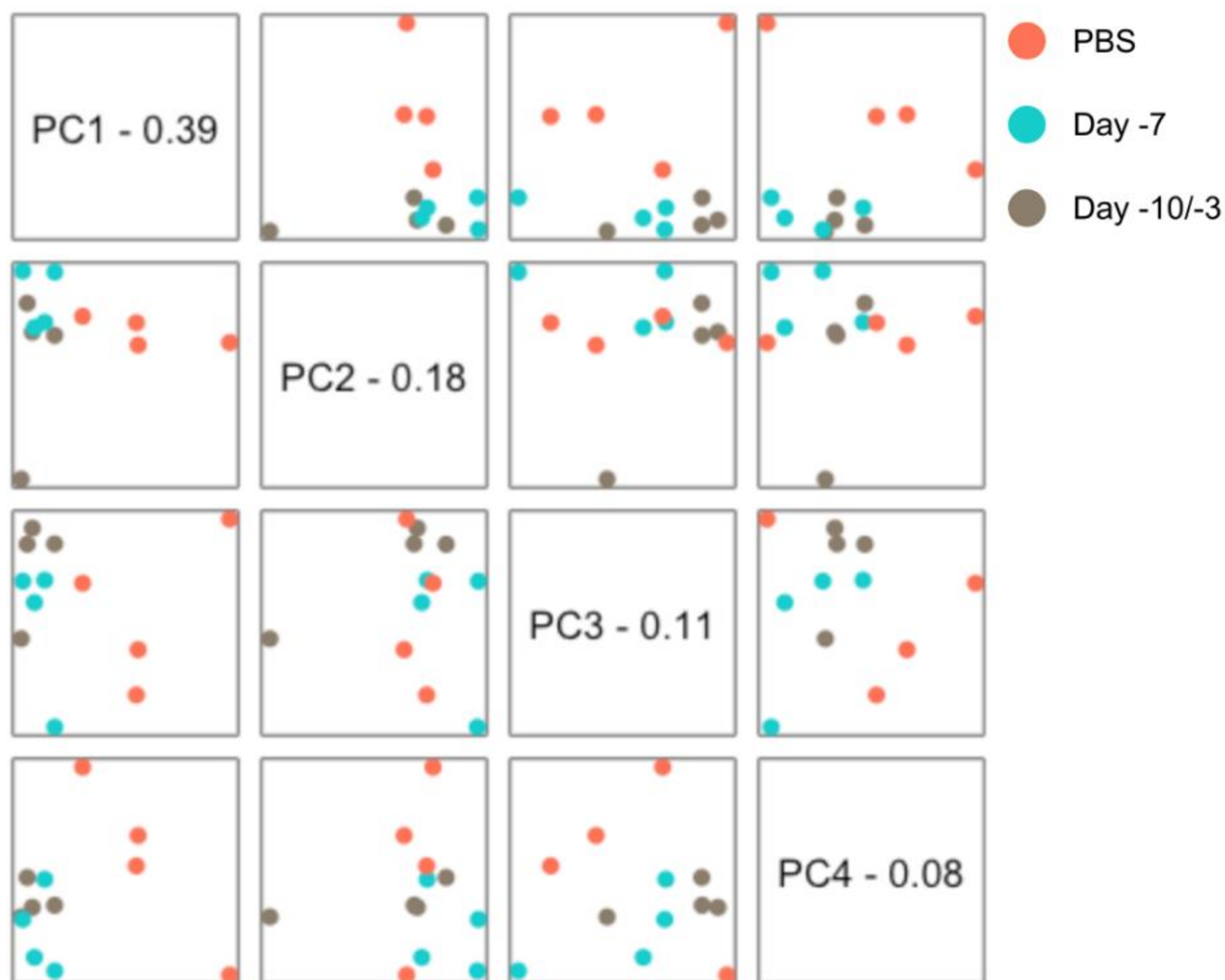

Figure 9. Principal component analysis (PCA) displays differences between the three treatment groups. The first four principal components of the gene expression data are plotted against each other, and coloring is set by group.

When normalized to the PBS treatment group, the samples treated with DMPA displayed differential gene expression. Over three hundred genes displayed a statistically significant change in expression. Of those genes, the log2(fold change) of the expression following treatment ranged from -7.62 to 5.02, which would be -196.72 to 32.447-fold.

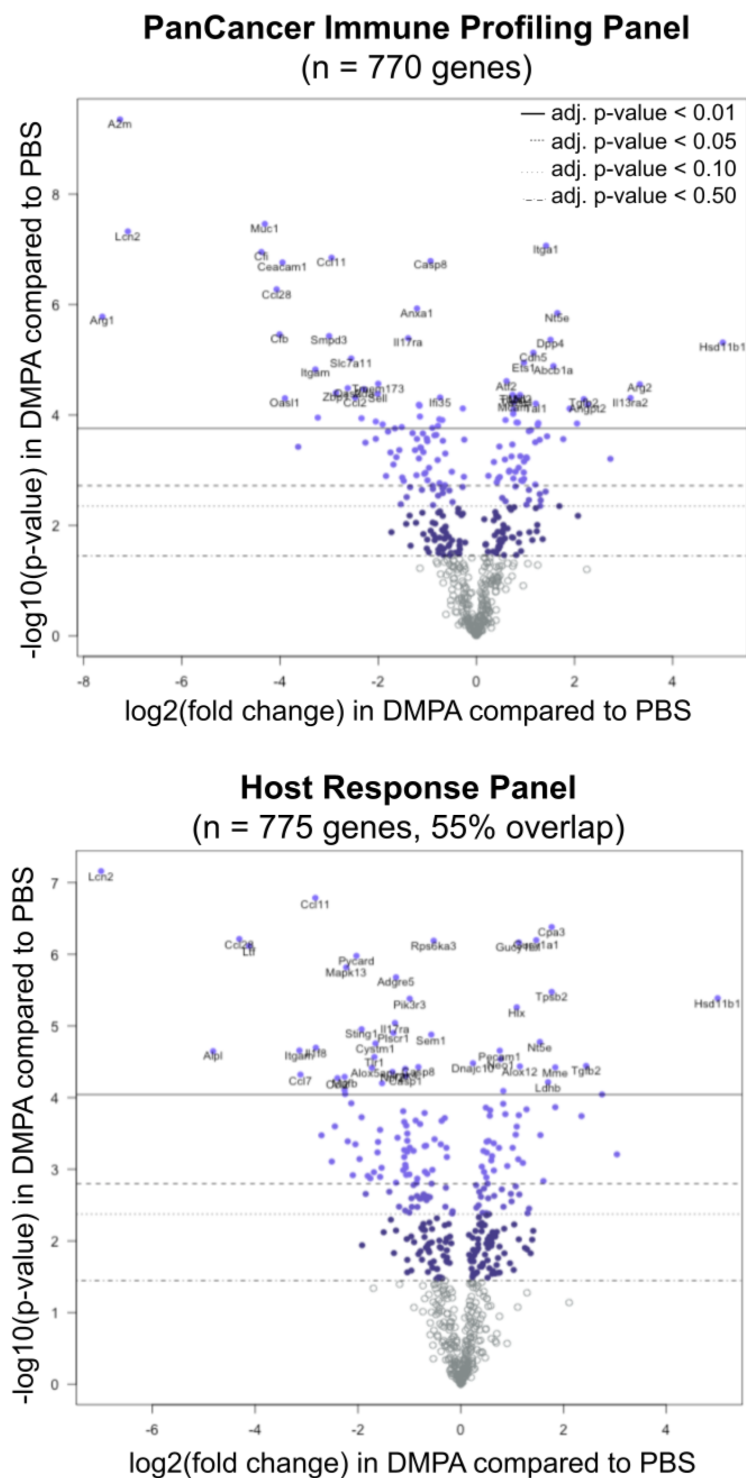

Figure 10. Volcano plots display the differential gene expression of DMPA-treated mice compared to PBS-treated mice using the PanCancer Immune Profiling Panel and the Host Response Panel (NanoString Technologies). The panels account for 770 and 775 genes, respectively. There is a 55% overlap between panels, genes that appeared on both panels

changed similarly. Adjusted p-values range from $p < 0.50$ to $p < 0.01$. Named genes have an adjusted p-value < 0.01 and a false discovery rate < 0.05.

We noted that genes appearing on both panels displayed consistent changes in expression. The overlap between the two panels served as internal validation, indicating that the observed alterations in gene expression accurately reflect the characteristics of our samples, increasing our confidence in these results.

IV. DMPA reduces the expression of genes involved in maintaining the immune barrier

Our next steps involved the assessment of the functional roles and pathways attributed to our top candidate genes, specifically those with an adjusted p-value < 0.01 and a false discovery rate < 0.05. To elucidate the biological relevance of these differentially expressed genes, we conducted a literature review. By analyzing scientific literature relating to the genes within the context of their biological function, relationship to the female genital tract, or role in infection and immunity, we can parse the importance of our top candidate genes. This approach gave us insights into the potential immune mechanisms and processes underlying the observed gene expression changes in response to DMPA.

In our analysis, a significant proportion of the highly downregulated genes were identified as integral components of the innate immune system. Notably, these included genes essential for the functioning of key innate immune mechanisms such as the complement system (e.g. *Cfb*, Complement Factor B, -4.01 log2 fold change, p = 1.71E-05), barrier function (e.g. *Muc1*, Mucin 1, -4.31 log2 fold change, p = 4.33E-07), components of the inflammasome (e.g. *Sting1*, Stimulator of Interferon Genes, -2.01 log2 fold change, p = 7.22E-06), and mediators of nutritional immunity (e.g. *Lcn2*, Lipocalin 2, -7.1 log2 fold change, p = 3.68E-07). This makes sense due to the ability to be infected with a pathogen being based on the function of the innate

immune system. Additionally, we observed a decrease in the expression of major cytokines and chemokines (e.g. *Ccl28*, Chemokine (C-C) motif ligand 28, -4.07 log2 fold change, p = 5.17E-06) crucial for orchestrating the immune response, further highlighting the broad impact of DMPA treatment on immune modulation. We also found upregulation in some genes that are associated with immunosuppression and adaptive immunity.

