Nippostrongylus brasiliensis and herpes simplex virus-2 co-infections: Impact on female genital tract microRNA and immune profiles, and potential role in cervical carcinogenesis

By

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PREFACE

The study described in this thesis entitled "***Nippostrongylus brasiliensis* and herpes simplex virus-2 co-infections: Impact on female genital tract microRNA and immune profiles, and potential role in cervical carcinogenesis**" was carried out at the Discipline of Medical Microbiology, School of Laboratory Medicine and Medicine Sciences, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, under the supervision of Prof Zilungile L. Mkhize Kwitshana and Dr Pragalathan Naidoo. The animal experiments were conducted at the Institute of Infectious Disease and Molecular Medicine, University of Cape Town, South Africa, under the supervision of Prof William G.C. Horsnell and Dr Alisha Chetty.

The study described in this thesis is original work conducted and reported by the author. The study has not been used in any form, by any person or submitted to any tertiary institution for the award of a degree or diploma. Some of the work has been published in accredited journals in accordance with the thesis guidelines of the University of KwaZulu-Natal. This thesis does not include any data, pictures, graphs, or other information emanating from other people's work unless they are duly acknowledged.

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DECLARATION 1: PLAGIARISM

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DECLARATION 2: PUBLICATIONS

The publications that constitute this thesis, as well as those to which I contributed during the course of this PhD study, are listed below.

List of publications emanating from this PhD study:

1. **Pillay, R**., Naidoo, P. & Mkhize-Kwitshana, Z. L. 2024. Herpes simplex virus type 2 in sub-Saharan Africa and the potential impact of helminth immune modulation. *Frontiers in Cellular and Infection Microbiology,* 14**,** 1471411.

2. **Pillay, R**., Naidoo, P., Duma, Z., Bhengu, K., Mpaka - Mbatha, M., Nembe-Mafa, N. & Mkhize-Kwitshana, Z. L. 2024. Potential Interactions Between Soil-Transmitted Helminths and Herpes Simplex Virus Type II: Implications for Sexual and Reproductive Health in Sub-Saharan African. *Biology,* 13**,** 1050.

3. **Pillay, R**., Naidoo, P. & Mkhize-Kwitshana, Z. L. 2025. Exploring microRNA-Mediated Immune Responses to Soil-Transmitted Helminth and Herpes Simplex Virus Type 2 Co-Infections. *Diseases,* 13**,** 6.

4. **Pillay, R**., Naidoo, P. & Mkhize-Kwitshana, Z. L. 2025. Characterization of microRNA Expression Profiles of Murine Female Genital Tracts Following Nippostrongylus brasiliensis and Herpes Simplex Virus Type 2 Co-Infection. *Microorganisms,* 13**,** 1734.

5. **Pillay, R**., Naidoo, P. & Mkhize-Kwitshana, Z. L. 2025. Exploring Gene Expression Changes in Murine Female Genital Tract Tissues Following Single and Co-Infection with Nippostrongylus brasiliensis and Herpes Simplex Virus Type 2. *Pathogens,* 14**,** 795.

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2. Mpaka-Mbatha, M. N., Naidoo, P., Islam, M. M., Singh, R., Bhengu, K. N., Nembe-Mafa, N., **Pillay, R**., Duma, Z., Niehaus, A. J. & Mkhize-Kwitshana, Z. L. 2023. Immunological interaction during helminth and HIV co-infection: Integrative research needs for sub-Saharan Africa. *South African Journal of Science,* 119**,** 1/2.

3. **Pillay, R**., Mkhize-Kwitshana, Z. L., Horsnell, W. G. C., Icke, C., Henderson, I., Selkirk, M. E., Berkachy, R., Naidoo, P., Niehaus, A. J., Singh, R., Cunningham, A.F. & Shea, M. K. 2023. Excretory-secretory products from adult helminth Nippostrongylus brasiliensis have in vitro bactericidal activity. *Journal of Medical Microbiology,* 72.

4. Mkhize-Kwitshana, Z. L., Naidoo, P., **Pillay, R**., Lin, Z. & Dlamini, Z. 2025. Chapter Sixteen - Virally induced colorectal cancer drug resistance. *In:* Dlamini, Z. (ed.) *Colorectal Cancer Treatment Resistance.* Academic Press.

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PRESENTATIONS AT CONFERENCES AND SYMPOSIUMS

1. National

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2. International

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DEDICATION

I dedicate this thesis to my mother

Mimi Thungaveloo

"You are the wind beneath my wings"

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Philippians 4:13: *"I can do all things through Christ who strengthens me".*

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ABBREVIATIONS

AAMS	Alternatively activated macrophages
ANOVA	Analysis of variance
APCs	Antigen-presenting cells
ATG7	Autophagy related 7
BCG	Bacille Calmette-Guérin
bp	Base pair
BP	Biological process
CAMK1D	calcium/calmodulin-dependent protein kinase ID
CAP-miRSeq	Comprehensive Analysis Pipeline for miRNA-Sequencing data
CC	Cellular component
CCL11	C-C motif chemokine ligand 11
CCR2	C-C chemokine receptor type 2
CHPC	Centre for High Performance Computing
CT	Threshold cycle
CX3CR1	CX3C chemokine receptor 1
DAMPs	Danger associated molecular patterns
DCs	Dendritic cells
DE	Differentially expressed
DEGs	Differentially expressed genes
DGCR8	DiGeorge syndrome critical region 8
DIPLOMICS	DIstributed PLatform in OMICS
dLNs	Draining lymph nodes
EBV	Epstein Barr Virus
ELISA	Enzyme-linked immunosorbent assay
EMT	Epithelial-to-mesenchymal transition
EPG5	ectopic P-granules autophagy protein 5 homolog
ESP	Excretory-secretory products
EVs	Extracellular vesicles
FDR	False discovery rate
FGT	Female genital tract
FOXP3	Forkhead box P3
GO	Gene Ontology

HCMV	Human cytomegalovirus
HCV	Hepatitis C virus
HHV-8	Human herpesvirus 8
HIV	Human immunodeficiency virus
HNRNPA3	Heterogeneous nuclear ribonucleoprotein A3
HPV	Human papillomavirus
HSV-1	Herpes simplex virus type 1
HSV-2	Herpes simplex virus type 2
IFNs	Interferons
IgA	Immunoglobulin A
IgE	Immunoglobulin E
IgG	Immunoglobulin G
ILCs	Innate lymphoid cells
IL	Interleukin
iNOS	Inducible nitric oxide synthase gene
IPA	Ingenuity Pathway Analysis
IRAK1	Interleukin receptor associated kinase 1
IRGs	Immune-related genes
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KSHV	Kaposi’s sarcoma-associated herpesvirus
LAMP	Loop-mediated isothermal amplification
LAT	Latency-associated transcript
LCs	Langerhans cells
LDLR	low-density lipoprotein receptor
LMICs	Low- and middle-income countries
LogFC	Logarithmic fold change
LP	Lamina propria
LRP8	LDL Receptor Related Protein 8
MCC	Maximal clique centrality
MF	Molecular function
MGI	Mouse Genome Informatics
MMP-16	Matrix metalloproteinase 16
MHV-68	Murine gammaherpesvirus
MNV	Murine norovirus
MOI	Multiplicity of infection

mRNA	Messenger RNA
miRNA	MicroRNA
NAFLD	Non-alcoholic fatty liver disease
Nb	*Nippostrongylus brasiliensis*
NETs	Neutrophil extracellular traps
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGS	Next generation sequencing
NK	Natural killer
NO	Nitric oxide
NTDs	Neglected tropical diseases
PAMPs	Pathogen-associated molecular patterns
PBMCs	Peripheral blood mononuclear cells
PC	Preventive chemotherapy
PCR	Polymerase chain reaction
pDC	Plasmacytoid dendritic cells
PEDV	Porcine epidemic diarrhoea virus
PFU	Plaque-forming units
PPI	Protein-protein interactions
Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PPD	Purified protein derivative
PRRs	Pattern recognition receptors
PRRSV	Porcine reproductive and respiratory syndrome virus
Pre-SAC	Preschool-aged children
RIN	RNA Integrity Number
RISC	RNA induced silencing complex
RSV	Respiratory syncytial virus
RT-qPCR	Real time quantitative polymerase chain reaction
SAC	School-aged children
shRNA	Short hairpin RNA
SAMRC	South African Medical Research Council
SEM	Standard error of mean
SOCS1	Suppressor of cytokine signalling-1
STAT1	Signal transducer and activator of transcription 1
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins

SSA	Sub-Saharan Africa
STH	Soil-transmitted helminth
STHs	Soil-transmitted helminths
STIs	Sexually transmitted infections
STVIs	Sexually transmitted viral infections
TB	Tuberculosis
TGF-β	Transforming growth factor beta
Th1	T-helper 1
Th2	T-helper 2
Th17	T-helper 17
TLR	Toll like receptor
TNF-α	Tumour necrosis factor-alpha
Treg	T regulatory cells
TSLP	Thymic stromal lymphopoietin
UCT	University of Cape Town
WHO	World Health Organisation
WNV	West Nile virus

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ABSTRACT

Background:

Co-infections involving soil-transmitted helminths (STHs) and viral pathogens such as herpes simplex virus type 2 (HSV-2) remain poorly characterized, despite their overlapping prevalence in sub-Saharan Africa and other resource-poor regions. The immunomodulatory effects of STHs and the oncogenic potential of HSV-2 raise pertinent questions about whether STHs may alter host immunity and susceptibility to HSV-2 and influence molecular pathways associated with cervical cancer. MicroRNAs (miRNAs) are post-transcriptional regulators of gene expression, which play crucial roles in immune modulation, inflammation, and cancer-related pathways. This study aimed to characterize miRNA and mRNA expression profiles in the murine female genital tract (FGT) following single and co-infection with *Nippostrongylus brasiliensis* (*Nb*), a murine hookworm, and HSV-2, to elucidate potential molecular mechanisms by which helminth-induced immunoregulation may influence HSV-2 immunity and pathways implicated in cervical cancer.

Methods:

A comprehensive literature review was first conducted to assess existing knowledge on STH/HSV-2 co-infections and highlighted the biological plausibility and epidemiological significance of STH/HSV-2 co-infections and informed the conceptual framework for this investigation. The review also emphasised the potential role of miRNAs in regulating immune and disease pathways in the context of STH/HSV-2 co-infections. Experimentally, female BALB/c mice were either singly infected with *Nb* or HSV-2, co-infected with both pathogens, or left uninfected. Total RNA was extracted from FGT tissues and high-throughput next-generation sequencing was used to characterise miRNA and mRNA expression profiles in the FGT. Differential expression analysis was conducted using edgeR and limma packages in R. Ingenuity Pathway Analysis (IPA) was used to predict canonical pathways, biological functions, and to identify key immune and cancer-related pathways associated with differentially expressed (DE) miRNAs. Selected DE miRNAs were validated by RT-qPCR. RNA-sequencing (mRNA) data were functionally analysed using g: Profiler and visualized through protein–protein interaction networks constructed in Cytoscape, with hub gene and module identification via CytoHubba and MCODE.

Results:

MiRNA sequencing revealed eight DE miRNAs in *Nb*-infected FGT tissues (e.g., mmu-miR-218-5p, mmu-miR-449a-5p, mmu-miR-497a-3p, mmu-miR-144-3p), and nine DE miRNAs in HSV-2-infected tissues (e.g., mmu-miR-451a, mmu-miR-376a-3p, mmu-miR-205-3p, mmu-miR-103-

3p), relative to uninfected controls. Notably, only one miRNA (mmu-miR-199a-5p) was DE in co-infected tissues, suggestive of helminth-induced immunomodulation and antagonism of HSV-2-associated immune and transcriptomic responses. Several DE miRNAs identified have established roles in immune regulation and carcinogenesis within the female reproductive tract. IPA revealed enrichment of immune-related pathways such as neutrophil degranulation, interleukin (IL)-4 and IL-13 signaling, natural killer cell signaling, and ISGylation signaling. Cancer was predicted as a significantly enriched disease, particularly in the co-infected group. Complementary mRNA profiling demonstrated 368 DE genes in *Nb*-infected tissues (356 upregulated, 12 downregulated), with significant expression changes in Th2-related immune genes including C-C motif chemokine ligand 11 (*Ccl11*), C-C chemokine receptor type 2 (*Ccr2*), and CX3C chemokine receptor 1 (*Cx3cr1*). HSV-2-infected tissues yielded 140 DE genes (121 upregulated, 19 downregulated), with alterations in genes implicated in immune function [e.g., low-density lipoprotein receptor (*Ldlr*), calcium/calmodulin-dependent protein kinase ID (*Camk1d*), LDL Receptor Related Protein 8 (*Lrp8*), ectopic P-granules autophagy protein 5 homolog (*Epg5*)] and in cell cycle and sterol biosynthesis pathways. In contrast, co-infected tissues did not exhibit significant differential gene expression compared to uninfected controls, further supporting the potential immunomodulatory effect of *Nb* on anti-HSV-2 immune and transcriptomic responses.

Conclusion:

This study provides the first comprehensive analysis of miRNA and mRNA expression profiles in the murine FGT following *Nb* and HSV-2 single and co-infections, revealing distinct transcriptional and post-transcriptional signatures associated with helminth and viral infection. The suppression of transcriptomic responses during co-infection underscores the potential of helminth-mediated immunomodulation to alter anti-HSV-2 immunity and its potential to contribute to inflammation- and cancer-related pathways. These findings fill a critical gap in understanding the molecular interplay between STHs and HSV-2 and provide the groundwork for future mechanistic studies, including functional validation and translational research. The results are relevant for diagnostics, vaccine strategies, integrated disease control in co-endemic regions, and in understanding the potential links between co-infection and cervical cancer risk.

Keywords: Soil-transmitted helminths/STHs, *Nippostrongylus brasiliensis*, Herpes simplex virus type 2/HSV-2, co-infection, female genital tract, immunity, miRNAs, mRNAs, implications for cervical cancer risk

CHAPTER ONE

INTRODUCTION

1.1 Background

Sub-Saharan Africa (SSA) carries a disproportionate burden of infectious diseases, including a wide range of sexually transmitted infections (STIs). Among these, herpes simplex virus type 2 (HSV-2) is one of the most prevalent STIs. HSV-2 is a neurotropic virus and the leading cause of genital ulcers and genital ulcer disease worldwide (World Health Organisation, 2016). Globally, over 491.5 million individuals, or approximately 13.2% of the population, are infected with HSV-2, with women in SSA disproportionately affected (James et al., 2020, Harfouche et al., 2021).

HSV-2 primarily infects the genital mucosa, where it replicates within keratinocytes of the epithelium. The infection follows a well-characterized course comprising primary infection, latency, and periodic reactivation. In immunocompetent individuals, primary infections are often asymptomatic or mild, presenting with non-specific symptoms such as fever, myalgia, lymphadenopathy, and dysuria (Schiffer and Corey, 2013, Mathew and Sapra, 2024). In symptomatic cases, painful vesicular lesions or genital ulcers, the clinical hallmark of HSV-2, occur in approximately 10 - 25% of individuals and may persist for up to three weeks (World Health Organisation, 2016, Mathew and Sapra, 2024).

A defining feature of HSV-2 is its ability to establish latency in sensory ganglia, with periodic reactivation and viral shedding at the genital mucosa (Chan et al., 2011). In addition to its ability to establish latency, the lack of an effective vaccine or curative treatment means that HSV-2 causes lifelong infections, with transmission possible even during asymptomatic viral shedding (Jiang et al., 2016, World Health Organisation, 2016).

Importantly, HSV-2 infection is associated with epithelial disruption and chronic inflammation, which increase the risk of acquiring or transmitting other STIs, most notably human immunodeficiency virus (HIV) (James et al., 2020, Harfouche et al., 2021). Moreover, HSV-2 has been implicated in cervical carcinogenesis, particularly as a co-factor in the presence of persistent high-risk human papillomavirus (HPV) infection (Smith et al., 2002, Zhao et al., 2012, Li and Wen, 2017, Zhang et al., 2023). While HPV is the primary etiological agent of cervical cancer, HSV-2 may enhance HPV oncogenicity by promoting chronic inflammation, epithelial injury, and genomic instability. Proposed mechanisms include the facilitation of HPV entry, impairment of immune responses, induction of DNA damage, promotion of HPV replication, and

disruption of vaginal microbiota (Al-Daraji and Smith, 2009, Uysal et al., 2022, Sausen et al., 2023). HSV-2/HPV interactions are especially relevant in SSA, where limited access to HPV vaccination and cervical cancer screening contributes to a disproportionately high burden of disease (Sung et al., 2021).

In parallel, SSA has a high prevalence of neglected tropical diseases (NTDs), including soil-transmitted helminth (STH) infections. STHs are intestinal parasites that disproportionately affect individuals in impoverished settings where poor sanitation and inadequate healthcare access drive transmission (Hotez and Kamath, 2009). Globally, over 1.5 billion individuals are infected with STHs, with the highest burdens observed in tropical and subtropical regions, particularly SSA, China, Asia, and South America (World Health Organisation, 2024). The most common STHs, *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, and *Ancylostoma duodenale*, cause chronic infections, and approximately 300 million people suffer from heavy infections that contribute to severe morbidity and over 150,000 deaths annually (Hotez et al., 2006).

Though distinct in transmission routes and pathogenesis, the distribution of HSV-2 and STHs overlaps geographically, making co-infections in endemic areas highly plausible. Such co-infections may have significant immunological and clinical implications, particularly for female reproductive health (Chetty et al., 2020).

HSV-2 and STHs induce opposing immune responses. HSV-2 elicits a strong T-helper 1 (Th1)-mediated immune response, characterized by pro-inflammatory cytokines, interferon production, and recruitment of neutrophils and antigen-presenting cells to the genital mucosa (Chew et al., 2009, Chan et al., 2011). In contrast, STH infections trigger T-helper 2 (Th2) and regulatory immune responses, characterized by elevated levels of interleukins (IL)-4, IL-5, and IL-13, as well as the expansion of regulatory T and B cell populations. These responses are accompanied by the release of immunosuppressive mediators IL-10 and transforming growth factor-beta (TGF-β), which collectively contribute to a highly immunomodulatory environment (McSorley and Maizels, 2012, Harris and Loke, 2017, Rapin and Harris, 2018).

Notably, STH-induced immunomodulation is not confined to the gastrointestinal tract; it can influence mucosal immune responses at distal sites, including the female genital tract (FGT). Chronic STH infections are known to suppress antiviral Th1 responses, potentially compromising mucosal immunity and increasing susceptibility to secondary infections, including viral STIs such as HIV and HPV (Chetty et al., 2020). For instance, STH infections have been linked to increased HIV viral loads, lower $CD4^+$ counts, and higher HPV prevalence (Mkhize-Kwitshana et al., 2011,

Adeleke et al., 2015, Gravitt et al., 2015). Recently, studies in Peru and Togo demonstrated that STH-infected women exhibited a 60% higher HPV prevalence and an increase in cervical Th2 cytokines (Gravitt et al., 2015, Omondi et al., 2022), suggesting that STH-driven immunomodulation may hinder viral clearance and potentially increase the risk of cervical neoplasia. While direct associations between STH infections and cancers of the female reproductive tract remain limited, systemic STH-induced immunomodulatory effects may influence disease progression in the presence of oncogenic viruses by compromising antiviral immunity and fostering a tumour-conducive microenvironment in the FGT (Chetty et al., 2020).

Despite the overlapping distribution of HSV-2 and STHs in endemic regions, STH/HSV-2 co-infections are underexplored, particularly in comparison to STH/HIV and STH/HPV co-infection studies (Pillay et al., 2024). This may be due, in part, to the asymptomatic nature of HSV-2, which results in underdiagnosis and underreporting (Mathew and Sapra, 2024). However, there is emerging evidence that STH-induced immunomodulation can exacerbate HSV-2-related pathology (Chetty et al., 2021).

A recent experimental study found that *Nippostrongylus brasiliensis* (*Nb*), the murine counterpart of human hookworm, induced a Th2 immune profile in the vaginal tract, which was associated with increased ulceration and worsened HSV-2 pathology. This effect was driven by IL-5 and eosinophilic inflammation and occurred independently of IL-4 receptor alpha signaling (Chetty et al., 2021).

At the molecular level, the host response to STH/HSV-2 co-infection may be orchestrated by complex regulatory networks involving microRNAs (miRNAs) and messenger RNAs (mRNAs). MiRNAs are small, non-coding RNA molecules that regulate gene expression post-transcriptionally by targeting mRNAs for degradation or translational repression (Bernardo et al., 2012). Host-derived miRNAs are critical for modulating normal immune responses, while host- and pathogen-derived miRNAs are implicated in various infectious and inflammatory diseases (Gonzalez Plaza, 2016, Chandan et al., 2020), including STH and HSV-2 infections (Entwistle and Wilson, 2017, Dass et al., 2023). Moreover, dysregulated miRNAs are increasingly implicated in the development of cancers, including those affecting the female reproductive tract (Nothnick, 2012, Logan and Hawkins, 2015, Duică et al., 2020, Chauhan et al., 2024).

Similarly, changes in mRNA expression offer insight into host gene expression and immune activation during infection, providing insight into disease progression and immune dysregulation at a transcriptional level (Bhassu and Sareshma, 2021). In the context of STH/HSV-2 co-infection,

FGT-specific miRNA and mRNA profiling may reveal key mechanisms by which STHs alter HSV-2-specific immune responses and modulate susceptibility to disease. Advances in high-throughput sequencing and bioinformatics allow for comprehensive analyses of these complex interactions to identify key molecular pathways involved in host-pathogen interactions, immune regulation, and disease progression (Bernardo et al., 2012, Bhassu and Sareshma, 2021).

Understanding the immunological interplay between STHs and HSV-2 at a molecular level is of particular importance in SSA, where these infections are highly prevalent and health systems are under-resourced. Co-infections may complicate clinical management, exacerbate morbidity, and increase susceptibility to other STIs, infertility, and malignancy (Chetty et al., 2020, Pillay et al., 2024). Despite their potential significance, the immunological consequences of STH/HSV-2 co-infections remain poorly characterized, representing a critical gap in the current literature.

1.2 Study Rationale

Although the individual effects of STHs and HSV-2 infections on host immunity have been well studied (Chew et al., 2009, Chan et al., 2011, McSorley and Maizels, 2012, Harris and Loke, 2017, Rapin and Harris, 2018), there is a paucity of research examining the impact of STH/HSV-2 co-infection, particularly in the context of immune regulation and disease progression within the FGT. The potential of STH-induced immunomodulation to alter anti-HSV-2 immune responses remains largely unknown. Furthermore, how STH/HSV-2 co-infections impact post-transcriptional regulatory mechanisms, particularly miRNA-mediated gene regulation, and their potential contribution to cervical carcinogenesis, has not yet been investigated.

This study sought to address this gap by investigating the impact of acute *Nb* and HSV-2 co-infection on the miRNA and mRNA expression profiles of the murine FGT. By comparing changes in miRNA and mRNA expression during single and co-infection, this study sought to elucidate whether *Nb*-induced immunomodulation may alter HSV-2-induced immune responses, and potentially influence pathways associated with cervical cancer progression.

The findings from this work provide novel insights into the molecular mechanisms of co-infection, with broader implications for understanding immune regulation at mucosal surfaces, improving infection control strategies, and informing future integrated treatment and vaccine development efforts targeting STHs and HSV-2.

1.3 Aim

The overarching aim of this study was to investigate how co-infection with the murine hookworm, *Nb*, modulates miRNA and mRNA expression in the FGT of mice, and to determine whether these transcriptomic changes influence (i) immune responses to HSV-2 and (ii) molecular pathways implicated in cervical carcinogenesis.

1.4 Study Hypothesis

Co-infection with the hookworm *Nb* alters the miRNA and mRNA expression profiles of the murine female genital tract, reflecting an immunomodulatory environment that alters host immune responses to HSV-2 and influences molecular pathways implicated in cervical cancer development.

1.5 Objectives

The study objectives and sub-objectives are described below:

Objective 1: To conduct a literature review and advocate for research into STH/HSV-2 co-infections.

a. To conduct a comprehensive literature review on STH/HSV-2 co-infections, highlighting current knowledge gaps, synthesising emerging evidence, and advocating for increased research efforts into STH/HSV-2 co-infections and their implications for female reproductive health.

Objective 2: To conduct miRNA profiling of the murine FGT, identification, and validation of differentially expressed (DE) miRNAs.

a. To characterise and compare the expression of miRNAs in murine FGT tissues following acute single and co-infection with *Nb* and HSV-2 using next-generation sequencing (NGS).
b. To validate a subset of DE miRNAs using real-time quantitative PCR (RT-qPCR).

Objective 3: To predict the functional significance of DE miRNAs by using bioinformatics tools to predict immune-related gene targets, regulatory interactions, and potential involvement in cervical cancer-associated molecular pathways.

a. To identify immune-related target genes of DE miRNAs using bioinformatics tools.
b. To predict miRNA-mRNA regulatory interactions associated with host immunity using bioinformatics tools.

c. To identify the potential involvement of DE miRNAs in molecular pathways linked to cervical carcinogenesis using bioinformatics tools.

Objective 4: To conduct mRNA profiling of the murine FGT, identify differentially expressed genes (DEGs) and conduct functional annotation.

a. To characterise and compare mRNA expression in the murine FGT following acute single and co-infection with *Nb* and HSV-2 using NGS technology.
b. To identify DEGs, hub genes, and functional gene modules involved in immune responses and disease-related pathways using bioinformatics tools.

1.6 Overview of Study Design and Methodology

This exploratory study was designed to examine the impact of acute *Nb* and HSV-2 co-infection on the miRNA and mRNA expression profiles of the murine FGT in the context of host immunity. By characterising and comparing changes in miRNA and mRNA expression during single and co-infection, this study sought to elucidate whether *Nb*-induced immunomodulation may alter immune responses to HSV-2, and potentially influence pathways associated with cervical cancer. To achieve this, a well-established female BALB/c mouse model was used (Chetty et al., 2021), and FGT tissue samples were analysed using NGS and various bioinformatics approaches were employed for downstream analyses of DE miRNAs and mRNAs.

The BALB/c mouse model was selected due to its established use in immunological studies and well-characterised immune responses to STH/HSV-2 co-infection (Chetty et al., 2021). Typically, BALB/c mice exhibit a Th2-biased immune response, which makes them suitable for studying the immune dynamics of STH infections. *Nb* is widely used in murine models and is a robust model for human hookworm, due to similarities in its life cycle, ease of infection, and ability to elicit Th2 immune responses in the host (Camberis et al., 2003, Bouchery et al., 2017). Following subcutaneous inoculation, infective third-stage *Nb* larvae (L3) undergo a tissue migratory phase, penetrating the skin and migrating via the bloodstream to the lungs. After traversing the pulmonary alveoli, the larvae ascend the trachea, are swallowed, and reach the small intestine, where they mature into adult worms. The infection is self-resolving, with adult worms typically expelled within 10 - 13 days (Camberis et al., 2003).

Similarly, BALB/c mice have been used extensively in HSV-2 pathogenesis, antiviral immunity, and vaccine development studies, allowing for reliable infection of the FGT and studying innate and adaptive immune responses elicited by HSV-2 (Linehan et al., 2004, Parr et al., 1994, Byers

et al., 2012, McLean et al., 2012, Marshak et al., 2014, Chetty et al., 2021). Female mice are used to model vaginal HSV-2 infection after they reach sexual maturity, between 5 and 6 weeks of age. The BALB/c strain is preferred due to its ease of use and enhanced susceptibility to HSV-2. At puberty, female mice experience cyclical fluctuations in sex hormones, progressing through four estrous stages: proestrus, estrous, metestrus, and diestrus. As these hormonal changes influence the host's vulnerability to HSV-2, exogenous progesterone administration is required to synchronize the estrous cycle and reduce inter-animal variability. Additionally, progesterone treatment induces thinning of the vaginal epithelium, thereby enhancing viral uptake (Linehan et al., 2004, Parr et al., 1994, Byers et al., 2012, McLean et al., 2012, Marshak et al., 2014, Chetty et al., 2021). To model HSV-2 infection in mice, progesterone-treated female mice are anaesthetized and intravaginally inoculated with HSV-2. The virus initially infects the vaginal epithelium, leading to epithelial damage, genital inflammation, and ulcer formation, which are typically observed by day 3 post-infection. As the infection progresses, the virus disseminates to adjacent urogenital and gastrointestinal tissues, including the vulva, urethra, bladder, and rectum. Clinically, this manifests as perianal swelling, urinary retention, and constipation. In severe infection, the disease advances to the development of purulent genital lesions, neurological involvement marked by hind limb paralysis, and death (Marshak et al., 2014).

As highlighted earlier, a previously described murine model was employed in this study (Chetty et al., 2021). Female BALB/c mice aged 6-10 weeks were randomly allocated to four experimental groups (n = 6 per group): (1) *Nb*-only infection, (2) HSV-2-only infection, (3) co-infection with *Nb* and HSV-2, and (4) uninfected controls. To synchronize the estrous cycles, all mice received a subcutaneous injection of 2 mg Depo-Provera seven days prior to experimental procedures. On day 7, mice in Groups 1 and 3 were subcutaneously inoculated with 500 third-stage *Nb* larvae (L3). Subsequently, on day 14, mice in Groups 2 and 3 were intravaginally inoculated with HSV-2. All mice were euthanized two days after HSV-2 challenge, and FGT tissues (excluding ovaries) were harvested for comparative analysis of infection-associated changes in miRNA and mRNA expression profiles. Next-generation sequencing (NGS) was used to assess both miRNA and mRNA expression, and was selected for its sensitivity, accuracy, and ability to provide a global, unbiased characterisation of miRNA and mRNA expression (Bernardo et al., 2012, Bhassu and Sareshma, 2021). miRNA sequencing was used to identify DE miRNAs, and mRNA sequencing (RNA-sequencing) was concurrently conducted. This approach allowed for a comprehensive analysis of infection-associated transcriptomic changes in the murine FGT.

Following sequencing, various bioinformatics tools were used to process and analyze the large datasets generated through NGS. This involved quality control, alignment, normalization, and

differential expression analyses using established pipelines (Sun et al., 2014, Jiang et al., 2024). Additionally, miRNA-mRNA interaction prediction tools and databases were employed to construct regulatory networks related to miRNA targets and their immunological relevance. These analyses allowed for the identification of key target genes and miRNAs potentially linked to immune-related and cervical cancer-related pathways. Functional enrichment analyses, including core expression analysis, Gene Ontology, Kyoto Encyclopaedia of Genes and Genomes (KEGG), and Reactome pathway analysis were also conducted to identify biological processes and signaling pathways modulated by infection.

Taken together, this integrative approach allowed for comprehensive analyses of the molecular responses to single and co-infection within the FGT, offering novel insights into how *Nb* and HSV-2 co-infection may influence immunity and disease progression in the FGT.

All experimental procedures were conducted in strict compliance with regulatory ethical standards guiding animal research (National Health Research Ethics Council, 2024). Approval to conduct this study was granted by the Animal Research Ethics Committee of the University of KwaZulu Natal (approval number: AREC/00005911/2023). The details of the experimental procedures and methods employed in this study are comprehensively described in the Materials and Methods Sections of the publications presented in Chapters 3 and 4.

1.7 Thesis Outline

This thesis is presented in publication format and adheres to the guidelines for thesis submission by publication as stipulated by the University of KwaZulu Natal. The thesis comprises five chapters. Five articles have been published and are formatted according to the journal requirements. The chapters of the thesis are as follows:

Chapter 1: This chapter includes the introduction to the study, comprising the background, study rationale, aims, objectives, overview of the study design and methodology, and the thesis outline.

Chapter 2: This chapter addresses Objective 1 of this study.

Objective 1: To conduct a literature review and advocate for research into STH/HSV-2 co-infections.

a. To conduct a comprehensive literature review on STH/HSV-2 co-infections, highlighting current knowledge gaps, synthesising emerging evidence, and advocating for increased research efforts into STH/HSV-2 co-infections and their implications for female reproductive health.

The chapter comprises three publications:

Publication 1: *Herpes simplex virus type 2 in sub-Saharan Africa and the potential impact of helminth immune modulation*. [Published in Frontiers in Cellular and Infection Microbiology Journal]

Publication 2: *Potential Interactions Between Soil-Transmitted Helminths and Herpes Simplex Virus Type II: Implications for Sexual and Reproductive Health in Sub-Saharan African.* [Published in MDPI Biology]

Publication 3: *Exploring microRNA-Mediated Immune Responses to Soil-Transmitted Helminth and Herpes Simplex Virus Type 2 Co-Infections.* [Published in MDPI Diseases]

Chapter 3: This chapter addresses Objectives 2 and 3 of this study.

Objective 2: To conduct miRNA profiling of the murine FGT, identification, and validation of differentially expressed (DE) miRNAs.

a. To characterise and compare the expression of miRNAs in murine FGT tissues following acute single and co-infection with *Nb* and HSV-2 using next-generation sequencing (NGS).
b. To validate a subset of DE miRNAs using real-time quantitative PCR (RT-qPCR).

Objective 3: To predict the functional significance of DE miRNAs by using bioinformatics tools to predict immune-related gene targets, regulatory interactions, and potential involvement in cervical cancer-associated molecular pathways.

a. To identify immune-related target genes of DE miRNAs using bioinformatics tools.
b. To predict miRNA-mRNA regulatory interactions associated with host immunity using bioinformatics tools.
c. To identify the potential involvement of DE miRNAs in molecular pathways linked to cervical carcinogenesis using bioinformatics tools.

The chapter comprises Publication 4: *Characterisation of microRNA expression profiles of murine female genital tracts following Nippostrongylus brasiliensis and Herpes Simplex Virus type 2 co-infection.* [Published in MDPI Microorganisms]

Chapter 4: This chapter addresses Objective 4 of this study.

Objective 4: To conduct mRNA profiling of the murine FGT, identify differentially expressed genes (DEGs) and conduct functional annotation.

a. To characterise and compare mRNA expression in the murine FGT following acute single and co-infection with *Nb* and HSV-2 using NGS technology.

b. To identify DEGs, hub genes, and functional gene modules involved in immune responses and disease-related pathways using bioinformatics tools.

The chapter comprises Publication 5: *Exploring gene expression changes in murine female genital tract tissues following single and co-infection with Nippostrongylus brasiliensis and Herpes Simplex Virus type 2.* [Published in MDPI *Pathogens*]

Chapter 5: This chapter focuses on the study's synthesis, conclusion, and directions for future research.

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CHAPTER TWO
LITERATURE REVIEW

2.1 Herpes simplex virus type 2 in sub-Saharan Africa and the potential impact of helminth immune modulation

Herpes simplex virus type 2 (HSV-2) and helminths, including schistosomiasis and soil-transmitted helminths, pose significant public health challenges, particularly in sub-Saharan Africa (SSA). The high prevalence and overlapping geographical distribution of both pathogens means that a large proportion of the sub-Saharan African population may be co-infected, with potential consequences for HSV-2 outcomes. It is well established that helminth-induced immune modulation influences the outcomes of bystander infectious and non-infectious diseases. However, given its predominantly asymptomatic nature, how helminths influence HSV-2 is understudied when compared to priority sexually transmitted viral infections, such as HIV and HPV (Chetty et al., 2020). Concernedly, epidemiological and immunological evidence on HSV-2 and helminth co-infections are grossly lacking, and whether helminths alter host immune responses to HSV-2 remains unclear. It is imperative that the true burden of HSV-2 and helminth co-infections is assessed, and the potential impact of helminth-induced immune modulation on HSV-2 outcomes is determined. Such studies are timely and necessary for developing and implementing preventative and therapeutic strategies aimed at mitigating the burden of both infections in SSA. To address this critical gap in knowledge, a perspective piece entitled: **"Herpes simplex virus type 2 in sub-Saharan Africa and the potential impact of helminth immune modulation"** was published in **Frontiers in Cellular and Infection Microbiology.** This perspective piece sought to articulate the relevance of exploring the potential impact of helminth-driven immune modulation on HSV-2 outcomes. By drawing from existing evidence of helminth-induced immune modulation on priority diseases in SSA and emphasizing gaps in our current understanding of HSV-2 and helminth co-infections, it aimed to stimulate pertinent discussions in this neglected area of research. The published PDF version, which is formatted according to the journal's requirements, is shown below.

1. **Pillay, R**., Naidoo, P. & Mkhize-Kwitshana, Z. L. 2024. Herpes simplex virus type 2 in sub-Saharan Africa and the potential impact of helminth immune modulation. *Frontiers in Cellular and Infection Microbiology,* 14. (Impact Factor = 4.6) **[Presented as per Frontiers in Cellular and Infection Microbiology Journal format requirements]**

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Herpes simplex virus type 2 in sub-Saharan Africa and the potential impact of helminth immune modulation

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Herpes simplex virus type 2 (HSV-2) and helminth infections are among the most widespread infectious diseases in sub-Saharan Africa (SSA). Helminths are known to modulate host immune responses and consequently impact the severity and outcomes of unrelated diseases, including allergies, autoimmune conditions, and infectious diseases. In this way, helminths may modulate essential immune responses against HSV-2 during co-infection and may alter susceptibility to and pathology of HSV-2. However, the epidemiology of STH/HSV-2 co-infections is understudied, and whether helminths influence the host immune response to HSV-2 is not well understood. In this perspective piece, we briefly examine the current knowledge on helminth immune modulation of important pathogens that are endemic to SSA, arguing that it is important to explore HSV-2 and helminth co-infections to elucidate potential interactions between HSV-2 and helminths. This is particularly relevant in SSA, where both pathogens are highly prevalent.