Gene	Log2(FoldΔ)	Relevant Function
Arg1	-7.62	
A2m	-7.26	Innate: Complement
Lcn2	-7.1	Innate: Nutritional Immunity
Alpl	-4.91	
Cfi	-4.38	Innate: Complement
Muc1	-4.31	Innate: Barrier
Ltf	-4.21	Innate: Nutritional Immunity
Ccl28	-4.07	Cytokines/Chemokines
Cfb	-4.01	Innate: Complement
Ceacam1	-3.95	Innate: Barrier
Itgam	-3.23	Innate: Complement
Smpd3	-3	
Ccl11	-2.95	Cytokines/Chemokines
Slc7a11	-2.55	Innate: Nutritional Immunity
Mapk13	-2.31	Innate: Inflammasome
Pycard	-2.11	Innate: Inflammasome
Sting1	-2.01	Innate: Inflammasome
Cystm1	-1.74	
Plscr1	-1.39	Innate: Inflammasome
Il17ra	-1.39	Cytokines/Chemokines
Adgre5	-1.34	
Anxa1	-1.21	Innate: Barrier
Casp1	-1.15	Innate: Inflammasome
Pik3r3	-1.07	
Casp8	-0.934	Innate: Inflammasome
Sem1	-0.648	
Rps6ka3	-0.601	
Ets1	0.969	
Cdh5	1.16	
Gucy1a1	1.39	
Itga1	1.42	Adaptive
Dpp4	1.51	Adaptive
Nt5e	1.65	Adaptive
Hsd11b1	5.02	

Figure 11. The heat map displays the name and log2 fold change of genes found to have differential expression changes following DMPA treatment with a p-value < 0.01 and a false discovery rate < 0.05. The relevant function is listed based on host immune pathways or components associated with the gene.

To validate this data, we used Gene Set Analysis to make volcano plots that highlight genes that NanoString had tagged as being part of a specific biological pathway (38). As expected, many of the differentially expressed genes were marked as being related to the innate immune system. We also saw confirmation of our findings for processes including Chemokine Receptors, Cytokine Receptors, and Complement. Gene Set Analysis was not available for all our functions of interest.

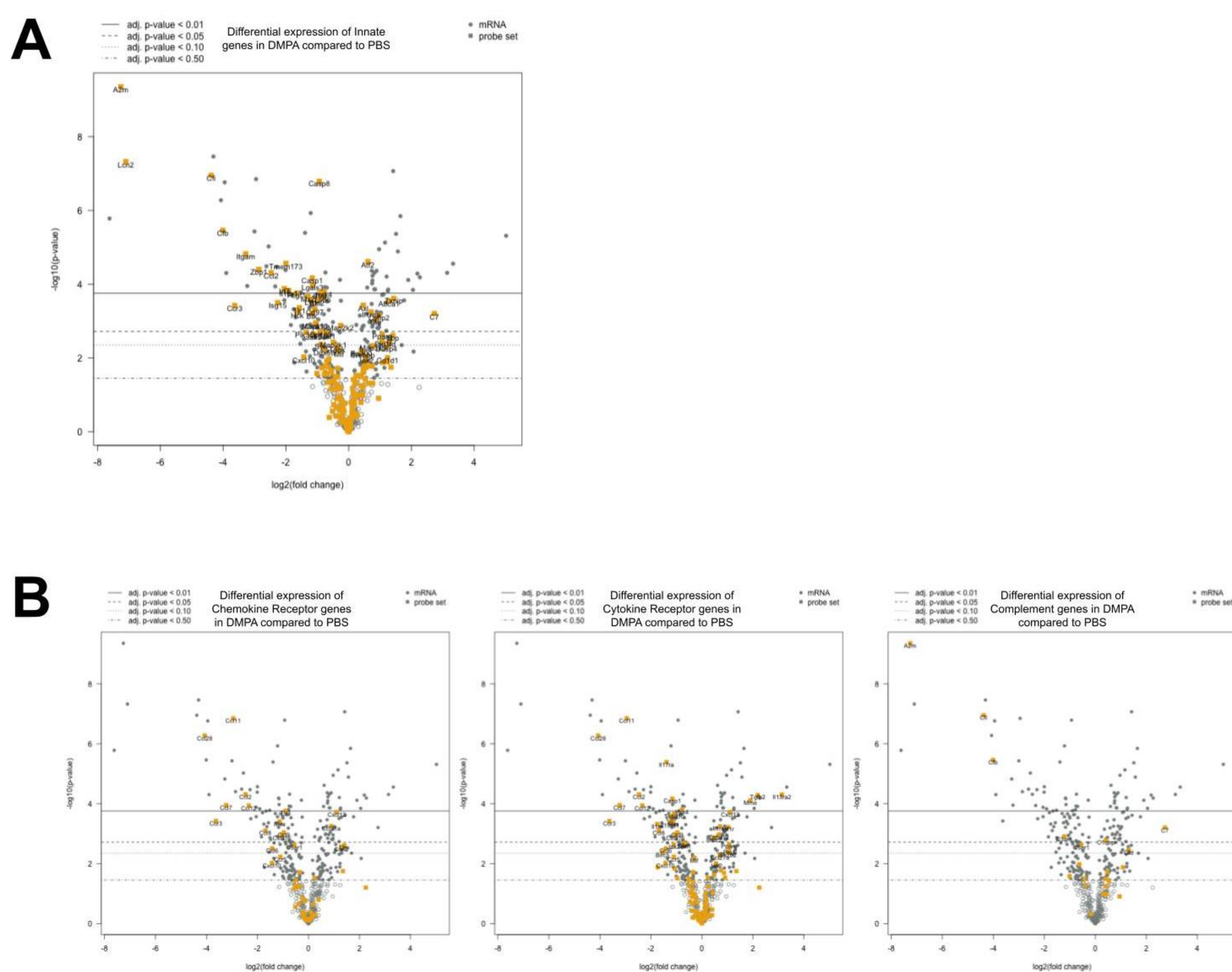

Figure 12. Volcano plots show differential gene expression with genes from the probe set of interest highlighted in yellow using Gene Set Analysis. (A) Differentially expressed innate genes of DMPA-treated mice compared to PBS-treated mice using the PanCancer Immune Profiling Panel. (B) Differentially expressed genes of DMPA-treated mice compared to PBS-treated mice using the PanCancer Immune Profiling Panel, from left: chemokine receptor genes, cytokine receptor genes, complement genes.

Of the 34 most differentially expressed, 24 genes were interferon-stimulated genes, according to the Interferome database (87). This means that they can be expressed following stimulation with interferons, which are a group of signaling proteins that modulate immune function. Specifically, they were regulated by both Type I and Type II Interferon. Type I interferon is expressed in response to viral infections (88). The Interferon-Stimulated Genes (ISGs) induced by type I interferon are associated with suppressing viral replication and increasing the expression of immune signaling proteins. Type II interferon, which consists of IFN-γ, is associated with controlling intracellular pathogens.

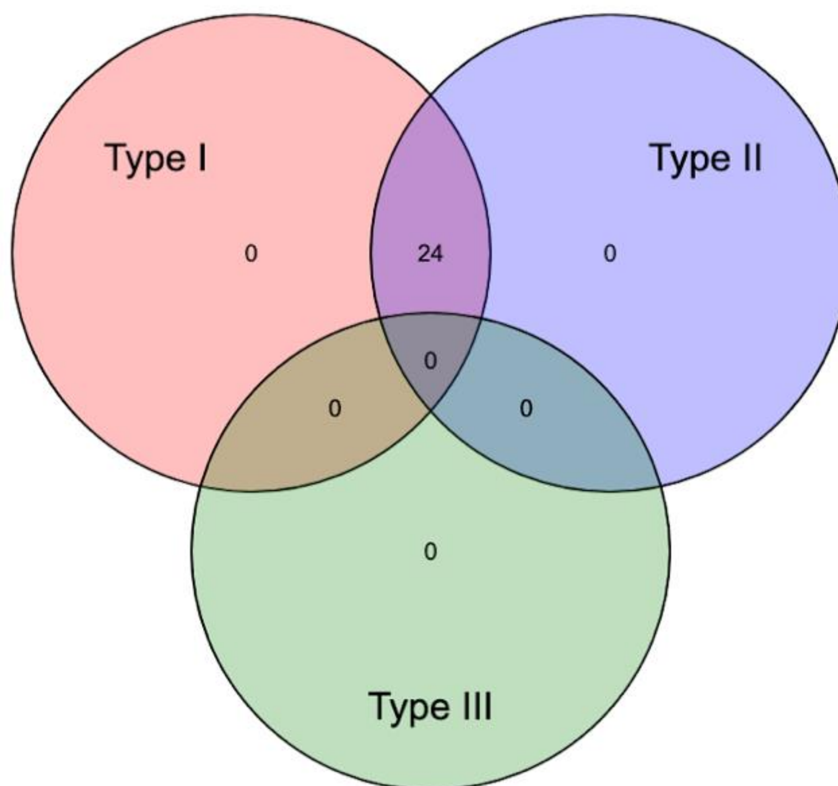

Figure 13. Venn diagram shows the number of genes regulated by one or more interferon (IFN) types.

Additionally, we used the Interferome database for our analysis of the association of top candidate Interferon-Stimulated Genes (ISGs) with biological processes (87). Our findings

revealed a statistically significant correlation between the differentially expressed genes and the host response to lipopolysaccharide, a component commonly associated with gram-negative bacteria (89). This substantiates our literature review and provides valuable insights for potential future research.