KEYWORDS

sub-Saharan Africa, HSV-2, helminths, co-infection, HSV-2 outcomes, helminth immune modulation

1 Introduction

Sub-Saharan Africa (SSA) bears a disproportionate and overlapping burden of Herpes simplex virus type 2 (HSV-2) (James et al., 2020) and helminth infections (Hotez and Kamath, 2009), therefore HSV-2 and helminth co-infections may likely occur. HSV-2, also known as genital herpes, is among the most common sexually transmitted viral infections

(STVIs) (World Health Organisation, 2016). Helminths, including schistosomiasis and soil-transmitted helminthiasis, are common and widespread parasitic worm infections, particularly in SSA (Hotez and Kamath, 2009). Immunologically, helminth infections elicit potent T helper 2 (Th2) and immune modulatory responses, which dampen opposing T helper 1 (Th1) and T helper 17 (Th17) immune responses (Mcsorley and Maizels, 2012). In this way, helminth-induced immune modulatory effects are known to modulate host immune responses to unrelated pathogens, including important STVIs such as HIV (Mkhize-Kwitshana et al., 2011) and human papillomavirus (HPV) (Gravitt et al., 2015; Omondi et al., 2022). This in turn alters the pathology and clinical outcomes of important infections. In view of this, helminth immune modulation may potentially alter HSV-2 pathology and outcomes in individuals with HSV-2 and helminth co-infections. As effective host immunity to HSV-2 is primarily mediated by a Th1 response, helminth-induced Th2 and immune modulatory responses may hypothetically contribute to more severe outcomes of HSV-2. Nevertheless, there is a significant lack of evidence to validate this hypothesis. Moreover, despite their overlapping distribution in SSA, little is known about co-infections between helminths and HSV-2. Studying the epidemiological and immunological dynamics of these infections may help identify potential interactions and novel therapeutic interventions.

2 Method for literature search

A literature search was performed using search engines including Google Scholar, Google, PubMed, Web of Science and Science Direct to retrieve studies related to helminths and HSV-2 single and co-infections and their respective host immune responses. The search terms used included: "helminths", "helminths and immune responses", "HSV-2", "HSV-2 and immune responses", "helminths and HSV-2 co-infection". In addition, to retrieve articles related to HSV-2 and/or helminths and their associations with infectious diseases endemic to SSA, the following search terms were used: "HSV-2 co-infections", "HSV-2 and HIV", "HSV-2 and HPV", "HSV-2 and cervical cancer", "HSV-2, HPV, and cervical cancer", "helminth co-infections in Africa", "helminths and malaria", "helminths and TB", "helminths and HIV", "helminths and HPV", "helminths and COVID-19". The review focussed on the following article types, published in English: (i) review articles, (ii) human studies conducted in SSA, and (iii) experimental studies, where applicable. No year restrictions were applied.

3 Herpes simplex virus type 2 infections

HSV-2, which causes genital herpes, is a human DNA virus belonging to the Herpesviridae family and alpha subfamily (Chan et al., 2011). It is one of the most prevalent STVIs worldwide. An estimated 23.9 million incident HSV-2 infections were reported among individuals aged 15 – 49 years worldwide in 2016. Moreover, in 2016, the global prevalence was an estimated 13.2%, which equated to 491.5 million infections (James et al., 2020). There are significant differences in HSV-2 prevalence between continents, regions, and countries. Notably, the highest rates of infection are reported in countries with poor socio-economic conditions and under-resourced health facilities, particularly countries within SSA. HSV-2 seroprevalence in SSA is estimated at 33%, which is considerably higher compared to other global regions, such as estimated seroprevalences of 7% in Europe and 17% in the Americas (James et al., 2020). There are also considerable differences in HSV-2 prevalence between the subregions of SSA. Higher HSV-2 infection levels were recorded in Eastern Africa and Southern Africa, followed by Central Africa and Western Africa. Importantly, in SSA, nearly 50% of women and more than 25% of men were reportedly infected with HSV-2, demonstrating that women have a two-fold higher risk of infection (Harfouche et al., 2021).

HSV-2 transmission occurs via sexual contact with HSV-2-infected individuals during active viral shedding. The virus primarily targets the genital mucosa, replicating within keratinocytes of the genital epithelium. The natural progression of HSV-2 infection comprises three distinct phases: primary infection, latent infection, and reactivation. In immunocompetent individuals, most primary HSV-2 infections are self-limiting and asymptomatic, or may manifest as mild, non-specific symptoms (Schiffer and Corey, 2013). Symptomatic genital herpes is characterised by fever, body aches, lymphadenopathy, and dysuria, which resolve within 10 to 14 days. In addition, the classic feature of HSV-2 infection, occurring in 10 - 25% of initial infections, are painful genital vesicles or ulcers, which last approximately 3 weeks (World Health Organisation, 2016; Mathew Jr. and Sapra, 2024). Primary infection is followed by the latent phase, where the virus establishes latency in the sensory neurons and ganglia, leading to lifelong infection in humans. Cycles between latent and reactivated infection lead to recurrent symptoms, including genital lesions, genital ulcer disease, subclinical infections, and asymptomatic viral shedding (Chan et al., 2011). During reactivation, the virus travels along sensory nerves to the genital mucocutaneous site, replicates and forms herpetic lesions (Mathew Jr. and Sapra, 2024). Symptomatic recurrences typically occur within a year after initial HSV-2 infection and are less severe than the primary episode. In rare cases, systemic complications including recurrent meningitis, hepatitis and pneumonitis can occur during primary or reactivated infection, particularly in immunocompromised individuals due to AIDS, organ transplantation or chemotherapy. Although also rare, neonatal HSV infection occurs following viral transmission during childbirth and, when untreated, is associated with high mortality (>80%) and neurological morbidity (Schiffer and Corey, 2013; Mathew Jr. and Sapra, 2024).

Importantly, HSV-2 infection is characterised by intermittent viral shedding from the genital mucosa, in both symptomatic and asymptomatic individuals (World Health Organisation, 2016). Asymptomatic viral shedding from HSV-2-infected individuals contributes to the high prevalence of HSV-2. Diagnosis of HSV-2 infection is based on clinical presentation and laboratory detection of the HSV-2 virus, its viral proteins or genetic material, or HSV-2 specific

antibodies. Several laboratory techniques may be used including: (i) viral culture from swab or needle aspirations; (ii) serology, such as enzyme-linked immunosorbent assays (ELISA) and Western blot assays to detect HSV-2 specific antigens and/or antibodies; (iii) and molecular-based methods, such as polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP) to detect the virus. Each of these methods has its benefits and drawbacks (Nath et al., 2021). However, these laboratory techniques require specialized equipment and personnel, and are therefore not accessible to a large proportion of populations, particularly in low- and middle-income countries (LMICs). LMICs face significant challenges with their healthcare systems and majority of their populations have limited access to healthcare facilities, leading to many undiagnosed HSV-2 infections. Moreover, because of its asymptomatic and/or non-specific clinical presentation, HSV-2 is often undiagnosed, or diagnosis and proper treatment may be delayed (Mathew Jr. and Sapra, 2024). Collectively, these factors exacerbate the spread of infection and increase the likelihood of co-infections with other prevalent pathogens.

Moreover, despite its high prevalence, there are currently no preventative or curative treatments for HSV-2 infection, and an effective vaccine is yet to be developed. Currently three approved classes of drugs are used to alleviate symptoms by targeting viral DNA replication and suppressing reactivation of HSV-2. These are acyclic guanosine analogues, acyclic nucleotide analogues, and pyrophosphate analogues. The common drugs from these classes include acyclovir, valacyclovir, cidofovir, and foscarnet. Acyclovir is the gold standard for treatment of HSV infections (Jiang et al., 2016). Unfortunately, because of its latent nature, HSV-2 causes lifelong infections and antivirals do not eliminate or prevent viral shedding. Concerningly, the emergence of antiviral drug resistance following long-term use and among immunocompromised patients has been reported, underscoring the urgent need to develop newer and effective therapeutic strategies (Jiang et al., 2016).

4 Immune responses during HSV-2 infection

Host immunity against HSV-2 involves components of the innate and adaptive immune responses that recognise, target, and lyse virally infected cells (Chan et al., 2011). The innate arm of immunity is critical as it forms the initial non-specific defence against HSV-2 infection and stimulates the adaptive immune response. The adaptive immune response plays an important role in viral clearance and development of long-term memory (Chan et al., 2011; Zhu et al., 2014).

The innate immune response is triggered by interaction between the virus and innate immune cells through pattern recognition receptors (PRRs), which detect pathogen-associated molecular patterns (PAMPs), such as viral DNA. The main PRRs, toll-like receptors (TLRs), occur on innate immune cells, including mononuclear phagocytes, dendritic cells (DCs), and natural killer (NK) T cells (Chew et al., 2009; Chan et al., 2011). Specific TLRs, such as TLR2 (Kurt-Jones et al., 2004), TLR3 (Zhang et al., 2007), TLR5 (Nazli et al., 2009), and TLR9 (Lund et al., 2006) have been shown to contribute to anti-HSV-2 innate immune responses. Following viral recognition and TLR activation, immune cells produce type I interferons (IFNs), thus stimulating an antiviral state through the activation of the RNA-dependent protein kinase (PKR) pathway via IFN-α1 transgene (Carr et al., 2005). Four main antigen-presenting cell (APC) subsets, Langerhans cells (LCs), $CD14^-$ lamina propria (LP)-DCs, $CD14^+$ LP-DCs, and macrophages, have been shown to regulate the antiviral state in the vaginal mucosa by polarizing $CD4^+$ and/or $CD8^+$ T cells through the expression of migration receptors (Duluc et al., 2013). Type I IFNs, mainly IFN-α and IFN-β, also promote an antiviral state by inhibiting translation and promoting the degradation of viral mRNA. In addition, type I IFNs support dendritic cell maturation and IL-15 production, which is needed for NK cell proliferation and survival. NK cells, in turn, secrete IFN-γ, and induce apoptosis of virally infected cells through the release of perforin and granzyme B. IFN-γ further enhances the anti-HSV-2 innate response by activating inducible nitric oxide synthase (iNOS). TLRs stimulate the production of proinflammatory cytokines, including IL-1, IL-6, and TNF-α, which contribute to the anti-HSV-2 innate immune response (Chan et al., 2011).

The adaptive immune response comprises cell-mediated and humoral responses. Adaptive immunity is triggered by the innate immune response, and promotes viral clearance and development of long-term memory (Zhu et al., 2014). During cell-mediated immunity, $CD4^+$ T cells are recruited to the infection site and are activated by MHC class II antigen presentation on APCs. Activated $CD4^+$ T cells release IFN-γ, stimulating epithelial cells to produce chemokines CXCL9 and CXCL10. A chemokine-driven gradient is created, which attracts cytotoxic $CD8^+$ T cells to the infection site and stimulates nitric oxide (NO) release from epithelial cells and APCs. HSV-2-specific $CD8^+$ T cells release IFN-γ and kill infected cells through perforin and fas-mediated pathways (Chan et al., 2011). Regulatory T cells (Tregs), which are known to suppress pathogen-associated immunity and tissue damage, have been found to play a role in HSV-2 infection. For example, mice depleted of Tregs, had reduced IFN levels in their draining lymph nodes (dLNs) and infection sites. In addition, there was delayed migration of NK cells, DCs, and T cells to infection sites, and increased proinflammatory chemokine levels in dLNs (Lund et al., 2008).

Lastly, during humoral responses, B cells are recruited to the infection site and are activated by $CD4^+$ T cells to produce antibodies, IgA and IgG. However, the roles of B cells and antibodies remain debatable, given that HSV-2 viral glycoproteins have been shown to evade antibody-mediated protection [reviewed in (Chew et al., 2009; Chan et al., 2011; Zhu et al., 2014)].

5 Associations between HSV-2 and HIV, HPV, and cervical cancer

There are strong biological and epidemiological associations between HSV-2 and HIV; HSV-2 has been shown to be a key driver of the HIV epidemic in SSA, by biologically enhancing HIV acquisition and transmission by almost three-fold (James et al.,

2020; Harfouche et al., 2021). HIV prevalence is highly prevalent in individuals co-infected with HSV-2 (Looker et al., 2017, 2020). Moreover, co-infection with HSV-2 has been shown to increase HIV viral shedding in genital secretions (Todd et al., 2013) and is associated with accelerated HIV disease progression (Lingappa et al., 2010; Reynolds et al., 2012). The underlying biological mechanisms by which HSV-2 increases the risk of HIV infection, include a compromised genital epithelium barrier, an influx and increased number of target cells in genital tissue for HIV entry, decreased innate mucosal immunity, and chronic mucosal inflammation (Zhu et al., 2014).

Several studies have explored the potential association between HSV-2 and human papillomavirus (HPV), particularly in terms of co-infection (Francis et al., 2018; Taku et al., 2021; Uysal et al., 2022) and the potential risk for cervical cancer development (Smith et al., 2002; Zhao et al., 2012; Martin Luther et al., 2014; Li and Wen, 2017; Yousif Elemam et al., 2018; Zhang et al., 2023).

Studies have shown that HSV-2-infected individuals are more likely to have concurrent HPV infections; co-infection is reported more frequently in regions with high prevalences of both viruses, and particularly among women (Francis et al., 2018; Taku et al., 2021; Uysal et al., 2022). HSV-2 infection has been associated with an increased risk of acquiring HPV. Biologically, inflammation and disruption of genital epithelial barriers caused by HSV-2 genital ulcers, can facilitate the transmission of other viruses, including HPV (Sausen et al., 2023).

It is well known that persistent infection with high-risk strains of HPV, mainly HPV-16 and HPV-18, is the primary cause of cervical cancer (De Sanjosé et al., 2018). What is less understood, however, is the role of HSV-2 in cervical carcinogenesis. In 2020, cervical cancer affected an estimated 604,000 women and accounted for 342,000 deaths globally. SSA has the highest cervical cancer incident and mortality rates, and cervical cancer is the leading cause of cancer-related deaths among women in SSA (Sung et al., 2021). This highlights that despite the availability of an effective vaccine against HPV, cervical cancer is still a significant public health concern, particularly in SSA. Moreover, this suggests that several other factors may contribute to cervical cancer pathogenesis.

Whether HSV-2 infection alone, or in conjunction with HPV, impacts the development of cervical cancer remains debatable, with studies yielding conflicting results (Sausen et al., 2023). However, there is some evidence to suggest that co-infection with HSV-2 may increase the risk of HPV-related cervical cancer (Smith et al., 2002; Zhao et al., 2012; Martin Luther et al., 2014; Li and Wen, 2017; Yousif Elemam et al., 2018; Zhang et al., 2023). Several mechanisms by which HSV-2 may contribute to cervical cancer development have been described: (i) HSV-2-associated genital ulcers may facilitate HPV entry to the basal layer; (ii) HSV-2 induces an inflammatory response, which may impair T helper cell mediated immune responses; (iii) HSV-2 acts directly on host cellular DNA, and induces the production of nitric oxide, resulting in cellular DNA damage; (iv) HSV-2 infection accelerates replication of HPV and its integration of viral DNA sequences; (iv) both HSV-2 or HPV infections may induce immunological and microbiological changes, such as dysbiosis of the vaginal microbiota. Such changes could potentially create a conducive environment for HPV persistence, HSV-2 reactivations, and progression to cervical cancer (Al-Daraji and Smith, 2009; Uysal et al., 2022; Sausen et al., 2023). Thus, establishing the role of HSV in cervical carcinogenesis may have important implications for mitigating the occurrence of cervical cancer. Should HSV-2 contribute to cervical cancer, then its timely diagnosis and treatment could become a potential therapeutic avenue to curb the disease.

Apart from the significant burdens of HSV-2, HIV, HPV, and cervical cancer in SSA, the continent bears a substantial burden of other infectious diseases, such as tuberculosis (TB), malaria, as well as neglected tropical diseases (NTDs), such as helminths (Hotez and Kamath, 2009). Given this geographic overlap of multiple infectious diseases, the occurrence of concurrent infections is highly likely, with profound consequences for individual and public health in SSA. Herein, we hypothesise the potential impact of helminth immune modulation on HSV-2 pathology in SSA.

6 Helminth infections

Helminths are endemic to SSA and contribute to approximately 85% of the continent's NTD burden. Helminths are associated with extreme poverty, causing chronic and insidious infections that negatively impact child development, pregnancy outcomes and economic productivity (Hotez and Kamath, 2009). Most infections are caused by the four major soil transmitted helminths (STHs) (*Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus* and *Ancylostoma duodenale*) (World Health Organisation, 2024b). According to the World Health Organisation (WHO), approximately 232 million pre-school and school-aged children residing in the WHO African region are at risk of infection (World Health Organisation, 2024c). Other vulnerable groups include women of reproductive age, and adults working on tea farms and in mines (World Health Organisation, 2024b).

STHs are transmitted via faecal contamination of food and environmental sources. Individuals become infected with *Ascaris lumbricoides* and *Trichuris trichiura* infections when they ingest embryonated eggs found in contaminated water or food, and with hookworms, when infective larvae penetrate the skin. STH have complex lifecycles, sometimes requiring multiple hosts to successfully complete their developmental stages, which comprise larval migration through one or more host tissues, maturation into adult worms, reproduction, and the excretion of new eggs into the environment (Bethony et al., 2006). STHs require similar diagnostic methods and respond to the same treatment. Large-scale efforts to reduce STH-associated morbidity in at-risk groups residing in endemic regions, include STH preventative chemotherapy using the benzimidazole anthelmintics, albendazole (400 mg) and mebendazole (500 mg) (World Health Organisation, 2024b).

Human schistosomiasis is a parasitic disease caused by trematodes of the genus *Schistosoma*. There are two major forms of disease, intestinal and urogenital schistosomiasis. Three main species of schistosomes infect human beings, *Schistosoma mansoni* and *Schistosoma japonicum* (intestinal schistosomiasis), and *Schistosoma haematobium* (urogenital schistosomiasis) (Colley et al., 2014). It is estimated that at least 264 million people

worldwide are infected with *Schistosoma* spp (World Health Organisation, 2024a). Schistosomiasis is associated with significant morbidity and mortality. More than 90% of the infections occur in Africa, an estimated two thirds of infections are caused by *Schistosoma haematobium* (Santos et al., 2021).

Schistosomiasis transmission occurs when infected individuals contaminate freshwater sources with faeces or urine containing parasite eggs, which then hatch into larvae (cercariae) in the water. Infection occurs when larvae, released by aquatic snails, penetrate the skin during contact with contaminated water. Within the human host, larvae mature into adult schistosomes and migrate through the blood vessels. Adult schistosomes pair up and colonise the blood vessels for many years, where they produce eggs. Some of these eggs are excreted through faeces or urine, continuing the parasite's lifecycle, while others become lodged in the intestines or liver (*Schistosoma mansoni* and *Schistosoma japonicum)*, or walls of the urinary tract and bladder (*Schistosoma haematobium)* (Odegaard and Hsieh, 2014; Colley et al., 2014). Moreover, the embedded eggs induce a chronic, distinct immune-mediated granulomatous response that has local and systemic pathological consequences (Colley et al., 2014).

Standard diagnostic methods for schistosomiasis include detection of viable eggs in urine or stool samples, or tissue biopsies, using techniques such as microscopy and the Kato-Katz method. However, these methods suffer from low sensitivity, and the true burden of schistosomiasis may be underestimated (Colley et al., 2014). Schistosomiasis control focuses on periodic, large-scale treatment of at-risk populations with praziquantel, to reduce morbidity. While praziquantel is safe to administer and effective against adult schistosomes, poor efficacy against immature schistosome larvae is reported (Colley et al., 2014).

7 Immune responses during helminth infections

Helminths and their human hosts have co-evolved over many centuries; thereby helminth parasites have developed several mechanisms to ensure their longevity in infected hosts. Typically, helminth-induced tissue injury stimulates a Th2 host immune response, which supports wound repair and reduces tissue inflammation caused by helminths as they migrate through different host tissues and organs. Initial helminth-induced tissue injury activates the innate immune response, where the release of danger associated molecular patterns (DAMPS) stimulate epithelial cells to release alarmin cytokines [IL-25, IL-33, and thymic stromal lymphopoietin (TSLP)]. IL-25 and IL-33 stimulate innate cells to produce Th2-associated cytokines (IL-4, IL-5, and IL-13), while TSLP suppresses the production of IL-12, a major Th1 cytokine, and supports dendritic cell maturation (Harris and Loke, 2017; Rapin and Harris, 2018). The host adaptive immune response to helminth infection is important for stimulating the development of Th2 cells and their associated cytokines, helminth expulsion, and preventing re-infection (Harris and Loke, 2017). Helminth-induced Th2 responses can downregulate Th1 and Th17 immune responses and their associated cytokines, such as IL-12, IFN-γ, IL-17, IL-23, and TNF-α. Moreover, helminths can dampen Th1 and Th2 host immune responses through the expansion of regulatory cell populations ($FOXP3^+$ T regulatory and B regulatory cells) and alternatively activated macrophages (AAMs). Collectively, these regulatory cell populations stimulate the release of immunosuppressive cytokines, IL-10, and tumour growth factor (TGF-β), thus creating a hyporesponsive or tolerant environment in the infected host that promotes helminth survival and limits host tissue damage (Mcsorley and Maizels, 2012).

8 Bystander effects of helminth immune modulation on important pathogens

The immune modulatory effects of helminths can influence a range of unrelated bystander conditions and infections. The influence of helminth immune modulation on inflammatory conditions, such as allergies and autoimmune diseases, has been described. According to the hygiene hypothesis, the absence of helminths in developed regions, due to improved sanitation and hygiene, has led to an increase in allergic conditions and autoimmune diseases, compared to helminth-endemic regions (Maizels and Mcsorley, 2016). In support, evidence emerging from Africa, demonstrate decreased prevalence of atopy (Van Den Biggelaar et al., 2000) and decreased levels of autoreactive antibodies in helminth-infected individuals (Mutapi et al., 2011).

Helminth immune modulation has been shown to influence susceptibility to and the clinical course of infectious diseases that are endemic to SSA, with varying outcomes. Some key examples from studies conducted in SSA are provided here.

Studies of helminth-malaria co-infection have yielded conflicting results. Helminth co-infection was associated with a higher prevalence of non-severe malaria (*Plasmodium falciparum* or *Plasmodium vivax*) (Degarege et al., 2012; Babamale et al., 2018), and with a higher intensity of *Plasmodium falciparum* (Babamale et al., 2018). Moreover, individuals co-infected with STHs and *Plasmodium falciparum* had more pronounced malnutrition, anaemia and lower body weight, suggesting that co-infection with STHs exacerbates malnutrition, anaemia, low body weight status (Degarege et al., 2010). STHs are associated with anaemia and nutritional deficiencies (Stephenson et al., 2000; Mpaka-Mbatha et al., 2022). STHs may suppress appetite due to gut inflammatory responses mediated by the infected host. Some STHs (*Ascaris lumbricoides* and hookworms) secrete inhibitors of pancreatic enzymes, which may directly impair host nutrient absorption in the small intestine (Cappello, 2004). Hookworms colonise the small intestine, where they feed on blood. High intensity hookworm infections are associated with iron deficiency anaemia, particularly in children and women of reproductive age, who are malnourished or have low iron levels (Loukas et al., 2021).

Helminth immune modulation has also been shown to influence *Mycobacterium tuberculosis*. A strong association between helminth infection and active TB was reported in a

cohort of Ethiopian patients (Elias et al., 2006), suggesting that helminth immune modulation impairs host immune responses to TB. Moreover, in helminth-infected individuals, purified protein derivative (PPD) and T cell responses were impaired in response to natural immunisation and bacille Calmette-Guérin (BCG) vaccination, respectively, but improved following anthelmintic treatment (Elias et al., 2001). Chronic helminth infection was associated with reduced BCG efficacy and correlated with increased levels of TGF-β (Elias et al., 2008).

Sexually transmitted viral infections are highly endemic to SSA. Given the significant burden of HIV in SSA, many studies have focussed on helminth-HIV interactions. In this context, a high prevalence of helminths was reported among HIV-infected individuals (Mkhize-Kwitshana et al., 2011; Ivan et al., 2013; Abossie and Petros, 2015). In helminth/HIV co-infected individuals, $CD4^+$ counts were lower (Adeleke et al., 2015), immune cells were dysregulated, and HIV viral loads were higher (Mkhize-Kwitshana et al., 2011). In addition, hookworm infection was correlated with a higher risk of HPV infection, higher HPV viral loads, and distinct mixed Type 1/Type 2 immune profiles in the vaginal tracts of helminth/HPV co-infected women (Gravitt et al., 2015; Holali Ameyapoh et al., 2021; Omondi et al., 2022).

Schistosoma haematobium infection, which causes urogenital schistosomiasis, can profoundly impact reproductive health. In chronically infected women, vaginal pathology is associated with itching, bleeding, pain, discharge, genital lesions, genital tumours, and ectopic pregnancies (Hegertun et al., 2013; Norseth et al., 2014). In men, urogenital schistosomiasis is associated with pathology of the seminal vesicles, dysuria, haematuria, pelvic pain, and infertility (Kayuni et al., 2019; Roure et al., 2024). Importantly, *Schistosoma haematobium* is classified as a group 1 carcinogen; the correlation between urogenital schistosomiasis and bladder cancer has been previously described (Salem et al., 2011; Khaled, 2013; Ishida and Hsieh, 2018). Urogenital schistosomiasis is also associated with an increased risk of HIV infection (Looker et al., 2017), particularly in women (Kjetland et al., 2006; Ndhlovu et al., 2007; Downs et al., 2011). Biologically, chronic inflammation and tissue damage caused by *Schistosoma haematobium* eggs, enhanced immune activation, and genital lesions in the female reproductive tract, are thought to increase the risk of HIV, by increasing viral entry points and the number of target cells at the infection site. Additionally, *Schistosoma haematobium*-associated Th2 immune response may suppress the Th1 responses needed for effective anti-HIV immunity (Chetty et al., 2020).

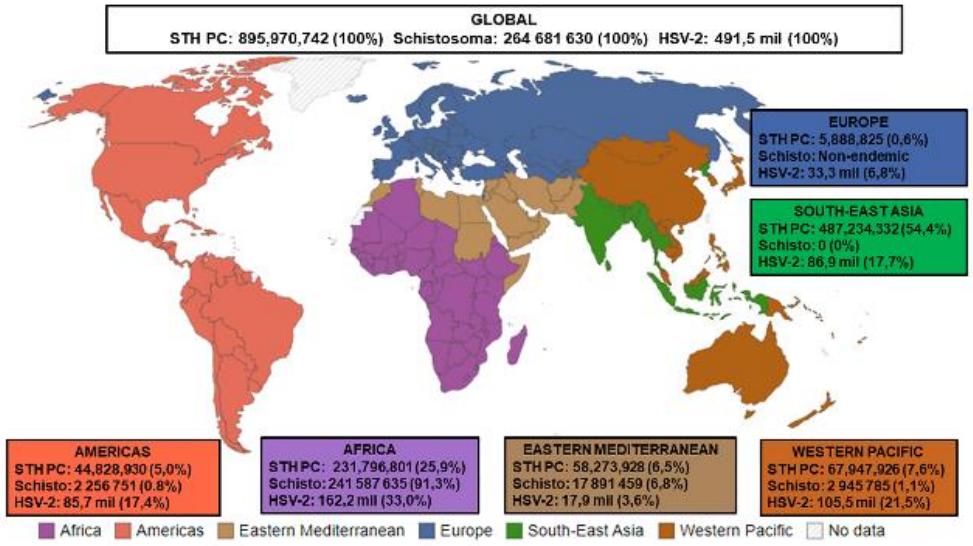

FIGURE 1
Estimated numbers (n) and proportions (%) of STH PC required for pre-SAC and SAC, Status of Schistosomiasis in endemic countries, and HSV-2 infections. STH PC: Soil-transmitted helminths Preventive Chemotherapy [Data presented is the estimated number of Pre-school Aged (Pre-SAC) and School-Aged Children (SAC) requiring PC for STHs in 2022[a]; % Proportion = Estimated number of STH PC/Estimated number of Global STH PC x 100]; Schisto: Status of Schistosomiasis in endemic countries in 2022[b]; % Proportion = Estimated number of Schistosoma/Estimated number of Global Schistosoma PC x 100]; HSV-2: Herpes Simplex Virus Type II [Data presented is the estimated number of people within the 15-49 year age group that were infected with HSV-2 in 2016[c]; % Proportion = Estimated number of HSV-2/Estimated number of Global HSV-2 x 100, Source of WHO regions map[d]. [a]Source: Adapted from (World Health Organisation, 2024c). [b]Source: Adapted from (World Health Organisation, 2024a). [c]Source: Adapted from (James et al., 2020). [d]Source: Adapted from (Our World in Data, 2023).

When holistically evaluating the effects of helminth immune modulation, it is important to mention that helminths may have beneficial effects on concurrent infections. This was reported during the recent COVID-19 pandemic; helminth co-infection (*Hymenolepis nana, Schistosoma mansoni* and *Trichuris trichiura*) mitigated COVID-19 severity in patients from Africa (Wolday et al., 2021).

Taken together, it is evident that different factors determine whether helminths have beneficial or detrimental effects on concurrent bystander infections. These include the helminth species, the worm burden, type of concurrent infection/condition, tissue tropism, and the timing and niche of infection (Schlosser-Brandenburg et al., 2023).

9 Discussion: potential implications of helminth immune modulation on HSV-2

Considering the studies described above, it is plausible that helminth immune modulation may influence HSV-2 outcomes in SSA. Given the substantial prevalence and geographic overlap of HSV-2 and helminths in SSA, it is likely that co-infections occur (Figure 1). However, no studies have examined the influence of helminth immune modulation on HSV-2 co-infection in humans. However, in a very recent murine study, Chetty et al. (2021) demonstrated that acute, self-limiting infection with *Nippostrongylus brasiliensis*, a murine intestinal helminth that is closely related to the human hookworms, induced a classic Th2 immune response in the female genital tract (FGT). FGT immune responses to subsequent genital HSV-2 were impaired, leading to enhanced HSV-2 pathology. This enhanced pathology was dependent on IL-5 and associated with increased levels of IL-33, group 2 innate lymphoid cells (ILC2s), and accumulation of eosinophils in the FGT (Chetty et al., 2021). The exact mechanisms by which *Nippostrongylus brasiliensis* influences the FGT, a site it does not colonise, need to be further explored. It has been suggested that helminth-derived excretory-secretory products may play a key role in inducing immune cell trafficking and Th2 responses in uncolonized tissues, such as the FGT (Zarek and Reese, 2021). Importantly, Chetty et al. (2021) demonstrate the systemic effects of helminth infection on FGT immune responses to HSV-2 infection, which supports previously observed clinical correlations between STHs and viral infections in vaginal tissue (Gravitt et al., 2015; Omondi et al., 2022). Thus, their novel findings provide some insight into how helminths may alter HSV-2 pathology in co-infected individuals and provides a basis for future human studies. Given that helminths stimulate Th2 and immune modulatory responses, whereas protection from HSV-2 requires a Th1 response, we hypothesise that helminths may compromise host immunity to HSV-2 during co-infection. This could enhance susceptibility to HSV-2, promote viral persistence and pathology, and impair responses to HSV-2 treatment. Therefore, helminth-HSV-2 co-infections could have significant consequences for sexual and reproductive health, such as increased risk of acquiring other sexually transmitted infections, infertility, and cancer progression (Chetty et al., 2020). The ensuing negative impact on inadequately resourced healthcare systems in SSA would be substantial. Furthermore, it may be necessary to consider how helminth immune modulation may impact HSV-2 treatment efficacy; this factor may need to be considered when designing appropriate treatment strategies and potential HSV-2 vaccines. We argue that helminth/HSV-2 co-infections are grossly understudied, therefore their potential impact on public health is underestimated. To address this gap, we believe that human studies exploring helminth/HSV-2 co-infections in SSA are warranted. Moreover, we believe that evaluating and managing these infections using holistic and integrated approaches may advance the WHO 2030 Sustainable Development Goals aimed at eliminating NTDs and sexually transmitted infections (World Health Organisation, 2022a, 2022).

10 Concluding remarks

HSV-2 and helminth co-infections may commonly occur in SSA. However, their potential interactions are grossly understudied and poorly understood. There is a significant paucity of data on HSV-2 and helminth co-infections, and whether helminth immune modulation influences HSV-2 pathology is unclear. We assert that epidemiological and immunological studies on HSV-2 and helminth co-infections are needed to fully understand the interplay between these pathogens. Moreover, evidence from such studies could prove relevant as they may inform the therapeutic management of HSV-2 and helminths in co-endemic regions such as SSA.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

RP: Conceptualization, Writing – original draft, Writing – review & editing. PN: Supervision, Writing – review & editing. ZM: Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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2.2 Potential Interactions Between Soil-Transmitted Helminths and Herpes Simplex Virus Type II: Implications for Sexual and Reproductive Health in Sub-Saharan African

Sub-Saharan Africa (SSA) bears a disproportionate burden of soil-transmitted helminths (STHs) and sexually transmitted infections (STIs), including herpes simplex virus type 2 (HSV-2). Considering this epidemiological overlap, co-infections are likely and may profoundly impact sexual and reproductive health (Chetty et al., 2020). Immunologically, STHs and HSV-2 elicit opposing immune responses – HSV-2 typically induces a pro-inflammatory T-helper 1 (Th1) response, whereas STHs stimulate a potent T-helper 2 (Th2) and anti-inflammatory phenotype in the infected host (Chetty et al., 2020). Additionally, the immunomodulatory effects of STHs could potentially alter HSV-2 pathology by dampening Th1 responses, leading to enhanced viral replication, more frequent viral reactivations, and more severe clinical symptoms (Chetty et al., 2021). If this is indeed the case, sexual and reproductive health, as well as clinical management practices and therapeutic strategies could be profoundly impacted. Therefore, a nuanced understanding of the immunological interactions between STHs and HSV-2 is required. Focussed epidemiological and immunological data could potentially inform public health policies, leading to integrated control measures that address both STHs and HSV-2 infections simultaneously. This is particularly relevant in SSA and co-endemic regions, where targeted interventions can significantly mitigate the overall disease burden. This is discussed in the review paper entitled: "**Potential Interactions Between Soil-Transmitted Helminths and Herpes Simplex Virus Type II: Implications for Sexual and Reproductive Health in Sub-Saharan African",** which was published in **MDPI Biology**. This paper sought to outline the epidemiology of STHs and HSV-2 in SSA and review current knowledge on host immune responses to both pathogens. By evaluating the immunomodulatory effects of STHs on other viral pathogens, this paper hypothesised how STHs may influence HSV-2 outcomes, and how their co-occurrence may have broader implications for sexual and reproductive health in SSA, including pertinent sexual and reproductive health issues, such as HIV, cervical cancer, and infertility. The paper emphasized the clinical significance of STH/HSV-2 co-infections and the need for focused research and integrated strategies for their detection and management in SSA. The published PDF version, which is formatted according to the journal's requirements, is shown.

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Review

Potential Interactions Between Soil-Transmitted Helminths and Herpes Simplex Virus Type II: Implications for Sexual and Reproductive Health in Sub-Saharan African

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Simple Summary: In sub-Saharan Africa (SSA), soil-transmitted helminths (STHs) and herpes simplex virus type II (HSV-2) infections are widespread, and co-infections are likely to occur. It is against this backdrop, that we examine the potential interactions between STHs and HSV-2. STHs and HSV-2 induce opposing host immune responses—HSV-2 triggers a proinflammatory T-helper 1 (Th1) immune response, whereas STHs induce dominant anti-inflammatory T-helper type 2 (Th2) immune responses. It is well known that STHs have bystander effects on unrelated conditions by downregulating Th1 and T-helper type 17 (Th17) immune responses. In this way, STHs may potentially compromise essential anti-HSV-2 Th1 responses, leading to enhanced susceptibility to HSV-2 and HSV-2 pathology. Surprisingly, there is a significant lack of epidemiological and immunological studies on STH-HSV-2 co-infections in humans. Thus, we highlight the need for studies that focus on STH-HSV-2 co-infections in SSA.

Abstract: Sub-Saharan Africa (SSA) bears a disproportionate and overlapping burden of soil-transmitted helminths (STHs) and sexually transmitted viral infections. An estimated 232 million pre-school and school-aged children in SSA are vulnerable to STH infections. Together with this, SSA has a high prevalence of herpes simplex virus type II (HSV-2), the primary cause of genital herpes. Studies have examined the immunological interactions between STHs and human immunodeficiency virus and human papillomavirus during co-infections. However, epidemiological and immunological studies on STH-HSV-2 co-infections are lacking, therefore their impact on sexual and reproductive health is not fully understood. STH-driven Th2 immune responses are known to downregulate Th1/Th17 immune responses. Therefore, during STH-HSV-2 co-infections, STH-driven immune responses may alter host immunity to HSV-2 and HSV-2 pathology. Herein, we provide an overview of the burden of STH and HSV-2 infections in SSA, and host immune responses to STH and HSV-2 infections. Further, we emphasize the relevance and urgent need for (i) focused research into the interactions between these important pathogens, and (ii) integrated approaches to improve the clinical detection and management of STH-HSV-2 co-infections in SSA.

Keywords: soil-transmitted helminths; herpes simplex virus-2; co-infection; immunological interactions; sub-Saharan Africa

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1. Introduction

Sub-Saharan Africa (SSA) has the second largest population size of approximately 1.3 billion people, comprising 15.5% of the total global population [1]. The continent bears a disproportionate burden of sexually transmitted viral infections [human immunodeficiency virus (HIV), human papillomavirus (HPV), and herpes simplex virus type II (HSV-2) [2] as well as soil-transmitted helminth (STH) infections [3]. The most prevalent STHs are *Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus*, and *Ancylostoma duodenale*. These STHs typically cause chronic and asymptomatic infections and are associated with significant morbidity [4]. The co-endemicity of STHs and sexually transmitted viral infections can have significant consequences for sexual and reproductive health. While there is evidence of how STHs influence HIV [5], and HPV [6–8], the effect of STHs on HSV-2 is poorly understood. Insight into the effect of STHs on HSV-2 co-infections is relevant, particularly in SSA, where both pathogens are highly prevalent. Herein, we provide an overview of the burden of STH and HSV-2 infections. Drawing from existing literature on the local and/or systemic effects of STHs on viral co-infections, we postulate the potential effects of STHs on HSV-2 during co-infection. Finally, we offer recommendations for future studies and therapeutic interventions aimed at addressing STH-HSV-2 co-infections and their impact on sexual and reproductive health in SSA.