Biological Processes

Term Name	Term Definition	Gene Count	p Value*
response to lipopolysaccharide	Any process that results in a change in state or activity of an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a lipopolysaccharide stimulus; lipopolysaccharide is a major component of the cell wall of gram-negative bacteria. [GOC:add, ISBN:0721601464]	5	1.04E-5
apoptotic process	A programmed cell death process which begins when a cell receives an internal (e.g. DNA damage) or external signal (e.g. an extracellular death ligand), and proceeds through a series of biochemical events (signaling pathways) which typically lead to rounding-up of the cell, retraction of pseudopodes, reduction of cellular volume (pyknosis), chromatin condensation, nuclear fragmentation (karyorrhexis), plasma membrane blebbing and fragmentation of the cell into apoptotic bodies. The process ends when the cell has died. The process is divided into a signaling pathway phase and into an execution phase, which is triggered by the former. [GOC:dhl, GOC:ecd, GOC:go_curators, GOC:mtg_apoptosis, GOC:tb, ISBN:0198506732, PMID:18846107, PMID:21494263]	4	0.0222
proteolysis	The hydrolysis of proteins into smaller polypeptides and/or amino acids by cleavage of their peptide bonds. [GOC:bf, GOC:mah]	4	0.0135
regulation of apoptotic process	Any process that modulates the occurrence or rate of cell death by apoptotic process. [GOC:jl, GOC:mtg_apoptosis]	4	4.8E-4
cell cycle	The progression of biochemical and morphological phases and events that occur in a cell during successive cell replication or nuclear replication events. Canonically, the cell cycle comprises the replication and segregation of genetic material followed by the division of the cell, but in endocycles or syncytial cells nuclear replication or nuclear division may not be followed by cell division. [GOC:go_curators]	3	0.0112
response to drug	Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a drug stimulus. A drug is a substance used in the diagnosis, treatment or prevention of a disease. [GOC:jl]	3	0.0211
response to stress	Any process that results in a change in state or activity of a cell or an organism (in terms of movement, secretion, enzyme production, gene expression, etc.) as a result of a disturbance in organismal or cellular homeostasis, usually, but not necessarily, exogenous (e.g. temperature, humidity, ionizing radiation). [GOC:mah]	3	0.00271

Figure 14. The table describes the biological process, description, number of genes (within the list of top hits) associated with the function, and the p-value listed from lowest to highest.

DISCUSSION

I. Overview of findings

Depo Provera (depot medroxyprogesterone acetate, DMPA) is a synthetic form of progesterone. It is widely used clinically as a hormonal contraceptive and is included on the World Health Organization's Model List of Essential Medicines (1). In humans, it is associated with an increase in HIV acquisition (9). In mice and non-human primates, it has been shown to increase susceptibility to simian-human immunodeficiency virus, *Mycobacterium tuberculosis*, Zika virus, Herpes Simplex Virus-2, and *Chlamydia* (3-8). Despite this documented increase in infection, including sexually transmitted infection, the cell and molecular mechanism(s) for how DMPA alters susceptibility to pathogens is unknown. We hypothesized that DMPA weakens the immune barrier in the female genital tract. It was our goal to characterize how DMPA alters gene expression and morphology in the murine female genital tract.

First, we found that DMPA increases *Chlamydia trachomatis* bacterial burden in mice three days post-infection, with some PBS-treated mice falling below the limit of detection. This shows that DMPA is required for reliable *Chlamydia* infection *in vivo* and mice may be considered resistant to infection in the absence of DMPA treatment.

Next, we have shown that DMPA causes significant thinning of the epithelium throughout the female genital tract. We used microscopy and ImageJ to quantitatively analyze the epithelium in the murine female genital tract, seven days after DMPA treatment. In the lower genital tract, we saw thinning of the stratum basalis epithelial layer and a complete absence of the *stratum corneum* layer following DMPA treatment. In the upper genital tract, we found the columnar epithelium to be thinned.

Taking a broad approach by using NanoString to measure the expression of over 1100 murine genes in the uterus following DMPA treatment, we found significant differential gene expression. Specifically, we found downregulation of many innate immune-related genes including those related to the inflammasome, complement, barrier maintenance, and nutritional immunity. We also found upregulation in genes associated with immunosuppression and the adaptive immune system.

Based on the extensive morphological changes, and downregulation of key innate immune components, we find that DMPA is weakening the immune barrier of the murine female genital tract. This is supported by our finding of an increase in the bacterial burden of *Chlamydia trachomatis* in DMPA-treated mice, as well as the documented increase in susceptibility to a variety of mucosal pathogens. Our data support a model where DMPA weakens both the physical barrier and the immune barrier functions to allow reliable *Chlamydia* infection in the murine female genital tract. This also may explain why DMPA use in humans is associated with an increase in sexually transmitted infections.

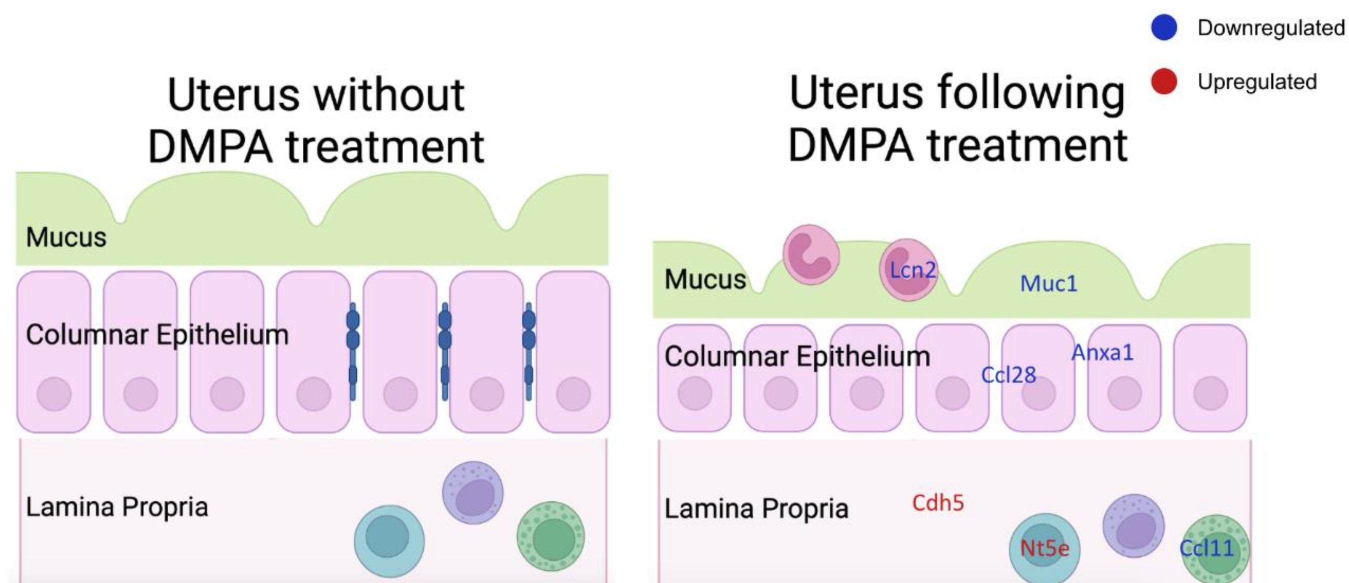

Figure 15. The working model displays morphological and transcription changes in the FGT associated with DMPA treatment. The columnar epithelium thinned and relevant differentially expressed gene names affixed to their related structures including the epithelial cells and professional immune cells such as neutrophils, B cells, and T cells which are important for the immune response within the FGT.

II. Possible mechanisms of action for DMPA's effects on the immune barrier

The immunosuppressive effects seen with DMPA usage are not found with other hormonal contraceptives (41). There is no significant association noted between oral contraception use and sexually transmitted infection. These effects are also independent of endogenous progesterone as similar effects are not found in naturally cycling people or animals with high progesterone (41). In fact, DMPA is required for mice to be infected with genital Herpes, and their resistance to lethal HSV challenge is retained following treatment with a non-DMPA saline suspension of progesterone (44). While modest differences in infectivity cannot be ruled out, it appears that DMPA is unique and specific in modulating the host response to infection. Therefore, it is crucial to look at the characteristics that make DMPA different from other hormonal contraceptives.

DMPA is a synthetic progestin (1). Steroid hormones bind to receptors in the host cell nucleus, which then bind to DNA sequences to alter transcription. For therapeutic action, progestins typically mimic the actions of natural progesterone by binding to the progesterone receptor. DMPA works by inhibiting the production of gonadotropin to prevent ovulation and follicular maturation while thinning the epithelium. However, the negative side effects seen following DMPA treatment are not recapitulated through natural progesterone or other progestins (40, 41). This suggests that DMPA exhibits off-target effects, likely based on acting outside of the intended progesterone receptor. One way that DMPA may be mediating this increase in susceptibility is through the mediation of other steroid receptors including the glucocorticoid receptor (8, 45). DMPA has partial GR agonist activity and has been shown to alter the expression of GR-regulated genes (46).

Glucocorticoids are steroid hormones produced by the adrenal glands; they bind to the glucocorticoid receptor (GR) which is present in almost every vertebrate animal cell (47). Common glucocorticoid drugs include prednisone and dexamethasone. These are used to reduce immune function activities including inflammation for the treatment of conditions caused by an overactive immune system, such as asthma and arthritis. Prolonged glucocorticoid therapy is associated with an increased risk for various infections, including a five-fold higher risk of developing *Mycobacterium tuberculosis* (48, 49).

The tuberculosis field has been interested in how glucocorticoids and GR-signaling impact infection risk (48, 49). In peripheral blood mononuclear cells, it has been shown that DMPA can act as a GR ligand for transactivation and transrepression, meaning that DMPA could activate or inhibit the transcription of GR-regulated genes (47). Transactivation and

transrepression have previously been shown to be an important anti-inflammatory pathway for glucocorticoids (50).

While the research into this connection is promising, there are many unanswered questions. Thus far, these studies have been conducted in cell lines - mechanistic *in vivo* and *in vitro* studies are critical for understanding how DMPA may act through the GR to induce effects such as modulation of the immune response. Further, physiologically relevant concentration for dose-dependent effects is not clear and it is possible that the GR-like effects would not be found in human DMPA users.