2. Literature Search

A literature search was conducted on PubMed and Google Scholar to identify studies related to STH and HSV-2 mono- and co-infections and their impact on host immunity. The following search terms were used: "soil-transmitted helminths", "soil-transmitted helminths and immune responses", "HSV-2", "HSV-2 and immune responses", and "soil-transmitted helminths and HSV-2 co-infection". Experimental studies, human studies, and review articles in English were retrieved. No year or region restrictions were applied.

3. STH Infection Burden

Helminths are macroparasitic worms that have infected humans for thousands of years. Evidence of their clinical manifestations has been documented in Hippocrates, Egyptian, and Bible records, and helminth eggs and protozoan cysts have been identified in coprolites and other naturally and/or artificially preserved fossils [9,10]. Helminths are grouped into two major categories: nematodes or roundworms and platyhelminths or flatworms. Nematodes comprise STHs, also known as intestinal helminths, and filarial parasites. While STHs cause intestinal infections, filarial parasites cause lymphatic filariasis (elephantiasis) and onchocerciasis (river blindness) [9]. Flatworms comprise trematodes (flukes) and cestodes (tapeworms) [10,11].

STHs are widely distributed parasitic worms, causing more than 1.5 billion human infections globally. Most infections occur in East Asia, China, SSA, and the Americas [3]. The three main medically important STHs are roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), and hookworms (*Necator americanus* and *Ancylostoma duodenale*) [3,12]. Table 1 summarizes the classification, biology, and global prevalence of the major human STHs discussed in this review.

Table 1. Classification, biology, and global prevalence of the major human STHs in SSA.

STH	Global Prevalence	Classification and Biology	References
Ascaris lumbricoides	772–892 million	- Roundworm. - Disease in humans: Ascariasis. - Classification: (i) Domain: Eukaryota, (ii) Kingdom: Animalia, (iii) Subkingdom: Metazoa, (iv) Phylum: Nematoda, (v) Class: Secernentea, (vi) Order: Ascaridida, (vii) Family: Ascarididae, (viii) Genus: *Ascaris*, (viii) Species: *Ascaris lumbricoides*. - Morphology: (i) Unsegmented, cylindrical and elongated bodies. (ii) Worms are white to pinkish in colour. (iii) Adult male: 15–31 cm by 2–4 mm. (iv) Adult female: 20–35 cm by 3–6 mm and produce 240,000 eggs/day. Lifecycle: Embryonated eggs from contaminated soil, food, or water are ingested, moulting into L1, then L2 larvae that penetrate the small intestine. Larvae then migrate through the liver and lungs, where they mature to L3 larvae, ascend the trachea, are swallowed, and re-enter the intestine. There, they moult into L4 larvae and mature into adult worms that reproduce, releasing thousands of eggs per day. Embryonated eggs and adults are viable for several years.	[13–18]
Trichuris trichiura	429–508 million	- Whipworm. - Disease in humans: Trichuriasis. - Classification: (i) Domain: Eukaryota, (ii) Kingdom: Animalia, (iii) Subkingdom: Metazoa, (iv) Phylum: Nematoda, (v) Class: Adenophorea, (vi) Order: Trichocephalida, (vii) Family: Trichuridae, (viii) Genus: *Trichurus*, (ix) Species: *Trichuris trichiura*. - Morphology: (i) Whip-like appearance. The anterior end is thin and thread-like, while the posterior end is thicker and contains most of the reproductive organs. Males have a coiled tail, whereas females have a straight posterior end. (ii) Worms are white to pinkish in colour. (iii) Adult male: 30–45 mm by 0.1–0.5 mm. (iv) Adult female: 35–55 mm by 0.1–0.5 mm and produce 3000–10,000 eggs/day Lifecycle: Unembryonated eggs from contaminated feces develop into infective eggs in the soil. Infection begins when contaminated soil, food, or water is ingested. The eggs hatch in the small intestine, and L1 larvae migrate to the large intestine, where they penetrate epithelial cells at the crypt base. After several moults, they mature into whip-like adult worms, which can live for years. Eggs remain viable in soil for months under favorable conditions.	[13–16,19–21]
Necator americanus and *Ancyclostoma duodenale*	406–480 million	- Hookworms. - Disease in humans: Necatoriasis and Ancylostomiasis, respectively. - Classification: (i) Domain: Eukaryota, (ii) Kingdom: Animalia, (iii) Subkingdom: Metazoa, (iv) Phylum: Nematoda, (v) Class: Secernentea, (vi) Order: Strongylida, (vii) Family: Uncinariidae or Ancylostomidae, (viii) Genus: *Necator* or *Ancylostoma*, (ix) Species: *Necator americanus* or *Ancylostoma duodenale*. - Morphology: (i) Both species have a simple cylindrical body with a curved, hook-like anterior end. (ii) Worms are pale, translucent, or white. (iii) Adult male: 6–11 mm by 0.4–0.6 mm. (iv) Adult female: 10–13 mm by 0.4–0.6 mm and produce 10,000–20,000 (*Ancylostoma duodenale*) and 5000–10,000 (*Necator americanus*) eggs/day. Lifecycle: Eggs released in the host's feces hatch into rhabditiform (L1) larvae in soil. These larvae feed on organic matter and moult twice to become infective filariform (L3) larvae, which penetrate human skin. Following skin entry, they migrate through the bloodstream to the lungs, ascend the respiratory tract, and are swallowed. In the small intestine, they mature into adult worms, attach to the intestinal lining, and feed on blood. Female worms produce thousands of eggs daily, completing the cycle. Differences in the lifecycles of hookworms: *Necator americanus* remains confined to the intestines after entry and survives for several years, while *Ancylostoma duodenale* larvae can enter dormancy in tissues and reactivate later. Additionally, *Ancylostoma duodenale* can be transmitted through oral ingestion of larvae, unlike *Necator americanus*, which is exclusively acquired via skin penetration. Adult *Ancylostoma duodenale* worms typically survive for several months.	[13–16,20,22,23]

Key drivers of STH infection include poverty, overcrowded living spaces, insufficient clean water resources, poor hygiene practices, and inadequate sanitation [3]. In endemic regions, humidity and warm soil temperatures favor the development of non-fertile eggs into either fertile eggs (*Ascaris lumbricoides* and *Trichuris trichiura*) or infective larvae (hookworms) [24]. Infection with roundworms (*Ascaris lumbricoides*) and whipworms (*Trichuris trichiura*) occurs when embryonated eggs from contaminated water or food sources are ingested. In hookworm infections (*Necator americanus* and *Ancylostoma duodenale*), infective larvae penetrate the skin. Once inside the host, the parasites undergo several developmen-

tal stages, including migration of larvae through host tissues, maturation into adult worms, mating, and release of eggs back into the environment via the feces of the infected host [12].

Typically, STHs cause asymptomatic infections and are associated with higher morbidity than mortality [4]. Chronic infections and heavy worm burdens are associated with malnutrition and anemia, stunted physical growth, and impaired intellectual, cognitive, and educational development [12]. Severe complications associated with heavy worm burdens include rectal prolapse (trichuriasis), intestinal blockage or perforation, and duct blockages that may lead to biliary or pancreatic disease (ascariasis). Each year, children die from intestinal obstructions or STH-associated complications that require surgical intervention, which is not always available in resource-poor regions of SSA [25–27].

Although highly prevalent, STHs are classified as neglected tropical diseases (NTDs) due to three distinguishing features: (i) infections primarily occur in resource-poor tropical and subtropical developing countries, (ii) infections are chronic and insidious in nature, and (iii) the impact of infection burden on educational and economic development has not been quantified [12,14]. In SSA, STHs are the most widespread NTDs, contributing to 85% of the NTD burden [28]. Table 2 summarizes the prevalence of STHs in the different countries in SSA.

Table 2. Prevalence of STHs in SSA.

Countries in SSA	Total Population, *n*	STH Prevalence	
		n	%
Nigeria	232,679,478	48,681,440	20.9
Democratic Republic of Congo	109,276,265	26,830,345	24.6
Ethiopia	132,059,767	27,233,348	20.6
United Republic of Tanzania	68,560,157	24,470,870	35.7
Uganda	50,015,092	19,696,574	39.4
South Africa	64,007,187	13,672,166	21.4
Angola	37,885,849	4,886,509	12.9
Mozambique	34,631,766	12,223,289	35.3
Cameroon	29,123,744	3,493,226	12.0
Madagascar	31,964,956	7,117,949	22.3
Malawi	21,655,286	7,837,505	36.2
Kenya	56,432,944	5,524,519	9.8
Zimbabwe	16,634,373	553,600	3.3
Zambia	21,314,956	4,577,679	21.5
Rwanda	14,256,567	4,218,568	29.6
Sierra Leone	8,642,022	1,189,047	13.8
Togo	9,515,236	2,544,074	26.7
Guinea	14,754,785	2,676,150	18.1
Cote d'Ivoire	31,934,230	2,543,761	8.0
Burundi	14,047,786	969,250	6.9
Benin	14,462,724	1,959,363	13.5
Senegal	18,501,984	1,339,379	7.2
Central African Republic	5,330,690	1,528,494	28.7
Liberia	5,612,817	830,898	14.8
Namibia	3,030,131	330,850	10.9

Table 2. *Cont.*

Countries in SSA	Total Population, *n*	STH Prevalence	
		n	%
Congo	6,332,961	839,886	13.3
South Sudan	11,943,408	779,808	6.5
Chad	20,299,123	552,311	2.7
Gabon	2,538,952	465,348	18.3
Lesotho	2,337,423	387,421	16.6
Comoros	866,628	277,694	32.0
Equatorial Guinea	1,892,516	387,783	20.5
Cabo Verde	524,877	143,054	27.3
Guinea-Bissau	2,201,352	383,629	17.4
Botswana	2,521,139	43,831	1.7
Sao Tome and Principe	235,536	83,988	35.7
Gambia	2,759,988	53,098	1.9
Eswatini	1,242,822	20,097	1.6
Ghana	34,427,414	No preventative chemotherapy required	No preventative chemotherapy required
Niger	27,032,412	No preventative chemotherapy required	No preventative chemotherapy required
Overall prevalence, *n* (%)	1,163,487,343 (14.2) [a]	231,346,801 (19.9) [b]	

[a] % Overall prevalence (total population) = total population in sub-Saharan Africa/world population as of 8 November 2024 (8,187,059,938) × 100. Source: [1,29]. [b] % Overall prevalence (Helminths prevalence) = total helminths prevalence in sub-Saharan Africa/total population in sub-Saharan Africa × 100. Source: [29,30].

Those most vulnerable to STHs include preschool-aged children (pre-SAC; 1–4 years), school-aged children (SAC; 5–14 years), girls and women of reproductive age, pregnant and/or breastfeeding women, and people with high-risk occupations, such as miners or tea-pickers living in STH-endemic regions [3].

To mitigate the risk of infection, the World Health Organisation (WHO) introduced widespread deworming and preventive chemotherapy (PC) programs in STH-endemic regions. The regular administration of anthelmintic drugs, primarily mebendazole and albendazole, to at-risk children, reduces both the number of moderate- and heavy-worm burden infections, and the overall prevalence of STH infections [12,31].

There has been a significant improvement in the WHO efforts to prevent and eliminate STHs in SSA. For example, one of the WHO 2020 NTD Roadmap targets was to treat a minimum of 75% of at-risk SAC (5–14 years) in helminth-endemic regions where infection prevalence exceeded 20%. In SSA, approximately 70% of at-risk children received regular anthelmintic treatment by 2018, and there was a notable reduction in the prevalence of STH infection in SAC, from 44% in 2000 to 13% in 2018 [32]. Despite this progress, several barriers may hinder the successful elimination of STHs. For example, recurring infections with *Ascaris lumbricoides* and *Trichuris trichiura* have been reported [33]. In addition, interruptions, such as those caused by the recent COVID-19 pandemic, hinder the successful implementation of PC programs. To focus on control measures for COVID-19, some countries in SSA, including South Africa, Malawi, Botswana, Namibia, Lesotho, Eswatini, and Gabon, did not implement their PC for SAC in 2021 [31]. Importantly, sustainable and effective PC programs require an integrated approach that combines clean water supplies, improved sanitation, health education, behavior change, and the regular implementation of PC [12,31–33].

4. HSV-2 Infection Burden

The Herpesviridae family comprises three subgroups, namely alpha-, beta-, and gamma-herpesviruses. Herpes simplex virus (HSV) is a double-stranded, human alphaherpesvirus, with two known serotypes: HSV type I (HSV-1) and HSV type II (HSV-2). HSV-1 infection primarily causes corneal keratitis and/or oral blisters, whereas HSV-2 infection is associated with genital herpes and genital ulcer disease [34].

HSV-2 is among the most prevalent sexually transmitted viral infections worldwide. In 2016, the total number of HSV-2 infections among people aged 15–49 years worldwide, was approximately 491.5 million (13.2% global prevalence). In 2016, the annual incidence of HSV-2 among people aged 15–49 years worldwide, was approximately 23.9 million [35]. HSV-2 prevalence varies significantly across the WHO regions. Infection rates are notably highest in areas with low socio-economic status and limited healthcare resources, particularly within SSA. The estimated HSV-2 seroprevalence in SSA is 33%, substantially exceeding rates in other regions, such as 7% in Europe and 17% in the Americas (Table 3). There is also considerable variation in HSV-2 prevalence among SSA subregions, with the highest rates observed in Eastern and Southern Africa, followed by Central and Western Africa. Moreover, infection is more prevalent among women than men, both in Africa and globally [35,36].

Table 3. HSV-2 prevalence in the WHO regions.

WHO Region	Prevalence, *n* (%) [a]
Africa	162,200,000 (33.0)
The Americas	85,700,000 (17.4)
Eastern Mediterranean	17,900,000 (3.6)
Europe	33,300,000 (6.8)
South-East Asia	86,900,000 (17.7)
Western Pacific	105,500,000 (21.5)
Total	491,500,000 (100)

Estimated numbers (*n*) and proportions (%) of HSV-2 across the WHO regions [data presented are the estimated number of people (*n*) within the 15–49-year age group that were infected with HSV-2 in 2016] [a]; % proportion = estimated number of HSV-2/estimated number of total HSV-2 × 100. [a] Source: Adapted from [35].

HSV-2 infects the genital mucosa and replicates in keratinocytes that line the epithelium, leading to genital lesions [37]. Furthermore, by establishing latency in sensory neurons and ganglia, HSV-2 can evade the immune system and antivirals, thereby causing lifelong infections [38]. Although HSV-2 primarily causes self-limiting and asymptomatic infections, both symptomatic and asymptomatic individuals actively shed the virus during reactivation periods. Lifelong reactivation of genital lesions commonly occurs in symptomatic individuals [39]. Rarely, HSV-2 is associated with life-threatening conditions, such as herpes simplex encephalitis in newborns and immunocompromised individuals [39].

Despite its high prevalence, there are currently no prophylactic or curative treatments for HSV-2. While antiviral treatment with drugs, such as acyclovir, reduces recurrent HSV-2 infections, they are ineffective in eliminating viral shedding. Furthermore, resistance to antivirals, especially in immunocompromised individuals, has been reported [40].

HSV-2 is recognized as a critical driver of the HIV epidemic in various regions, most notably in SSA, where the highest HSV-2 and HIV infection burdens are recorded [41]. For example, Bradley et al. [42] reported that among individuals infected with HSV-2, the HIV prevalence was 41%, whereas among those without HSV-2 infection, the HIV prevalence was 6% [42]. This striking epidemiological association between HSV-2 and HIV infection has significant public health consequences. HSV-2 infection is also known to increase the risk of transmitting and acquiring HIV infection by almost three-fold. Biologically, HSV-2 infection facilitates HIV transmission by causing genital ulcers, which compromise the

integrity of the genital mucosal barrier. In addition, HSV-2 infections cause increased trafficking of Th1 $CD4^+$ cells to the infected genital site, thereby increasing the number of available target cells for HIV entry [43,44]. Individuals with incident HSV-2 infection are more vulnerable to HIV infection [45,46]. Furthermore, the interactions between HSV-2 and HIV are bidirectional. HSV-2 co-infection promotes HIV genital shedding [47] and may accelerate the progression of HIV disease [48,49], whereas HIV co-infection associates with increased occurrence and quantity of HSV-2 viral shedding [50].

HSV-2 has also been linked to cervical cancer; however, its role in cervical carcinogenesis is poorly understood. To date, a few studies have examined the association between HSV-2 and cervical cancer, with conflicting results [51]. There is considerable disparity in the global distribution of cervical cancer [52,53], and resource-poor regions across the world (South-East Asia, Melanesia, SSA, and South America) carry approximately 90% of the cervical cancer disease burden. SSA has the highest incidence of cervical cancer cases and the highest cervical cancer mortality rates. In addition, most cancer-related deaths among women in SSA are due to cervical cancer [53].

Longstanding infection with oncogenic or high-risk HPV strains (mainly HPV-16 and HPV-18) is the leading risk factor for cervical cancer [54,55]. However, in 90% of women, high-risk HPV infection does not progress to cervical cancer [54]. This implies that co-factors, such as environmental, host, and viral factors, may act jointly with HPV to contribute to cervical carcinogenesis. Other well-known risk factors for cervical cancer include smoking, multiple sexual partners, long-term oral contraceptive use, and HIV infection [52,54]. Because of its persistent nature and ability to reactivate from latency, HSV-2 may cause abnormal cellular DNA replication and genetic mutations. Therefore, persistent infection with HSV-2 could lead to abnormal differentiation and proliferation of the cervical epithelium, which may lead to cervical cancer [56]. Furthermore, HSV-2 promotes ulceration and inflammation of the genital epithelium and induces an influx of Th1 $CD4^+$ cells to the infected site. In addition to promoting the acquisition of HIV, HSV-2-induced genital ulceration may facilitate infection with oncogenic strains of Epstein–Barr virus (EBV) and HPV [57].

There is epidemiological evidence supporting the association between HSV-2 and cervical cancer [58–60]. Li and Wen [60] reported that HSV-2 infection was linked to the occurrence of cervical cancer, even after controlling for oncogenic HPV as a confounding factor. In addition, their study found that co-infection with HPV and HSV-2 correlated with a higher relative risk for cervical cancer compared to a single infection with either virus [60]. Similarly, Zhao et al. [58] observed that while HSV-1 was not associated with cervical cancer, HSV-2 infection—either alone or in combination with HPV—was linked to cervical carcinogenesis [58]. Koanga et al. [59] further demonstrated that HSV infection promoted cervical inflammation and abnormalities in infected women, suggesting it may serve as a co-factor in cervical carcinogenesis [59]. These findings underscore the potential link between HSV-2 and cervical carcinogenesis, which warrants further investigation. Given its high prevalence and associations with HIV and HPV, HSV-2 represents a significant public health challenge, particularly in SSA.

5. Immune Response to STHs

STHs exhibit notable differences in their routes of infection and lifecycles and demonstrate distinct variations in tissue tropism. Hookworms, for example, infect the human host via skin penetration, whereas *Ascaris lumbricoides* and *Trichuris trichiura* are orally infective [15,16]. Moreover, STHs have complex lifecycles, comprising different stages. For example, the larval stages of *Ascaris lumbricoides* complete extra-intestinal migratory phases during which larvae hatch in the intestine, and migrate through the liver and lungs, before finally settling in the small intestine, where they develop into adult worms and reproduce. As they transit through the different tissues, they induce local inflammation, cellular remodeling, and immune modulation. Similarly, the hookworms (*Ancylostoma duodenale* and *Necator americanus*) undergo extra-intestinal migration via blood circulation and the

lungs, before establishing themselves in the small intestine. In contrast, *Trichuris trichiura* does not exhibit tissue migration, establishing infection in the large intestine [15,16]. STH developmental stages and tissue migrations occur over time, spanning weeks to years, depending on the STH species and its specific mammalian host. Thus, host immunity is often shaped differently based on the tissue involved or possibly the STH's lifespan [61]. These differences contribute to varying clinical outcomes as observed across STH infections. In addition, the pathological effects of STHs are linked to the worm burden and whether the infection is acute or chronic [61]. Despite these species-specific differences, STHs have been shown to influence and regulate the host immunity (i.e., parasite-specific immunoregulation), by typically inducing dominant anti-inflammatory Th2 and immunomodulatory responses. STH-driven immune responses reduce STH-associated inflammation and favor the long-term survival of both the host and parasite. A dominant Th2 phenotype consequently downregulates Th1 and Th17 proinflammatory immune responses, limits worm burden, and promotes healing of damaged host tissue [62]. Secretion of alarmin cytokines, [Interleukin (IL)-25, IL-33, and thymic stromal lymphopoietin (TSLP)], initiates a coordinated Th2 response. IL-25 and IL-33 subsequently activate Th2 innate lymphoid cells (ILC2s), which secrete IL-5 and IL-13. TSLP suppresses IL-12, a Th1 response cytokine, and supports dendritic cell (DC) maturation [63]. IL-4, IL-5, and IL-13 amplify the Th2 response within the intestine and stimulate immunoglobulin-E (IgE) and immunoglobulin-G (IgG) antibody production by activated B cells. Furthermore, Th2 cytokines recruit and activate various myeloid cells (basophils, eosinophils, macrophages, and mast cells). Alternatively-activated macrophages (AAMs) and eosinophils attempt to trap and kill the worms as they migrate through host tissues [63]. Worm expulsion is further promoted by the "weep and sweep" mechanism, which includes increased mucus secretion by goblet cells and the concerted action of various Th2 cytokines (IL-3, IL-4, IL-5, IL-13, and IL-9), $CD4^+$ T cells, ILC2s, and myeloid cells (basophils, eosinophils, and mast cells) [64].

During chronic STH infections, immunomodulation is further enhanced by regulatory T (Treg) cells, which secrete immunosuppressive cytokines, such as IL-10 and transforming growth factor beta (TGF-β) [62] (Figure 1).

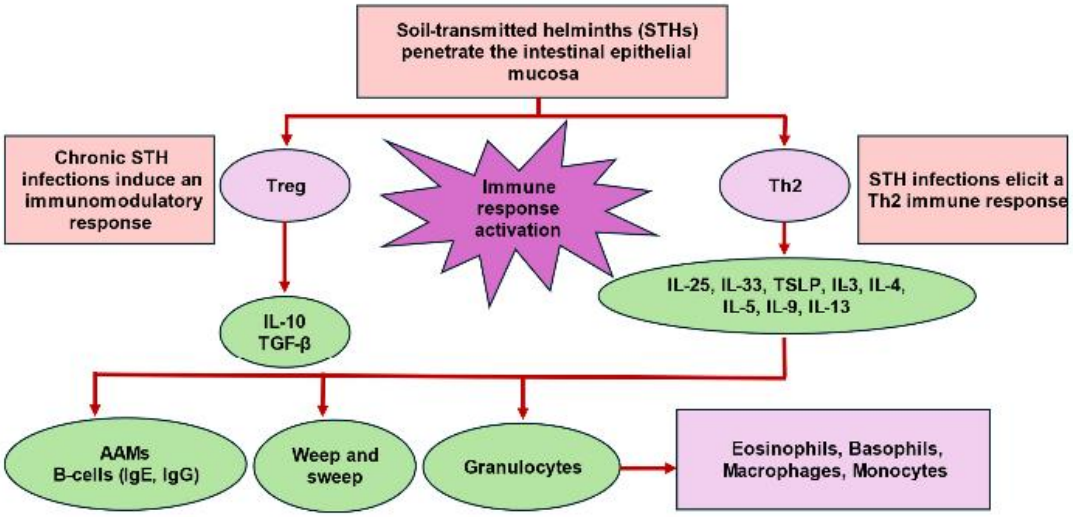

Figure 1. Illustration of the immune response to STHs. Footnote: AAMs: alternatively-activated macrophages; IgE: immunoglobulin E; IgG: immunoglobulin G; IL: interleukin; STH: soil-transmitted helminth; TGF-β: transforming growth factor beta; Th2: T-helper type 2 cells; Treg: regulatory T cells; TSLP: thymic stromal lymphopoietin.

Moreover, STHs alter host immunity, directly or indirectly, by employing several strategies, including (i) the induction of host resistance and tolerance responses, (ii) secretion of immunomodulatory products, such as excretory-secretory products (ESP), and (iii) interaction with the intestinal microbiome [65]. Interestingly, the STH-induced immune effects are not limited to the tissues they transit through or colonize, but extend to distal parts of the body, leading to systemic immunomodulation. Importantly, opposing immune responses are directed against STHs (i.e., Th2 and immunomodulatory) versus viral and bacterial pathogens (i.e., Th1 and Th17 immunity), and these responses broadly inhibit one another. Consequently, this may lead to varied outcomes in terms of host health and susceptibility to co-infections. Epidemiological data also indicate an inverse relationship between STHs and the incidence of allergies, autoimmune diseases, and metabolic disorders. Additionally, STHs may alter responses to vaccines [reviewed in [61,66,67]].

Co-infections with multiple helminths are also common, particularly in endemic regions. As observed in animal studies, poly-helminth infections can impact the outcome of each infection in several ways: (i) one helminth infection can suppress the immune response to a concurrent helminth infection; (ii) the timing of each infection impacts the resulting immunopathological effects; and (iii) host susceptibility or resistance may be altered by the co-infecting helminth, independent of immunity developed through immunization [67].

6. Immune Response to HSV-2

An effective Th1 proinflammatory immune response against HSV-2 requires multiple coordinated mechanisms of both innate and adaptive components of the immune system (Figure 2).

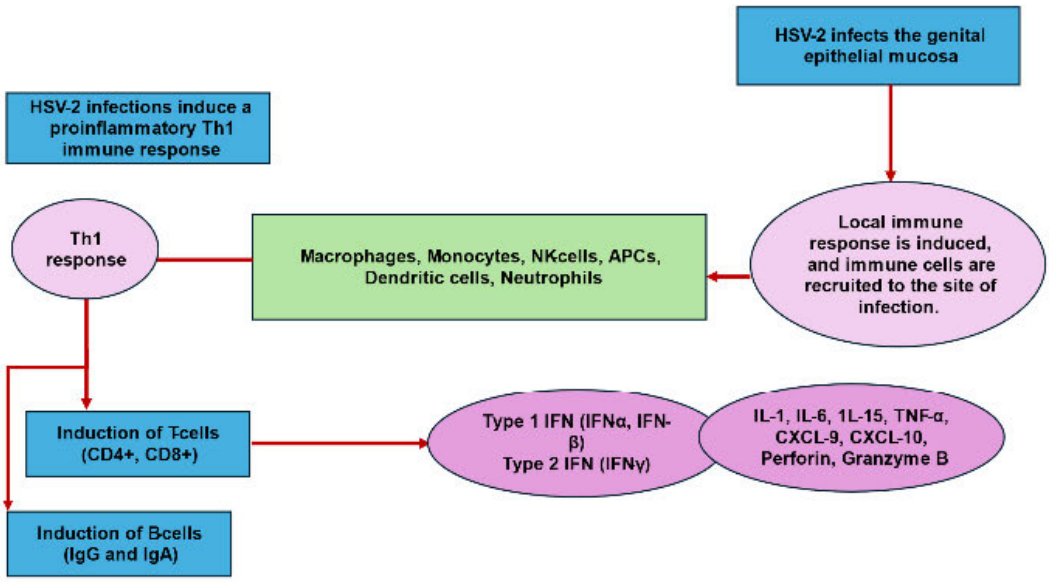

Figure 2. Illustration of the immune response to HSV-2 infection. Footnote: APCs: antigen-presenting cells; CXCL: chemokine (C-X-C motif) ligand; HSV-2: herpes simplex virus type II; IFN: interferon; IFN-α: interferon alpha; IFN-β: interferon beta; IFN-γ: interferon gamma; IL: interleukin; NK: natural killer; Th1: T-helper type 1 cells; TNF-α: tumor necrosis factor alpha.

Firstly, innate immunity is activated and is essential for early control of the virus and for initiating the adaptive arm of immunity [68,69]. Following infection, toll-like receptors (TLRs) present on infected genital epithelial cells detect and bind to HSV-2-specific

pathogen-associated molecular patterns (PAMPs) [69]. Several immune cells participate in the innate response to HSV-2 infection. These include antigen-presenting cells (APCs), plasmacytoid dendritic cells (pDC), neutrophils, macrophages, monocytes, and natural killer (NK) cells [39]. Once activated, TLRs stimulate the synthesis of type I interferons (IFN-α and IFN-β), which inhibit translation and promote the degradation of HSV-2 mRNA. TLRs also stimulate the production of proinflammatory cytokines (IL-1, IL-6, and TNF-α). Simultaneously, type I IFNs stimulate the maturation of dendritic cells (DC) and IL-15 synthesis, which supports NK cell production and survival. NK cells secrete IFN-γ, a type II interferon, perforin and granzyme B, which subsequently stimulate apoptosis of HSV-2-infected cells. IFN-γ further enhances the anti-HSV-2 response through the activation of the inducible nitric oxide synthase gene (iNOS gene) [69].

Secondly, both cell-mediated and humoral components of adaptive immunity are essential for viral clearance. Cell-mediated immunity is initiated by an influx and activation of Th1 $CD4^+$ T cells at the infected genital site. Th1 $CD4^+$ cells are activated when they bind to MHC class II receptors present on local APCs, such as pDCs. Following activation, $CD4^+$ T cells secrete IFN-γ, which stimulates genital epithelial cells to release the chemokines, CXCL9 and CXCL10, thereby creating a chemokine gradient. Subsequently, cytotoxic CD8+ T cells are attracted to the infected genital mucosa, and cause epithelial cells and APCs to release nitric oxide (NO) [69]. $CD8^+$ T cells also secrete IFN-γ, which further promotes apoptosis of HSV-2-infected cells via perforin- and fas-mediated cytolytic mechanisms [70].

During the humoral-mediated immune response, activated B-cells release antibodies (IgG and IgA). However, how antibody-mediated responses contribute to HSV-2 immunity is debatable because viral glycoproteins released by HSV-2 evade antibody-mediated protection. Lastly, although Treg cells occur at the infected site, their precise role in HSV-2 clearance is yet to be clearly defined [68,69].

7. Impact of STHs on HSV-2 and Other Viral Co-Infections

As highlighted earlier, STHs induce systemic immunomodulatory responses that have downstream effects on bacterial, protozoan, and viral co-infections. In the context of STH-viral co-infections, studies have examined the effects of STHs on enteric, respiratory, hepatotropic, and sexually transmitted viruses (reviewed recently by [67,71]). Despite the significant burden and geographic overlap of STHs and HSV-2, there are no epidemiological and immunological studies on human STH-HSV-2 co-infections in SSA. Therefore, the true impact of STH-HSV-2 co-infections remains unclear. In this section, we have focused on STH-viral co-infections. Drawing from recent murine and human studies, where available, we provide key examples of the influence of STHs on viral co-infections, emphasizing the existing research gaps in relation to human STH-HSV-2 co-infections.

Co-infection with STHs may lead to beneficial or detrimental outcomes for viral infections. Moreover, STH and viral interactions may occur in a bidirectional manner, subsequently affecting both pathogens [67]. To date, our understanding of how STHs impact viral co-infections is shaped primarily by studying animal models, which provide valuable mechanistic insight into STH-viral co-infections.

Animal models of STHs, such as *Nippostrongylus brasiliensis*, *Heligmosomoides polygyrus*, *Ascaris suum*, *Trichinella spiralis*, and *Trichuris muris*, are widely used because of their close similarities in lifecycle to their human counterparts [72].

In the case of STH-enteric viral co-infections, STHs impair host immune responses to enteric viruses. For example, acute infection with *Heligmosomoides polygyrus* and *Trichinella spiralis* exacerbated enteric murine norovirus (MNV) in co-infected mice [73]. This impaired antiviral response was independent of changes in the intestinal microbiota, dependent on STAT6-induced activation of alternative macrophages (AAM), and was partially restored following neutralization of Ym1, a molecule of AAM [73]. Similar results were observed in mice co-infected with *Heligmosomoides polygyrus* and West Nile virus (WNV). This phenotype was associated with enhanced WNV pathology, increased gut dysmotility and permeability, compromised $CD8^+$ T cell responses, and higher mortality. Poorer

outcomes in co-infected mice were mediated by STH-induced Th2-specific tuft cell and IL25/IL4 signaling pathways [74]. Co-infection with *Heligmosomoides polygyrus* enhanced the replication and shedding of murine astrovirus [75].

STH co-infection has also been shown to impair host immune responses to some respiratory viruses. As an example, poorer outcomes were observed in mice co-infected with *Ascaris suum* and vaccinia virus; this enhanced pathology was associated with the ablation of $CD8^+$ T cells, significantly reduced IFN-γ-secreting $CD4^+$ T cells, higher viral loads, and increased mortality. Moreover, co-infected mice had markedly fewer *Ascaris* lung-stage larvae, suggesting a two-way interaction between *Ascaris suum* and the vaccinia virus [76]. In support of these findings, *Ascaris*-infected pigs were more susceptible to respiratory viral infection [77]. In a human cohort of Colombian infants, *Ascaris*-specific IgE levels and TLR-4 gene polymorphisms were associated with more severe respiratory syncytial virus (RSV) bronchiolitis [78].

Interactions between herpesvirus and STHs have also been studied. In one study, acute infection with *Heligmosomoides polygyrus* or *Schistosoma mansoni eggs*, a trematode helminth, mediated the reactivation of human Kaposi's sarcoma-associated herpesvirus in vitro [79]. Consistent with these findings, in vivo co-infection with *Heligmosomoides polygyrus* or *Schistosoma mansoni eggs* reactivated murine gammaherpesvirus (MHV-68) infection from latency. MHV-68 reactivation required a "two-signal" mechanism involving IL-4-induced STAT6 activation and IFN-γ-blockage [79].

In some instances, STHs are beneficial to the outcomes of viral co-infections, particularly enhancing protection against respiratory viruses. *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* enhanced the host immune response to murine gammaherpesvirus 4 (MHV-4) respiratory infection through an IL-15-driven expansion of virtual memory $CD8^+$ T cells [80]. In another study, co-infection with *Heligmosomoides polygyrus* conferred antiviral protection against pulmonary RSV; this STH-induced protection was associated with reduced pulmonary inflammation and pathology in co-infected mice, and was driven by microbiota-dependent production of type 1 IFN [81]. *Trichinella spiralis* also enhanced protection against influenza virus by reducing virus-associated lung inflammation in co-infected mice, without altering viral replication or clearance [82]. More recently, studies have focused on the effects of STHs on the novel SARS-CoV-2 virus and its associated disease COVID-19. In some cases, SARS-CoV-2/COVID-19 are associated with exaggerated proinflammatory Th1 responses that may lead to life-threatening severe acute respiratory failure and systemic inflammatory syndrome. It has been hypothesized that STH-induced anti-inflammatory and immunosuppressive Th2 responses may downregulate SARS-CoV-2/COVID-19-associated Th1 responses, thus mitigating severe SARS-CoV-2 and COVID-19 outcomes in STH-endemic regions [83]. Moreover, STH-induced immunomodulation may reduce the expression of ACE-2, a critical receptor for SARS-CoV-2 entry into cells, thereby potentially impairing viral entry and replication [83]. In support of these hypotheses, helminth co-infection (*Hymenolepis nana, Schistosoma mansoni,* and *Trichuris trichiura*) mitigated COVID-19 severity in patients from Africa [84].

The distribution and prevalence of STHs and sexually transmitted viral infections overlap geographically. For example, the high prevalence of STHs in HIV-infected individuals [85–87] and HPV-infected women [6] has been reported. In addition, previous studies have demonstrated that STHs regulate host immune mechanisms related to sexually transmitted viral co-infections [6,8,85,88–90]. Previous studies have demonstrated a link between STHs and HIV co-infections, including lower $CD4^+$ counts [88], dysregulated immune cells, and higher HIV viral loads [85]. In the case of HPV, Gravitt et al. [6] reported that HPV infection was more prevalent in older women, aged 30–45 years, who were co-infected with STHs. Notably, although STHs neither enter nor reside in the vaginal tract, the authors detected IL-4, a Th2 cytokine, in the cervical fluids collected from STH-infected women. In addition, they reported that IL-4 levels were positively associated with other cytokines typically produced during antihelminth immune responses (IL-5, IL-8, IL-10, IL-21, IL-25, and IL-31), suggesting that higher HPV prevalence among STH-infected women may

be driven by STH-induced immunomodulation, which may impair effective host anti-HPV control [6]. Similarly, in other human studies, hookworm infections were correlated with a higher risk of HPV infection [7], higher HPV viral loads, and distinct mixed Type 1/Type 2 immune profiles in the vaginal tracts of women with HPV and hookworm co-infections [8]. Conversely, human cervical cells infected with *Nippostrongylus brasiliensis* in vitro, demonstrated decreased uptake of HPV16 pseudovirions, reduced cell migration, and lower expression levels of vimentin and N-cadherin, which are key epithelial-to-mesenchymal transition (EMT) markers used to evaluate cancer progression [91]. In addition, in vivo infection of mice with *Nippostrongylus brasiliensis* was associated with reduced vimentin expression in the vaginal tract, thereby further demonstrating the parasite's potential to impair cervical cancer progression [91]. When taken together, these co-infection studies demonstrate both favorable and unfavorable effects of STHs on HPV and/or cervical cancer [6,91], however, they are insufficient to conclusively define the role of STHs in HPV co-infection and cervical cancer. Therefore, further studies on the influence of STHs on female genital tract-specific immunity to HPV and the development of cervical cancer are needed.