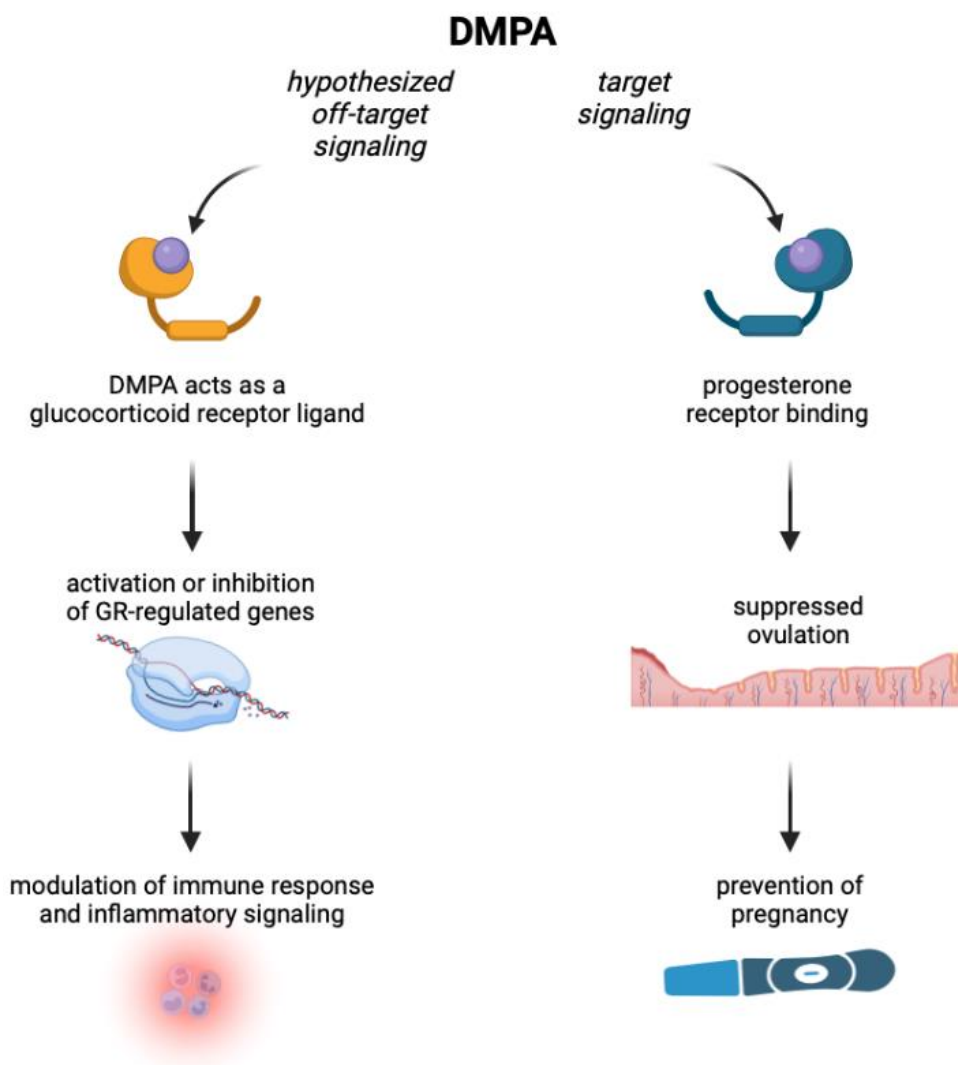

Figure 16. Graphical representation of the progesterone receptor target signaling that aims to suppress ovulation, as compared to the hypothesized off-target glucocorticoid receptor signaling which may have immunomodulatory effects.

Given the downregulation of innate, and specifically inflammasome and inflammatory marker-related genes it is likely that DMPA treatment leads to a reduction in the inflammatory response. In cases of infection, inflammation can be beneficial, as it can alert the body to the presence of microbial invaders (20). Additionally, it acts as a signal to recruit immune cells to the site of infection, facilitating the elimination of pathogens and the restoration of homeostasis. By dampening the immediate immune response to damage or pathogens, DMPA treatment might inadvertently create an environment conducive to pathogen entry and replication.

III. Limitations

A. Differences between the human and murine immune systems and responses

While I have previously described the benefits of utilizing a mouse model to study the effects of DMPA on the immune barrier in the female genital tract, it is important to contextualize how this affects the significance of our results. A better understanding of the documented susceptibility to mucosal pathogens will be useful for epidemiological research including the treatment and prevention of sexually transmitted infections (10). There are significant obstacles to clinical investigation into the efficacy of DMPA use, particularly in areas with high levels of HIV infection and transmission (29-32). Experimental design using mice as a pre-clinical model is well-positioned to generate relevant data that can be used independently or for human health-based hypotheses. Additionally, the mouse model allows us to explore a long-held experimental standard in the field of *Chlamydia* research, which also has implications for public health, given that the mouse model is used for *Chlamydia* vaccine development (3).

Our results support the findings in humans and mice that there is a reduction in the epithelial barrier and an increase in susceptibility to mucosal infection (26, 27, 34, 39). The specific genes and pathways, as well as the magnitude of differential expression or infection, would need to be studied in humans to draw specific conclusions.

Interestingly, there was some discordance between our results and studies of the impact of DMPA on gene expression in human ectocervical biopsies. For example, we found downregulation of C3 (complement component 3, -1.49 log2 fold change following DMPA treatment). C3 is arguably the most crucial protein in the complement system, as it acts by initiating complement activation, amplifying the complement pathway, and triggering the activation of the terminal pathway, resulting in the formation of the membrane attack complex (21). In studies of human tissue, increased levels of progesterone, both due to exogenous treatment with DMPA and endogenous origin, resulted in increased expression of C3 protein (13).

One potential reason for differences between human and murine studies is evolutionary (51). The immune systems of mice and humans are evolving in response to different selective pressures. In the wild, mice are on the ground and constantly exposed to diverse microbial populations. There are several examples of viruses and bacteria (Herpes Simplex Viruses, *Shigella* spp.) that cannot naturally establish infection in mice despite being infectious to humans (44, 52). Arguably, *Chlamydia trachomatis* may be an example of this phenomenon given our finding of significantly reduced bacterial burden in the absence of DMPA treatment.

Though our results may not be directly translational, studying the effects of DMPA treatment is incredibly relevant to public health and host-pathogen research. Given that DMPA is necessary to establish a mouse model of *Chlamydia* infection, our knowledge of *Chlamydia*

pathogenesis *in vivo* is directly influenced by the effects of DMPA (33, 36, 37). Critical evaluation of DMPA's impacts on the immune system is crucial for this field and future study of infectious susceptibility to other relevant infections such as HIV.

B. NanoString and evaluation of RNA expression

The targeted view garnered through NanoString has given us valuable information as to how DMPA impacts gene expression. Further research using RNASeq would provide more comprehensive data and may be of use for a holistic approach.

IV. Description of individual differentially expressed genes

One of the most important elements of this work was curating and evaluating the differentially expressed genes to compile our "top hits." First, I normalized the gene expression to compare the DMPA treatment groups to the PBS treatment group and then I segmented the genes with a p-value < 0.01 and a false discovery rate < 0.05. The log2 fold change of the gene expression following treatment ranged from -7.62 to 5.02, which would be -196.72 to 32.447-fold. I then conducted literature reviews of these top candidate genes to determine relevant functions and themes.

The largest magnitude fold change was found in the Arg1 (arginase 1, -7.62 log2 fold change, $p = 3.64E-06$) protein-coding gene. Arginase is the terminal enzyme of the urea cycle (53). It is a characteristic marker of M2 macrophages which are known for their anti-inflammatory and wound healing effects. A role for arginase outside of the urea cycle has been found for mammary glands, macrophages, and mucosa of the small intestine. Uterine arginase activity was shown to be increased following the administration of β-estradiol. Inhibition of uterine arginase activity was associated with embryonic arrest (54). This activity fits with

DMPA's function as a contraceptive. However, there are known to be additional effects stemming from arginase's use in immune cells. Arginine metabolism regulates innate and adaptive immune responses (55). It is involved in an antimicrobial effector pathway for polymorphonuclear leukocytes (PMN). Following cell death, arginase is released from the PMN and depletes surrounding arginine. This causes the suppression of T and NK cell proliferation as well as cytokine secretion, with the process being implicated with immunosuppression during chronic inflammatory states and tumor growth.

Hsd11b1 (11β-hydroxysteroid dehydrogenase type 1, 5.02 log2 fold change, p = 8.15E-06) was our most upregulated gene. This gene encodes an enzyme that catalyzes the conversion of the stress hormone cortisol to the inactive metabolite cortisone (56). Hsd11b1 expression is induced in monocytes upon their differentiation to macrophages. Further, Hsd11b1 has been shown to contribute to local immunosuppression by regulating the activity of myeloid cells. Hsd11b1 plays a significant role in the early response to an inflammatory stimulus, which promotes later resolution. It is interesting to note that Hsd11b1 is responsible for converting glucocorticoids into their active forms within tissues and, in the uterus, it has been observed that Hsd11b1 is positively regulated by progesterone. This suggests that Hsd11b1 may play a role in the modulation of the inflammatory response following DMPA treatment.

V. Description of differentially expressed pathways

A. Complement

The complement system is part of the innate immune system (14). Complement proteins opsonize pathogens, meaning they coat them with antibodies and other complement proteins, allowing them to be more easily destroyed by phagocytes. It induces an inflammatory response

to fight off infection. Downregulation of components of the complement system, such as that seen with DMPA, has implications for infectious susceptibility.

A2m (alpha-2-macroglobulin, -7.26 log2 fold change, p = 8.42E-09) was a major hit. The protein encoded by this gene has roles as a protease inhibitor (trypsin, thrombin, collagenase) and cytokine transporter (57). Alpha-2-macroglobulin can inhibit inflammatory cytokines, disrupting inflammatory cascades. It is locally produced by macrophages, fibroblasts, and epithelial cells. *In vitro*, A2m enhances the migration of polymorphonuclear cells. Additionally, A2m is produced by the stromal component of endometrial tissue and may have a role in implantation (58). Alpha-2-macroglobulin may modulate protease-generating reactions that take place adjacent to the endothelial surface, thereby protecting the vascular endothelium (57). A2m is part of the complement system and undergoes proteolysis, a conformational change, and exposes an internal thioester that binds and traps active protease - similarly to C3.

Cfb (complement factor b, -4.01 log2 fold change, p = 1.71E-05) is an important component of the initiation and amplification of the alternative pathway of the complement system. Upon cleavage of the Cfb protein by Cfd, an active serine protease subunit is formed which then binds to C3b to form the C3 convertase of the alternative pathway (59). C3 convertase then goes on to cleave C3 into C3a and C3b, of which C3b can go on to opsonize microbial pathogens or can bind to Bb in a positive feedback loop, amplifying the alternative pathway (21, 59). Prior studies using mouse and rat models have shown that Cfb is a hormonally regulated protein (60). Treatment of these animal models with exogenous progesterone suppresses the expression of the Cfb protein. However, human studies in which endometrial tissue was examined have shown that expression of Cfb proteins localized in the glandular

epithelial cells of the FGT is increased during DMPA treatment as well as during the luteal phase of the menstrual cycle (61).

The Cfi (complement factor I, -4.38 log2 fold change, p = 6.76E-07) gene encodes the complement factor I protein, a serine protease most commonly produced by hepatocytes and monocytes (62). The protein acts by cleaving the ɑ-chain of complement factor 3b and complement factor 4b proteins, deactivating them, and blocking the amplification of the complement cascade (63).

Itgam (integrin alpha M chain, -3.23 log2 fold change, p = 1.79E-05) is part of the heterodimeric integrin alpha-M beta-2 (64). It is associated with the promotion of antibody-dependent or independent effector cell functions. Itgam enables complement component C3b binding activity and is involved in regulating the uptake of complement-coated particles and pathogens. It plays a role in the adhesive interactions of monocytes, macrophages, and granulocytes as well as regulating neutrophil migration. Past studies have found this gene to be upregulated with the levonorgestrel intrauterine system (65).

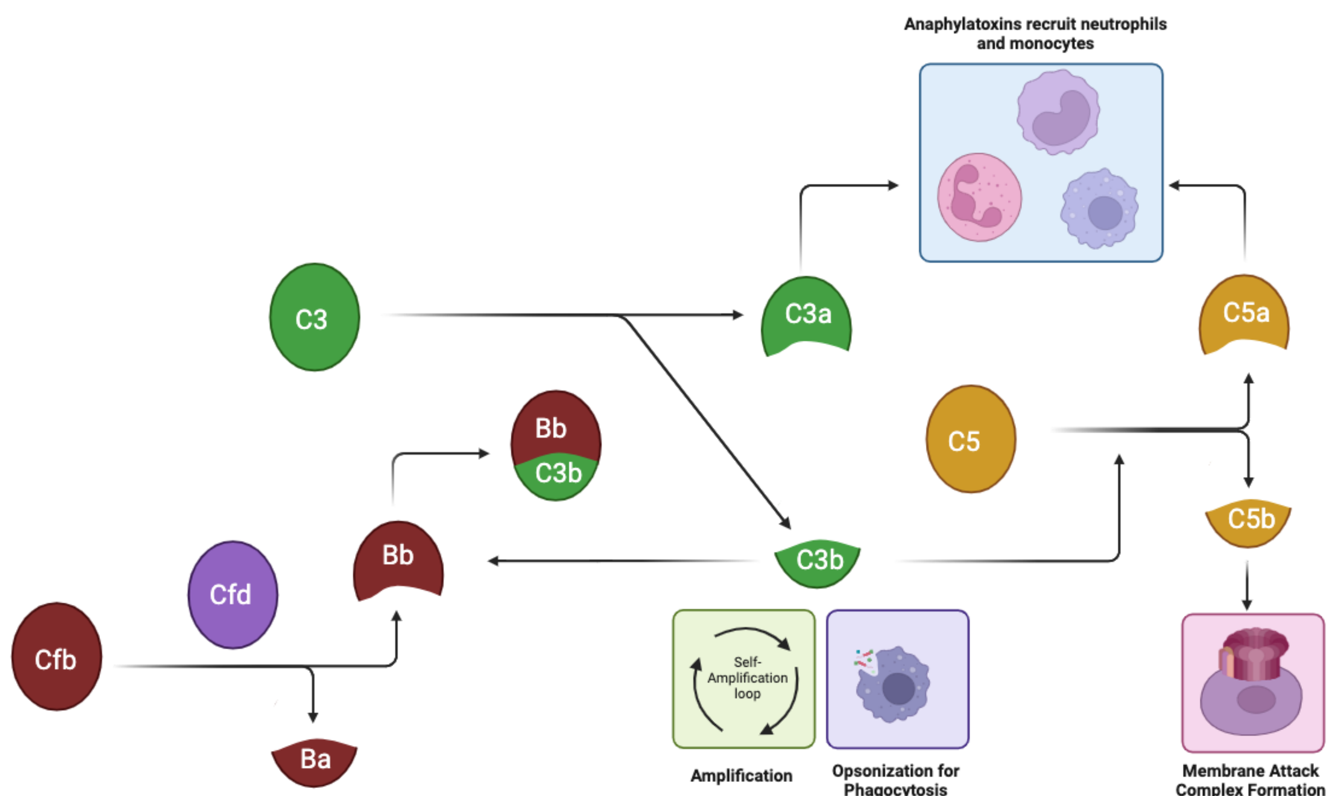

Figure 17. An overview of the complement cascade. This highlights the importance of differentially expressed genes such as C3 and Cfb.

B. Inflammasome

The cGAS-STING pathway senses the presence of cytosolic DNA from bacteria and viruses, initiating a type I interferon response in mammals. This process is induced by Sting1 (stimulator of interferon genes, -2.01, log2 fold change, p = 7.22E-069) which is downregulated in the DMPA-treated mice. Prior studies using Sting1 knockout mice have shown a lethal susceptibility to HSV-1 infection as well as a reduced T-cell mediated response to plasmid DNA vaccination (66). Following *Chlamydia* infection, Sting1 deficient mice displayed a higher incidence of hydrosalpinx, suggesting that the type I interferon response may have a protective capacity in host innate immunity (67). The STING pathway is relatively novel, and the literature is rapidly evolving but its relationship with hormones, and more specifically synthetic hormones like DMPA, has yet to be investigated.

Many inflammasome receptors require the protein ASC (Apoptosis-associated speck-like protein containing a CARD, -2.11 log2 fold change, p = 7.42E-06) which is encoded by the Pycard gene (68). Pycard is downregulated in our treatment groups. This pathway mediates the activation of NF-κB and caspase-1 for bacterial pathogen-induced host cell death, and the production of proinflammatory cytokines IL-1β and IL-18 (68, 69). Caspase-1 itself is slightly downregulated following DMPA treatment (caspase-1, -1.15 log2 fold change, p = 4.15E-05). Multiple components of the NLRP3 inflammasome are downregulated, signifying a general reduction in the inflammatory response which could be crucial in understanding DMPA's immunosuppressive effects.

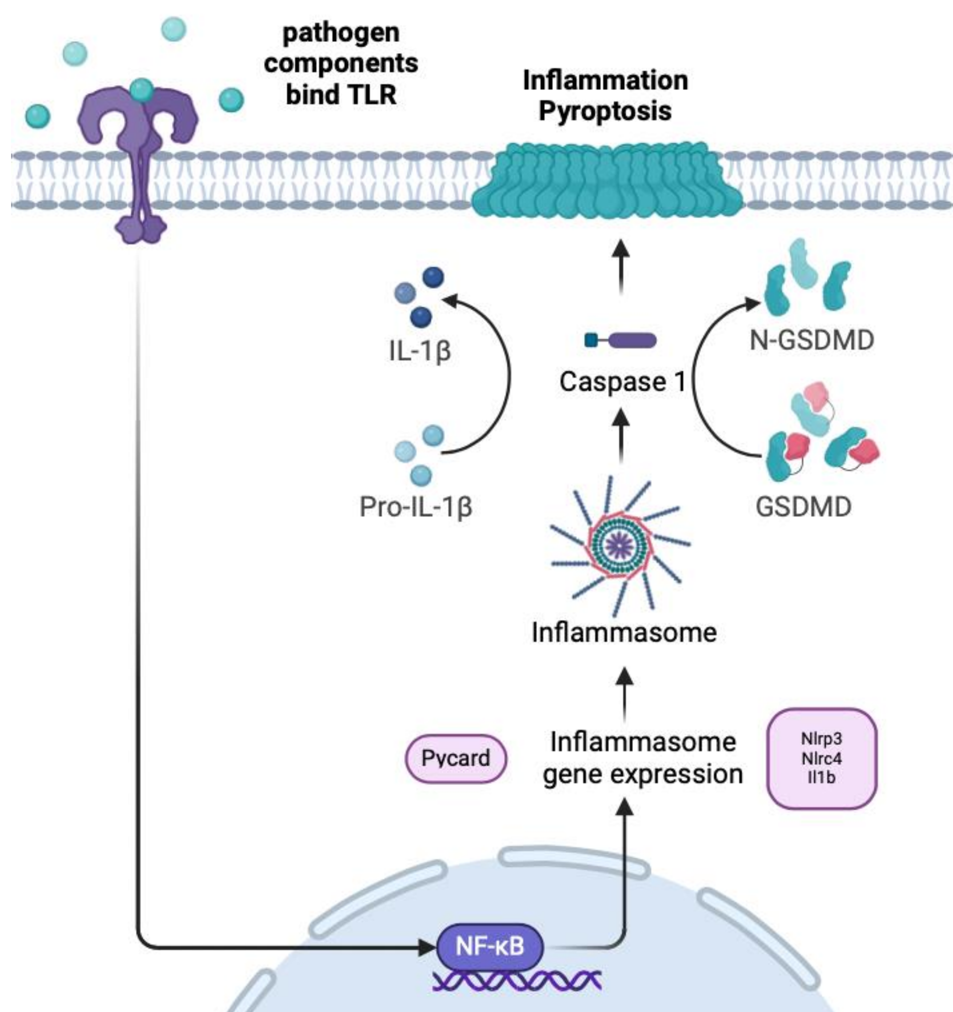

Figure 18. An overview of the inflammasome. This demonstrates the importance of differentially expressed genes such as Pycard and Casp1.