In summary, the above studies highlight important features of STH-viral co-infections: (i) interactions may occur in a bidirectional manner with potential consequences for both STHs and viruses; and (ii) STHs may have favorable or unfavorable effects on viral co-infections; whether co-infection outcomes are beneficial or detrimental to the host depends on one or more key factors, including the type of STH, the stage of STH lifecycle, tissue tropism, the type of viral infection, and the timing and site/s of infection [67,71]. Considering these factors, it is likely that interactions between STHs and HSV-2 are complex because various factors may contribute to their interactions.

However, there is a scarcity of epidemiological and immunological studies on the impact of STH-induced Th2 immune responses on host immunity to HSV-2 infection, and currently only data from a single animal study exists [89]. In their murine model, Chetty et al. [89] demonstrated that acute intestinal infection with *Nippostrongylus brasiliensis*, which closely resembles human hookworms, stimulated a Th2 profile in the vaginal tract. This acute *Nippostrongylus brasiliensis* infection was associated with enhanced vaginal ulceration and pathology following HSV-2 co-infection. Although this enhanced HSV-2 pathology was independent of IL-4 receptor alpha (IL4ra), it was driven by IL-5 and associated with higher eosinophil counts and IL-33 levels in the vaginal tract [89]. The findings by Chetty et al. [89] suggest that the systemic STH-induced Th2 immune profile, detected in the murine vaginal tract, impaired host immunity to HSV-2, thereby exacerbating HSV-2 pathology. In support, Oh et al. [92] reported that dysbiosis of vaginal microbiota led to increased levels of IL-33 in the murine vagina, which subsequently impaired antiviral responses to HSV-2. Importantly, Chetty et al. [89] demonstrated that although STHs neither enter nor reside in the vaginal tract during their lifecycles, they were able to induce potent Th2 immune responses specific to the vaginal tract. These findings are consistent with previously observed clinical correlations between STHs and sexually transmitted viral infections in vaginal tissue [6,8]. The underlying mechanisms by which *Nippostrongylus brasiliensis* influences the vaginal tract need further investigation. Mechanisms such as the ability of STHs to induce host resistance and tolerance responses, alter the microbiome, as well as the immunomodulatory capabilities of their ESP, may likely be contributory factors. For example, it has been suggested that STH-derived ESP may promote immune cell trafficking and Th2 responses in uncolonized tissues, such as the vaginal tract [93]. We argue that the work by Chetty et al. [89] provides a foundation for future human studies, raising important questions regarding (a) the mechanisms by which STHs elicit such changes in the non-colonized vaginal tract, and (b) the implications for anti-HSV-2 immunity, HSV-2 progression and pathology in STH-HSV-2 co-infected individuals. Given that STHs stimulate Th2-biased immune responses, whereas protection from HSV-2 requires a Th1 response, we hypothesize that STHs may compromise host immunity to HSV-2 during co-infection (Figure 3). This could enhance susceptibility to HSV-2, promote viral

persistence and pathology, and impair responses to HSV-2 treatment. Likewise, STH-HSV-2 co-infections could have significant consequences for sexual and reproductive health, such as increased risk of acquiring other sexually transmitted infections, infertility, and cancer progression [94]. In such scenarios, poorly resourced healthcare systems in SSA, in terms of diagnosis, treatment, and long-term management, would be negatively impacted. In addition, no studies have examined the effects of HSV-2 co-infection on STH lifecycle, such as larval migration, adult worm establishment, persistence in the intestine, and anti-STH immunity. These are important research gaps that warrant further investigation to understand the association between STHs and HSV-2 and their impact on overall health.

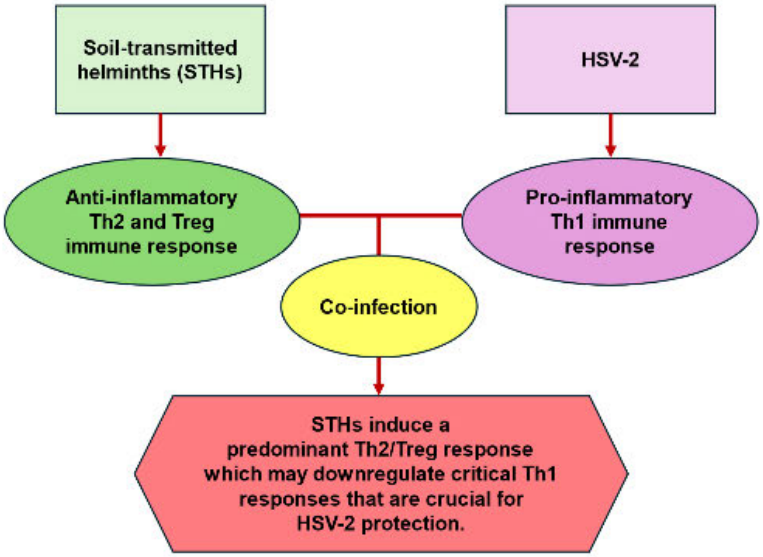

Figure 3. Illustration of potential immune response during STH-HSV-2 co-infection. Footnote: HSV-2: herpes simplex virus type II; STHs: soil-transmitted helminths; Th1: T-helper type 1 cells; Th2: T-helper type 2 cells; Treg: regulatory T cells.

8. STHs Eradication and HSV-2 Management Experiences in Africa

The WHO-recommended HSV-2 treatment regime includes (i) primary HSV-2 infection, (ii) recurrent HSV-2 infection, and (iii) frequent and/or severe recurrent HSV-2 infection. The mainstay HSV-2 antivirals include acyclovir and valacyclovir [95], which inhibit nucleoside analog-polymerase and pyrophosphate analog-polymerase and act against all types of herpesviruses [96]. Without an effective vaccine for HSV-2, these recommended antivirals act by reducing the number of recurrent HSV-2 infections but cannot eliminate HSV-2 shedding. Furthermore, drug resistance to antivirals due to chronic use and among immunocompromised individuals has been reported [40].

Treatment recommendations by the WHO for STH-endemic regions include the use of broad-spectrum anthelmintics. Mebendazole (500 mg) and albendazole (400 mg) are administered orally as single doses to treat STHs [3]. These benzimidazole agents bind to parasite β-tubulin and inhibit parasitic microtubule polymerization, with subsequent death of worms within several days [12]. In regions where STHs and schistosomiasis are co-endemic, the WHO recommends that praziquantel (for schistosomiasis) and mebendazole/albendazole are safely administered together. This is currently being done, particularly in SSA, where most of these infections occur [97].

Anthelmintics are affordable, effective in treating infections, safe, and easy to administer at regular intervals [12]. However, STH PC efforts are hindered by frequent re-infections

that occur because of the high numbers of STH eggs and larvae in the environment [33]. In addition, there are growing concerns about drug-resistant helminths, which may further reduce the efficacy of anthelmintics [98–100]. As an alternative to anthelmintics, anti-STH vaccines are considered effective strategies to combat STHs in the long term. Despite several efforts in anti-STH vaccine development over the last decade, there are currently no approved human anti-STH vaccines [reviewed in [101,102]]. Moreover, the development of effective anti-STH vaccines is thwarted by several challenges. These include the complex biology and lifecycles of STHs, multiple stages, and stage-specific antigen expression within the same host, and the immunomodulatory properties of STHs [101,102].

Due to the significant burden of HIV/AIDS in SSA, research into the effect of anthelmintic treatment on host immunity to sexually transmitted viral infections has primarily focused on HIV. For example, deworming of STH-HIV co-infected individuals was shown to decrease HIV viral loads and improve $CD4^+$ cell counts [87,103,104]. However, data on STH co-infection with other viruses, particularly HSV-2, are lacking. For STH-HSV-2 co-infections, there are currently no general guidelines for therapeutic interventions other than treating each infection following diagnosis [67]. To date, the effect of anthelmintic treatment on HSV-2 co-infection has not been investigated. Likewise, the effect of HSV-2 treatment on STH co-infection has yet to be investigated. However, the interactions between STHs and HSV-2 may need to be considered for both anti-HSV-2 treatment and deworming programs. As previously mentioned, STH co-infections can alter the immune response in a manner that supports the onset and progression of concurrent HSV-2 infections [89]. Thus, it follows that treatment of concurrent HSV-2 might be more effective if the STH infection has been treated prior to, or in parallel, if there are no concerns about adverse drug interactions. Therefore, long-term studies are needed to determine the safety and efficacy of anthelmintic treatment to control STHs in HSV-2 co-infected individuals. Moreover, as there is currently no evidence, targeted studies investigating the impact of therapeutic interventions in STH-HSV-2 co-infections are needed to develop consensus guidelines for the treatment of co-infections. In SSA, integrated treatment and management approaches that actively address STH-HSV-2 co-infections may help to effectively mitigate the burden of STHs and HSV-2 and improve overall sexual and reproductive health.

9. Research Implications and Future Directions

HSV-2 prevalence is higher in SSA than in other regions of the world [35]. However, despite its significant disease burden, there are no effective vaccines to curb HSV-2 transmission. We argue that there is a need for ongoing efforts to identify effective preventive and curative strategies for HSV-2 infection.

The extent and impact of STH-HSV-2 co-infections in SSA is understudied. There are several outstanding research questions regarding whether STHs have beneficial, harmful, or no effects on anti-HSV-2 immunity and HSV-2 pathology. Moreover, whether HSV-2 co-infection has an impact on the STH lifecycle, such as larval migration, adult worm establishment, persistence in the intestine, and anti-STH immunity, has yet to be determined. Although host immune responses to single STH and HSV-2 infections have been extensively examined, human studies that demonstrate whether STH-induced immunity modulates HSV-2-specific immune responses are lacking. It is plausible that the opposing Th1 and Th2 immune responses elicited in HSV-2 and STH infections, respectively, may influence the outcome of HSV-2 infection. Therefore, there is a significant need for studies that examine the burden of STHs and HSV-2 co-infections in SSA. In addition, we assert that multifaceted studies should be undertaken to explore the (a) effects of acute/chronic STH infection on HSV-2 co-infection, (b) identification of STH/HSV-2-specific biomarkers that may serve as diagnostic and/or prognostic markers of infection, (c) impact of STH co-infections on HSV-2 pathology in the reproductive tract, and the implications thereof for susceptibility to HSV-2, and (d) treatment outcomes in STH-HSV-2 co-infections. Focused research on STH-HSV-2 co-infections could provide valuable insights into the mechanistic and immunological interactions between these pathogens. A better understanding of these interactions could

inform policymaking and practices in co-endemic regions. For example, screening for HSV-2 in STH-endemic regions might improve patient outcomes by managing co-infections that could exacerbate HSV-2. In addition, the integration of deworming programs with HSV-2 interventions in co-endemic regions could reduce overall disease burden and transmission. Moreover, the investigation of the immune pathways affected by STH-HSV-2 co-infections could help identify new therapeutic targets and guide the development of prevention and control interventions that specifically address co-infected populations. With this view, we assert that integrated approaches that include (1) early diagnosis of STH and HSV-2 single and co-infections, (2) mass deworming programs, (3) ongoing surveillance, (4) health education programs for vulnerable communities, (5) provision of proper sanitation and clean water, (6) development of sensitive diagnostic tools, (7) allocation of resources for multifaceted research, (8) identification of new therapeutic agents, (9) the development of prophylactic vaccines for STH and HSV-2, and (10) the development of consensus guidelines for the treatment of co-infections, are required.

10. Conclusions

Host immunity to HSV-2 requires a proinflammatory Th1 immune response, which may be dampened by STHs, which typically elicit opposing Th2 immune responses. Furthermore, chronic STH infections induce immunomodulatory responses that can further reduce immune responses to HSV-2, thereby potentially promoting susceptibility to and enhanced pathology of HSV-2. Given that STHs are widespread in SSA, where HSV-2 is also highly prevalent, the immunomodulatory effects of STHs may influence host immunity to HSV-2. Insight into the interactions between these two important pathogens may improve diagnostic and treatment approaches, guide the design of potential vaccines, and inform policymaking and practices for the clinical detection and management of STHs and HSV-2 in SSA.

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2.3 Exploring microRNA-Mediated Immune Responses to Soil-Transmitted Helminth and Herpes Simplex Virus Type 2 Co-Infections

In recent years, our understanding of the regulatory roles of microRNAs (miRNAs) has significantly improved. MiRNAs are single-stranded, non-coding RNAs that regulate gene expression at a post-transcriptional level (Bernardo et al., 2012). It is now well understood that miRNAs regulate a wide range of biological processes, including host immunity. Moreover, dysregulated miRNA expression is observed in various infectious and non-infectious diseases (Gonzalez Plaza, 2016, Chandan et al., 2020). Similarly, dysregulated expression of miRNAs, both host- and pathogen-derived, and their immune-related functions, have been reported in soil-transmitted helminth (STH) and herpes simplex virus type 2 (HSV-2) infections (Entwistle and Wilson, 2017, Dass et al., 2023). However, little is known about how miRNAs regulate the immune response to STH/HSV-2 co-infection. Exploring the regulatory role of miRNAs in this scenario provides a novel lens through which to understand the immunological interactions between these pathogens. Recent advances in molecular biology, such as high throughput sequencing and access to bioinformatics tools, provide new avenues for studying these potentially complex immunological interactions at the level of miRNA expression. Insights into specific miRNA expression profiles and the genes they regulate during STH/HSV-2 co-infections can help identify novel biomarkers of infection and therapeutic strategies aimed at mitigating infection burdens in co-endemic regions. This is discussed in the review article entitled: **"Exploring microRNA-Mediated Immune Responses to Soil-Transmitted Helminth and Herpes Simplex Virus Type 2 Co-Infections"**, which was published in **MDPI Diseases**. This article discussed the potential influence that STH co-infection may have on host immunity to HSV-2. Specifically, the article explored how miRNAs contribute to both STH and HSV-2 infections and offered insights into how miRNAs may potentially mediate immune responses to STH/HSV-2 co-infections. Through this integrative approach, the article sought to offer a comprehensive framework for future research into STH/HSV-2 co-infections. The published PDF version, which is formatted according to the journal's requirements, is shown below.

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Review

Exploring microRNA-Mediated Immune Responses to Soil-Transmitted Helminth and Herpes Simplex Virus Type 2 Co-Infections

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Abstract: Over the last two decades, the field of microRNA (miRNA) research has grown significantly. MiRNAs are a class of short, single-stranded, non-coding RNAs that regulate gene expression post-transcriptionally. Thereby, miRNAs regulate various essential biological processes including immunity. Dysregulated miRNAs are associated with various infectious and non-infectious diseases. Recently co-infection with soil-transmitted helminths (STHs) and herpes simplex virus type 2 (HSV-2) has become a focus of study. Both pathogens can profoundly influence host immunity, particularly in under-resourced and co-endemic regions. It is well known that STHs induce immunomodulatory responses that have bystander effects on unrelated conditions. Typically, STHs induce T-helper 2 (Th2) and immunomodulatory responses, which may dampen the proinflammatory T-helper 1 (Th1) immune responses triggered by HSV-2. However, the extent to which STH co-infection influences the host immune response to HSV-2 is not well understood. Moreover, little is known about how miRNAs shape the immune response to STH/HSV-2 co-infection. In this article, we explore the potential influence that STH co-infection may have on host immunity to HSV-2. Because STH and HSV-2 infections are widespread and disproportionately affect vulnerable and impoverished countries, it is important to consider how STHs may impact HSV-2 immunity. Specifically, we explore how miRNAs contribute to both helminth and HSV-2 infections and discuss how miRNAs may mediate STH/HSV-2 co-infections. Insight into miRNA-mediated immune responses may further improve our understanding of the potential impact of STH/HSV-2 co-infections.

Keywords: microRNAs; soil-transmitted helminths; herpes simplex virus-2; co-infection; immunological interactions; host immunity; immunomodulation

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1. Introduction

MiRNAs are a class of small, single-stranded, non-coding RNA molecules known to regulate gene expression at a post-transcriptional level. By binding to their target mRNAs, miRNAs regulate a wide range of essential physiological functions, including immunity. Moreover, dysregulated miRNA expression is a hallmark of various human infections and

disease processes. In this way, miRNAs may serve as potential biomarkers in the diagnosis and prognosis of various infections and as novel therapeutic targets [1].

Soil-transmitted helminths (STHs) are among the most prevalent parasitic infections. Globally more than 1.5 billion humans are infected, particularly those living in under-resourced tropical and subtropical countries across sub-Saharan Africa, the Americas, China, and East Asia. Limited access to clean water, proper sanitation, and healthcare facilities exacerbates the risk and transmission of infection. The three major groups of STHs are roundworms (*Ascaris lumbricoides*), whipworms (*Trichuris trichiura*), and hookworms (*Necator americanus* and *Ancylostoma duodenale*) [2].

A significant epidemiological feature of under-resourced countries is their high burden of sexually transmitted viral infections including herpes simplex virus type II (HSV-2), which causes genital herpes [3]. In 2016, more than 491.5 million people (13.2%) worldwide, aged between 15 and 49 years, were reportedly infected with HSV-2; most infections were reported in the WHO African region [4].

It is well recognised that STHs stimulate dominant Th2 immune responses that down-regulate Th1 and Th17 immune responses. Moreover, they possess potent strategies to modulate host immunity to unrelated infections [5]. By employing the following strategies, STH modulate immunity: (i) they induce host resistance and tolerance responses, (ii) they secrete immunomodulatory products such as excretory-secretory products (ESP), and (iii) they interact with the intestinal microbiome [6]. HSV-2 infection, on the other hand, triggers a proinflammatory Th1 immune response [7].

The immunomodulatory capabilities of STHs have been shown influence the outcomes of concomitant infections, which occur commonly in under-resourced countries [8]. Moreover, STH co-infections have been shown to influence immune responses to sexually transmitted viruses, including human papillomavirus (HPV) [9,10], human immunodeficiency virus (HIV) [11–13], and HSV-2 [14]. Given the overlapping incidences of STH and HSV-2 infections in under-resourced countries [15], the likelihood of STH/HSV-2 co-infections is high, with potential implications for host immunity. Because STHs are associated with Th2 and immunomodulatory responses, their immunomodulatory effects may potentially dampen the Th1 responses triggered by HSV-2. Surprisingly, very little is known about the epidemiology of STH/HSV-2 co-infections or the immunological interactions between these pathogens. Moreover, how miRNAs may contribute to these potential immunological interactions is undetermined. This dearth of information highlights a significant gap in our understanding of the impact of STH/HSV-2 co-infections in under-resourced countries. Exploring the role of miRNAs in immune responses to STH and HSV-2 single and co-infections could provide valuable insight into various key aspects of host immunity.

In this article, we consider how STH co-infection may suppress essential anti-HSV-2 immune responses, thereby potentially exacerbating HSV-2 severity. Specifically, the potential role of miRNAs in shaping immune responses to STH/HSV-2 co-infection is explored. We highlight the need for studies that focus on STH/HSV-2 co-infections and discuss future work needed to elucidate the role of miRNAs in immune responses elicited by these pathogens during co-infection. Such work may contribute to alleviating the burden of STHs and HSV-2 infections in under-resourced countries.

2. Overview of Soil-Transmitted Helminth Infections and Host Immune Responses

Helminths are widely distributed parasitic worms that compose of two major phyla: the Nematoda (roundworms) and Platyhelminths (flatworms). STHs belong to Nematoda,

while Platyhelminths are divided into two classes, cestodes (tapeworms) and trematodes (flukes) [16–19]. In this article, we have focussed on STHs.

Infections with STHs, also known as intestinal helminths, are among the most prevalent helminth infections, worldwide [2]. The four most prevalent STH species are *Ascaris lumbricoides* (roundworms), *Trichuris trichiura* (whipworm), and *Ancylostoma duodenale* and *Necator americanus* (hookworms). These infections disproportionately affect vulnerable and impoverished countries, where access to clean water, proper sanitation, and healthcare facilities is limited [2,20]. While most infections are mild and asymptomatic, STHs are associated with significant morbidity due to the chronicity of infections. Severe infections are associated with malnutrition, anaemia, and impaired physical and mental growth [19–21].

STHs are primarily transmitted via faecal contamination of the environment and food sources in areas that lack clean water and sanitation facilities. Infection occurs following ingestion of embryonated eggs from contaminated water or food sources (*Ascaris lumbricoides* and *Trichuris trichiura)* or penetration of the skin by infective larvae (*Necator americanus* and *Ancylostoma duodenale)*. STHs have complex lifecycles comprising various developmental stages, including migration of larvae through host tissues, maturation into adult worms, reproduction, and faecal excretion of eggs into the environment via an infected host [19–21].

STHs have coevolved with their hosts over many centuries, consequently developing several mechanisms to undermine host immunity. Regardless of their routes of infection and lifecycles, STHs typically elicit a dominant Th2 immune response in their hosts. STH-induced Th2 immune responses control inflammation, promote repair of damaged tissue, and support host tolerance. In this way, STHs establish long-standing and insidious infections with low host mortality but high morbidity [6,22,23]. A STH-driven Th2 phenotype is initiated by alarmin cytokines interleukin (IL)-25 and IL-33 and thymic stromal lymphopoietin (TSLP). Several co-ordinated immunological and physiological responses follow, driven by the release of Th2 cytokines (IL-4, IL-5, IL-9, and IL-13). There is an amplification of alternatively activated macrophages, increased levels of parasite-specific immunoglobulin (Ig), non-specific IgE, eosinophils, basophils, and degranulation of mast cells. Smooth muscle contraction and goblet cell hyperplasia contribute to the "weep and sweep" mechanism required for STH expulsion. STH-induced Th2 immune responses typically downregulate Th1 and Th17 inflammatory cytokines. Additionally, in chronic STH infections, anti-inflammatory cytokines [IL-10 and transforming growth factor-β (TGF-β)] and regulatory cell populations (forkhead box P3 (FoxP3)-expressing regulatory T cells and B cells) are produced, thus creating an environment that favours parasite survival [6,23–25]. STH infections rarely occur in isolation, particularly in under-resourced regions, and co-infections with bacterial, protozoan, and viral pathogens occur commonly. Consequently, STH-induced immunomodulation has bystander effects on host immune responses to a range of pathogens, as well as allergies, autoimmune diseases, and inflammatory conditions, with subsequent beneficial or detrimental outcomes for co-infected hosts [5,6,8].

3. Overview of HSV-2 Infection and Host Immune Responses

HSV-2 is a double-stranded human DNA virus and is a member of the Herpesviridae family and alpha-herpesvirus subfamily [26].

HSV-2 infects keratinocytes that line genital mucosa, leading to genital lesions [27]. Infections are primarily self-limiting and asymptomatic but may cause life-threatening complications in newborns and immunocompromised individuals. HSV-2 causes lifelong infections due to its ability to establish latency in sensory neurons and ganglia [28]. Reactivation of genital lesions occurs commonly in symptomatic individuals and viral shedding

by asymptomatic and symptomatic individuals facilitating the spread of infection [7]. Importantly, HSV-2 can promote the acquisition of other sexually transmitted infections and is considered a key driver of HIV transmission [4,29]. Thus, HSV-2 represents a significant global public health concern.

The anti-HSV-2 immune response is complex, involving both the innate and adaptive components of immunity. Innate immunity is vital for the initial control of the virus and mediates an effective adaptive immune response. Following primary HSV-2 infection, the toll-like receptor (TLR) signalling pathway is stimulated and produces type 1 interferon (IFN), mainly IFNα and IFNβ. Type 1 IFNs activate innate cells such as natural killer cells and plasmacytoid dendritic cells and stimulate production of Th1 inflammatory cytokines IL-1, IL-6, and tumour necrosis factor-α (TNF-α). Natural killer cells also stimulate cytokine production and kill virally infected cells, and plasmacytoid dendritic cells produce type 1 IFN. Adaptive immunity against HSV-2 comprises humoral and cellular responses. In particular, $CD4^+$ and $CD8^+$ T cells are important for clearing infection through the production of IFNγ [30,31]. However, the virus has evolved strategies to evade host immunity and establishes latency in sensory neurons and ganglia, thereby causing lifelong infections [28]. Currently there are no vaccines for HSV-2. The mainstay drugs used to treat HSV-2 infection include acyclovir, valacyclovir, and famciclovir. However, these drugs are ineffective in eliminating viral shedding and are associated with side effects and limited bioavailability [32]. Drug resistance to antivirals has also been reported [33]. Therefore, new and more effective therapeutic approaches are needed.

4. Soil-Transmitted Helminth and HSV-2 Co-Infection: A Potential Role for miRNAs

Recently, the interaction between STH and HSV-2 has become a focus of study. In their landmark study, Chetty et al. (2021) demonstrated that acute infection with *Nippostrongylus brasiliensis*, a murine hookworm with a similar lifecycle to the human hookworms *Ancylostoma duodenale* and *Necator americanus*, systemically enhanced vaginal pathology in mice co-infected with HSV-2. Vaginal pathology was associated with an IL-5-driven Th2 response that resulted in accumulation of eosinophils and enhanced ulceration in the vaginal tracts of co-infected mice [14]. Their findings suggest that STH co-infection may potentially impair anti-HSV-2 immune responses and exacerbate HSV-2 pathology, thus negatively impacting female reproductive health. Given the geographical overlap of STH and HSV-2, it is plausible that co-infections occur in individuals living in co-endemic regions, with subsequent consequences for HSV-2 pathology. STH co-infection could influence the outcome of HSV-2 by inducing Th2 responses that modulate anti-HSV-2 Th1 responses. No epidemiological evidence has been reported; however, it remains possible that STH/HSV-2 co-infections may contribute to altered pathology, immune responses, or transmission dynamics, highlighting the need for focussed studies that investigate their clinical and public health implications.

While we postulate that STH/HSV-2 co-infection may exacerbate HSV-2 pathology, it is important to consider other key role players that may contribute to host immune responses and infection outcomes. In this regard, appropriate host immune responses to either pathogen requires the co-ordinated control of specific genes. Recently, the central role of miRNAs in regulating immune responses to various infectious and non-infectious diseases has been recognised [34–36]. In this way, miRNAs may regulate important aspects of host immunity to STH/HSV-2 co-infection.

5. MiRNA Biogenesis

MiRNAs are a class of small, non-coding, single-stranded RNAs, of approximately 22 nucleotides in length. They regulate the expression of their target mRNAs at a post-transcriptional level [1]. The lin-4 and let-7 miRNAs were first described in 1993 in *Caenorhabditis elegans* and were shown to be essential for normal development of the nematode [37]. By regulating the expression of their target mRNAs, miRNAs participate in various essential biological processes including normal development, differentiation, metabolism, and immunity. Moreover, miRNAs have been identified in a diverse range of pathogens, including STHs [38–43] and HSV-2 [44–48]. Dysregulated miRNA expression is a hallmark of various infections and disease processes. Thus, miRNAs may serve as key biomarkers in the diagnosis and prognosis of various conditions and may potentially serve as novel therapeutic targets [1].

The biogenesis of miRNAs has been comprehensively reviewed elsewhere [1,49]. Briefly, miRNA biogenesis is a complex process involving several sequential steps that result in mature, functional miRNAs. Biogenesis may occur via one of two pathways: the canonical and non-canonical pathways. The canonical pathway forms the main pathway by which miRNAs are synthesized (Figure 1). It begins in the nucleus where miRNA genes are transcribed by RNA polymerase II to form an initial transcript, called the primary miRNA (pri-miRNA). A pri-miRNA, which is typically several hundred nucleotides long, forms a hairpin structure and is usually capped and polyadenylated. The pri-miRNA is then recognized and processed in the nucleus by the Drosha-DGCR8 (DiGeorge syndrome critical region 8) complex. Here, Drosha cleaves the pri-miRNA near the base of the hairpin, forming a smaller hairpin structure called the precursor miRNA (pre-miRNA). The pre-miRNA is then exported from the nucleus to the cytoplasm via the exportin-5/Ran-GTP complex. Further processing of the pre-miRNA occurs in the cytoplasm, where the enzyme Dicer cleaves the pre-miRNA near its hairpin loop to form a double-stranded miRNA/miRNA duplex [1].

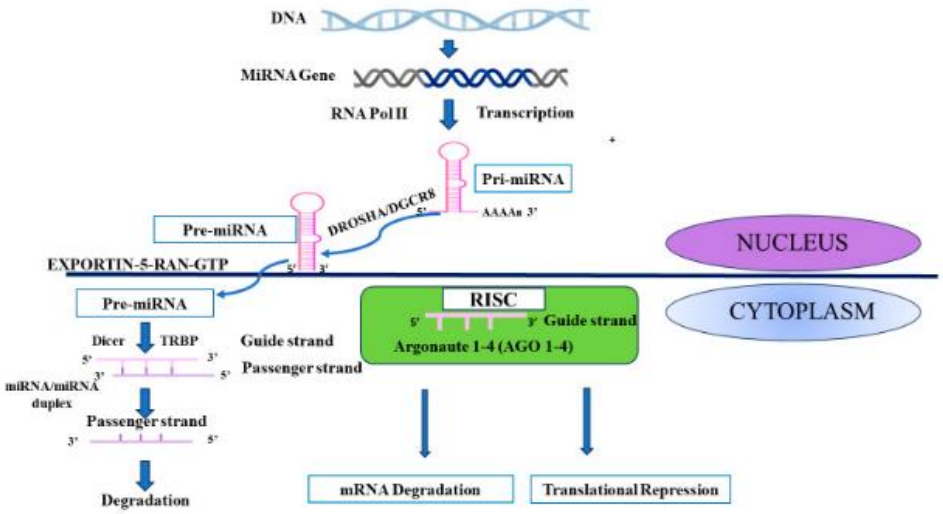

Figure 1. Schematic diagram of the biogenesis of miRNAs via the canonical pathway from the transcription of pri-miRNAs to the translational repression/mRNA degradation. Sequential steps are described in text.

The non-canonical and canonical pathways differ by one or more steps. Specifically, the enzymes Drosha and DGCR8 are essential for miRNA biogenesis to occur via the canonical pathway. Therefore, in their absence, miRNA biogenesis follows the non-canonical pathway. Several non-canonical miRNA biogenesis pathways have been described [49]. Briefly, non-canonical miRNA biogenesis comprises Drosha/DGCR8-independent and Dicer-independent pathways. In the Drosha/DGCR8-independent pathway, pre-miRNAs resemble Dicer substrates. Examples of Drosha/DGCR8-independent pathway include the following: (i) mirtrons, which are formed from mRNA introns during splicing and (ii) 7-methylguanosine (m7G)-capped pre-miRNAs, which are exported directly to the cytoplasm via exportin 1, without Drosha cleavage. In contrast, Dicer-independent miRNAs are processed by Drosha from endogenous short hairpin RNA (shRNA) transcripts. These pre-miRNAs need AGO2 to mature in the cytoplasm, as they are much shorter than Dicer substrates. Pre-miRNAs are then loaded onto AGO2, followed by AGO2-dependent slicing of the 3p strand, and finally, 3′-5′ trimming of the 5p strand to complete maturation [49].

Once formed, the double-stranded miRNA/miRNA duplex comprises a mature miRNA strand (guide strand) and a complementary miRNA* strand (passenger strand). The mature miRNA or guide strand is then preferentially selected and loaded onto the RNA-induced silencing complex (RISC) and attaches to one of four Argonaute proteins (Argonaute-1, Argonaute-2, Argonaute-3, and Argonaute-4) [50]. The miRNA guides RISC to its target messenger RNA (mRNA), and the miRNA within the RISC binds to target mRNAs through complementary base pairing. This binding can lead to translational repression, mRNA degradation, or a combination of both, depending on the degree of complementarity and other factors [1]. In mammals, mRNA degradation is dependent on Argonaute-2, which possesses enzymatic slicer activity. Alternatively, Argonaute-1, Argonaute-3, and Argonaute-4 inhibit mRNA translation without mRNA degradation. The seed sequence, which is made up of the first 6-8 bases of the miRNA molecule, determine the specificity of miRNA binding with their complementary mRNA sequences [50]. By binding to their target mRNAs, miRNAs regulate gene expression required for various essential biological processes such as normal development, differentiation, metabolism, and immunity. Importantly, miRNAs are pleiotropic; that is, a single miRNA may target numerous mRNAs. Furthermore, a single mRNA may be regulated by several miRNAs. In this way, miRNAs and their target mRNAs form complex regulatory networks that must be carefully deciphered when determining the specific roles and targets of miRNAs [1].

6. Innate and Adaptive Immune Cells and Pathways Mediated by miRNAs

As key players in host immunity, miRNAs regulate the survival and functioning of host innate and adaptive immune cells, as well as the signalling pathways involved in host immune responses to infections [35,51]. In innate immunity, miRNAs form regulatory networks and mediate the functions of innate immune cells including granulocytes [52], macrophages [53], dendritic cells [54], and natural killer cells [55]. In adaptive immunity, miRNAs regulate the development, differentiation, selection, and function of T-cells [56] and B-cells [57]. Several miRNAs, such as miR-10a, miR-17-92 cluster, miR-21, miR-29, miR-145, miR-146a, miR-155, miR-181, and miR-223, play key roles in the maturation, proliferation, differentiation, and activation of various innate and adaptive immune cells [comprehensively reviewed by [35,51]].

7. Immune Cells and Pathways Mediated by miRNAs in Soil-Transmitted Helminth Infections

Animal models of STHs are widely studied primarily because of their similarities in life cycle to their human counterparts [58]. Animal models such as *Nippostrongylus brasiliensis*,

Heligmosomoides polygyrus, *Ascaris suum*, *Trichinella spiralis*, and *Trichuris muris* have been useful in elucidating important aspects of host–STH interactions and host immunity. In this section of the article, we will provide an overview of the host- and helminth-derived miRNAs reported in STH infections using animal models, with an emphasis on their roles in modulating immune responses. While we have primarily focussed on STH infections, in some instances, we have also reported on miRNAs isolated from other helminth species known to cause human infections.

7.1. Immune Cells and Pathways Mediated by Host-Derived miRNAs in Soil-Transmitted Helminth Infections

The role of host miRNAs in regulating crucial components of anti-STH immunity has been recently described [50]. For example, miRNAs regulate the biology and/or function of intestinal epithelial cells, innate lymphoid cells (ILCs), Th2 cells, and regulatory T (Treg) cells during anti-STH immunity: (i) The intestinal epithelial barrier forms an important interface for host–helminth interaction by serving as a physical barrier, detecting parasites and mounting initial host immune responses. Epithelial cells also differentiate into secretory goblet cells which produce increased amounts of mucous during STH infection to facilitate parasite expulsion [50]. MiRNAs have been shown to support the integrity and function of intestinal epithelial cells during anti-STH immunity. Biton and colleagues (2011) reported that mice deficient of the enzyme Dicer were more susceptible to *Trichuris muris* infection than their wildtype counterparts. Dicer deletion compromised the epithelial barrier and led to decreased goblet cell production and reduced expression of resistin-like molecule beta (RELMβ), a Th2 antiparasitic cytokine. The authors also showed that inhibition of miR-375 prevented goblet cell differentiation and TSLP induction in response to IL-13 [59]. (ii) Innate lymphoid cells (ILC2) play essential roles in anti-STH immunity, mainly by producing Th2 cytokines IL-5, IL-9, and IL-13 [24]. MiR-155 regulates appropriate responses by ILC2. In mice infected with *Nippostrongylus brasiliensis*, miR-155 was upregulated in ILC2, while miR-155-deficient mice displayed impaired IL-33-driven ILC2 responses. In vitro, miR-155 promoted ILC2 survival by protecting ILC2 from apoptosis [60]. (iii) Similarly, Okoye and colleagues (2014) [61] demonstrated that miR-155 and miR-146a regulate Th2 cells during anti-STH immunity. Th2 cells drive Th2 responses by releasing key cytokines IL-4, IL-5, IL-9, and IL-13 [24]. Several miRNAs regulate the normal development of Th2 cells, including miR-19a, miR-24, and miR-27 [62]. In the context of anti-STH immunity, T-cell-intrinsic miR-155 promoted Th2 immunity to *Heligmosomoides polygyrus*, by decreasing Sphingosine-1-phosphate-receptor 1 gene (*S1pr1*) expression, which supports the egress of lymphocytes from lymphoid tissue. In contrast, miR-146a inhibited Th2 immunity. T-cell-intrinsic miR-146a prevented overt Th1/Th17 skewing by regulating Th1- and Th17-cell differentiation. The deletion of miR-146a in T-cells was associated with a mixed Th1/Th2/Th17 response and enhanced susceptibility to *Trichuris muris* or *Heligmosomoides polygyrus* infection [61]. More recently, Entwistle and colleagues used a murine model of *Heligmosomoides polygyrus* to study the miRNAome of the intestine during acute reactivation of Th2 immune responses. The authors identified several upregulated host miRNAs, including miR-99a-5p, miR-155-5p, and miR-148a-3p, during active worm expulsion. In addition, miR-99a-5p and miR-155-5p levels were increased in the lungs of mice following allergen-induced acute exacerbation. While pharmacological inhibition of these miRNAs, either individually or in combination, did not compromise antihelminth immunity, the blocking of miR-99a-5p or miR-155-5p was associated with a significant reduction in allergen-induced exacerbation in the lungs [63]. (iv) STH infection induces the production of FoxP3-expressing Treg cells, which modulate host immunity via the production of anti-inflammatory cytokines, IL-10, and TGFβ. Thereby, Tregs reduce STH-mediated inflammation and pathology, creating a conducive environment for STH survival [24]. MiRNAs regulate important aspects of

Treg differentiation, function, and survival. MiRNAs such as miR-21, miR-31, miR-24, and miR-210 are known to regulate FoxP3 expression and Treg development [64,65]. In addition, FoxP3 activates miR-155 and miR-146a, by suppressing the suppressor of cytokine signalling-1 (SOCS1) and the signal transducer and activator of transcription 1 (Stat1) effector pathways, respectively [66,67]. In the context of antihelminth immunity, following infection with the trematode, *Schistosoma mansoni*, miR-182 expression was required for Tregs to suppress Th2-associated inflammation, through the regulation of transcription factor *Cmaf* [68]. More recently, it was reported that *Ascaris suum* infection modulated host-derived extracellular vesicle (EV) miRNA expression profiles; the differentially expressed miRNAs were associated with immune-related cells, including Tregs, B cells, and dendritic cells [69].

7.2. Immune Cells and Pathways Mediated by Helminth-Derived miRNAs in Soil-Transmitted Helminth Infections

The development of high-throughput technologies, such as next-generation sequencing and microarray profiling, have enabled the discovery of novel helminth-derived miRNAs. To date, miRNAs have been identified in at least 35 helminth species, including nematodes, trematodes, and cestodes of clinical and veterinary importance [70]. Comparative studies show that some miRNAs are conserved across diverse organisms, while others are helminth-specific and novel [71]. To date, helminth-specific miRNAs from various helminth species have been identified in infected hosts, including *Schistosoma japonicum* [72,73], *Schistosoma mansoni* [74], *Onchocerca volvulus* [75,76], *Loa loa* [77], *Onchocerca ochengi* [76,77], *Nippostrongylus brasiliensis* [43], *Heligmosomoides polygyrus* [38], *Necator americanus* [41], *Ascaris lumbricoides* and *Ascaris suum* [39], *Trichuris suis* [40], and *Trichuris muris* [42]. Studies have also shown that the expression patterns of helminth-derived miRNAs differ during developmental stages, between sexes, and within specific cells and tissues [78–81].

Extracellular vesicles (EVs) are recognized as crucial contributors to cell–cell communication during host–parasite communication and participate in immunomodulatory functions. EVs are membrane-bound bodies secreted by various parasites, including helminths, that contain a repertoire of proteins, lipids, and RNAs, including miRNAs [82]. EVs and their miRNA cargos have been characterized in nematodes, cestodes, and trematodes [reviewed by [82–84]. Recent reviews by others have highlighted the role of helminth-derived EVs and their miRNA cargos in modulating host immunity [82,85–87]. In support of this, several research groups have studied the immunomodulatory capabilities of helminth-derived EVs [43,88,89]. For example, a previous animal study reported that EVs secreted by the intestinal nematode, *Heligmosomoides polygyrus*, suppressed macrophage activation by downregulating Th1- and Th2-associated molecules and inhibited IL-33 alarmin receptor subunit ST2. Moreover, following vaccination with the helminth EVs, mice developed strong antibody responses, which conferred protection against infection [88]. In another study, *Nippostrongylus brasiliensis*-derived EVs, but not *Trichuris muris*-derived EVs, were shown to reduce gut inflammation in EV-treated mice. In mice treated with *Nippostrongylus brasiliensis*-EVs, reduced gut inflammation was associated with decreased levels of proinflammatory cytokines and increased levels of IL-10 [43]. It has been shown that *Ascaris suum* EVs inhibited tumour necrosis factor (TNF-α) levels in lipopolysaccharide-stimulated peripheral blood mononuclear cells (PBMCs), compared to nonvesicle fractions, which showed no inhibitory activity [89].

An important component of helminth EVs is miRNAs. Studies have demonstrated a role for EV-associated miRNAs in host–parasite interactions and their roles in host innate and adaptive immunity during helminth infection [38,90–95]. For example, Buck et al. (2014) demonstrated that synthetic mimics of *Heligmosomoides polygyrus* miRNAs not only shared sequence similarities to host miRNAs but were able to modulate cytokine

production in host mammalian epithelial cells. EV-derived miRNAs from the trematode, *Fasciola hepatica*, have been characterized and associated with potential immunomodulatory functions [90,91]. Similarly, during infection with *Fasciola hepatica*, fhe-miR-125b, a conserved miRNA, was incorporated into a host Argonaut protein (Ago-2) within macrophages and negatively regulated the production of inflammatory cytokines [95]. In other studies, EV-derived miRNAs with potential host immune targets, such as IL-13, IL-25, and IL-33, have been reported in *Ascaris suum* [92]. The in vitro uptake of *Schistosoma japonicum* EVs, containing miR-125b and bantam, led to increased macrophage proliferation and TNF-α production through the regulation of their respective targets *Pros1, Fam212b, and Clmp* [93]. In vitro, *Schistosoma mansoni* EVs containing miR-10 regulated MAP3K7 and NF-κB activity and led to impaired Th2 differentiation [94]. The same authors identified helminth-specific miRNAs within the Peyer's patches and mesenteric lymph nodes of *Schistosoma mansoni*-infected mice [94].

Taken together, these studies highlight molecular mechanisms by which helminths exploit host cells and their miRNA machinery, revealing an important aspect of cross-species communication used by helminths to modulate host immunity.

8. Immune Cells and Pathways Mediated by miRNAs in HSV-2 Infections

In this section of the article, we provide an overview of the HSV-2- and host-derived miRNAs reported in HSV-2 infections, with an emphasis on their roles in modulating immune responses.

8.1. Immune Cells and Pathways Mediated by HSV-2-Derived miRNAs in HSV-2 Infection

Similarly, many human DNA viruses encode and express unique viral miRNAs (vmiRNAs). Among these are alpha-herpesviruses [Herpes Simplex Virus type 1 (HSV-1) and HSV-2], beta-herpesviruses [human cytomegalovirus (HCMV)], and gamma-herpesviruses [Epstein Barr Virus (EBV) and human herpesvirus 8 (HHV-8)] [96]. In HSV-2, 18 pri-miRNAs and 24 mature miRNAs have been reported. HSV-1 and HSV-2 are closely related, and several miRNAs are conserved between the viruses, bearing more that 70% similarity between their seed sequences [46,47]. VmiRNAs participate in host–viral interactions by regulating host biological functions including cell proliferation, differentiation, apoptosis, and the cell cycle. Thereby, viruses establish infection and produce viral progeny. In addition, vmiRNAs have been reported to contribute to the establishment of latent infection either by regulating metabolic processes or immune responses of host cells [reviewed in [96–99]]. VmiRNAs can function as orthologs of host miRNAs, thereby regulating their expression. Moreover, vmiRNAs have developed mechanisms to avoid detection by the host immune system [32].

Interactions between HSV-2 vmiRNAs, host miRNAs, and/or target mRNAs can influence HSV-2 replication, immune evasion, latency, and pathology. A characteristic feature of HSV-2 is its ability to establish latent infection in its host. During latency, only the latency-associated transcript (LAT) is expressed. HSV-2 vmiRNAs located within the LAT include miR-H2, miR-H3, miR-H4, miR-H7, miR-H9, miR-H10, and miR-H24 [44,45,47,100]. Studies have shown that, to regulate latency and promote immune evasion, (i) HSV-2-miR-H2 targets *ICP0*, a gene involved in viral replication, lytic infection, and reactivation, and (ii) HSV-2-miR-H1, HSV-2-miR-H3, and HSV-2-miR-H4 target the lytic neurovirulence factor *ICP34.5* [44,45]. In contrast, HSV2-miR-H6, which is associated with HSV2-LAT, was shown to play a role in reactivation of the virus from latency [48]. Taken together, these findings suggest that HSV-2 LAT-encoded vmiRNAs contribute to viral latency and reactivation by regulating their viral targets.

8.2. Immune Cells and Pathways Mediated by Host-Derived miRNAs in HSV-2 Infection

In addition to HSV-2 vmiRNAs, host miRNAs (also known as cellular miRNAs) contribute to the establishment of initial infection, reactivation, and viral latency [reviewed in [96,98,99]]. As highlighted earlier, the TLR signalling pathway is initiated during primary HSV-2 infection and is crucial for effective innate and adaptive responses. Host miRNAs have been shown to regulate the TLR pathway during HSV-2 infection and contribute to HSV-2 pathology [101]. Following HSV-2 infection of guinea pigs, six miRNAs (miR-592, miR-1245b-5p, miR-150, miR-342-5p, miR-1245b-3p, and miR-124) were upregulated and correlated with the downregulation of key TLR-associated genes [101]. In another study, host miR-36 was shown to regulate infection of haemopoietic and endothelial cells with the closely related herpesviruses: Kaposi's sarcoma-associated herpesvirus (KSHV), EBV, and HSV-2 [102]. Host miRNAs have also been reported to suppress viral replication and promote latency of HSV-2. For example, host miR-138 targeted host mRNAs *OCT-1*, *FOXC1*, as well as HSV-2 *ICP0*, *UL19*, and *UL20* genes, demonstrating that HSV-2 exploits host neuron-specific miRNAs to suppress lytic genes and favour latency [100]. More recently, it was reported that HSV-2 infection of THP-1 macrophages led to the upregulation of immune-associated miRNAs (miR-374a, miR-29b, miR-195, and miR-181a) and the downregulation of their corresponding mRNA targets (IL-10, AKT1, P13K, and AKT2). Suppression of these mRNA targets subsequently led to their inability to inhibit autophagy. In addition, the authors showed that combinatorial miRNA exposure of the THP-1 macrophages prior to HSV-2 infection reduced viral replication and the release of new virions [103].

In summary, host- and pathogen-derived miRNAs are critical regulators of immune responses to helminth and HSV-2 infections, regulating the activation, differentiation, and functions of innate and adaptive cells and signalling pathways of immunity. Therefore, miRNAs contribute significantly to the complexity of host–pathogen interactions, and insight into their mechanisms are required to fully understand these host–pathogen interactions.

9. Soil-Transmitted Helminth Immunomodulation of Respiratory, Enteric, and Sexually Transmitted Viral Infections

Experimental and epidemiological studies have shown that STH-induced immunomodulation may influence immune responses to enteric [104,105], respiratory [106–108], and sexually transmitted viral infections [9–11,13,14,109,110] with varying outcomes. STH co-infection also modulates host immunity to major global pathogenic bacteria and protozoans, which is reviewed elsewhere [6,8]. Here, we provide a brief overview of STH-induced modulation of pathogenic viruses. In the context of STH and enteric viral co-infections, *Heligmosomoides polygyrus* promoted susceptibility to the West Nile virus (WNV) and associated with enhanced intestinal pathology, translocation of intestinal bacteria, and impaired CD8+ T cell responses [105]. Similarly, *Trichinella spiralis* promoted infection with an enteric murine norovirus and associated with alterations in intestinal microbiota and STAT-6-dependent activation of alternative macrophages [104].

In contrast, STH co-infections are beneficial to the outcomes of some respiratory viruses. For example, in mice co-infected with *Heligmosomoides polygyrus*, there was reduced lung disease and inflammation following respiratory syncytial virus [107]. *Trichinella spiralis* reduced influenza virus-induced lung inflammation and associated with lower levels of tumour necrosis factor (TNF) and reduced cellular recruitment in mice co-infected with influenza A virus [106]. More recently, co-infection with *Schistosoma mansoni* or *Trichuris trichiura* was associated with reduced COVID-19 severity in patients infected with SARS-CoV-2 [108].

STHs may also influence the outcomes of sexually transmitted viral infections. Individuals co-infected with STHs and HIV had lower $CD4^+$ counts [109], dysregulated immune cells and higher HIV viral loads [11], and altered Th1/Th17 cytokine gene expression [13]. Similarly, hookworm infections increased the risk of HPV infection [110] and correlated with higher HPV viral loads and distinct mixed Type 1/Type 2 vaginal cytokine profiles in women co-infected with hookworm and HPV [10]. Conversely, in vitro infection of cervical cells with *Nippostrongylus brasiliensis* led to reductions in the uptake of HPV16 pseudo-virions, cell migration, and expression of vimentin and N-cadherin [111]. Following infection with the parasite in vivo, there was reduced vimentin expression in the murine vaginal tract, suggesting the parasite's ability to impair cervical cancer progression [111]. As highlighted earlier, Chetty and colleagues (2021) demonstrated that acute infection of mice with *Nippostrongylus brasiliensis* correlated with enhanced vaginal ulceration following subsequent HSV-2 infection, suggesting that the parasite impairs anti-HSV-2 responses required to adequately control infection. Notably, the authors demonstrated that although the STH neither enters nor resides in the female genital tract, it induced a potent Th2 immune response specific to the female genital tract that altered vaginal immune responses to HSV-2 [14]. Although STH co-infections could be linked to reduced immunity to HSV-2 in endemic regions, there is a significant paucity of data on STH/HSV-2 co-infections. Further studies on HSV-2 pathology in STH-endemic regions are needed to fully elucidate the clinical and public health implications of STH/HSV-2 co-infections.

10. Potential Role of miRNAs in Mediating STH/HSV-2 Co-Infections

Looking ahead, the findings by Chetty and colleagues [14] raise several questions regarding the mechanisms by which STH induce a Th2 phenotype in the female genital tract. Furthermore, while there is no definitive evidence regarding STH/HSV-2 co-infections in humans, their findings are suggestive of significant interactions between STHs and HSV-2 and provide a basis for future work. Because STHs and HSV-2 disproportionately affect vulnerable and marginalized communities, it is important to understand how STHs may impact HSV-2 severity. It would be of interest to examine the miRNA expression profile during STH/HSV-2 co-infection and how miRNAs contribute to host immunity during co-infection. To our knowledge, no studies have explored the miRNA profiles during STH/HSV-2 co-infection. However, having described the role that miRNAs play as mediators of immunological responses during single STH and HSV-2 infections, it follows that miRNAs would contribute to the host immune response during STH/HSV-2 co-infection. Although speculative, we contend that miRNA regulation could potentially occur via different mechanisms:

(i) Modulation of host immune responses: As discussed earlier, STH and HSV-2 infections elicit opposing immune responses. HSV-2 requires a robust Th1 immune response for effective viral control [7]. Consequently, host-derived immune-related miRNAs, such as miR-138 [100], miR-36 [102], miR-374a, miR-29b, miR-195, and miR-181a [103], are dysregulated following HSV-2 infection. On the other hand, STHs typically induce Th2-mediated and immunomodulatory responses that suppress Th1-mediated responses [5]. Host-derived miRNAs have been shown to regulate important aspects of anti-STH immunity, including the biology/function of intestinal epithelial cells [e.g., miR-375] [59], ILCs [e.g., miR-155] [60], Th2 cells [e.g., miR-155, miR-146a, miR-19a, miR-24 and miR-27] [62], and Treg cells [e.g., miR-21, miR-31, miR-24, miR-210, miR-182] [64,65,68]. Therefore, in STH/HSV-2 co-infections, several miRNAs could mediate these opposing immune responses. For example, host-derived miRNAs, such as miR-146a and miR-155, are known to regulate inflammation and immune responses, following the recognition of pathogens by TLRs. Specifically, miR-146a regulates the NF-κB pathway by downregulating TRAF6

and IRAK1, which are key adaptors in the TLR signalling pathway. miR-146a also reduces the production of proinflammatory Th1 cytokines, such as TNF-α, IL-6, and IFN-γ [35]. Moreover, studies have shown that the coactivation of miR-146 and miR-155 is regulated by the NF-κB signalling pathway and may contribute to a negative feedback loop, protecting the host against an excessive TLR4 response [53,112,113]. The combined upregulation of miR-146a and miR-155, as previously reported in helminth infections [61,114], may dampen inflammation and reduce anti-HSV-2 responses. In addition, STH-derived EVs and miRNAs may suppress proinflammatory Th1 cytokines, which are critical for HSV-2 control, thereby promoting increased viral replication and persistence. Simultaneously, STH-derived EVs containing miRNAs could modulate key host innate immune signalling pathways, such as the TLR signalling or NF-κB activation to suppress inflammation. This immune suppression could indirectly promote HSV-2 replication and pathology.

(ii) Regulation of viral replication: HSV-2-derived miRNAs, such as HSV-2-miR-H1, HSV-2-miR-H2, HSV-2-miR-H3, and HSV-2-miR-H4, regulate important aspects of viral replication, latency, and reactivation [44,45]. These viral miRNAs might act synergistically with STH-mediated immunomodulation to promote chronic infection. Similarly, certain host-derived miRNAs, such as host miR-138 target viral genes (e.g., HSV-2 *ICP0*, *UL19*, and *UL20*), limit viral replication [100]. However, during STH/HSV-2 co-infections, STHs may suppress these antiviral miRNAs indirectly by promoting an immunosuppressive environment.

In these scenarios, we hypothesise that pathogen-derived and host-derived miRNAs may modulate the host immune response during STH/HSV-2 co-infection, potentially favouring Th2 over Th1 immunity, thus influencing HSV-2 replication, latency, and pathology (Figure 2). Nevertheless, the miRNA profiles and their roles in STH/HSV-2 co-infections remain unexplored, highlighting opportunities for research on STH/HSV-2 co-infections with a particular emphasis on miRNAs.

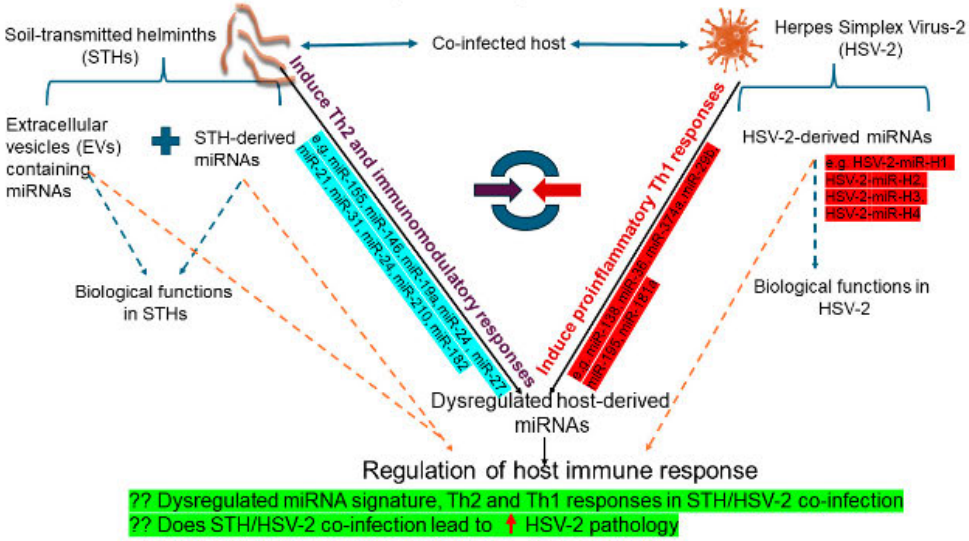

Figure 2. Schematic representation of potential miRNAs involved in regulating STH/HSV-2 co-infection. Host- and pathogen-derived miRNAs have been shown to regulate important biological functions, including aspects of both innate and adaptive immunity, during single STH and HSV-2 infections. Examples of relevant miRNAs are included in the figure. The miRNAs highlighted in blue have been shown to regulate anti-STH immunity, and the miRNAs highlighted in red are known to regulate anti-HSV-2 immunity (as explained in full in text).

11. Opportunities and Future Directions

As highlighted earlier, helminth-derived miRNAs have been identified in infected hosts and play a role in modulating host immunity and infection. Furthermore, dysregulated miRNA expression is a hallmark of various infections and disease processes [1]. Given the emerging role of miRNAs as diagnostic and prognostic biomarkers, this creates a new avenue for exploration. Specifically, circulating miRNAs possess several attractive features that make them potential diagnostic or prognostic biomarker candidates: (i) They have a broad spectrum of mRNA targets, whereby a single miRNA can regulate multiple mRNA targets and their associated cellular pathways. (ii) They are tissue- and disease-specific, expressing signature patterns specific to a tissue or disease context. (iii) They are highly stable and can withstand freeze-thaw cycles and enzymatic degradation. (iv) They can be detected noninvasively in a range of body fluids such as blood, urine, and saliva [1].

In addition to their diagnostic potential, miRNAs can be leveraged for potential therapeutic purposes. MiRNAs are appealing therapeutic tools because of the ability of a single miRNA to regulate a wide range of mRNA targets and cellular pathways. Briefly, the goal of miRNA-based therapeutics is to reverse dysregulated miRNA expression profiles that are associated with disease processes. MiRNAs and the cellular pathways associated with diseases can be manipulated, by either enhancing or restoring the levels of endogenous miRNAs needed to suppress disease or reducing or inhibiting the function of miRNAs that promote disease. To achieve this, nucleic acids including synthetic miRNAs (miRNA mimics), recombinant vectors with miRNA-encoding sequences, and oligonucleotide-based inhibitors (anti-miRs/miRNA inhibitors) may be used [115]. However, the field of miRNA-based therapeutics is still in its infancy, and several challenges must still be resolved before its successful implementation. Such challenges include identifying the correct administration routes, ensuring in-body stability, targeting the appropriate tissue and cell types, and achieving the overall intended intracellular effects [115].

Given their features such as small size, high sequence similarity, and at times low expression, highly sensitive and specific techniques are required to identify and quantify miRNAs. Methods to detect and study miRNAs such as hybridization (northern blot, microarrays, bead-based profiling), amplification (RT-qPCR), next-generation sequencing, and enzyme-based methods have been recently reviewed [116]. Importantly, miRNAs are embedded within complex regulatory networks and deciphering their specific roles may be challenging. To overcome this, the use of next-generation sequencing techniques and bioinformatics tools to analyse miRNA and mRNA datasets can help unravel these complex networks [117]. Finally, these techniques can be leveraged to transform our understanding of host immunity to STH/HSV-2 single and co-infections. Moreover, elucidating the roles of miRNAs in STH/HSV-2 co-infection may help uncover essential genes and pathways involved in mediating effective host immunity. This could provide novel diagnostic or prognostic biomarkers of infection. Exploring the molecular and immunological basis of STH/HSV-2 co-infections may also uncover novel mechanisms that can be targeted for therapeutic purposes.

12. Conclusions

STHs and HSV-2 infections disproportionally affect under-resourced countries, thus representing significant public health challenges. Exploring potential STH/HSV-2 co-infections in these settings is necessary to mitigate the potential negative impact of co-infections. MiRNAs are critical mediators of host immune responses to pathogens. Moreover, they may be exploited by pathogens to enhance pathogen function and survival. Given that miRNAs mediate host responses to STH and HSV-2 infections, it follows that miRNA dysregulation could contribute to susceptibility to infection and subsequent pathol-

ogy. However, miRNA expression associated with STH/HSV-2 co-infection and their underlying mechanisms are largely unknown. Studies relating to miRNA expression during STH/HSV-2 co-infection are now needed to understand (i) the molecular mechanisms that underpin STH/HSV-2 co-infections and (ii) how dysregulation of miRNAs may contribute to the course of infection. Understanding the roles of miRNAs in host immune responses to STH/HSV-2 co-infection could help in identifying essential genes and pathways involved in facilitating effective host immunity. However, miRNAs are pleiotropic, and they are embedded within complex regulatory networks. Therefore, deciphering their specific roles may be challenging. Techniques such as high-throughput sequencing and bioinformatics analyses of large miRNA and mRNA datasets may help in unravelling these complex networks. This could provide new diagnostic or prognostic biomarkers or novel therapeutic approaches for these important pathogens.

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CHAPTER THREE
RESULTS

3.1 Characterization of microRNA expression profiles of murine female genital tracts following *Nippostrongylus brasiliensis* and Herpes Simplex Virus type 2 co-infection

The dominant T-helper 2 (Th2) immune phenotype elicited by soil-transmitted helminths (STHs) are known to extend beyond the primary site of infection, impacting immunity to unrelated infections at distal sites, including the female genital tract (FGT) (Chetty et al., 2020). Understanding the bystander effects of STHs on FGT immunity is important in the context of sexually transmitted infections (STIs). STH-driven immune modulation has been shown to alter murine FGT immune responses to HSV-2, leading to enhanced HSV-2 pathology (Chetty et al., 2021). However, the molecular mechanisms underlying these changes in the FGT are less understood. In particular, the role of miRNAs, which are integral post-transcriptional mediators of immune responses and cellular signaling pathways, has not been previously studied. Exploring miRNA expression profiles can provide deeper insights into the FGT response to STH/HSV-2 co-infection; changes in miRNA expression profiles during STH/HSV-2 co-infections may uncover unique molecular mechanisms of pathogen synergy or antagonism. Therefore, to further explore the impact of STH/HSV-2 co-infection on the FGT, the article entitled "**Characterization of microRNA expression profiles of murine female genital tracts following *Nippostrongylus brasiliensis* and Herpes Simplex Virus type 2 co-infection"**, which was published in **MDPI *Microorganisms***, is presented below. This exploratory study focused on FGT miRNA expression profiles following acute single infection with *Nippostrongylus brasiliensis* (*Nb*) or HSV-2 or *Nb*/HSV-2 co-infection using a BALB/c murine model. This study provides novel insights into the expression of murine FGT miRNAs during infection with *Nb* and/or HSV-2 by providing miRNAs associated with host immune responses and disease processes. In addition, the study leveraged bioinformatics tools to predict the possible gene targets and pathways associated with differentially expressed miRNAs during single and co-infection.

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Characterization of microRNA Expression Profiles of Murine Female Genital Tracts Following *Nippostrongylus brasiliensis* and Herpes Simplex Virus Type 2 Co-Infection

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Abstract

Soil-transmitted helminths (STHs) and Herpes Simplex Virus type 2 (HSV-2) are highly prevalent infections with overlapping distribution, particularly in resource-poor regions. STH/HSV-2 co-infections may impact female reproductive health. However, many aspects of STH/HSV-2 co-infections, including the role of microRNAs (miRNAs) in regulating female genital tract (FGT) immunity and their potential contribution to pathologies such as chronic inflammation, impaired mucosal defense, and reproductive tract cancers remain unclear. In this study we investigated the miRNA expression profiles in murine FGT tissues following single or co-infection with *Nippostrongylus brasiliensis* (*Nb*) and HSV-2 and explored predicted miRNA-mRNA targets and pathways. An analysis of miRNA sequencing data was conducted to determine differentially expressed (DE) miRNAs between infected FGT tissues and uninfected controls. Ingenuity Pathway Analysis was conducted to predict the immune-related target genes of the DE miRNAs and reveal enriched canonical pathways, top diseases, and biological functions. Selected representative DE miRNAs were validated using RT-qPCR. Our results showed a total of eight DE miRNAs (mmu-miR-218-5p, mmu-miR-449a-5p, mmu-miR-497a-3p, mmu-miR-144-3p, mmu-miR-33-5p, mmu-miR-451a, mmu-miR-194-5p, and mmu-miR-192-5p) in the comparison of *Nb*-infected versus uninfected controls; nine DE miRNAs (mmu-miR-451a, mmu-miR-449a-5p, mmu-miR-144-3p, mmu-miR-376a-3p, mmu-miR-192-5p, mmu-miR-218-5p, mmu-miR-205-3p, mmu-miR-103-3p, and mmu-miR-200b-3p) in the comparison of HSV-2-infected versus uninfected controls; and one DE miRNA (mmu-miR-199a-5p) in the comparison of *Nb*/HSV-2 co-infected versus uninfected controls (p-value < 0.05, $|\log FC| \geq 1$). Core expression analysis showed that, among other canonical pathways, the DE miRNAs and their predicted mRNA targets were involved in neutrophil degranulation, interleukin-4 and interleukin-13 signaling, natural killer cell signaling, interferon alpha/beta signaling, and ISGylation. Additionally, cancer was predicted as one of the significantly enriched diseases, particularly in the co-infected group. This is the first study to provide insights into the FGT miRNA profiles following *Nb* and HSV-2 single and co-infection, as well as the predicted genes and pathways they regulate, which may influence

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host immunity and pathology. This study highlights the role of miRNAs in regulating FGT immunity and pathology in the context of STH/HSV-2 co-infection.

Keywords: microRNAs; soil-transmitted helminths; herpes simplex virus type 2/HSV-2; *Nippostrongylus brasiliensis*; co-infection

1. Introduction

Soil-transmitted helminths (STHs) and Herpes Simplex Virus type 2 (HSV-2) are widespread infections with overlapping geographical distribution, particularly in resource-poor countries [1,2]. Given their shared geographical distribution, STH/HSV-2 co-infections may impact host immunity and subsequently affect female reproductive health. However, studies investigating STH/HSV-2 co-infections in co-endemic regions remain limited.

Herpes Simplex Virus type 1 (HSV-1) and type 2 (HSV-2) are members of the *Herpesviridae* family, a group of double-stranded DNA viruses capable of establishing lifelong latency. While both HSV-1 and HSV-2 can cause genital herpes, HSV-2 is the primary etiological agent, with an estimated 491.5 million infections reported globally [1,3]. HSV-2 infection, a common sexually transmitted infection, is primarily asymptomatic but may be characterized by recurrent painful genital ulcers. Importantly, HSV-2 infection promotes both susceptibility to and transmission of other sexually transmitted infections, especially HIV [1]. Moreover, HSV-2 has been proposed as a co-factor in the development of cervical cancer, potentially enhancing the oncogenic effects of other agents such as HPV by promoting epithelial disruption and chronic inflammation, impairing local immune responses, and facilitating viral transmission and persistence [4].

Effective viral control requires a robust T-helper type 1 (Th1) immune response, involving innate interferon (IFN)-mediated natural killer cell activity, followed by adaptive immunity via IFN-γ-secreting $CD4^+$ and $CD8^+$ T cells [5].

STHs, which include roundworms, whipworms, and hookworms, cause over 1.5 billion human infections worldwide. STH infections are most prevalent in tropical and subtropical regions, affecting communities that lack clean water, proper sanitation, and good hygiene practices [2]. STHs typically induce a dominant T-helper type 2 (Th2) immune response and promote immunoregulatory cell populations (Treg or Th3). Both Th2 and Th3 populations suppress Th1 responses. This contrasts with the Th1-induced immunity required for viral control, highlighting the antagonistic nature of the immune responses elicited by STHs and HSV-2. The two arms of the immune system counter-regulate each other such that a predominance of Th2 would downregulate Th1 responses [6,7]. Consequently, STH infections cause systemic immunological changes that influence the outcomes of a range of unrelated co-infections, including important sexually transmitted viral co-infections, such as HIV [8–10] and HPV [11,12]. Interestingly, these systemic immunological effects extend to distal uncolonized sites, including the female genital tract (FGT). STHs have been shown to alter female fertility [13] and FGT immunity [12] and may contribute to a tolerant microenvironment that supports the progression of virally induced cancers [14,15]. Other mechanisms include chronic inflammation and immune suppression induced through Th3 regulatory cells, which may negatively affect the immune surveillance of transformed oncogenic cells, thus promoting their uncontrolled proliferation [16].

The question of how STH-induced immune responses influence HSV-2 immune control is less explored but relevant due to the potential immunological interactions between these pathogens. Recently, infection with *Nippostrongylus brasiliensis* (*Nb*), a murine hookworm, was shown to compromise immunity to subsequent genital HSV-2 and

exacerbated HSV-2 pathology in the murine FGT [17]. This suggests that STH-induced immune modulation may suppress protective Th1 responses against HSV-2, potentially worsening the course of HSV-2 infection, with serious implications for female sexual and reproductive health. However, many aspects of STH/HSV-2 co-infections remain poorly understood, particularly the role of microRNAs (miRNAs) in mediating host immune responses and pathology. To date, no studies that demonstrate how miRNAs regulate immunity or contribute to disease outcomes in the context of STH/HSV-2 co-infection have been identified.

MiRNAs, which are a class of small, non-coding RNA molecules, are important mediators of gene expression and function. By binding to complementary sequences on target messenger RNAs (mRNAs), miRNAs either inhibit mRNA translation or lead to mRNA degradation [18]. In this way, miRNAs participate in a diverse range of physiological functions, including the regulation of host immune responses. MiRNAs are also involved in disease pathogenesis; dysregulated miRNA expression has been linked to diverse disease contexts, such as infectious diseases, inflammatory diseases, allergies, and cancers. Given their essential multifaceted regulatory roles in physiological and disease processes, miRNAs hold diagnostic and prognostic potential and may serve as therapeutic targets for various diseases [19].

In HSV-2 infections, both host- and HSV-2-derived miRNAs influence HSV-2 infection by regulating viral replication, immune evasion, latency, and various aspects of anti-HSV-2 innate and adaptive immune responses [20,21]. Similarly, in STH infections, host- and helminth-derived miRNAs regulate crucial aspects of antihelminth immunity, influencing intestinal epithelial cell function and the activation of antihelminth innate and adaptive immune cells [22].

Moreover, dysregulated miRNA expression has been implicated in the pathogenesis of various cancers, including those of the female reproductive system. Dysregulation of miRNAs has been linked to cervical, endometrial, and ovarian cancers, influencing key cancer-related pathways such as apoptosis, proliferation, differentiation, and metabolism [23].

The interplay between host-, HSV-2-, and STH-derived miRNAs may profoundly shape the pathogenesis and clinical outcomes of co-infections by modulating immune responses. To better understand STH/HSV-2 co-infections, it is essential to investigate how miRNAs regulate gene expression and immune signaling pathways and how their dysregulation may contribute to chronic inflammation, impaired mucosal immunity, and the progression of FGT-related diseases, including cancer.

In recent years, advances in next-generation sequencing (NGS) platforms and bioinformatics tools have enabled more comprehensive analyses of miRNA functions, interactions, and regulatory networks. NGS offers several advantages compared to traditional methods, such as enhanced sensitivity and higher sequencing depths, which facilitate the identification of numerous host- and pathogen-specific miRNAs [24]. Sequencing approaches have been previously used to characterize HSV-2 [25–28] and STH miRNAs [29–33]. In the present study, we used an NGS approach to identify differentially expressed (DE) host miRNAs in the FGT tissues of BALB/c mice singly and co-infected with *Nb* and HSV-2. The results of this study will deepen our understanding of the roles of miRNAs in STH/HSV-2 co-infection. Moreover, insights from this work may lead to novel therapeutic miRNA targets and pathways, aimed at mitigating the potential effects of co-infections and improving strategies for infection control.

2. Materials and Methods

2.1. Ethical Approval

Approval to conduct this study was granted by the Animal Ethics Committee of the University of Cape Town (UCT) (approval number: FHS AEC REF NO: 021_012) and the

Animal Research Ethics Committee of the University of KwaZulu Natal (approval number: AREC/00005911/2023).

All experimental procedures were conducted at the Institute of Infectious Diseases and Molecular Medicine, UCT, in compliance with the Section 20 dispensation to conduct animal experiments, which was obtained from the South African Department of Agriculture, Land Reform and Rural Development [reference number: 12/11/1/7/1 (6151KL)]. Only researchers accredited by the South African Veterinary Council performed the experimental procedures.

2.2. Experimental Models

2.2.1. Animals

All experiments were conducted as previously described [17]. Female BALB/c mice (Age, 6–10 weeks old; weight, 18–20 g) were bred and maintained under specific pathogen-free conditions at the Research Animal Facility, UCT, South Africa. Mice were group-housed in individually ventilated cages within the same room of the BSL II facility, with no interaction between groups. Food and water were available ad libitum.

Mice were randomly assigned to four experimental groups: (1) singly infected with *Nb*, (2) singly infected with HSV-2, (3) co-infected with *Nb* and HSV-2, and (4) uninfected control groups. Each group comprised 6 mice ($n = 6$).

The estrous cycles of all mice were synchronized by subcutaneously administering 2 mg of Medroxyprogesterone Acetate (Depo Provera®) seven days prior to the start of experimental procedures. As pubescent female mice have variable susceptibility to HSV-2 infection, exogenous progesterone was required to synchronize their estrous cycles and reduce experimental variation. Additionally, progesterone modifies the thickness of the vaginal epithelium and the expression of HSV-2 entry receptors, enhancing viral uptake [34].

Seven days later, mice in Groups 1 and 3 were injected subcutaneously in the neck scruffs with 500 L3-stage *Nb* larvae. Seven days post-*Nb* infection, mice in Groups 2 and 3 were infected intravaginally with HSV-2 Strain G. All mice were euthanized two days post-HSV-2 infection. The FGT tissues (excluding ovaries) were collected, preserved in RNAlater (ThermoFisher Scientific, Cat. No.: AM7021, Waltham, MA, USA), stored at 4 °C overnight, and then transferred to a −80 °C freezer until further analysis. A schematic overview of the experimental design employed in this study is shown in Figure 1.

2.2.2. *Nb* Maintenance and Infection

Nb maintenance and infection were conducted as previously described [17]. Briefly, *Nb* was propagated in male Wistar rats via subcutaneous infection with 5000 L3-stage *Nb* larvae. Thereafter, faecal samples were collected during peak periods of *Nb* egg excretion, which occurs between days 6 and 8 post-infection. To prepare faecal cultures, a faeces/charcoal mix was placed on wet-raised filter paper, and the hatched L3-stage *Nb* larvae that migrated to the edge of the filter paper were collected by gentle washing with water. These L3-stage *Nb* larvae were quantified using a dissecting microscope (Leica M80 Stereo Microscope, Leica Microsystems GmbH, Wetzlar, Germany), followed by resuspension in an appropriate volume of distilled water for use in subsequent infections. Each mouse in Groups 1 and 3 was subcutaneously infected in the scruff of the neck with 500 L3-stage *Nb* larvae suspended in 200 µL of sterile phosphate-buffered saline (PBS) using a 21G needle (Becton, Dickinson & Company, Franklin Lakes, NJ, USA). Infections were administered seven days after Depo-Provera treatment and seven days before viral infection.