C. Barrier

As described, we noted significant differences in the FGT barrier at the morphological and transcription levels. Genes associated with barrier maintenance and function were also downregulated following DMPA treatment.

To access host cells in the mucosa, pathogens must first navigate the protective mucus layer (15). This layer is made up of mucins, including Muc1 (mucin 1, -4.31 log2 fold change, p = 2.81E-07). Muc1 can also regulate chemokine secretion from epithelial cells and plays a role in regulating inflammation in the mucosa (70). Muc1-deficient mice exhibit altered infection kinetics and increased susceptibility and severity following infection with *Pseudomonas*, influenza, *C. jejuni*, and *H. pylori*. This demonstrates that mucins and the mucus barrier are an integral part of the immune response and that downregulation of Muc1 may play an important role in DMPA's increased susceptibility to mucosal pathogens.

D. Nutritional Immunity

Following DMPA treatment, there is a reduction in genes associated with nutritional immunity, which could result in increased nutrients for bacteria, hence decreasing the host's resistance to them (22). Lcn2 (lipocalin-2, -7.1 log2 fold change, p = 5.56E-07) plays a crucial role in the innate immune response as it inhibits the ability of Gram-negative bacteria to scavenge free iron by blocking their catecholate-type siderophores (71). It is expressed by macrophages and epithelium in response to inflammation and has been shown to correlate with the extent of intestinal inflammation; this may connect to differences in the inflammatory response following DMPA treatment (72). During the acute phase response to infection, bacteria

encounter Toll-like receptors (TLRs) on host immune cells. Once activated, these TLRs then induce the production of Lcn2 (73). Following expression and secretion, Lcn2 can restrict bacterial growth by sequestering the siderophore. Lcn2-deficient mice have been shown to have a 1000-fold higher bacterial burden than wild-type mice following *E. coli* infection, demonstrating its importance in protecting against bacterial infection (73).

Ltf (lactotransferrin, -4.21 log2 fold change, p = 1.71E-06) binds iron at mucosal surfaces and is associated with the regulation of iron homeostasis, host defense against microbial infections, anti-inflammatory activity, and the regulation of cellular growth and differentiation (74). It has a role in body secretions with antimicrobial activity such as milk, colostrum, tears, urine, saliva, and cervicovaginal fluid, as a non-specific part of the immune system. Lactoferrin is released by neutrophils during inflammation (75). It acts on DNA and RNA viruses including herpes simplex viruses 1 and 2 and HIV (74). This gene is estrogen-inducible with its regulation under the influence of sex hormones (76). Ltf is robustly expressed in the uterine epithelium of adult mice, suggesting that the downregulation following treatment with DMPA may be the result of observed changes of the uterine epithelium. This could implicate Ltf in DMPA's immunomodulatory effects through reduction of iron binding, barrier function, and antimicrobial secretions.

E. Cytokines and Chemokines

Ccl28 (chemokine (C-C) motif ligand 28, -4.07 log2 fold change, p = 5.17E-06) is a mucosa-associated epithelial chemokine that is active in the intercellular region (77). It enhances IgA immune response and has been shown to act against HIV infection in mice (78). *In vitro*, it demonstrates antimicrobial action and is implicated in the immune responses of mucosal membranes and saliva secretion (77). The action of this protein to recruit immune cells and

enhance immune response, including against HIV infection, may implicate it in action against *Chlamydia* infection (78). Its significant downregulation in DMPA-treated mice may relate to their increased susceptibility to infection.

VI. Next steps and future directions

A. Validation and relative contribution

Our immediate next steps are to validate our top hits. We are currently utilizing reverse transcription-quantitative polymerase chain reaction (RT-qPCR) which measures RNA levels to corroborate the RNA expression data obtained from our NanoString panels. Additionally, we are using western blotting to evaluate the protein expression for these genes. Furthermore, to assess the observed changes in gene expression at the translational level, we are conducting western blots to evaluate protein expression levels for the identified candidate genes.

While we found a broad downregulation of innate immune components, it is important to delineate the relative contribution of our top candidate genes. We plan to use mice that have one of our differentially expressed candidate genes ‘knocked out’ or deleted. By assessing the morphological changes in the female genital tract and susceptibility to *Chlamydia* infection in mice lacking these candidate genes, we aim to understand the functional roles of these genes in maintaining the immune barrier.

B. Investigation of morphological changes

Our investigation revealed widespread morphological alterations throughout the murine female genital tract in response to DMPA treatment. These findings have broad implications, including perturbations in tissue homeostasis and increased exposure to pathogens. The observed thinning of the epithelial layer may compromise the integrity of the mucosal barrier, thereby facilitating the entry of pathogens. Increased intestinal epithelial permeability, or “leaky gut,” is

associated with the development of intestinal disease and cancer - demonstrating the importance of epithelial barrier integrity (79).

Additionally, local FGT effects could influence a person's risk of acquiring STIs, including HIV (42). Changes in the genital tract such as thinning of the epithelium, cervical ectopy, or alterations in the number or permeability of target cells can affect the ability of pathogens to enter and replicate within the cell.

Studying uterine epithelial permeability is important to understand its implications for reproductive health and susceptibility to infection (5). Investigating the factors influencing uterine epithelial barrier integrity could provide valuable insights into the development of reproductive disorders and inform strategies for preserving reproductive health.

The effects of epithelial thinning could be examined using fluorescein (FITC) permeability assays, where the dye is administered to assess tissue permeability over time (80). This would provide valuable insights into the epithelial barrier function and how DMPA modulates barrier integrity in the female genital tract. Additionally, given the differential expression of cytokines, chemokines, and immune cell markers combined with observed changes in the female genital tract, it is important to evaluate the cellular composition in these tissues following DMPA treatment. Utilizing flow cytometry would give valuable insight into characterizing the immune cell populations in the female genital tract and how they may be impacted by DMPA.

C. Mechanistic studies

Although the project had the primary objective of revealing the underlying cellular and molecular mechanisms responsible for the immunosuppressive effects of DMPA, there is still work to be done to answer this question. There are many unanswered questions regarding the

drug's impact on the immune system that require further investigation. Further evaluation of these mechanisms can help to improve our understanding of DMPA's effects on the immune system.

For example, investigating the glucocorticoid and progesterone receptor signaling can help to reveal how the glucocorticoid-like effects may be influencing immunosuppression in the mucosa (45). We can gain a better understanding of DMPA's ability to activate the glucocorticoid receptor by analyzing both transactivation and transrepression transcriptional activities of the GR. This could be evaluated through the use of RT-qPCR and western blot to measure glucocorticoid and progesterone receptor levels as well as GR-regulated genes following DMPA treatment.

Additionally, the signaling pathways that DMPA modulates to induce differential gene expression and changes in pro-inflammatory responses are unknown. Key signaling pathways that regulate the innate immune response, such as the RAS-MAPK and PI3K-AKT-mTOR pathways, are known to interact with the glucocorticoid receptor (81). RAS-MAPK is involved in initiating inflammation by inducing inflammatory cytokine production; recent studies have shown that GR can regulate RAS-dependent signaling and the activation of RAS (82, 83). Additionally, one of the mechanisms of action for glucocorticoids is through NF-κB repression as GR binds directly to NF-κB to inhibit inflammatory gene expression (84). It is likely that DMPA is directly or indirectly interacting with pro-inflammatory and immune signaling pathways to modulate the host response. This hypothesis is further supported by findings of downregulation of genes associated with these pathways following DMPA treatment in this study (e.g. *Mapk13*, mitogen-activated protein kinase 13, -2.31 log2 fold change, $p = 1.36E-05$) and others (34). By conducting RT-qPCR and western blot analyses of genes in these pathways,

we can gain an understanding of the connections between signaling events and the phenotypic outcomes that we see with DMPA treatment.

VII. Significance of the drug and the need for informed consent

DMPA suppresses ovulation and is used clinically as a hormonal contraceptive that offers discreet and highly effective birth control with injections administered once every three months (1). Its inclusion on the World Health Organization's Model List of Essential Medicines underscores its global significance in reproductive healthcare (2). With over thirty-five million individuals relying on DMPA worldwide, particularly in regions such as sub-Saharan Africa where it accounts for half of all contraceptives used, its accessibility bears direct benefits (1). Access to safe contraceptives offers direct benefits, allowing individuals to exert control over their reproductive health. This, in turn, leads to a decrease in unintended pregnancies, maternal and infant morbidity, and the risk of vertical disease transmission (2).

Injectable hormonal contraceptives are one of the most effective methods of contraception available today, provided that they are used correctly (2). For women in developing countries, injectable forms of contraception are a popular choice due to their practicality and effectiveness. They offer a high level of effectiveness and are reliably reversible as fertility is not permanently impaired. Clinical trials indicate that the rate of pregnancy is less than 1 per 100 women in the first year, making them an essential part of any integrated family planning service provided by a clinic or other health facility.

Hormonal birth control is more than 99% effective at preventing pregnancy but poses the risk of side effects. Multiple epidemiological studies have shown an association between DMPA use and an increased risk of HIV infection (9, 29-32). This risk is especially pertinent for women in sub-Saharan Africa, where HIV remains a leading cause of death. Notably, studies controlling

for variables such as frequency of intercourse, age, and number of sexual partners have reported statistically significant increases in HIV infection rates among DMPA users (41). Concerningly, infection with inflammatory STIs, including *Chlamydia*, may also increase the risk of HIV acquisition (85). It is crucial to understand the impact of DMPA on mucosal susceptibility to HIV-1 and other sexually transmitted infections, as millions of women require protection from unintended pregnancy and infection. Consistent use of condoms is highly recommended to prevent the spread of sexually transmitted infections, especially among people who use hormonal contraception (2).