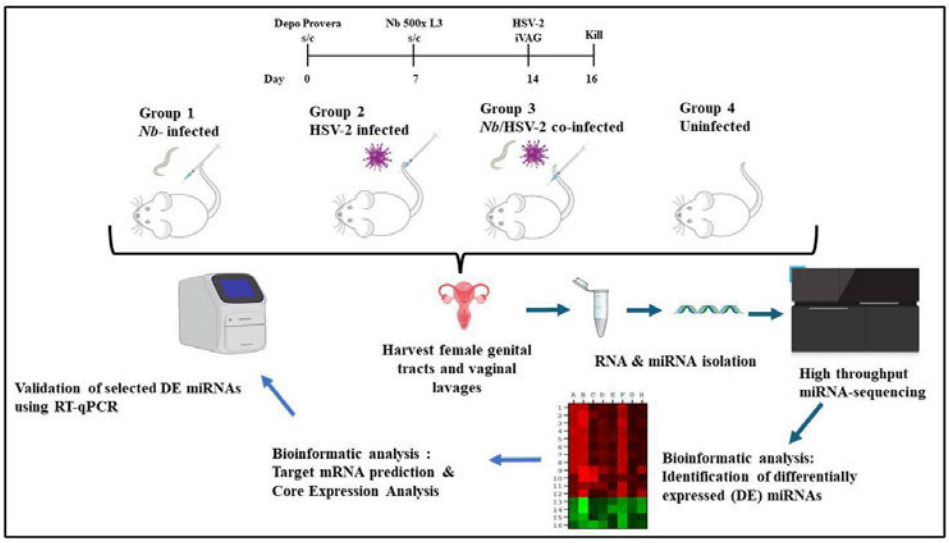

Figure 1. Experimental design: A murine model was used to investigate whether miRNAs in the female genital tract (FGT) are differentially expressed (DE) following acute single or co-infection with *Nippostrongylus brasiliensis* (*Nb*) and HSV-2. Female BALB/c mice aged 6-10-week-old were randomly assigned to four experimental groups (n = 6 mice per group): (1) singly infected with *Nb*, (2) singly infected with HSV-2, (3) co-infected with *Nb* and HSV-2, and (4) uninfected control groups. To synchronize the estrous cycles, all mice were given a subcutaneous injection of 2 mg Depo Provera® seven days before the start of experimental procedures. On day 7, mice in Groups 1 and 3 were injected subcutaneously with 500 L3-stage *Nb* larvae. On day 14, i.e., 7 days after *Nb* infection, mice in Groups 2 and 3 were intravaginally challenged with HSV-2. All mice were sacrificed two days after HSV-2 infection. The FGT tissues (excluding ovaries) from each group were isolated and studied comparatively to identify DE miRNAs associated with infection-induced immunity. Target mRNA prediction and core expression analysis were conducted, and a subset of DE miRNAs was randomly selected for validation by RT-qPCR. (Drawn in PPT by author. Free images sourced from https://bioicons.com/ and https://pixabay.com/).

2.2.3. Virus

Human herpesvirus 2 strain G (HSV-2, ATCC, VR-734) was cultured in Vero cells at a multiplicity of infection (MOI) of 0.1, as previously described [17]. Two to three days post-infection, both cells and supernatants were harvested, and viral titers were quantified using a plaque assay. Viral stocks were aliquoted and stored at −80 °C until needed.

For HSV-2 infection, mice were anaesthetized via intraperitoneal injection with xylazine (10 mg/kg) and ketamine (100 mg/kg) using a 27 G needle. Each mouse in Groups 2 and 3 was intravaginally challenged with 5×10^5 plaque-forming units (PFU) of HSV-2 strain G in a total volume of 5 µL using a Gilson P10 (Gilson, Inc., Middleton, WI, USA) pipette fitted with a sterile 10 µL filter tip. The pathogen was administered once. Vaginal lavages were collected at two days post-HSV-2 infection. This was performed by washing the vaginal vault 10 times with 50 µL of RNAlater; this washing step was performed three times. To assess the severity of HSV-2-associated illness, mice were monitored by clinical characterization and scored from 0 to 5 as follows: 0 (no pathology), 1 (slight genital/perianal erythema), 2 (genital/perianal swelling and erythema), 3 (genital lesions and/or visible weight loss), 4 (hind limb paralysis and/or purulent lesions), and 5 (premoribund), as previously described [17].

2.3. Total RNA Extraction and Quality Control

The murine FGT tissues (excluding ovaries) were isolated, preserved in RNAlater (ThermoFisher Scientific, Cat. No.: AM7021, Waltham, MA, USA), stored at 4 °C overnight, and then transferred to a −80 °C freezer until further analysis. FGT tissues were homogenized in 360 µL RPL buffer (Qiagen, Cat. no./ID. 217684, Hilden, Germany) on ice at 30 s intervals using a handheld homogenizer set on medium speed. Total RNA, including miRNAs, was extracted from FGT tissues using the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen, Cat. no./ID. 217684, Hilden, Germany), according to the manufacturer's protocol. Total RNA was eluted in 15 µL of RNAse-free water. RNA concentration and purity were quantified by measuring absorbance at 260 and 280 nm on the NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA). Purified RNA samples were stored at −80 °C.

2.4. Library Preparation and Sequencing

Small RNA library preparation and sequencing were conducted using the South African Medical Research Council (SAMRC) Genomics Platform (Cape Town, South Africa). Total RNA was processed using the MGIEasy Small RNA Library Prep Kit (Cat. No.: 940-000196-00, MGI Tech Co., Ltd., Shenzhen, China) and the high-throughput MGI SP-100 sample preparation system, following the manufacturer's instructions. Briefly, 3′ and 5′ adapters were ligated to small RNAs, followed by reverse transcription and PCR amplification using barcoded primers. Libraries were then subjected to bead-based size selection and purification to enrich for miRNA-sized fragments. Library fragment sizes were assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Only RNA samples with RNA Integrity Numbers (RIN) ≥ 7 were included for sequencing. A total of 22 samples met this criterion and were distributed across the experimental groups as follows: *Nb*-infected (n = 6), HSV-2-infected (n = 5), co-infected with *Nb* and HSV-2 (n = 5), and uninfected controls (n = 6). Sequencing was performed on the DNBSEQ-G400RS platform using single-end 50 bp reads with the Small RNA FCL SE50 sequencing kit (Item No.: 1000019478, MGI Tech Co., Ltd., Shenzhen, China).

2.5. Bioinformatics Analysis

2.5.1. Detecting Host-Derived miRNAs and DE miRNAs

Bioinformatics analyses were performed with support from the DIstributed PLatform in OMICS (DIPLOMICS; Cape Town, South Africa). The Comprehensive Analysis Pipeline for miRNA-Sequencing data (CAP-miRSeq) was employed for adapter removal, quality trimming, read alignment, miRNA detection and quantification, and data visualization [35]. The quality of raw sequencing reads was evaluated using FastQC (version 0.12.1), and reads were aligned to the *Mus musculus* reference genome. Known and novel miRNAs were identified and annotated using the miRDeep2 mouse miRNA database. Differential expression analysis was conducted using the edgeR package (version 3.42.4) from Bioconductor (Release 3.17). This model uses empirical Bayes estimation and exact tests based on the negative binomial distribution [35]. DE miRNAs were defined based on a p-value < 0.05 and $|\log FC| \geq 1$, an approach previously used in infection-related miRNA studies [36,37]. The schematic workflow of the miRNA analysis is shown in Figure 2.

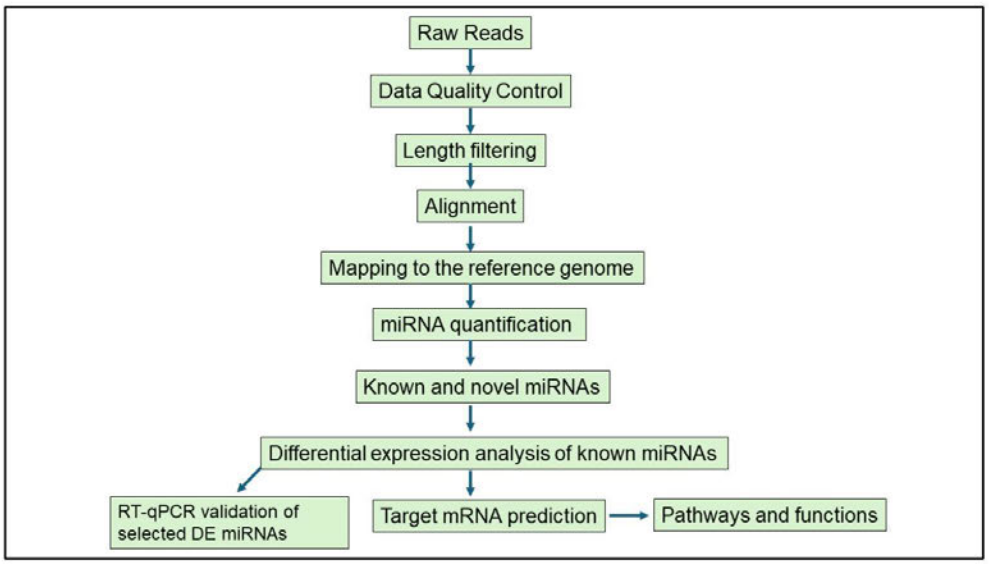

Figure 2. Schematic workflow of the bioinformatics analysis performed to study differentially expressed miRNAs in murine female genital tracts following single and co-infection with *Nippostrongylus brasiliensis* and HSV-2.

2.5.2. MiRNA Target Gene Prediction; Network and Core Expression Analysis

Ingenuity Pathway Analysis (IPA) (Qiagen, Redwood City, CA, USA) was employed to identify the predicted mRNA targets of the DE miRNAs. This was performed using the miRNA target filter tool within IPA, which associates DE miRNAs with their potential target genes by drawing information from data sources such as TargetScan, TarBase, miRecords, and IPA's knowledge base. These data sources include both experimentally validated miRNA-mRNA interactions and in silico predicted miRNA-mRNA interactions based on literature. This IPA incorporated the logarithmic fold changes and *p*-values of the DE miRNAs. Given that one miRNA may potentially regulate multiple mRNAs [18], to maintain relevance to this study, only mRNA targets involved in immune-related processes/pathways/diseases were analysed. This was performed by filtering all predicted mRNA targets using the following parameters: Source: All; miRNA Confidence Level: Experimentally Validated, High Predicted; Species: Mouse; Pathways: Immune system. To visualize predicted regulatory networks between the DE miRNAs and their mRNA targets, graphs were constructed using the network, overlay and build functions in IPA. Core expression analysis was then performed on all the mRNA targets and the DE miRNAs, with the workflow was set as follows: Core Analysis type: Expression Analysis.

2.6. Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

A subset of DE miRNAs was randomly selected for validation by RT-qPCR as follows: two miRNAs that were commonly DE in both the *Nb*-infected versus uninfected and HSV-2-infected versus uninfected comparisons, and one unique DE miRNA from each of the single-infection comparisons. In addition, the single DE miRNA identified in the co-infected versus uninfected group was validated. This approach was adopted to verify the miRNA-sequencing results and is consistent with previous studies that explored DE miRNAs in various infection models [37–39]. For the first-strand cDNA synthesis, reverse transcription (RT) was conducted using the miRCURY LNA RT Kit (Qiagen, Cat. No.

339340, Hilden, Germany), following the manufacturer's protocol. Briefly, 200 ng/µL of the extracted RNA was added to the RT reaction and cDNA was made using universal RT primers provided in the kit. All reactions were set up on ice, and aliquots of the cDNA were stored at −20 °C until required. RT-qPCR was performed on an ABI 7000 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). The miRCURY LNA SYBR Green PCR Kit (Qiagen, Cat. No. 339345, Hilden, Germany) was used in RT-qPCR reactions together with individual miRCURY LNA miRNA PCR (Qiagen, Hilden, Germany) primer assays, following the manufacturer's protocol. U6 (GeneGlobe ID/Cat. No.: YP02119464|Cat. No.: 339306, Qiagen, Hilden, Germany) was used as an internal control for miRNA template normalization. RT-qPCR reactions were set up for five DE miRNAs using primers—mmu-miR-192-5p (GeneGlobe ID/Cat. No.: YP00204099/339306, Qiagen, Hilden, Germany), mmu-miR-194-5p (GeneGlobe ID/Cat. No.: YP00204080/339306, Qiagen, Hilden, Germany), mmu-miR199a-5p (GeneGlobe ID/Cat. No.: YP00204494/339306, Qiagen, Hilden, Germany), mmu-miR-200b-3p (GeneGlobe ID/Cat. No.: YP00206071/339306, Qiagen, Hilden, Germany), and mmu-miR-218-5p (GeneGlobe ID/Cat. No.: YP00206034/339306, Qiagen, Hilden, Germany). Individual RT-qPCR assays were performed in triplicates with a total reaction volume of 10 µL. The PCR cycling profile was set as follows: 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 10 s, combined annealing/extension at 56 °C for 60 s, and melt curve analysis at 60–95 °C. Data were analysed using the Design and Analysis Software 2.8.0 (Applied Biosystems, Foster City, CA, USA). Expression levels of the miRNAs were measured using CT (threshold cycle). Relative expression levels of miRNAs were calculated by the $2^{-\Delta\Delta Ct}$ method.

2.7. Statistical Analysis

Statistical analysis was conducted using Graph Pad Prism software (version 8.0). The significant difference between two groups was analysed by one-way analysis of variance (ANOVA) using Student's *t*-test. All experimental data for qRT-PCR are expressed as mean ± standard error of mean (SEM). A p-value < 0.05 was considered as a significant difference.

3. Results

3.1. Animal Infection

In this study, we investigated FGT miRNA expression profiles of four different groups: (1) singly infected with *Nb*, (2) singly infected with HSV-2, (3) co-infected with *Nb* and HSV-2, and (4) uninfected control groups. Body weights and pathology scores were assessed in all mice over the course of the study. At day 2 post-HSV-2 infection, no visible signs of disease were detected, and there were no significant differences in body weight or pathology scores among the four groups. Supplementary Figure S1 shows the pathology scores for the infected groups.

3.2. Basic Characteristics of Libraries Obtained from miRNA-Sequencing

Of the 24 FGT tissue samples collected from mice across four experimental groups, 22 yielded RNA of sufficient quality (RIN ≥ 7) for miRNA-sequencing. Therefore, the distribution of sequenced samples was as follows: *Nb*-infected (n = 6), HSV-2-infected (n = 5), *Nb*/HSV-2 co-infected (n = 5), and uninfected controls (n = 6). Sequencing produced 373,2 million reads that were aligned to the reference genome. Length distribution analysis showed that reads of 21–24 bp were the most abundant. A summary of the numbers of reads aligned to the reference genome, precursor and mature miRNAs, as well as the number of miRNAs reads with ≥5× coverage detected for each sample per group is provided

in Supplementary Table S1. A heatmap illustrating the overall trend of miRNA expression across the four experimental groups is shown in Supplementary Figure S2.

3.3. *Differential Expression of miRNAs*

Three comparisons were made between FGT tissues of the infected versus uninfected groups: (1) *Nb*-infected compared to uninfected, (2) HSV-2-infected compared to uninfected, and (3) *Nb*/HSV-2 co-infected compared to uninfected. DE miRNAs in each comparison were identified (p-value < 0.05 and |logFC| ≥ 1). We found that within FGT tissues, 8 miRNAs were DE in the *Nb*-infected versus uninfected groups, 9 miRNAs were DE in the HSV-2-infected versus uninfected groups, and 1 miRNA was DE in the *Nb*/HSV-2 co-infected versus uninfected group (Figure 3). The DE miRNAs identified in each comparison are shown in Table 1.

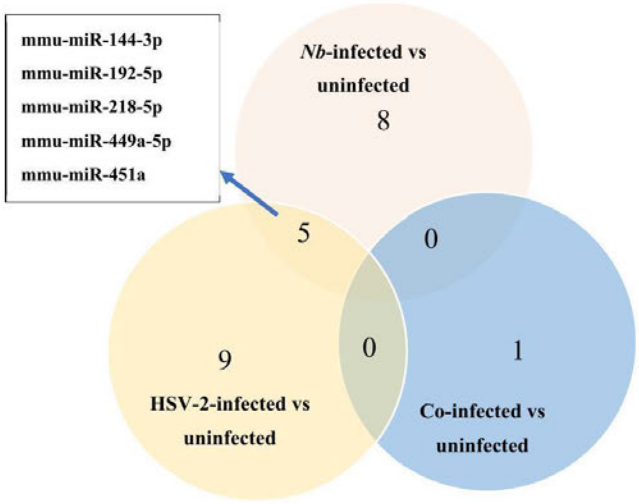

Figure 3. Venn diagram of the number of differentially expressed (DE) miRNAs identified using miRNA-sequencing in the *Nb*-infected versus uninfected, HSV-2-infected versus uninfected, and co-infected versus uninfected comparisons. Five common DE miRNAs were observed in the *Nb*-infected versus uninfected and HSV-2-infected versus uninfected comparisons.

Table 1. Differentially expressed microRNAs that were identified in each comparison.

***Nb*-Infected Versus Uninfected**			
Name of miRNA	**Log Fold Change (LogFC) ***	***p*-value ***	**Up/Downregulation**
mmu-miR-194-5p	−3.27	8.00×10^{-4}	Down
mmu-miR-218-5p	2.09	4.20×10^{-3}	Up
mmu-miR-449a-5p	2.17	4.30×10^{-3}	Up
mmu-miR-192-5p	−1.92	8.38×10^{-3}	Down
mmu-miR-497a-3p	1.43	1.57×10^{-2}	Up
mmu-miR-144-3p	1.51	2.65×10^{-2}	Up
mmu-miR-33-5p	1.34	4.05×10^{-2}	Up
mmu-miR-451a	1.06	4.44×10^{-2}	Up
HSV-2-infected versus Uninfected			
Name of miRNA	**Log Fold Change (LogFC) ***	***p*-value ***	**Up/Downregulation**
mmu-miR-192-5p	−2.94	3.48×10^{-4}	Down
mmu-miR-451a	1.88	1.37×10^{-3}	Up

mmu-miR-449a-5p	1.72	2.88×10^{-3}	Up
mmu-miR-218-5p	−1.54	9.12×10^{-3}	Down
mmu-miR-144-3p	1.71	9.73×10^{-3}	Up
mmu-miR-376a-3p	1.26	1.05×10^{-2}	Up
mmu-miR-205-3p	−1.46	1.91×10^{-2}	Down
mmu-miR-103-3p	−1.31	2.07×10^{-2}	Down
mmu-miR-200b-3p	−1.17	3.77×10^{-2}	Down
***Nb*/HSV-2 co-infected versus Uninfected**			
Name of miRNA	**Log Fold Change (LogFC) ***	***p*-value ***	**Up/Downregulation**
mmu-miR-199a-5p	−2.46	4.88×10^{-2}	Down

Footnote: * DE miRNAs were identified based on p-value < 0.05 and |logFC| ≥ 1.

A total of 8 DE miRNAs were identified in the *Nb*-infected compared to uninfected FGT tissues, of which 6 miRNAs were upregulated (mmu-miR-218-5p, mmu-miR-449a-5p, mmu-miR-497a-3p, mmu-miR-144-3p, mmu-miR-33-5p, and mmu-miR-451a) and 2 were downregulated (mmu-miR-194-5p and mmu-miR-192-5p). A total of 9 DE miRNAs were identified when the HSV-2-infected and uninfected FGT tissues were compared, of which 4 miRNAs were upregulated (mmu-miR-451a, mmu-miR-449a-5p, mmu-miR-144-3p, and mmu-miR-376a-3p) and 5 miRNAs (mmu-miR-192-5p, mmu-miR-218-5p, mmu-miR-205-3p, mmu-miR-103-3p, and mmu-miR-200b-3p) were downregulated. In the comparison between the co-infected and uninfected FGT tissues, only 1 DE miRNA (mmu-miR-199a-5p) was identified and was downregulated. We observed 5 common DE miRNAs in the *Nb*-infected versus uninfected and HSV-2-infected versus uninfected comparisons (mmu-miR-144-3p, mmu-miR-192-5p, mmu-miR-218-5p, mmu-miR-449a-5p, and mmu-miR-451a). Among these, mmu-miR-218-5p was upregulated in the *Nb*-infected versus uninfected comparison but downregulated in the HSV-2-infected versus uninfected comparison.

3.4. MiRNA Target Gene Prediction, Network and Core Expression Analysis

Potential mRNA targets of all the DE miRNAs in each comparison were predicted by Qiagen IPA. In brief, the DE miRNAs for each comparison were individually submitted to IPA. All predicted targets identified in IPA, and the corresponding DE miRNAs, were included in the subsequent core expression analysis. Multiple predicted mRNA target genes with known roles in host immunity were identified. Networks were constructed in IPA to visualize the predicted regulatory functions of the DE miRNAs (Figure 4, Supplementary Figures S3 and S4). A summary of the predicted targets of the DE miRNAs for each comparison is shown in Tables 2–4.

Table 2. *Nb*-infected versus uninfected: list of predicted immune-related targets for the differentially expressed miRNAs.

Name of miRNA	Predicted Immune-Related Targets	Predicted Regulatory Effect
mmu-miR-144-3p	*AP1S3, CTNNB1, EP300, HIF1A, ITCH, JUN, MAGT1, MAP3K8, MEF2A, MTOR, RAC1, RHOA, ROCK1, TNFSF11, UBE2D1, UBE2D3*	Predicted inhibition
mmu-miR-192-5p	*A1BG, DHX58, GM2A, NLRC5, RSAD2, STK3, ZEB1*	Predicted activation
mmu-miR-194-5p	*FASLG, GYG1, IL9, RAP2B, SUMO2, TAB3*	Predicted activation
mmu-miR-218-5p	*C5, CD200R1, COL1A1, DDX41, LMO7, NUP50, PIK3C2A, PLCG1, RAB6A, RICTOR, RNF41, RPS6KA3, SH3KBP1, SOCS3, SPSB1, UBE2H, VAMP7, VAT1*	Predicted inhibition
mmu-miR-33-5p	*EPX3, GCA*	Predicted inhibition
mmu-miR-449a-5p	*ADAM10, AP2A2, BCL2, BCL6, BTN1A1, C9, CANT1, CASP2, CCL22, CCND1, CD47, CD79A, CREB1, CSF1R, CTNNB1, DEFB124, DNM1L, FBX017, FCER1A, GRAP2, GSTO1, IL23R, IL6R, ISG20, MAP2K1, MAP3K3, MAPT, MUC5B, MYC, MYH9, NECTIN2, ORMDL3, PTPN4,*	Predicted inhibition * Predicted activation (*TP53*)

	*RNF4, ROCK1, SIAH1, SLC44A2, SMAD3, SNAP23, TOM1, TP53 *, TRAPPC1, TRIM21, TXN, UBE2L3, VAMP2, VAT1, VEGFA, WASF1*	
mmu-miR-451a	*ATF2, EDAR, MIF, RAC1, WASF1, YBX1*	Predicted inhibition
mmu-miR-497a-3p	*HSP90AB1, IL19*	Predicted inhibition

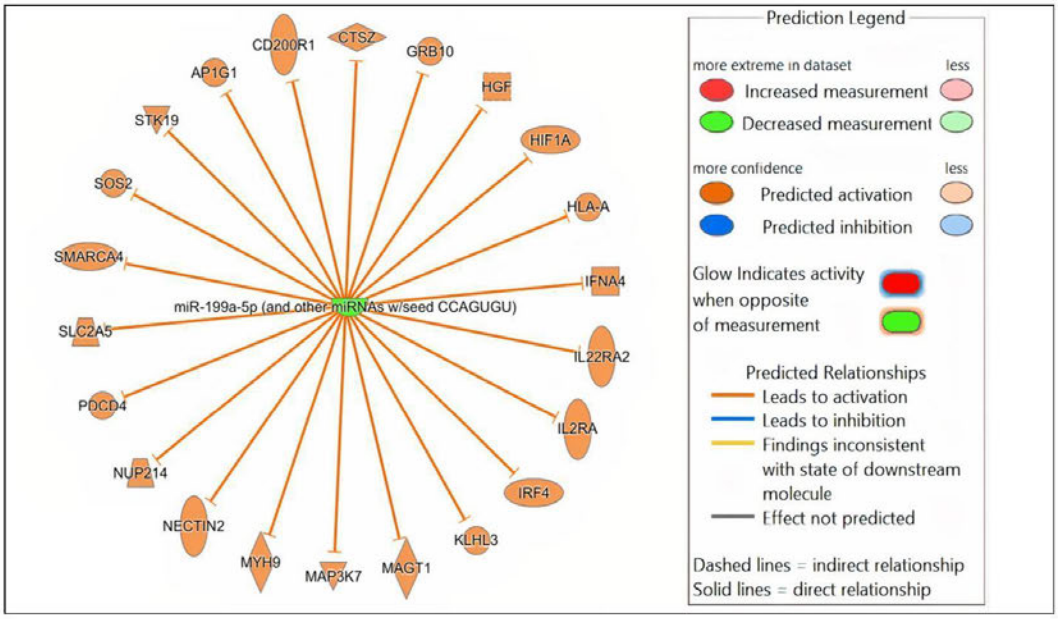

Figure 4. Network analysis. Representative graph showing the differentially expressed (DE) miRNAs-mRNA network in *Nb*/HSV-2 co-infection. The DE miRNA and the predicted targets and functional relation are represented as nodes and lines. The colour of the nodes represents their predicted expression status: orange (predicted activation) and blue (predicted inhibition). The different shapes of the nodes represent the nature of the molecules based on their activity, e.g., cytokines, enzymes, etc.

Table 3. HSV-2-infected versus uninfected: list of predicted immune-related targets for the differentially expressed miRNAs.

Name of miRNA	Predicted Immune-Related Targets	Predicted Regulatory Effect
mmu-miR-103-3p	*ARIH1, BTLA, CRKL, EEF2, FBXW11, ICOS, IL10RB, N4BP1, NUP58, PIK3CB, PRKCE, PTGS2, RAB10, SDCBP*	Predicted activation
mmu-miR-144-3p	*AP1S3, CTNNB1, EP300, HIF1A, ITCH, JUN, MAGT1, MAP3K8, MEF2A, MTOR, RAC1, RHOA, ROCK1, TNFSF11, UBE2D1, UBE2D3*	Predicted inhibition
mmu-miR-192-5p	*A1BG, DHX58, GM2A, NLRC5, RSAD2, STK3, ZEB1*	Predicted activation
mmu-miR-200b-3p	*AP1S2, ARIH1, CRKL, ELOC, MSN, PLCG1, PTEN, PTPN12, PTPN13, RAP1B, SEC23A, SNAP25, UBE2W, ZEB1*	Predicted activation
mmu-miR-205-3p	*PSMA5*	Predicted activation
mmu-miR-218-5p	*C5, CD200R1, COL1A1, DDX41, LMO7, NUP50, PIK3C2A, PLCG1, PRKG1, RAB6A, RICTOR, RNF41, RPS6KA3, SH3KBP1, SOCS3, SPSB1, UBE2H, VAT1*	Predicted activation
mmu-miR-449a-5p	*ADAM10, AP2A2, BCL2, BCL6, BTN1A1, C9, CANT1, CASP2, CCL22, CCND1, CD47, CD79A, CREB1, CSF1R, CTNNB1, DEFB124, DNM1L, FBXO17, FCER1A, GRAP2, GSTO1, IL23R, IL6R, ISG20, MAP2K1, MAP3K3, MAPT, MUC5B, MYC, MYH9, NECTIN2, ORMDL3, PTPN4, RNF4, ROCK1, SIAH1, SLC44A2,*	Predicted inhibition * Predicted activation (*TP53*)

	*SMAD3, SNAP23, TOM1, TP53 *, TRAPPC1, TRIM21, TXN, UBE2L3, VAMP2, VAT1, VEGFA, WASF1*	
mmu-miR-376a-3p	*CTSO, IL6, KIF5A, TRIM9*	Predicted inhibition
mmu-miR-451a	*ATF2, EDAR, MIF, RAC1, WASF1, YBX1*	Predicted inhibition

Table 4. *Nb*/HSV-2 co-infected versus uninfected: list of predicted immune-related targets for the differentially expressed miRNAs.

Name of miRNA	Predicted Immune-Related Targets	Predicted Regulatory Effect
mmu-miR-199a-5p	*AP1G1, CD200R1, CTSZ, GRB10, HGF, HIF1A, HLA-A, IFNA4, IL22RA2, IL2RA, IRF4, KLHL3, MAGT1, MAP3K7, MYH9, NECTIN2, NUP214, PDCD4, SLC2A5, SMARCA4, SOS2, STK19*	Predicted activation

All the DE miRNAs and their predicted targets for each group-wise comparison were used in IPA for the subsequent core expression analysis. For each group-wise comparison, target genes of upregulated and downregulated miRNAs were collectively analysed.

In the comparison of *Nb*-infected versus uninfected FGT tissues, immune-related canonical pathways were significantly enriched (p-value < 0.05). "Neutrophil degranulation" was the canonical pathway with the highest significance. Other immune-related canonical pathways, including "interleukin-4 and interleukin-13 signaling", were also significantly enriched. Among the top diseases and biological functions, "inflammatory response" (diseases and disorders), "cell-to-cell signaling and interaction" (molecular and cellular function), and "haematological system development and function" (physiological system development and function) were identified.

In the comparison of HSV-2-infected versus uninfected FGT tissues, "role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis" was the canonical pathway with the highest significance, followed by "neutrophil degranulation", and "hepatitis B chronic liver pathogenesis signaling pathway". Among the top diseases and biological functions, "inflammatory response" (diseases and disorders), "cell death and survival" (molecular and cellular function), and "haematological system development and function" (physiological system development and function) were identified.

In the comparison of *Nb*/HSV-2 co-infected versus uninfected FGT tissues, the most significant canonical pathway was the "ISGylation signaling pathway". Immune-related canonical pathways including "natural killer cell signaling", and "interferon alpha/beta signaling" were also significantly enriched. Among the top diseases and biological functions, "cancer" (diseases and disorders), "cell-to-cell signaling and interaction" (cellular and molecular function), and "haematological system development and function" (physiological system development and function) were identified. The top 5 canonical pathways, diseases, molecular and cellular functions, and physiological system development and functions of each comparison are shown in Tables 5–7. Networks of the top canonical pathway identified in each comparison are shown in Figures 5–7.

Table 5. *Nb*-infected versus uninfected: summary of core expression analysis.

Top Canonical Pathways		
Name	***p*-value ***	**Overlap †**
Neutrophil degranulation	1.60×10^{-18}	4.8% (23/477)
Role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis	1.16×10^{-16}	5.6% (19/337)
Hypoxia signaling in the cardiovascular system	3.49×10^{-16}	15.4% (12/78)
Interleukin-4 and interleukin-13 signaling	7.80×10^{-16}	11.7% (13/111)
NGF Signaling	2.47×10^{-15}	10.7% (13/121)
Top Diseases and Biological Functions		
Diseases and disorders		

Name	***p*-value range ****	**No. of Molecules ‡**
Inflammatory Response	1.85×10^{-8}–2.05×10^{-36}	87
Infectious Diseases	2.83×10^{-8}–1.73×10^{-29}	76
Organismal Injury and Abnormalities	3.24×10^{-8}–1.73×10^{-29}	107
Cancer	3.24×10^{-8}–9.38×10^{-21}	89
Haematological Disease	3.10×10^{-8}–2.56×10^{-20}	62
Molecular and Cellular Functions		
Name	***p*-value range ****	**No. of Molecules ‡**
Cell-To-Cell Signaling and Interaction	2.03×10^{-8}–3.36×10^{-29}	74
Cell Death and Survival	3.13×10^{-8}–1.61×10^{-28}	77
Cellular Development	3.24×10^{-8}–3.51×10^{-27}	80
Cellular Growth and Proliferation	3.24×10^{-8}–3.51×10^{-27}	81
Cellular Movement	3.31×10^{-8}–3.05×10^{-26}	70
Physiological System Development and Function		
Name	***p*-value range ****	**No. of Molecules ‡**
Haematological System Development and Function	3.15×10^{-8}–2.36×10^{-32}	80
Tissue Morphology	3.15×10^{-8}–2.36×10^{-32}	68
Haematopoiesis	2.06×10^{-8}–3.51×10^{-27}	47
Lymphoid Tissue Structure and Development	3.15×10^{-8}–3.51×10^{-27}	60
Tissue Development	3.24×10^{-8}–3.51×10^{-27}	66

Footnote: * Indicates how statistically significant the enrichment is for a particular pathway/function. Calculated using the right-tailed Fisher's exact test; ** Range of significant *p*-values for the diseases/functions grouped in a specific category; † Refers to the number of genes from the input dataset that are found in a pathway/function compared to the total number of genes known to be involved in that pathway/function; ‡ Refers to the count of molecules (genes/proteins) from the input dataset that are associated with a specific pathway/disease/function.

Table 6. HSV-2-infected versus uninfected: summary of core expression analysis.

Top Canonical Pathways		
Name	***p*-value ***	**Overlap †**
Role of Macrophages, Fibroblasts, and Endothelial Cells in Rheumatoid Arthritis	8.68×10^{-19}	6.5% (22/337)
Neutrophil degranulation	7.97×10^{-18}	5.0% (24/477)
Hepatitis B Chronic Liver Pathogenesis Signaling Pathway	4.28×10^{-17}	9.0% (17/188)
Hypoxia Signaling in the Cardiovascular System	6.96×10^{-17}	16.7% (13/78)
Hepatic Fibrosis Signaling Pathway	7.52×10^{-17}	5.3% (22/416)
Top Diseases and Biological Functions		
Diseases and disorders		
Name	***p*-value range ****	**No. of Molecules ‡**
Inflammatory Response	4.64×10^{-9}–4.75×10^{-37}	100
Infectious Diseases	8.67×10^{-10}–1.66×10^{-31}	86
Organismal Injury and Abnormalities	5.03×10^{-9}–1.66×10^{-31}	127
Cancer	5.03×10^{-9}–5.40×10^{-22}	108
Tumor Morphology	1.99×10^{-9}–3.40×10^{-20}	38
Molecular and Cellular Functions		
Name	***p*-value range ****	**No. of Molecules ‡**
Cell Death and Survival	4.92×10^{-9}–4.28×10^{-30}	89
Cell-To-Cell Signaling and Interaction	4.51×10^{-9}–1.61×10^{-28}	85
Cellular Movement	4.64×10^{-9}–4.39×10^{-28}	82
Cellular Development	5.00×10^{-9}–2.13×10^{-26}	99
Cellular Growth and Proliferation	5.00×10^{-9}–2.13×10^{-26}	98
Physiological System Development and Function		
Name	***p*-value range ****	**No. of Molecules ‡**

Haematological System Development and Function	4.64×10^{-9}–1.17×10^{-35}	90
Tissue Morphology	3.75×10^{-9}–1.17×10^{-35}	80
Immune Cell Trafficking	4.64×10^{-9}–2.46×10^{-27}	66
Haematopoiesis	1.06×10^{-9}–2.13×10^{-26}	53
Lymphoid Tissue Structure and Development	3.75×10^{-9}–2.13×10^{-26}	70

Footnote: * Indicates how statistically significant the enrichment is for a particular pathway/function. Calculated using the right-tailed Fisher's exact test; ** Range of significant *p*-values for the diseases/functions grouped in a specific category; † Refers to the number of genes from the input dataset that are found in a pathway/function compared to the total number of genes known to be involved in that pathway/function; ‡ Refers to the count of molecules (genes/proteins) from the input dataset that are associated with a specific pathway/disease/function.

Table 7. *Nb*/HSV-2 co-infected versus uninfected: summary of core expression analysis.

Top Canonical Pathways		
Name	***p*-value ***	**Overlap †**
ISGylation Signaling Pathway	3.25×10^{-8}	4.6% (5/109)
Glucocorticoid Receptor Signaling	4.96×10^{-7}	1.2% (7/600)
Hepatitis B Chronic Liver Pathogenesis Signaling Pathway	1.89×10^{-5}	2.1% (4/188)
Natural Killer Cell Signaling	2.55×10^{-5}	2.0% (4/203)
Interferon alpha/beta Signaling	3.75×10^{-5}	3.9% (3/76)
Top Diseases and Biological Functions		
Diseases and disorders		
Name	***p*-value range ****	**No. of Molecules ‡**
Cancer	5.15×10^{-3}–5.25×10^{-13}	21
Haematological Disease	5.15×10^{-3}–5.25×10^{-13}	17
Immunological Disease	4.90×10^{-3}–5.25×10^{-13}	18
Organismal Injury and Abnormalities	5.15×10^{-3}–5.25×10^{-13}	23
Inflammatory Response	5.15×10^{-3}–3.81×10^{-10}	20
Molecular and Cellular Functions		
Name	***p*-value range ****	**No. of Molecules ‡**
Cell-To-Cell Signaling and Interaction	5.15×10^{-3}–3.81×10^{-10}	14
Cellular Development	5.15×10^{-3}–6.04×10^{-10}	20
Cellular Growth and Proliferation	5.15×10^{-3}–6.04×10^{-10}	20
Cellular Function and Maintenance	5.15×10^{-3}–4.91×10^{-8}	16
Cellular Movement	5.15×10^{-3}–6.64×10^{-8}	15
Physiological System Development and Function		
Name	***p*-value range ****	**No. of Molecules ‡**
Haematological System Development and Function	5.15×10^{-3}–3.81×10^{-10}	18
Immune Cell Trafficking	4.29×10^{-3}–3.81×10^{-10}	14
Lymphoid Tissue Structure and Development	5.15×10^{-3}–3.63×10^{-9}	15
Tissue Morphology	4.94×10^{-3}–5.17×10^{-9}	16
Haematopoiesis	5.15×10^{-3}–3.17×10^{-7}	10

Footnote: * Indicates how statistically significant the enrichment is for a particular pathway/function. Calculated using the right-tailed Fisher's exact test; ** Range of significant *p*-values for the diseases/functions grouped in a specific category; † Refers to the number of genes from the input dataset that are found in a pathway/function compared to the total number of genes known to be involved in that pathway/function; ‡ Refers to the count of molecules (genes/proteins) from the input dataset that are associated with a specific pathway/disease/function.

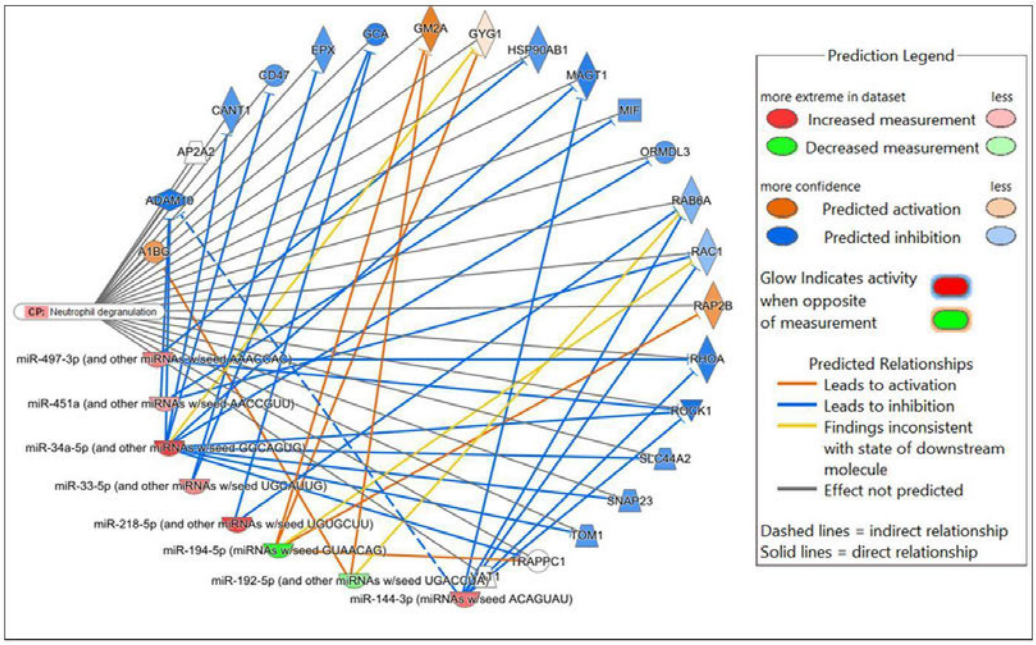

Figure 5. Network displaying miRNA-predicted mRNA targets involved in the neutrophil degranulation canonical pathway in the comparison of *Nb*-infected versus uninfected FGT tissues.

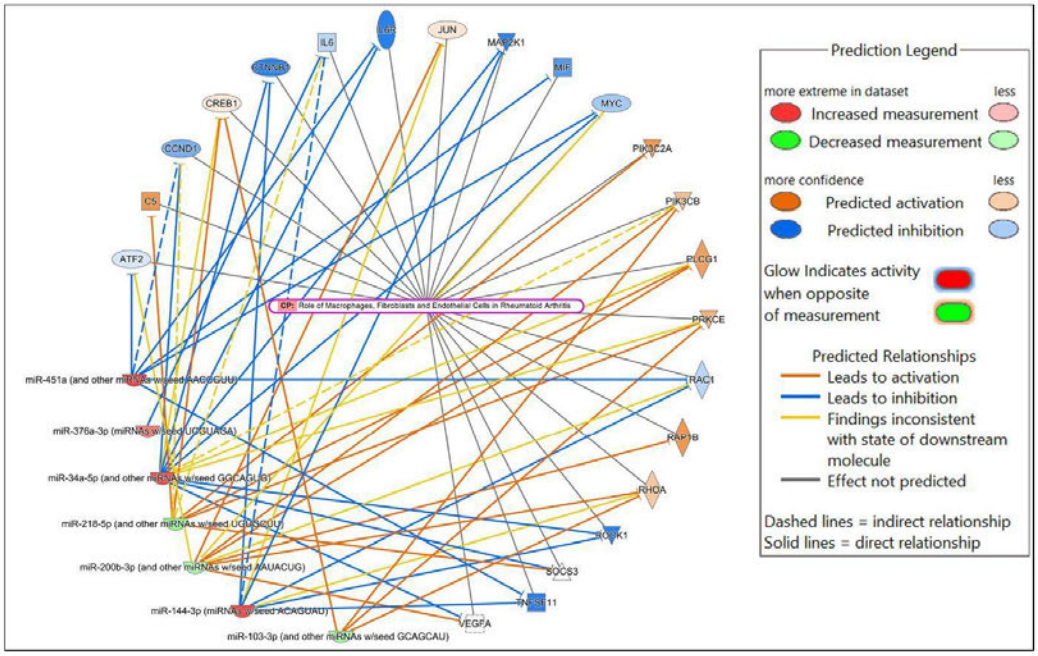

Figure 6. Network displaying miRNA-predicted mRNA targets involved in regulating the role of macrophages, fibroblasts and endothelial cells in rheumatoid arthritis canonical pathway in the comparison of HSV-2-infected versus uninfected FGT tissues.

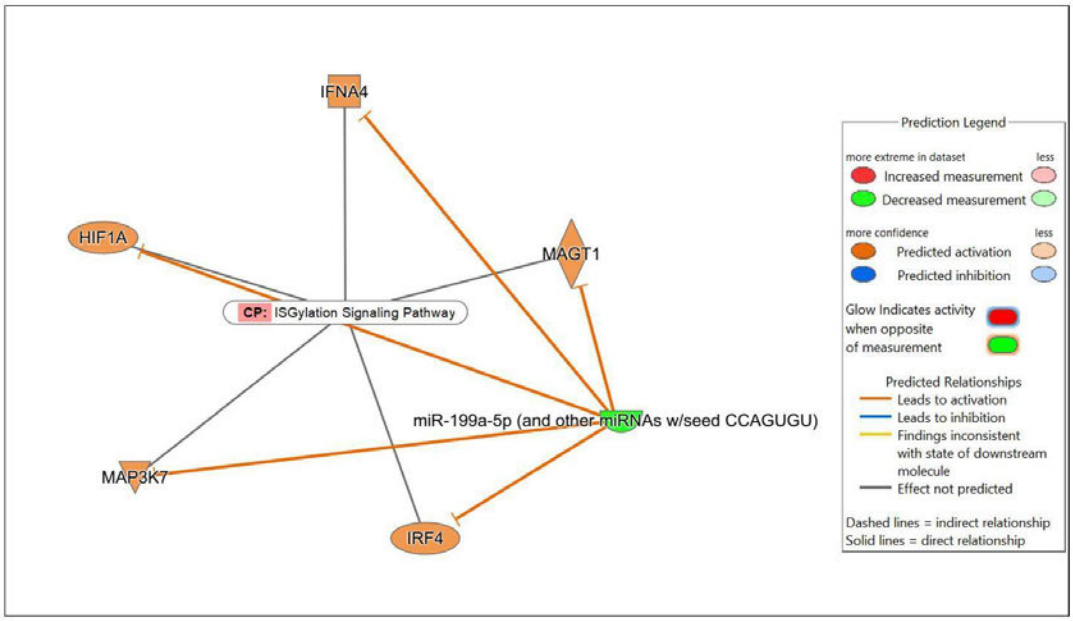

Figure 7. Network displaying miRNA-predicted mRNA targets involved in regulating the ISGylation signaling pathway in the comparison of *Nb*/HSV-2 co-infected versus uninfected FGT tissues.

3.5. Confirmation of DE miRNAs Using Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)

The RT-qPCR method was used to validate five selected DE miRNAs, comprising two common to both the *Nb*- and HSV-2-infected groups, one unique to each of the single infections, and the single DE miRNA in the co-infected group. These included mmu-miR-192-5p, mmu-miR-194-5p, mmu-miR-218-5p, mmu-miR-200b-3p, and mmu-miR-199a-5p. The RT-qPCR results exhibited a consistent trend in expression changes with those observed in the miRNA sequencing results, with significant results when comparing mmu-miR-218-5p (p-value = 0.0061) and mmu-miR-192-5p (p-value = 0.0004) in the *Nb*-infected versus uninfected groups. Significant values were also observed when comparing mmu-miR-218-5p (p-value = 0.0100) and mmu-miR-192-5p (p-value = 0.0006) in the HSV-2-infected versus uninfected groups, and mmu-miR-199a-5p (p-value = 0.0001) in the *Nb*/HSV-2 co-infected versus uninfected groups (Figure 8).

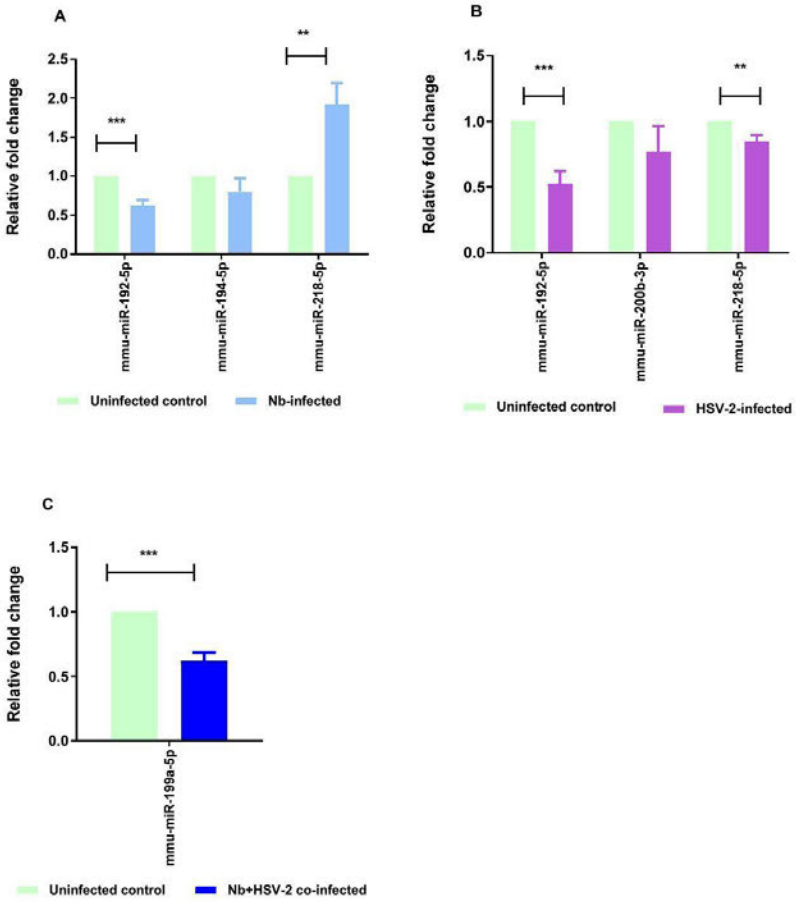

Figure 8. RT-qPCR validation. RT-qPCR was performed to validate the results of the miRNA-sequencing. Five miRNAs, representative of differentially expressed miRNAs from single *Nb* and HSV-2 infections and co-infection, (mmu-miR-192-5p, mmu-miR-194-5p, mmu-miR-218-5p, mmu-miR-200b-3p, and mmu-miR-199a-5p) were selected for validation. The following comparisons are shown: (**A**) *Nb*-infected versus uninfected, (**B**) HSV-2-infected versus uninfected, and (**C**) *Nb*/HSV-2 co-infected versus uninfected. The relative expression levels were normalized to U6, which was used as the internal control. The data are expressed as mean ± SEM, *** $p < 0.001$; ** $p < 0.01$.

4. Discussion

In this study, we investigated miRNA expression profiles in the murine FGT following single and co-infection with *Nb* and HSV-2. Differential expression analysis of miRNA-sequencing data revealed 8 DE miRNAs in *Nb*-infected, 9 in HSV-2-infected, and 1 in co-infected tissues compared to uninfected controls ($p < 0.05$, $|\log FC| \geq 1$). IPA predicted immune-related target genes and identified enrichment in pathways such as neutrophil degranulation, IL-4 and IL-13 signaling, natural killer cell signaling, interferon alpha/beta signaling, and ISGylation. Cancer was also identified as a significantly enriched disease category, particularly in the co-infected group. Selected miRNAs were validated by RT-qPCR, supporting the reliability of the sequencing data. A detailed discussion of

the immune- and pathology-associated DE miRNAs and predicted pathways identified in our study follows below.

4.1. Immune-Related DE miRNAs and Pathways

In this study, we investigated FGT miRNA expression profiles of four different groups: (1) singly infected with *Nb*, (2) singly infected with HSV-2, (3) co-infected with *Nb* and HSV-2, and (4) uninfected control groups. *Nb*, which is closely related to human hookworm, induces a transient, self-limiting infection characterized by a strong Th2 immune response. Clinical symptoms are mild and short-lived, with the infection typically resolving within 10–13 days [40]. In contrast, intravaginal HSV-2 infection in female mice, results in a localized infection that typically produces visible symptoms between days 3 and 5 post-infection [17,41]. In our study, the absence of overt clinical symptoms such as significant weight loss and pathology at day 2 post-HSV-2 infection is consistent with previous studies, which indicate that early-stage HSV-2 infection in murine models presents with minimal clinical manifestations [17].

We used NGS to characterize the miRNA profiles of FGT tissues of BALB/c mice singly infected with either *Nb* or HSV-2 or co-infected with both pathogens. We further identified the predicted target mRNAs of DE miRNAs and associated canonical pathways, diseases, and biological functions using IPA. Previous studies have only reported on the immunoregulatory roles of miRNAs during single infection with STHs [42–46] or HSV-2 [27,47,48], thus highlighting the critical role of miRNAs in regulating host cellular changes and immune responses during either infection. To our knowledge, this is the first study to examine miRNA expression profiles within murine FGT tissues following *Nb*/HSV-2 co-infection.

In this study, we demonstrated that miRNA expression profiles in the FGT were dysregulated following single infections with *Nb* or HSV2, as well as *Nb*/HSV-2 co-infection. Specifically, we observed both differences and similarities in the DE miRNA signatures based on the type of infection. During single *Nb* infection, 8 miRNAs (mmu-miR-194-5p, mmu-miR-218-5p, mmu-miR-449a-5p, mmu-miR-192-5p, mmu-miR-497a-3p, mmu-miR-144-3p, mmu-miR-33-5p, and mmu-miR-451a) were DE when compared to the uninfected control group. During single HSV-2 infection, 9 miRNAs (mmu-miR-192-5p, mmu-miR-451a, mmu-miR-449a-5p, mmu-miR-218-5p, mmu-miR-144-3p, mmu-miR-376a-3p, mmu-miR-205-3p, mmu-miR-103-3p, and mmu-miR-200b-3p) were DE when compared to the uninfected control group. We also observed 5 common DE miRNAs (mmu-miR-144-3p, mmu-miR-192-5p, mmu-miR-218-5p, mmu-miR-449a-5p, and mmu-miR-451a) during single *Nb* infection and single HSV-2 infection. Interestingly, among the common DE miRNAs, only miR-218-5p showed opposite expression patterns in response to single *Nb* infection and single HSV-2 infection.

Although most of the DE miRNAs identified in our study have not yet been described in the context of *Nb* or HSV-2 infections, we identified several miRNAs with established roles in immune responses and inflammation. Firstly, among the common DE upregulated miRNAs, miR-144-3p modulated immune responses to bacterial and viral infections by influencing processes such as autophagy, lipid metabolism, cytokine and chemokine production, and inflammation [49–52]. Upregulated levels of miR-449a-5p in the female reproductive tract have been reported in previous studies [53–55]. In other studies, miR-449a-5p was shown to regulate inflammatory responses in recipient T cells and promote apoptosis through the AKT signaling pathway [56,57]. Studies have reported that miR-451a plays a role in immune responses by modulation of neutrophil chemotaxis and macrophage M2 polarization [58,59]. Upregulated levels of miR-451a have been reported in sepsis and in response to both Gram-positive and Gram-negative microorganisms [60–62]. Previously, it was reported that miR-192-5p alleviated asthma-induced inflammation

in a murine model. Its overexpression was associated with decreased levels of ovalbumin-specific IgE, IL-4, IL-5, and IL-13, contributing to improved asthma outcomes by limiting airway remodeling and autophagy through the downregulation of matrix metalloproteinase (MMP)-16 and autophagy related 7 (ATG7) [63]. Similarly, upregulation of miR-192-5p was observed in serum exosomes of individuals with non-alcoholic fatty liver disease (NAFLD), where it was essential for activation of pro-inflammatory macrophages and NAFLD progression through the regulation of the Rictor/Akt/FoxO1 signaling pathway [64]. Serum miR-192-5p expression was significantly linked to various hepatitis B virus (HBV) infection marker levels and was identified as a biomarker for pegylated-IFN efficacy in chronic HBV treatment, thereby suggesting its role in HBV replication and antiviral immunity [65]. In a murine model of *Schistosoma japonicum* infection, miR-192-5p was highly abundant in the plasma extracellular vesicles of infected mice compared to uninfected mice [66]. In contrast, we observed a downregulation of miR-192-5p in both *Nb*-infected and HSV-2-infected FGT tissues and the predicted activation of several immune-related genes, including *A1BG*, *DHX58*, *GM2A*, *NLRC5*, *RSAD2*, *STK3*, and *ZEB1*. Among these, *ZEB1* has been described during helminth infection, where TLR9 stimulation increased *ZEB1* expression in cDC1 dendritic cells. *ZEB1* depletion led to reduced activation and production of IL-6, IL-10, and IL-12 and a shift in $CD4^+$ T helper cells toward a Th2 phenotype [67]. The role of *ZEB1* has been studied in HSV-1 infection; the viral protein *ICP0* promoted *ZEB1* and *ZEB2* degradation, resulting in an increased expression of the miR-183 cluster [68]. In Epstein–Barr Virus (EBV) infection, interaction of *ZEB1* with the ZV element regulated the transition between latency and lytic replication [69]. Therefore, in our study, the downregulation of miR-192-5p and its regulation of genes, such as *ZEB1*, may reflect an important component of innate immune responses during *Nb* and HSV-2 infections which requires further investigation.

In addition, we identified miRNAs known to modulate the NF-κB and Toll-Like Receptor (TLR) signaling pathways (e.g., mmu-miR-194-5p, mmu-miR-218-5p, mmu-miR-200b-3p). Previously it was reported that miR-194-5p exerted an indirect effect on NF-κB by targeting *TRIM23* and *C21ORF91*, genes which are required for NF-κB induction, thereby mitigating inflammation [70]. Increased expression of miR-194-5p was associated with decreased *TRAF6* levels and suppressed TNF-α production in palmitic acid-activated THP-1 monocytes, suggesting a negative regulation of the TLR4 signaling pathway [71]. Additionally, miR-194-5p directly targeted the *TLR4* gene, regulating cytokine production in response to *Salmonella* infection [72]. In this study, we observed the downregulation of miR-194-5p and the predicted activation of its immune-related genes, including *FASLG*, *GYG1*, *IL9*, *RAP2B*, *SUMO2*, and *TAB3*. Among these, *IL9* is a characteristic Th2 cytokine produced during innate immunity and known to confer protection against helminth infections [73–75], including *Nb* [76].

Similarly, the upregulation of miR-218-5p was inversely correlated with immune defense and inflammatory responses, NF-κB pathway modulation, antigen processing and presentation, and chemokine signaling [77]. In the present study, we observed an upregulation of miR-218-5p during single *Nb* infection. This finding is particularly interesting within the broader context of STH-induced immune modulation, as STHs, including *Nb*, are known to stimulate robust Th2 responses and immunomodulatory mechanisms that suppress pro-inflammatory Th1 immune pathways [6]. Our observation of upregulation of miR-218-5p, together with the predicted downregulation of its immune-related mRNA targets, suggests a potential role for this miRNA in mediating the immunomodulatory effects associated with *Nb* and warrants further investigation into its specific regulatory functions during *Nb*-associated immune responses. In contrast, we found that miR-218-5p was downregulated during single HSV-2 infection. Our findings are consistent with previous studies, which have demonstrated a role for miR-218-5p in viral infections. For

example, in porcine reproductive and respiratory syndrome virus (PRRSV), miR-218 downregulation facilitated viral replication through inhibition of type I IFN responses [78]. In porcine epidemic diarrhoea virus (PEDV), inhibition of heterogeneous nuclear ribonucleoprotein A3 (HNRNPA3) expression through miR-218-5p enhanced cellular lipid accumulation and viral replication [79].

Studies have shown that miR-200b-3p regulates viral replication. Our observation of decreased miR-200b-3p in the HSV-2-infected FGT tissues compared to uninfected controls is consistent with previous viral studies. For example, miR-200b-3p suppressed IFN-I production, driven by NF-κB and IRF3-mediated pathways, by directly targeting the *TBK1* gene. Its inhibition was associated with increased IFN-I production and broad-spectrum antiviral effects [80]. The miR-200 family, including miR-200b-3p, targeted *ZEB1* and *ZEB2*, suppressing the *BZLF1* gene and promoting EBV reactivation [81]. Decreased miR-200b-3p levels were associated with cytopathic inflammation during human cytomegalovirus (HCMV) infection, suggesting a role in the host response to viral pathogens [82].

Appreciable changes during co-infection were not observed in this study, since only 1 DE miRNA was identified when the co-infected group was compared to the uninfected group. Interestingly, miR-199a-5p was significantly downregulated when the co-infected group was compared to the uninfected group. The role of miR-199a-5p has not been previously investigated in co-infections. However, previous studies have shown that the upregulation of miR-199a-5p facilitated hepatitis C virus (HCV) replication via the PI3K/Akt, Ras/ERK and Wnt/β-catenin pathways [83]. Following HSV-1 infection, miR-199a-5p and miR-199a-3p suppressed *ARHGAP21*, thereby regulating *Cdc42* and impairing the secondary envelopment of HSV-1, demonstrating their antiviral activity [84]. miR-199a-5p-deficient mice showed enhanced inflammatory cell infiltration, pro-inflammatory cytokine expression and impaired intestinal barrier function following dextran sulphate sodium (DSS)-induced colitis, suggesting the anti-inflammatory role of miR-199a [85]. In helminth infections, downregulated levels of miR-199a-5p were reported in *Trichinella spiralis* infection [86]. Given the known antiviral and anti-inflammatory functions of miR-199a-5p, its downregulation in the co-infected FGT tissues observed in our study, may contribute to the enhanced vaginal pathology previously reported in *Nb*/HSV-2 co-infected mice [17]. Decreased levels of miR-199a-5p may potentially impair the host's ability to effectively regulate infection-associated inflammation associated with *Nb*/HSV-2 co-infection; however, the mechanisms by which this may occur requires further investigation.

Notably, the detection of only a single DE miRNA in the co-infected group, in contrast to several identified in the *Nb* and HSV-2 single infections, potentially reflects both biological and technical influences. Biologically, we hypothesize that the opposing immune responses induced by each pathogen (Th2-mediated polarization by *Nb* and a Th1-mediated immune response by HSV-2) may counteract one another, leading to a dampened immune state and limited miRNA dysregulation. This masking effect could substantially reduce the number of DE miRNAs identified, even though biologically meaningful regulatory dynamics are at play. In support, one compelling observation was the opposing expression of miR-218-5p in single *Nb* and HSV-2 infections. Moreover, according to IPA, miR-218-5p is predicted to regulate (i.e., either activate or inhibit) its immune-related target genes depending on its direction of expression. Such opposing regulation could lead to a net expression normalization during co-infection. However, technical factors must also be acknowledged, including small sample size, biological variability, and the analysis of acute infection at a single time point, all of which may limit the detection of subtle miRNA expression changes.

Taken together, our findings are suggestive of a complex interplay between *Nb* and HSV-2 and highlight the need for more integrated and functional analyses to fully characterize regulatory mechanisms during co-infection.

In support, computational analysis also revealed that the predicted target genes and DE miRNAs were statistically enriched in biological functions, including those associated with innate immunity. This implies that these miRNAs may contribute to critical FGT mucosal defenses by mobilizing innate immune responses during infection [87]. Core expression analysis of the DE miRNAs and their predicted targets has also identified the statistical over-representation of several canonical pathways previously implicated in the host response to infection, such as neutrophil degranulation, interleukin-4 and interleukin-13 signaling, interferon alpha/beta signaling, and ISGylation signaling pathways. For example, neutrophils play a protective role in helminth infections by limiting parasite survival and spread, mainly through the formation of neutrophil extracellular traps (NETs) and neutrophil degranulation. Neutrophils are the first cells to traffic infection sites, influencing the Th2 immune response by attracting other immune cells and priming macrophages toward an M2 phenotype [88–91]. Interleukin (IL)-4 and IL-13 are essential in Th2 immunity, enhancing resistance to helminths and neutralizing toxins by promoting Th2 and T follicular helper cell differentiation, IgE production, expansion of basophils and eosinophils, mast cell activation, M2 macrophage polarization, and goblet cell hyperplasia [92]. For example, IL-4 and IL-13 produced by innate immune cells were sufficient to promote effective Th2 immunity against *Nb* [93]. ISGylation, which is a post-translational modification involving the conjugation of ISG15, is induced by type I IFNs and plays an essential role in innate antimicrobial defense and immune regulation [94].

4.2. *Pathology-Related DE miRNAs and Pathways*

MiRNAs modulate key cellular processes, including proliferation, apoptosis, differentiation, and metabolism, that are frequently dysregulated in cancer. In female reproductive cancers, altered miRNA expression has been associated with ovarian, cervical, endometrial, and vulvar cancers. Depending on context and target genes, miRNAs may function as oncogenes or tumor suppressors [23].

In our study, cancer was predicted as one of the significantly enriched diseases, most notably in the co-infected group. This enrichment potentially reflects infection-induced miRNA dysregulation of immune- and inflammation-related signaling pathways commonly associated with carcinogenesis. Potential roles in cancer for some of the DE miRNAs observed in our study can be speculated based on previous literature and the results of our analyses. For example, several miRNAs identified in our study, including miR-451a, miR-449a-5p, miR-218-5p, miR-144-3p, miR-376a-3p, miR-199a-5p, miR-205-3p, miR-103-3p, and miR-200b-3p, have been previously implicated in female reproductive cancers [23]. Several are known tumor suppressors, including miR-192-5p [95,96], miR-194-5p [97], miR-451a [98], miR-218-5p [99], miR-144-3p [100], miR-199a-5p [101,102], miR-200b-3p [103], miR-205-3p [104], and miR-449a-5p [105,106], with dysregulation linked to enhanced proliferation, invasion, and poor prognosis in cervical, ovarian, or endometrial cancers. In contrast, miR-376a-3p was significantly elevated in ovarian cancer and associated with the clinical stages of disease [107], while miR-103-3p was associated with oncogenic roles in cervical cancer by promoting tumor cell survival [108].

In the context of our study, the DE miRNAs observed in the *Nb*-infected group suggests that STH-induced immune modulation may influence host miRNA networks in ways that overlap with cancer-related signaling. The concurrent downregulation of key tumor-suppressive miRNAs (miR-194-5p, miR-192-5p) and upregulation of others (miR-218-5p, miR-449a-5p miR-497a-3p, miR-144-3p, and miR-451a) could reflect *Nb*-induced immune modulation in the FGT. This immune modulation may influence miRNA expression in a context-dependent manner, potentially contributing to a microenvironment that supports malignant transformation [14,15].

Similarly, in the HSV-2-infected group, the downregulation of key tumor suppressors (miR-192-5p, miR-218-5p, miR-205-3p, and miR-200b-3p) may impair epithelial repair and promote a microenvironment favourable to carcinogenesis, while upregulated miRNAs (miR-451a, miR-449a-5p, and miR-144-3p) may reflect antiviral or tissue-protective responses.

Importantly, in the co-infected group, the significant downregulation of miR-199a-5p may reflect an immunological interaction between *Nb* and HSV-2, which contribute to pathology and epithelial stress. As highlighted earlier, considering the established antiviral, anti-inflammatory, and tumor-suppressive roles of miR-199a-5p, its reduced expression in co-infected FGT tissues may play a role in the increased vaginal pathology previously reported in *Nb*/HSV-2 co-infected mice [17]. It is also plausible that the loss of miR-199a-5p in this context may favour an increased risk for malignant transformation, highlighting the need for further mechanistic studies.

Taken together, our study provides evidence that FGT miRNA expression profiles during single and co-infection with *Nb* and HSV-2 are altered, and that these changes could influence the regulation of host immunity to either pathogen, while potentially overlapping with pathology-related pathways, such as cancer. While our core expression analysis suggests a link between miRNA regulation and cancer pathways during single, and more notably during co-infection, these findings remain speculative and require mechanistic validation.

5. Limitations of the Study and Future Work

While the murine model of *Nb* and/or HSV-2 infection replicates only certain aspects of human infection [6,109], our findings highlight the significant role of miRNA regulation and expression in single and co-infection settings. These insights may have broader implications for host immunity and pathogenesis for humans. Notably, our study provides evidence that miRNAs influence host immune responses and key biological pathways during single and co-infection with *Nb* and HSV-2. Despite the strengths of this work, including (a) an NGS approach and (b) single infection and co-infection, it has some limitations. The main limitation is the limited sample size. This limitation was addressed by confirming the miRNA expression of selected miRNAs using RT-qPCR and specific primers in a validation study. In addition, biological variation between individual mice could have confounded some of our observations. We also acknowledge that a comprehensive validation of all DE miRNAs is ideal to strengthen the reliability of the miRNA-sequencing results. However, due to resource constraints, it was not feasible to validate the full complement of DE miRNA identified through miRNA-sequencing. While the consistent expression trends observed in the subset of validated miRNAs using RT-qPCR support our miRNA-sequencing data, we acknowledge the need to expand the validation of DE miRNAs and their target genes and to confirm their mechanistic roles in our future work. Additionally, while our core expression analysis suggests a potential link between miRNA regulation and cancer-related pathways, these preliminary findings remain speculative and require validation through future mechanistic studies. Therefore, although our findings are insightful in the context of STH/HSV-2 co-infection, they should be validated through larger-scale investigations. Future research should include comprehensive analyses of miRNA expression profiles in response to each infection separately and in combination to better understand pathogen-specific miRNA expression changes. In addition, research on the impact of acute versus chronic infection on miRNA expression profiles and the prognostic potential of miRNAs is required. Though these were not within the scope of our current study, our present findings form a basis for such future studies. Our future studies will focus on validating target gene expression using RNA-sequencing and evaluation of the mechanistic roles of DE miRNAs using in vitro and in vivo models.

Elucidation of the mechanisms by which DE miRNAs regulate host immunity could lead to novel approaches to control or treat STHs and HSV-2 infections in co-endemic settings.

6. Conclusions

In summary, this study demonstrates that single and co-infection with *Nb* and HSV-2 are characterized by different miRNA expression patterns, which may underlie differences in host immune responses and infection-induced pathologies. Several miRNAs were found to be significantly dysregulated in both single and co-infection settings, including some not previously reported in co-infection models. Predicted target genes were associated with immune functions, and core expression analysis revealed strong associations with biological and immunological processes. In addition, the affected pathways also intersect with those implicated in important diseases, such as cancer. Overall, our findings suggest that the DE miRNAs identified in this study play central roles in immunity in the setting of single and co-infection with *Nb* and HSV-2, underscoring the importance of miRNA-focused research in understanding host immunity to and potential pathology associated with STH/HSV-2 co-infection.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/microorganisms13081734/s1, Supplementary Figure S1: Pathology scoring was assessed between the groups post-infection; Supplementary Table S1: Summary of the numbers of reads, precursor and mature miRNAs, and the number of miRNAs reads with ≥5x coverage detected for each sample in groups: A (Uninfected controls), B (singly infected with *Nb*), C (singly infected with HSV-2), and D (*Nb*/HSV-2 co-infected); Supplementary Figure S2: Heat map illustrating the overall trend of miRNA expression changes across the four groups: Samples A3-8 (Uninfected control), B1-6 (*Nb*-infected), C2-6 (HSV-2-infected), and D1,3-6 (*Nb*/HSV-2 co-infected); Supplementary Figure S3: Network showing predicted miRNA-target mRNA relationships in the comparison of *Nb*-infected versus uninfected controls; Supplementary Figure S4: Network showing predicted miRNA-target mRNA relationships in the comparison of HSV-2-infected versus uninfected controls.

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CHAPTER FOUR
RESULTS

4.1 Exploring gene expression changes in murine female genital tract tissues following single and co-infection with *Nippostrongylus brasiliensis* and Herpes Simplex Virus type 2

As highlighted earlier, soil-transmitted helminths (STHs) and herpes simplex virus type 2 (HSV-2) elicit opposing host immune responses – HSV-2 typically requires a robust T-helper 1 (Th1) pro-inflammatory response for effective anti-viral control, while STHs typically induce a dominant T-helper 2 (Th2) and anti-inflammatory phenotype. STH-driven Th2 immune responses are known to downregulate Th1 anti-viral responses with subsequent deleterious outcomes (Chetty et al., 2020). In the setting of STH/HSV-2 co-infection, these opposing immune responses may potentially impact host control of HSV-2 (Chetty et al., 2021). Gene expression changes, particularly at the primary site of HSV-2 infection, can help identify localized changes associated with the immune and disease pathways. In this context, studying the changes in gene expression profiles in the female genital tract (FGT) can improve our understanding of how the host responds to co-infection with both pathogens. The article entitled "**Exploring gene expression changes in murine female genital tract tissues following single and co-infection with *Nippostrongylus brasiliensis* and Herpes Simplex Virus type 2**" was published in **MDPI *Pathogens***. This aspect of work investigated changes in gene expression profiles in response to single and co-infection with *Nippostrongylus brasiliensis* (*Nb*) and HSV-2 in the FGT, using a BALB/c murine model. By leveraging high-throughput sequencing, the study aimed to delineate the gene expression signatures associated with each infection scenario. In addition, various bioinformatics tools were employed to predict the protein-protein interactions, hub genes and signaling pathways influenced by differentially expressed genes that may play a critical role in mediating host immunity. Collectively, the analyses focused on identifying key immune-related genes and disease pathways that are modulated during these infections, providing insights into how the FGT transcriptome changes in response to either *Nb* or HSV-2 or *Nb*/HSV-2 challenges.

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Exploring Gene Expression Changes in Murine Female Genital Tract Tissues Following Single and Co-Infection with *Nippostrongylus brasiliensis* and Herpes Simplex Virus Type 2

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Abstract

Background and Aim: The immunological interactions between soil-transmitted helminths (STHs) and herpes simplex virus type 2 (HSV-2), particularly in the context of co-infection, are poorly understood. Next-generation sequencing (NGS) offers a powerful approach to explore these complex immune responses and uncover potential therapeutic targets. This study leveraged NGS and bioinformatic tools to investigate transcriptional changes and immunological pathways in female genital tract (FGT) tissues of BALB/c mice acutely infected with *Nippostrongylus brasiliensis* (*Nb*), HSV-2, or co-infected. **Methods:** Total RNA was harvested from FGT tissues of BALB/c mice infected with *Nb*, HSV-2, co-infected with both pathogens, and uninfected controls. Differentially expressed genes (DEGs) were identified by comparing uninfected versus infected FGT tissues in R using edgeR and limma packages. Immune-related genes were identified by intersecting DEGs in each group-wise comparison with immune function gene sets derived from the Mouse Genome Informatics (MGI) database. Functional and pathway enrichment analyses were performed with g: Profiler and protein–protein interaction networks were built using the STRING database and visualized with Cytoscape. Key hub genes and significant gene modules were identified using the Cytoscape plugins CytoHubba and MCODE, followed by further functional analysis of these modules. **Results:** NGS analysis revealed distinct gene expression profiles in response to single infection with *Nb* or HSV-2, with both showing significant differences when uninfected controls were compared to infected FGT tissues at a 5% false discovery rate. Notably, there were no significant differences in gene expression profiles between uninfected and co-infected FGT tissues. In the comparison of uninfected versus *Nb*-infected FGT tissues, 368 DEGs were identified, with 356 genes upregulated and 12 downregulated. Several immune-related genes, such as *Ptprc*, *Ccl11*, *Ccr2*, and *Cx3cr1*, were significantly altered. Pathway analysis of DEGs, hub genes, and significant modules indicated modulation of immune and defense responses. Notably, *Nb* infection induced a robust Th2-dominant immune response in the FGT, with downregulation of pro-inflammatory genes. This likely reflects helminth-driven modulation that may impair protective Th1 responses and highlights the systemic impact of *Nb* on the FGT immunity. In the comparison of uninfected versus HSV-2-infected FGT tissues, 140 DEGs were identified, with 121 upregulated and 19 downregulated. Immune-related genes, including *Ldlr*,

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Camk1d, *Lrp8* and *Epg5*, were notably altered. HSV-2 infection led to early and predominant downregulation of immune genes, consistent with viral immune evasion strategies. In addition, functional analysis revealed enrichment in cell cycle and sterol biosynthesis pathways, suggesting that HSV-2 modulates host metabolism to support viral replication while influencing immune responses. In co-infection, no significant transcriptional changes were observed, potentially reflecting immune antagonism where *Nb*-induced Th2 responses may suppress HSV-2-driven Th1 immune responses. **Conclusions:** This preliminary study offers insights into the gene expression responses in the FGT to acute single and co-infection with *Nb* and HSV-2. Together, these findings reveal distinct transcriptomic changes in the FGT following *Nb* and HSV-2 infection, with co-infection potentially leading to immune antagonism and transcriptional equilibrium. This highlights the complex interplay between helminth- and virus-induced immune modulation in shaping FGT immunity. By leveraging NGS, this study highlights important immune-related pathways and serves as a foundation for further investigations into the mechanistic roles of DEGs in immunity to these pathogens, with potential implications for developing novel therapeutic strategies.