The research linking DMPA to sexually transmitted infection is concerning but does not necessarily outweigh the benefits. Instead, this is an issue of informed consent. DMPA users and their clinicians should have the full picture when using and prescribing this medication. In 2004, the Food and Drug Administration put out a Black Box warning for DMPA based on fracture and osteoporosis risk (1). This risk is now reflected in the medication guide that comes with every DMPA prescription. It is imperative that information regarding changes in susceptibility to sexually transmitted infections, including HIV, be equally prioritized in patient education materials accompanying DMPA prescriptions. Ensuring transparency regarding the potential risks associated with DMPA usage not only empowers individuals to make informed decisions about their reproductive health but also fosters trust between patients and healthcare providers. Additionally, further research and development should be employed to develop safe and reliable contraceptives as their popularity and use would considerably increase if they were further improved (2).

By conducting a thorough and detailed examination of the potential infection risk associated with DMPA use, we can provide healthcare providers with the necessary information

needed to have meaningful and informative discussions with their patients. The ultimate goal of this work is to promote patient safety and enhance the quality of reproductive healthcare delivery. Healthcare providers should be empowered with the information they need to deliver transparent information that is both informative and easy to understand. This approach will enable patients to make informed decisions regarding their reproductive healthcare, thereby reducing the risk of infection and other complications (9).

MATERIALS & METHODS

I. Outline of the experiments

We treated female C57BL/6 mice with 2.5 mg DMPA at either day -7 or day -10 and -3, which are both pre-treatments found in *Chlamydia* literature, or phosphate-buffered saline (PBS) as a negative control. C57BL/6 are wild-type and inbred to represent a single genotype, they are also the mouse strain used most widely in the *Chlamydia* field (34). Our negative control group was injected subcutaneously with PBS so that all experimental mice received a treatment. These mice did not receive any exogenous or synthetic hormones; therefore, they were all naturally cycling. All three experimental groups were sacrificed at day 0 when an infection would typically take place.

For evaluation of morphology, the entire murine female genital tract was excised. The tissues used for histopathology were analyzed for morphological changes. Differences in morphology will be investigated using hematoxylin and eosin (H&E) staining for visualization by microscopy. Representative images will be taken, and the epithelium be quantified under anonymized conditions using ImageJ.

Figure 19. An overview of the experimental approach used to evaluate morphology in the murine female genital tract.

For the gene expression analysis, the FGT was segmented, and the upper genital tract was analyzed for NanoString. The upper genital tract consists of the endocervix, bifurcated uterine horns, oviducts, and ovaries. To evaluate differential gene expression, the upper genital tract, rather than the entire female genital tract, was used because, *in vivo*, *Chlamydia* is inoculated transcervically. Therefore, the tissues used for our gene expression data would be comparable to an experimental infection and transcriptional changes would be directly relevant to the *Chlamydia* field. Differential gene expression of over 1100 genes will be surveyed using two commercially available NanoString panels. The PanCancer Immune Profiling Panel and Host Response Panel were chosen based on their close alignment with the genes that are potentially responsible for our phenotype of increases in susceptibility to infectious diseases.

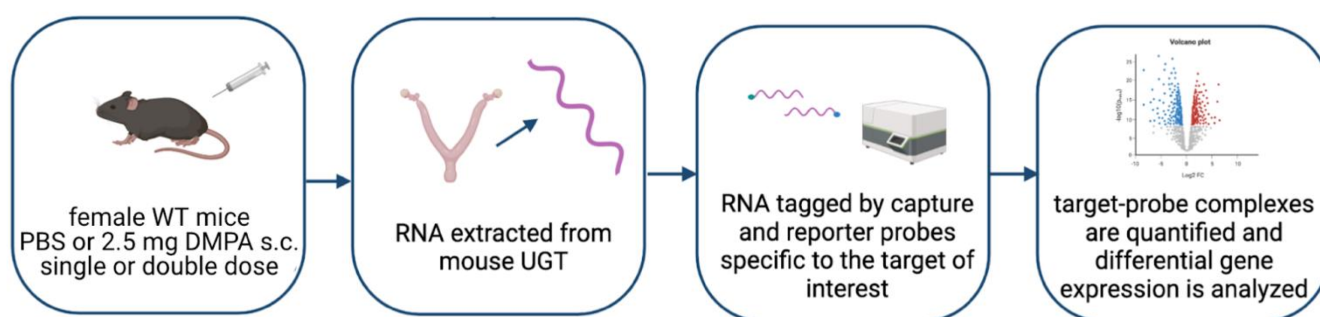

Figure 20. An overview of the experimental approach used in this experiment to evaluate gene expression in the murine female upper genital tract.

II. Formal methods

Mice. All mouse procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committees of Mount Holyoke College (#BR-62-0422). Female C57BL/6 mice were purchased from The Jackson Laboratory (USA), housed at Mount Holyoke College, and provided food and water ad libitum. The female genital tract was excised from all mice at 6 weeks.

DMPA treatment. Mice were injected subcutaneously with 2.5 mg of medroxyprogesterone acetate (Prasco Laboratories, Mason, OH, USA) in 100 µl starting at five weeks old. Control mice were injected with the same volume of sterile phosphate-buffered saline. Subcutaneous injections of DMPA commenced at either seven days or ten days and three days before sacrifice.

Bacterial challenge and quantification. *Chlamydia trachomatis* serovar L2 was cultured, inoculated into mice, and quantified as previously described [x]. Briefly, bacteria were propagated within McCoy cells (ATCC, Manassas, VA, USA) and released from the inclusion using sterile glass beads and sonication. Elementary bodies (EBs) were purified by density gradient centrifugation, stored at −80 °C, thawed immediately before use, and titered on McCoy cell monolayers to quantify inclusion forming units (IFU). 5 × 108 IFU of purified EBs were inoculated trans-cervically into the upper genital tract of female mice using a nonsurgical embryo transfer device (ParaTechs, Lexington, KY, USA). Mice were treated subcutaneously with 2.5 mg medroxyprogesterone acetate (Pfizer, New York, NY, USA) one week before infection and sacrificed three days post-infection. Bacterial burden in the upper genital tract was determined using quantitative PCR, shown previously to accurately reflect IFU (37). Total DNA was isolated from homogenized upper genital tracts using a DNeasy Blood and Tissue Kit (Qiagen, Beverly, MA, USA). *C. trachomatis* 16S DNA and mouse GAPDH DNA were amplified and quantitated on an AriaMX Real-Time PCR System (Agilent, Santa Clara, CA, USA) using specific primer pairs and probes (IDT, Coralville, IA, USA or Applied Biosystems, Waltham, MA, USA). The ratio of *C. trachomatis* to mouse DNA was calculated using standard curves generated from known amounts of purified *C. trachomatis* or mouse DNA.

NanoString/RNA isolation and gene expression analysis. N = 12 were sectioned at the cervix to separate the upper and lower genital tracts and sent to the Molecular Genetics Core at Boston Children's Hospital for NanoString. (Insert amount here) RNA was analyzed with the Host Response and PanCancer Immune Profiling probes set using a NanoString nCounter. Background correction was done using the mean of 20 negative control probes. Normalization was performed through the calculation of the geometric mean of positive controls and content normalization parameters. A quality check was conducted on the samples to detect lanes when the .5fm positive control is less than or equal to 2 standard deviations above the mean of the negative controls. No samples were flagged. The data was processed using nSolver software.

Microscopy and ImageJ. Tissues used for histology were fixed in 10% formalin before processing to paraffin, microtomy at 5µm, and hematoxylin and eosin by the Harvard Medical School Rodent Histopathology Core. Slides were imaged at 10x magnification on the Nikon Eclipse 50i. Using ImageJ, the simple columnar epithelium of the female upper genital tract was measured under anonymized conditions.

Statistical analysis. Statistical analysis was performed using Prism (GraphPad) and a P value of < 0.05 was considered significant. All bar graphs are shown as mean ± SEM, where **** $P < 0.0001$, *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, and N.S. indicates not significant by unpaired student's t-test. For NanoString data, statistical significance uses the Benjamini-Yekutieli False Discovery Rate takes into account the expectation that significant gene changes may be correlated with or dependent on each other, and gives an adjusted p-value.

APPENDIX I: METHODS

I. DMPA treatment.

Generic version of Depo-Provera - Contraceptive concentration 150mg/mL (medroxyprogesterone acetate injectable suspension, USP, Prasco Laboratories NDC 66993-370-83)

150 mg/mL stock → 25 mg/mL working concentration = 1:6 (six!) dilution

Stock: white, chalky liquid, 150 mg/mL at room temp, above dry bench closest to the refrigerator, use a syringe and needle to withdraw enough for use.

Preparation: Vortex well and frequently, precipitates easily. Dilute *1:6* with sterile PBS into a 50 mL conical or Eppendorf tube; administer 100 µL/mouse. Make more than needed. The working concentration is 25 mg/mL for 100 µL/mouse = 2.5mg/mouse. N = 5 @ 100 µL ≅ makes 700 µL.

Administer: Draw DMPA into the syringe and inject 100 µL subcutaneously (if it takes you a minute to set up in the mouse room or to get the manual restraint, you may need to vortex again. If the DMPA is in the syringe, be careful to vortex the side). Use a 1mL syringe and a 30-gauge needle to administer DMPA subcutaneously on the lower flank by injecting almost parallel to the belly. If you use a true 180-degree angle, the needle will go into the mouse's fur. Therefore, you need to use a slight angle (approximately 160-degree angle). Ensure that the gauge is facing towards you so that the smallest and sharpest part of the needle is being used. I have found that the 27-gauge needle may be more appropriate for larger experiments because the medication can go into suspension and get caught in the smaller gauge needle. On that note, it is important to continuously vortex the Eppendorf tube and needle.

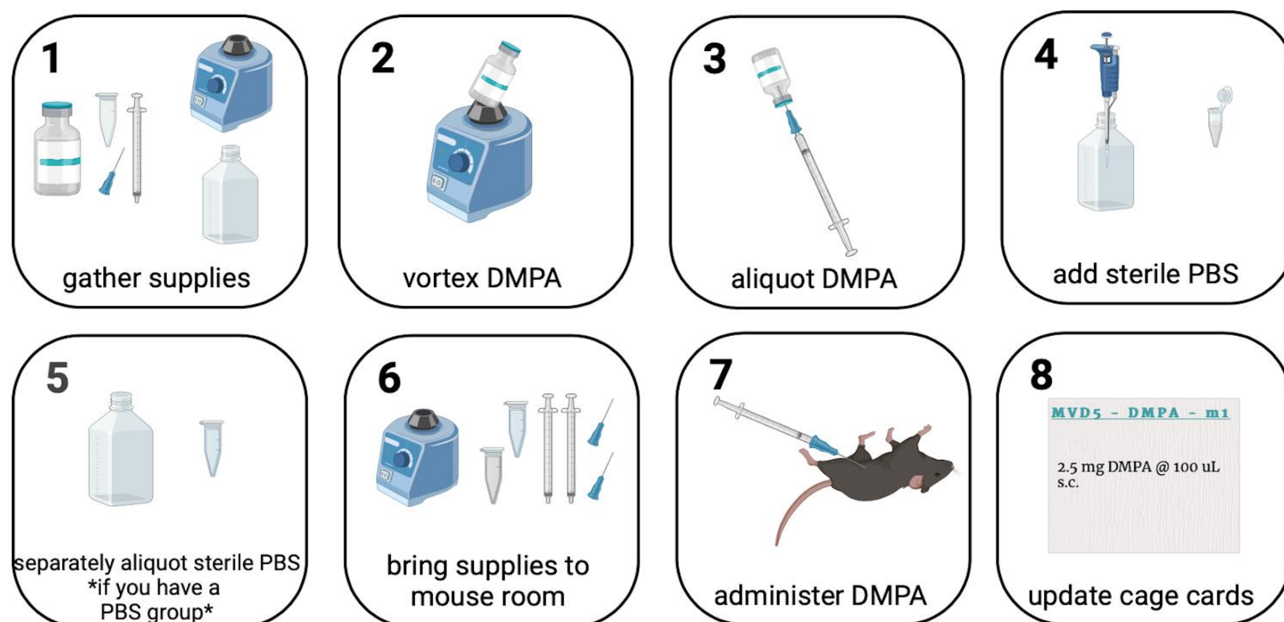

Figure 21. Instructions and visuals for DMPA preparation and administration. 1.) Gather needed supplies: DMPA (above bench, in the sharps area), sterile PBS (in the cell culture hood), vortex, needles & syringes (both to draw up the DMPA and to inject it). 2.) Vortex DMPA - we're still in the vial (this should be something you're checking, including between mice). 3.) Put the needle on the syringe, insert it into the vial, pull DMPA, and put it into an Eppendorf tube. Dilute 1:6 with sterile PBS. The working concentration is 25 mg/mL for 100 µL/mouse = 2.5mg/mouse. N = 5 @ 100 µL ≅ makes 700 µL. 4.) Add the correct amount of sterile PBS to the Eppendorf tube with DMPA and vortex. 5.) Make a separate aliquot of sterile PBS (for a PBS group). 6.) Walk to the mouse room. Bring with you: a working concentration of DMPA, sterile PBS, vortex, needles & syringes (if you need 2, bring 5-10). 7.) Draw DMPA into the syringe and inject 100 µL subcutaneously (if it takes you a minute to set up in the mouse room or to get the manual restraint, you may need to vortex again. If the DMPA is in the syringe, be careful to vortex the side). Do the same for the PBS group. 8.) Update cage cards (2.5 mg DMPA @ 100 µL s.c.)

II. NanoString and nSolver

We have raw NanoString data as two RCC files. These are titled MD-4708_Host_Response_RCC_20220819_171725.ZIP (Host Response) and MD-4708_RCC_20220817_172523.ZIP (PanCancer Immune Profiling) in the 2022 NanoString Data folder. To analyze them, you need two RLF files. These are saved as NS_Mm_HostResponse_v1.0.rlf (Host Response) and NS_Mm_CancerImm_C3400.rlf (PanCancer Immune Profiling) in the 2022 NanoString Data folder.

All NanoString analysis is done through nSolver. You will need to download from the NanoString website (nCounter® Data Analysis Solutions): nSolver for whatever computer type you use, nCounter Advanced Analysis Software, R3.3.2, and XQuartz.

Once everything is downloaded, you can upload the RCC and RLF files to nSolver (on the upper left side). Then you can establish a New Study, where you will be able to define a New Experiment. Here you will select from the RLF files (whether you want to analyze the PanCancer Immune Profiling Panel, Host Response Panel, etc.) and ensure that it includes the appropriate RCC files. You will be able to add annotations, if necessary, I use this feature if I want to analyze three or more different groups (e.g. DMPA day -7, DMPA days -10 and -3, and PBS). Background subtraction or background thresholding allows you to ensure negative controls or empty lanes are noted, but this is not needed for these samples.

Next, you will need to establish Normalization Parameters. There is a segment for Positive Control Normalization which are the genes established to be "Positive," the geometric mean can be used to calculate the normalization factor. I set it to flag lanes if the normalization factor is outside of the 0.3-3 range. Ensure that the Codeset Content includes all Endogenous genes while the Normalization Codes are all housekeeping genes. Again, I use the geometric mean to calculate the normalization factor, this time I flag lanes if it is outside of the 0.1-10 range. This will allow for the study of differential expression as we are minimizing flux between samples.

Then you will be able to build ratios for fold change estimation. Here is where you will determine the criteria for analysis. If there are two groups, you will be able to label one group as the base sample. If there are more than two groups (you will have previously needed to annotate

those), you can analyze by annotation. This will allow you to conduct Advanced Analysis to generate Volcano Plots, heat maps, PCA plots, and analyze specific functions.

Once normalized, select all samples that you wish to use for Advanced Analysis. Choose identifiers for your samples and use experiment annotations (covariates) for analysis. For analyses of these samples, adjust the categorical reference to the PBS treatment group. I have found that my results are faster, and the process is simplified if I normalize the data first, but this means that you have to do a "custom," rather than "quick," analysis and you need to deselect normalization from the modules that are running. Thus, treatment will be the selected predictor. After adjusting the parameters, you will be able to run the analysis (which takes time and computation). Choose Analysis Data, the other tabs will not support a graphical representation.

III. Microscopy

When setting up your slide, always start at 10x magnification. Adjusting the coarse focus on the microscope will get the slide into view. Once you are ready at 10x magnification, you will be able to adjust the fine focus to get the image as crisp as possible. This is something that you should take time to do as the image should be sharp, once the slide is in focus you cannot change the fine or coarse focus in between sequential images. As you adjust the brightness on the microscope, you will want to ensure that the lumen is white. If you turn the brightness too high, the image will be "overblown" and not of good quality as the tissue will be too bright. The tissue should be in color, not white as this would demonstrate that the brightness is too high. You can adjust the colors on the PixeLink settings.

For stitching images and creating a "map." Locate your region of interest using the microscope. Check that you have switched to the color camera and load PixeLink software. Click on "Play" to start the preview. Push the optical path-switching lever to initiate the video display

on the computer. Adjust the white balance numbers for red, green, and blue. Once you have established these features, including the brightness and focus on the microscope, do not change them between images. Identify your folder and save your file name in the following format:

Experimenterinitials-Treatmentmethod-mice#-MMDDYY_objectiveused_01.tif

e.g. MVD2-DMPA7-m2-051924_10x_01.tif

Ensure that your file format is a tif. Then, capture multiple images of the slide, making mental notes of regions already captured by using landmarks. It is helpful to take snapshots in this zigzag manner, and also take extra images outside of the area that you need to reduce the jagged edges of the image as much as possible (if you only image what you are interested in, there will be black edges around it and you will need to crop out important morphology to obtain a clear image).

IV. ImageJ

General troubleshooting: If in doubt go to Help and type what you're trying to do. The Fiji ImageJ download (free!) is preferable to the open-source web version. Flattening your image can be the answer when something is not right but you don't know why (Overlay → Flatten). Get in the habit of naming images with double digits if you are thinking of collecting more than ten (ex: 01 instead of 1). If you have too many pictures to scroll and confirm, uncheck "Confirm Files"

Camera	**Objective**	**Scale**
PixeLink	10X	100 µm = 156 pixels
	40X	100 µm = 622 pixels
	100X	100 µm = 1558 pixels

Figure 22. A table describing the pixels needed to set scale based on different objectives on the PixeLink camera. Adapted from the BIOL-230 Molecular Genetics & Cell Biology Lab Manual.

To adjust the color, go to 'Image' and then 'Adjust.' To adjust the orientation, go to 'Image' then 'Transform,' I would recommend selecting 'Show Preview.'

To add a scale bar to the image, select 'Analyze' and then 'Set Scale.' Use the table (Fig. 20) to adjust based on the objective used. Now the scale of the image has been set and the scale bar needs to be added. Select 'Analyze,' then 'Tools,' and pick 'Scale Bar.' The scale bar needs to be adjusted so that it is a round number. Once the scale bar is added, select 'Flatten' to preserve the scale bar on your image.

Pairwise Stitching is a key tool to stitch a few images together. Start by opening the images you are planning to stitch. Choose 'File' → 'Open' → 'Select.' You need at least two images open before you can attempt to stitch anything. Go to 'Plugins' → 'Stitching' → 'Pairwise stitching.'

Gridwise stitching works better than Pairwise if you have many images that you would like to stitch together into a map. When saving the images, if you are using 10 ≥ they must be saved with at least two-digit spaces (you cannot have 1 and 10, they have to be formatted as 01 and 10). Images must be saved as a file with only the images you are stitching, they cannot be "loose" or collected alongside miscellaneous content. Go to 'Plugins' → 'Stitching' → 'Grid/collection stitching.' I like to select "Unknown Position" as my type because it enables me to take the images as I see fit or have a bit of human error. On the next page, you should either search the directory for your file or copy the exact location of the file. Where it says "Output Textfile Name" type in your standardized collection naming system (including .tif) but replace the numbers with "ii" then choose linear blending for your fusion method. Change your

computational parameters to fit your preferences. Then select ‘Fuse and display.’ Press ok and the stitching will begin. Depending on your parameters this may take a lot of time and RAM

If your images are in color: go to ‘Image’ → ‘Type’ → ‘RGB Color.’ Otherwise, it will be saved as black and white. Then be sure to save your stitched image as a tiff.

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