Keywords: mRNA expression; soil-transmitted helminths; herpes simplex virus type 2/HSV-2; *Nippostrongylus brasiliensis*; co-infection; next-generation sequencing

1. Introduction

Globally, more than 491.5 million people are infected with herpes simplex virus type-2 (HSV-2) [1], while approximately 1.5 billion people are infected with soil-transmitted helminths (STHs) [2]. The highest prevalences of these infections are observed in countries with low socio-economic status and inadequately resourced healthcare facilities, most notably within sub-Saharan Africa [1,3]. Co-infection with HSV-2 and STHs is possible because both pathogens inhabit the same geographical regions, and they can co-exist within the same host [4]. However, immunological effects of co-infection with HSV-2 and STHs are poorly understood as epidemiological and immunological studies are lacking.

Primary infection with genital HSV-2 can manifest as painful genital lesions but is asymptomatic in most individuals [5]. Once infection is established, T-helper type 1 (Th1) HSV-2-specific immunity is induced, engaging both innate and adaptive immune mechanisms. During the early innate immune response, infected genital epithelial cells release type I interferons (IFN-α and IFN-β), which help suppress viral replication and attract immune cells to the site of infection. Natural killer (NK) cells target and eliminate virally infected cells early, and pro-inflammatory cytokines, such as IL-1, IL-6, and TNF-α, promote inflammation and recruit neutrophils and macrophages [6]. Dendritic cells and other innate immune cells detect the virus, activating adaptive immunity by presenting antigens to lymphocytes [7]. Adaptive immunity features IFN-γ-producing $CD4^+$ T cells, which boost macrophage and cytotoxic T cell activity, and $CD8^+$ T cells, which directly target and eliminate infected cells through viral antigen recognition [6]. Additionally, B cells produce neutralizing antibodies (IgG and IgA) to block viral entry and mediate antibody-dependent cellular cytotoxicity, collectively limiting viral spread. However, HSV-2 is capable of evading immune detection through several mechanisms, including suppressing MHC class I expression to reduce cytotoxic T lymphocyte activity and producing proteins that block type I IFN signaling, thereby dampening antiviral responses [6–8]. Additionally, during primary infection, the virus travels to the sensory neurons and ganglia, where it establishes lifelong latent infection. As a result, while the immune response can control

viral replication and limit reactivation, it is unable to completely eradicate the virus, leading to recurrent infections [5].

In contrast, STHs primarily induce a dominant T-helper type 2 (Th2) immune response, comprising both innate and adaptive immune mechanisms. The innate response to STHs comprises the release of cytokines, such as IL-4, IL-5, and IL-13, which drive eosinophilia, mast cell activation, and IgE production, which are key mechanisms required for the expulsion of parasites. IL-13 stimulates mucus production and the contraction of intestinal smooth muscles, aiding in parasite expulsion, while IL-5 enhances eosinophil activity to kill larvae or adult worms [9,10]. Additionally, adaptive immunity further supports this process by promoting Th2 cell development and associated cytokine secretion, facilitating parasite clearance and reducing the risk of reinfection [9]. Moreover, regulatory T and B cell populations, and alternately activated macrophages reduce excessive inflammation, creating a hyporesponsive and immune-tolerant environment by producing anti-inflammatory cytokines, such as IL-10, and transforming growth factor beta (TGF-β). Consequently, STHs often modulate host immunity to bystander infections [11].

STHs can modulate host immune responses to sexually transmitted viral infections. For example, in individuals co-infected with STHs and human immunodeficiency virus (HIV), the adverse effects associated with HIV may be intensified due to reduced $CD4^+$ cell counts, disrupted immune cell function, and elevated HIV viral loads [12,13]. Similarly, hookworm infection was linked to an increased risk of human papillomavirus (HPV) infection and elevated HPV viral loads, and mixed type 1/type 2 immune responses were observed in the vaginal tracts of STH/HPV co-infected women [14–16]. Co-infection with STHs and HSV-2, however, is less understood. Although limited, experimental studies have reported that in co-infected female mice, *Nippostrongylus brasiliensis* (*Nb*), a close relative of the human hookworms, exacerbated genital HSV-2. Co-infection with *Nb* and HSV-2 resulted in heightened vaginal ulceration and was associated with higher levels of IL-5 and IL-13 and the accumulation of eosinophils in the vaginal tract [17]. However, there is a lack of data on the immunological interactions at the mRNA gene expression level in STH/HSV-2 co-infected individuals. To address this gap and recognizing that the molecular mechanisms underpinning these interactions remain poorly understood, this preliminary study sought to investigate and identify the major transcriptomic features associated with *Nb* and HSV-2 single- and co-infection, using next-generation sequencing (NGS). Herein, we describe changes in gene expression profiles of the murine female genital tract following acute *Nb* and HSV-2 single and co-infection. Understanding the immunological interactions between STHs and HSV-2 at a genomic level is relevant to effective infection control and elimination.

2. Materials and Methods

2.1. Ethical Approval

Ethical clearance for the study was obtained from the Animal Ethics Committee at the University of Cape Town (UCT) (reference number: FHS AEC REF NO: 021_012) and the Animal Research Ethics Committee at the University of KwaZulu Natal (reference number: AREC/00005911/2023). Additionally, authorization under Section 20 dispensation to perform animal research at UCT was granted by the South African Department of Agriculture, Land Reform and Rural Development [reference number: 12/11/1/7/1 (6151KL)]. All experimental procedures were conducted at the Institute of Infectious Diseases and Molecular Medicine at the UCT by researchers certified by the South African Veterinary Council.

2.2. Experimental Models

2.2.1. Animals

All experiments in this study were conducted using female BALB/c mice (n = 6 per group; age, 6–10 weeks old; weight, 18–20 g). The mice were bred in-house under specific pathogen-free conditions at the UCT Research Animal Facility in South Africa. The mice were group-housed, with 6 mice per cage and food and water were provided *ad libitum*. A total of 24 mice were randomly sorted into four experimental groups, namely (1) singly infected with *Nb*, (2) singly infected with HSV-2, (3) co-infected with *Nb* and HSV-2, and (4) uninfected control groups.

All experimental procedures were conducted as previously described [17]. Firstly, to synchronize the estrous cycles, mice were subcutaneously treated with 2 mg Medroxyprogesterone Acetate (Depo Provera®) (Sigma-Aldrich, St. Louis, MO, USA) 7 days before the start of experimental procedures. Seven days later, mice were subcutaneously infected with 500 L3 *Nb* larvae. Seven days post-*Nb* infection, mice were infected with HSV-2 Strain G. All mice were sacrificed two days after HSV-2 challenge [17]. The FGT tissues (excluding ovaries) were isolated, preserved in RNAlater (Qiagen, Venlo, The Netherlands), kept at 4 °C overnight, and then stored at −80 °C until further analysis.

2.2.2. *Nippostrongylus brasiliensis* (Nb) Maintenance and Infection

Nb was maintained in male Wistar rats as previously described [17]. Here, rats were subcutaneously infected with a dose of 5000 L3 *Nb* larvae. Following infection, stool samples were collected between days 6 to 8, which correspond with the peak production of helminth eggs. To prepare stool cultures, a mixture of stool and charcoal was placed on a moistened and raised filter paper to allow hatched L3 *Nb* larvae to migrate to the outer surface of the filter paper, where they were then collected by gently rinsing off with water. These L3 *Nb* larvae were counted under a dissecting microscope and suspended in an appropriate volume of distilled water for use in subsequent mice infections. In mice, infection with *Nb* was performed 7 days following Depo-Provera administration and 7 days before viral infection. This was performed once by subcutaneous infection using a 21G needle and a dose of 500 L3 *Nb* larvae in a total volume of 200 µL of sterile phosphate-buffered saline (PBS). Following infection with *Nb*, mice were monitored daily for changes in body weight and clinical pathology scores as indicators of infection and to ensure animal welfare.

2.2.3. Virus

Human herpesvirus 2 strain G (HSV-2, ATCC, VR-734) was cultured in Vero cells at a multiplicity of infection (MOI) of 0.1, following previously described methods [17]. Two to three days later, both cells and supernatant were collected, and viral titers were measured by plaque assay. The viral aliquots were kept at −80 °C until further use.

For HSV-2 infection, mice were first anesthetized with Xylazine (10 mg/kg) + Ketamine (100 mg/kg) intraperitoneally using a 27G needle, and then infected with 5×10^5 plaque-forming units (PFUs) of HSV-2 strain G in a total volume of 5 µL, intravaginally, using a Gilson P10 pipette and sterile 10 µL filter pipette tip. The pathogen was administered once. To collect vaginal lavages, the vaginal vaults were flushed ten times with 50 µL RNAlater; this step was performed three times. Following infection with HSV-2, mice were monitored daily for changes in body weight and clinical pathology scores as indicators of infection and to ensure animal welfare. Parameters included body weight, coat condition, breathing, mobility, evidence of barbering, and visible signs of inflammation at the sites of infection. The severity of HSV-2-associated illness was assessed by clinical characterization and scored from 0 to 5 as follows: 0—no pathology observed; 1—slight genital/perianal

erythema; 2—genital/perianal swelling and erythema; 3—genital lesions and/or visible weight loss; 4—hind limb paralysis and/or purulent lesions; 5—premoribund [17].

2.3. Total RNA Extraction, Quantification, and Quality Control

The FGT tissues (excluding ovaries) of BALB/c mice were isolated, preserved in RNAlater (Qiagen, Venlo, The Netherlands), kept at 4 °C overnight, and then stored in a −80° C freezer until further analysis. FGT tissues were homogenized in 360 µL RPL buffer (Qiagen, Venlo, The Netherlands) on ice at 30 s intervals using a handheld homogenizer set on medium speed. Total RNA was extracted from the FGT tissues using the miRNeasy Tissue/Cells Advanced Micro Kit (Qiagen, Venlo, The Netherlands), which eliminates genomic DNA and yields RNA-enriched samples, according to the manufacturer's instructions. Total RNA was eluted in a final volume of 15 µL RNAse-free water. To assess the concentration and quality of RNA samples, absorbances at 260 and 280 nm were measured with the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Purified RNA samples were kept in a −80° C freezer.

2.4. Library Preparation and RNA Sequencing

RNA library preparation, quality assessment, and sequencing were performed by the South African Medical Research Council Genomics Platform (SAMRC Genomics Platform, Cape Town, South Africa). Poly (A) + RNA was isolated from total RNA using the Dynabeads mRNA Purification Kit (Thermo Fisher Scientific, Waltham, MA, USA). Libraries were constructed using the MGIEasy RNA Directional Library Prep Set (MGI Tech Co., Ltd., Shenzhen, China) according to the manufacturer's instructions. The concentration and quality of both purified mRNA and constructed libraries were assessed by Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA) and Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA), respectively. RNA integrity was confirmed using the RNA integrity number (RIN) via the Agilent 2100 Bioanalyzer system (Agilent, Santa Clara, CA, USA). All 24 RNA samples included in this study met the quality threshold, with RIN values greater than (>) 7. Sequencing was performed on the DNBSEQ-G400 system (MGI Tech Co., Ltd., Shenzhen, China) using a paired-end sequencing method (PE100) with the DNBSEQ-G400RS High-throughput Sequencing Set (FCL PE100) (MGI Tech Co., Ltd., Shenzhen, China).

2.5. Bioinformatics Analysis

Paired-end reads for the 24 samples were generated from the SAMRC Genomics platform and transferred to the Centre for High Performance Computing (CHPC) for subsequent data analysis. Bioinformatics analyses were performed by the DIstributed PLatform in OMICS (DIPLOMICS, Cape Town, South Africa). The RNA-sequencing data were processed using HTStream (v1.3.1) for quality control and preprocessing, which comprised removing adaptor sequences, contaminants, and low-quality reads to generate high-quality clean reads suitable for downstream analysis. A comprehensive MultiQC report was compiled using MultiQC (v1.21). No QC errors were encountered. RNA-sequencing reads were provided in fastq file format. Read alignment to the mouse reference genome was performed using STAR, and gene-level quantification was conducted. Normalization was carried out in R (version 4.3.3) using edgeR and limma packages.

2.6. Differentially Expressed Gene Analysis

The R packages, edgeR and limma, were used to analyse differential gene expression between two groups. Genes were considered significantly differentially expressed if they had an adjusted *p*-value less than (<) 0.05 and log fold change (LogFC) less than (<) −1 or

greater than (>) 1, where logFC represents log2 fold change. Volcano plots and heatmaps were generated to visualize the expression profiles of differentially expressed genes (DEGs).

2.7. Identification of Immune-Related DEGs, Functional and Pathway Enrichment Analysis

To identify immune-related genes within the cohorts of DEGs, gene sets associated with immune functions were retrieved from the Mouse Genome Informatics (MGI) database. These gene sets were then intersected with the DEGs in each group-wise comparison. Functional enrichment analyses of the DEGs were performed using the online tool g: Profiler (URL: https://biit.cs.ut.ee/gprofiler/gost, accessed on 4 March 2025). The analyses encompassed Gene Ontology (GO) analysis, which included terms related to biological process (BPs), cellular component (CC), and molecular function (MF), Kyoto Encyclopaedia of Genes and Genomes (KEGG) pathway enrichment analysis, and Reactome pathway analysis. Statistical significance was assessed using the hypergeometric test or Fisher's exact test, with multiple testing correction performed using the Benjamini–Hochberg false discovery rate (FDR) method. An FDR value < 0.05 was considered as significantly enriched. The online tool, SRplot (URL: https://www.bioinformatics.com.cn/en, accessed on 20 March 2025) was then used to visualize the DEG enrichment plots.

2.8. Protein–Protein Interaction Network Construction, Hub Gene and Functional Module Identification

For each group-wise comparison, the DEGs were uploaded to the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database (version 12.0) (URL: https://string-db.org/, accessed on 2 April 2025) to predict protein–protein interaction (PPI) networks with a high confidence interaction score of 0.7. Each node in the network represents a target gene. Each edge connecting nodes indicates predicted interactions, with edge thickness reflecting interaction strength. Hub genes, which play key roles in the network, were identified using the CytoHubba plug-in in Cytoscape software (version 3.10.3). Hub genes were calculated using the maximal clique centrality (MCC) algorithm, which is considered a highly sensitive and specific algorithm for identifying hub genes [18]. The top 10 genes with the highest MCC scores were identified as hub genes. Cytoscape was then used to visualize hub gene networks, highlighting both the hub genes and their interactions. Functional modules within these networks were identified using the MCODE plug-in within Cytoscape.

3. Results

3.1. Animal Infection

This study examined gene expression profiles in murine FGT tissues across four experimental groups: (1) singly infected with *Nb*, (2) singly infected with HSV-2, (3) co-infected with both *Nb* and HSV-2, and (4) uninfected controls. Throughout the study, body weight and clinical pathology scores were monitored to track infection-associated changes. In *Nb*-infected mice, transient weight loss (<10%) was observed between days 3 and 4 post-infection, with complete recovery evident by day 9, as was anticipated. At experimental endpoint (day 2 post-HSV-2 infection), no overt clinical signs were observed, and there were no statistically significant differences in body weight or pathology scores among the four experimental groups, indicating no observable signs of distress or disease, as was anticipated (Supplementary Figure S1A,B).

3.2. Identification of DEGs

RNA-sequencing was employed to conduct an in-depth analysis of the FGT gene expression profiles of BALB/c mice with *Nb* and HSV-2 single or co-infection. Next, differential expression analysis (LogFC < −1 or >1, adjusted p-value < 0.05) of the group-

wise comparisons of (a) uninfected versus *Nb*-infected FGT tissues, (b) uninfected versus HSV-2-infected FGT tissues, and (c) uninfected versus *Nb*/HSV-2 co-infected FGT tissues was performed.

In the comparison of uninfected versus *Nb*-infected FGT tissues, a total of 368 DEGs were identified, of which 356 genes were upregulated and 12 genes were downregulated (Supplementary File S1). The top 30 DEGs are listed in Table 1. A volcano plot of the DEGs and heatmap illustrating the expression patterns of the top 30 DEGs between uninfected versus *Nb*-infected FGT tissues are shown in Figure 1A,B.

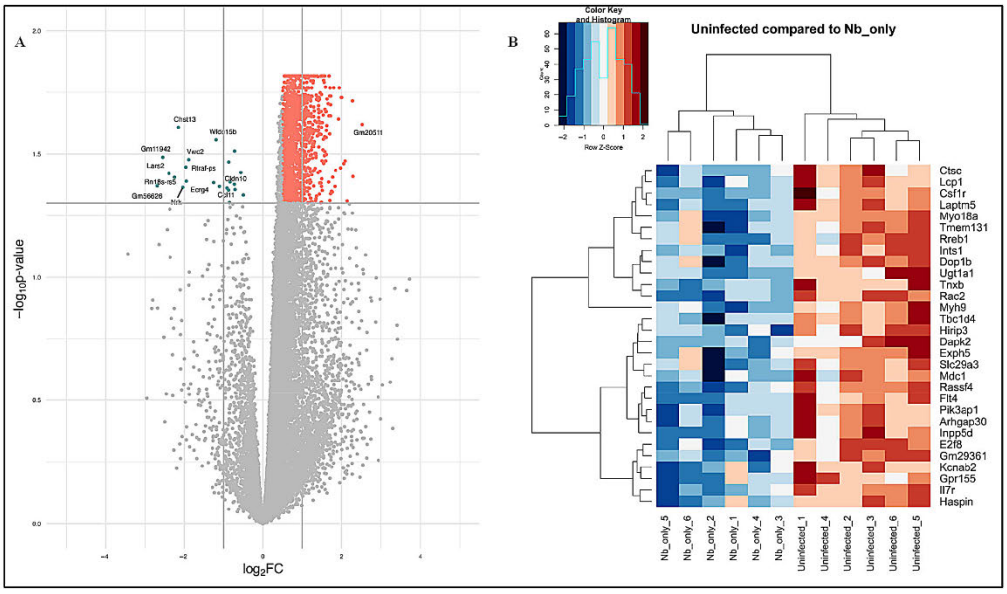

Figure 1. (**A**) Volcano plot of differentially expressed genes (DEGs) in uninfected versus *Nb*-infected FGT tissues. Orange dots represent significantly upregulated genes and blue dots represent significantly downregulated genes. Grey dots typically represent genes that do not meet the criteria for significant differential expression. (**B**) Heat map showing the top 30 DEGs in the comparison of uninfected versus *Nb*-infected FGT tissues. Z-score represents how much a gene's expression level in a particular sample deviates from the average expression level of that gene across all samples, measured in terms of standard deviations. A positive Z-score indicates higher-than-average expression, while a negative Z-score indicates lower-than-average expression. The colours on the heat map correspond to the expression levels of DEGs across all the samples with the orange colour representing a higher expression than the mean and the blue colour representing a lower expression than the mean.

In the comparison of uninfected versus HSV-2-infected FGT tissues, a total of 140 DEGs were identified, of which 121 genes were upregulated and 19 genes were downregulated (Supplementary File S2). The top 30 DEGs are listed in Table 2. A volcano plot of DEGs and heatmap illustrating the expression patterns of the top 30 DEGs between uninfected versus HSV-2-infected FGT tissues are shown in Figure 2A,B.

No DEGs were identified in the group-wise comparison of uninfected versus *Nb*/HSV-2 co-infected FGT tissues. This group was subsequently excluded from downstream bioinformatics analyses.

Table 1. Top 30 significant DEGs in the comparison of uninfected versus *Nb*-infected female genital tract tissues.

Gene ID	Gene Name	logFC	*p* Value	Adjusted *p* Value
ENSMUSG00000033016.17	*Nfatc1*	1.01	2.30×10^{-6}	0.02
ENSMUSG00000033220.8	*Rac2*	1.30	2.60×10^{-6}	0.02
ENSMUSG00000025017.11	*Pik3ap1*	1.55	6.32×10^{-6}	0.02
ENSMUSG00000029098.18	*Acox3*	1.02	9.09×10^{-6}	0.02
ENSMUSG00000038644.15	*Pold1*	1.02	1.63×10^{-5}	0.02
ENSMUSG00000039637.16	*Coro7*	1.05	1.73×10^{-5}	0.02
ENSMUSG00000033083.17	*Tbc1d4*	1.11	1.85×10^{-5}	0.02
ENSMUSG00000089960.2	*Ugt1a1*	1.27	2.14×10^{-5}	0.02
ENSMUSG00000029547.11	*Ints1*	1.16	2.14×10^{-5}	0.02
ENSMUSG00000041638.19	*Gcn1*	1.04	2.57×10^{-5}	0.02
ENSMUSG00000000631.21	*Myo18a*	1.14	3.02×10^{-5}	0.02
ENSMUSG00000003882.6	*Il7r*	1.54	3.37×10^{-5}	0.02
ENSMUSG00000032536.13	*Trak1*	1.08	3.37×10^{-5}	0.02
ENSMUSG00000028581.18	*Laptm5*	1.68	4.39×10^{-5}	0.02
ENSMUSG00000004099.17	*Dnmt1*	1.06	4.92×10^{-5}	0.02
ENSMUSG00000034584.4	*Exph5*	1.56	5.28×10^{-5}	0.02
ENSMUSG00000031004.9	*Mki67*	1.05	5.45×10^{-5}	0.02
ENSMUSG00000024621.17	*Csf1r*	1.68	5.55×10^{-5}	0.02
ENSMUSG00000026116.12	*Tmem131*	1.10	5.85×10^{-5}	0.02
ENSMUSG00000030560.18	*Ctsc*	1.32	6.20×10^{-5}	0.02
ENSMUSG00000022946.11	*Dop1b*	1.12	7.49×10^{-5}	0.02
ENSMUSG00000032380.10	*Dapk2*	1.37	7.63×10^{-5}	0.02
ENSMUSG00000020357.4	*Flt4*	1.48	7.64×10^{-5}	0.02
ENSMUSG00000061607.16	*Mdc1*	1.09	8.16×10^{-5}	0.02
ENSMUSG00000028931.13	*Kcnab2*	1.47	8.23×10^{-5}	0.02
ENSMUSG00000120290.1	*Gm56959*	1.08	8.40×10^{-5}	0.02
ENSMUSG00000026288.15	*Inpp5d*	1.67	8.97×10^{-5}	0.02
ENSMUSG00000022443.18	*Myh9*	1.29	9.65×10^{-5}	0.02
ENSMUSG00000020100.16	*Slc29a3*	1.16	9.69×10^{-5}	0.02
ENSMUSG00000048865.17	*Arhgap30*	1.50	9.84×10^{-5}	0.02

Table 2. Top 30 significant DEGs in the comparison of uninfected versus HSV-2-infected female genital tract tissues.

Gene ID	Gene Name	logFC	*p* Value	Adjusted *p* Value
ENSMUSG00000032193.10	*Ldlr*	2.27	9.96×10^{-7}	0.02
ENSMUSG00000031004.9	*Mki67*	1.24	5.82×10^{-6}	0.03
ENSMUSG00000039145.17	*Camk1d*	1.14	8.31×10^{-6}	0.03
ENSMUSG00000004356.9	*Utp20*	1.04	9.39×10^{-6}	0.03
ENSMUSG00000046179.18	*E2f8*	1.53	1.28×10^{-5}	0.03

Table 2. *Cont.*

Gene ID	Gene Name	logFC	*p* Value	Adjusted *p* Value
ENSMUSG00000024251.11	*Thada*	1.02	1.44×10^{-5}	0.03
ENSMUSG00000028613.16	*Lrp8*	1.28	2.26×10^{-5}	0.03
ENSMUSG00000055116.9	*Bmal1*	1.27	2.49×10^{-5}	0.03
ENSMUSG00000024660.10	*Incenp*	1.01	2.54×10^{-5}	0.03
ENSMUSG00000114934.2	*Gm48342*	1.52	3.35×10^{-5}	0.03
ENSMUSG00000024378.10	*Stard4*	1.25	3.38×10^{-5}	0.03
ENSMUSG00000093930.3	*Hmgcs1*	1.18	4.20×10^{-5}	0.03
ENSMUSG00000004099.17	*Dnmt1*	1.04	5.12×10^{-5}	0.03
ENSMUSG00000073529.10	*F830208F22Rik*	1.80	5.91×10^{-5}	0.03
ENSMUSG00000022351.15	*Sqle*	1.42	6.44×10^{-5}	0.03
ENSMUSG00000039835.17	*Nhsl1*	1.07	6.59×10^{-5}	0.03
ENSMUSG00000039840.9	*Epg5*	1.11	6.98×10^{-5}	0.03
ENSMUSG00000000631.21	*Myo18a*	1.06	7.31×10^{-5}	0.03
ENSMUSG00000030739.20	*Myh14*	1.23	7.35×10^{-5}	0.03
ENSMUSG00000021670.15	*Hmgcr*	1.05	7.51×10^{-5}	0.03
ENSMUSG00000035049.5	*Rrp12*	1.13	8.00×10^{-5}	0.03
ENSMUSG00000039087.18	*Rreb1*	1.17	8.25×10^{-5}	0.03
ENSMUSG00000022463.9	*Srebf2*	1.02	8.47×10^{-5}	0.03
ENSMUSG00000013629.17	*Cad*	1.02	8.78×10^{-5}	0.03
ENSMUSG00000001228.15	*Uhrf1*	1.18	9.33×10^{-5}	0.03
ENSMUSG00000046591.11	*Ticrr*	1.43	9.35×10^{-5}	0.03
ENSMUSG00000025278.10	*Flnb*	1.17	9.79×10^{-5}	0.03
ENSMUSG00000040721.10	*Zfhx2*	1.22	9.81×10^{-5}	0.03
ENSMUSG00000115783.3	*Bc1*	−1.93	9.84×10^{-5}	0.03
ENSMUSG00000034584.4	*Exph5*	1.42	1.01×10^{-4}	0.03

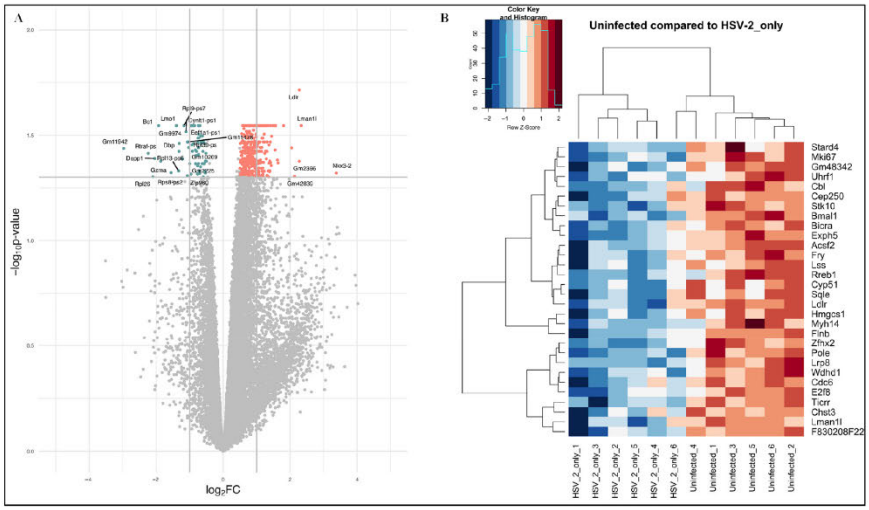

Figure 2. (**A**) Volcano plot of differentially expressed genes (DEGs) in uninfected versus HSV-2-infected FGT tissues. Orange dots represent significantly upregulated genes and blue dots represent

significantly downregulated genes. Grey dots typically represent genes that do not meet the criteria for significant differential expression. (**B**) Heat map showing the top 30 DEGs in the comparison of uninfected versus HSV-2-infected FGT tissues. Z-score represents how much a gene's expression level in a particular sample deviates from the average expression level of that gene across all samples, measured in terms of standard deviations. A positive Z-score indicates higher-than-average expression, while a negative Z-score indicates lower-than-average expression. The colours on the heat map correspond with the expression levels of DEGs across all the samples with the orange colour representing a higher expression than the mean and the blue colour representing a lower expression than the mean.

3.3. Identification of Immune-Related DEGs

To further identify immune-related genes within the cohorts of DEGs, gene sets associated with immune functions were retrieved from the Mouse Genome Informatics (MGI) database. These gene sets were then intersected with the DEGs in each group-wise comparison. In the uninfected versus *Nb*-infected FGT tissue comparison, 89 immune-related DEGs were obtained (Figure 3A, Table 3). In the uninfected versus HSV-2-infected FGT tissue comparison, 23 immune-related DEGs were obtained (Figure 3B, Table 3).

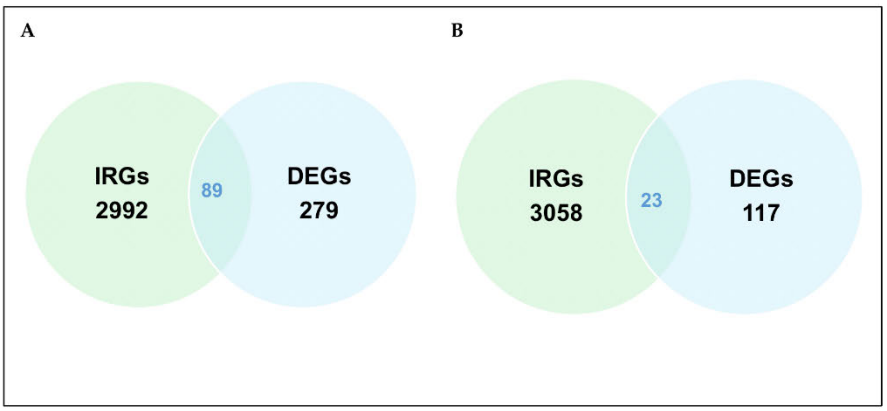

Figure 3. Venn diagram of total immune-related genes (IRGs) and differentially expressed genes (DEGs) in (**A**) uninfected versus *Nb*-infected FGT tissues and (**B**) uninfected versus HSV-2 FGT tissues.

Table 3. Immune-related DEGs in the uninfected versus (A) *Nb*-infected and (B) HSV-2-infected FGT tissues.

(A) Immune-Related DEGs: Uninfected Versus *Nb*-Infected FGT Tissues	(B) Immune-Related DEGs: Uninfected Versus HSV-2-Infected FGT Tissues
Nfatc1, Rac2, Pik3ap1, Myo18a, Il7r, Laptm5, Csf1r, Ctsc, Dapk2, Inpp5d, Myh9, Lcp1, Spn, Dock2, Itgb2, Cd74, Itgax, Nckap1l, Wdfy4, Csf2rb2, Ccr5, Ciita, Apbb1ip, Stk10, Cx3cr1, Chst3, Hcls1, Crtc3, Ptpro, Flnb, Ppargc1b, H2-Ab1, Cd180, Cbl, Adcy7, Vav1, Traf3, Pigr, Slc11a1, Dock8, Ccdc88b, Mpeg1, Zmiz1, C1qc, Prex1, Dtx1, Atp7a, Myo1f, Trim56, Elf4, Fgfr3, Trim62, Pld4, Bcl11b, Itga4, C1qb, Ctss, Wfdc15b, Tnfrsf11a, Cd68, Was, Ptprc, Adgre1, Ccr2, Dhx9, Lat2, C3ar1, Fcgr2b, Rab43, Naip2, Cd83, Tifab, Lyve1, Msmp, Plxna1, Spi1, Dtx4, Ikzf1, Itpkb, Rasal3, Csf2rb, Cd84, Sash3, Ccl11, C1qa, Myo1g, Epas1, Themis2, Tacr1	*Ldlr, Camk1d, Lrp8, Epg5, Myo18a, Flnb, Lmo1, Cbl, Stk10, Chst3, Bcl11b, Il7r, Spn, Atp7a, Foxn1, Zmiz1, Zbtb34, Dhx9, Myh9, Plec, Csf2rb2, Nkx3-2, Tnfrsf11a*

3.4. GO, KEGG and REACTOME Enrichment Analysis

To elucidate the significant biological processes, functional classification and specific signaling pathways associated with the immune-related DEGs, GO, KEGG and REACTOME enrichment analyses were performed. In the uninfected versus *Nb*-infected FGT tissue comparison, the top five enriched GO BP terms included "immune system process", "immune response", "regulation of immune system process", "leukocyte activation", and "response to stimulus". The top five GO CC terms were "cellular anatomical structure", "cellular component", "cell periphery", "plasma membrane", and "membrane". The top five GO MF terms were "protein binding", "binding", "molecular function", "immune receptor activity", and "cytokine receptor activity" (Figure 4). In addition, KEGG enrichment analysis revealed that these DEGs were enriched in several immune-related pathways including "chemokine signaling pathway", "B cell receptor signaling pathway", "Fc gamma R-mediated phagocytosis", "complement and coagulation cascades", and "cytokine-cytokine receptor interaction" (Figure 5A). Similarly, REACTOME enrichment analysis revealed that the immune-related DEGs were enriched in "Immune System", "Innate Immune System", "RAC2 GTPase cycle", "Interleukin-3, Interleukin-5 and GM-CSF signaling", and "Neutrophil degranulation" (Figure 5B).

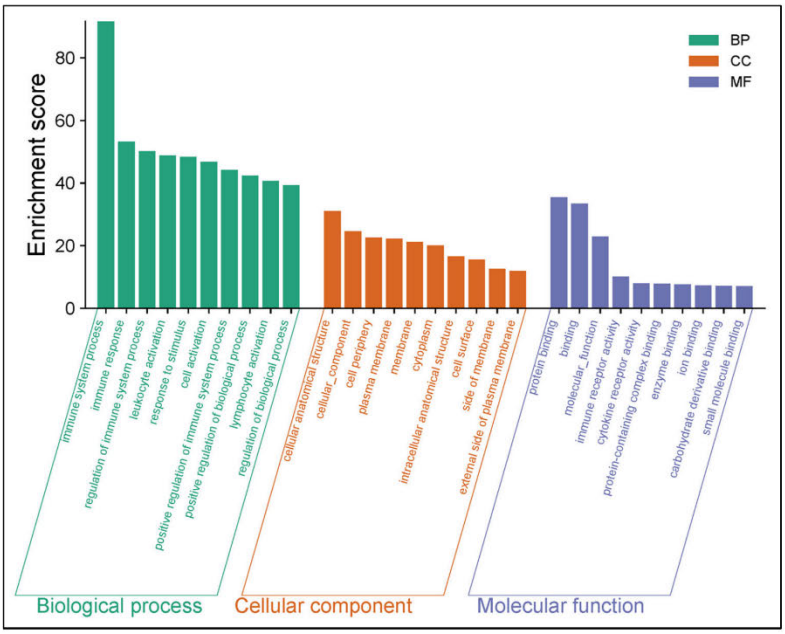

Figure 4. GO enrichment analysis of immune-related differentially expressed genes (DEGs) in the uninfected versus *Nb*-infected FGT tissues.

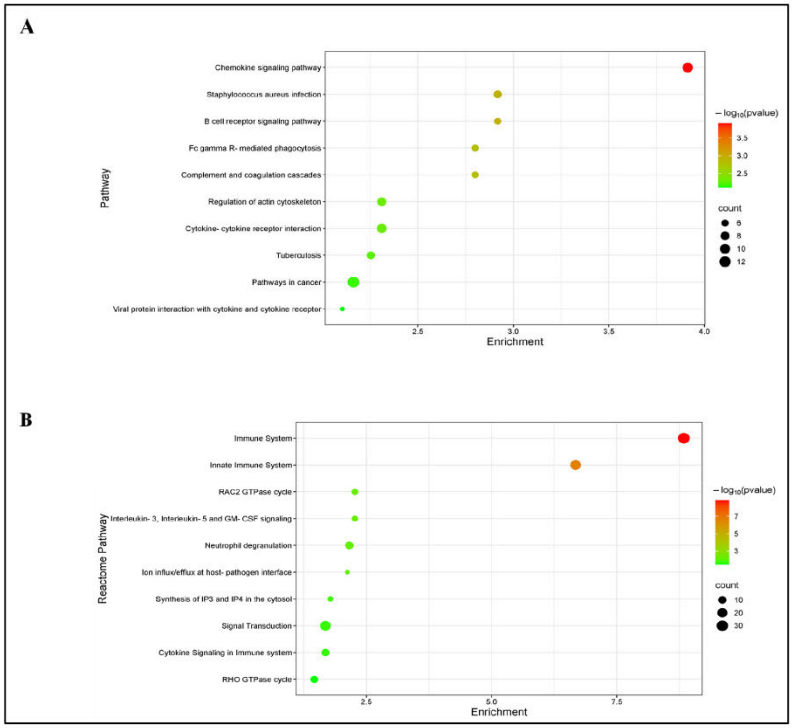

Figure 5. Immune-related differentially expressed genes (DEGs) in the uninfected versus *Nb*-infected FGT tissues: (**A**) KEGG enrichment analysis of immune-related DEGs and (**B**) REACTOME enrichment analysis of immune-related DEGs.

In the comparison of uninfected versus HSV-2-infected FGT tissues, the top five enriched GO BP terms for the immune-related DEGs included "immune system process", "regulation of immune system process", "leukocyte activation", "cell activation", and "multicellular organismal process". The top five GO CC terms were "cellular anatomical structure", "brush border", "intracellular organelle", "organelle", and "intracellular membrane-bounded organelle". The top five GO MF terms were "protein binding", "binding", "molecular function", "ion binding", and "small molecule binding" (Figure 6A). No enriched KEGG pathways were identified for these DEGs. The top REACTOME enriched pathways included "REACTOME root term", "immune system", "cargo recognition for clathrin-mediated endocytosis", "clathrin-mediated endocytosis", and "cytokine signaling in immune system" (Figure 6B).

Taken together, these findings indicate that immune-related DEGs were identified in comparisons between uninfected and infected FGT tissues (both *Nb*- and HSV-2-infected), and that these DEGs are involved in immunological and biological processes.

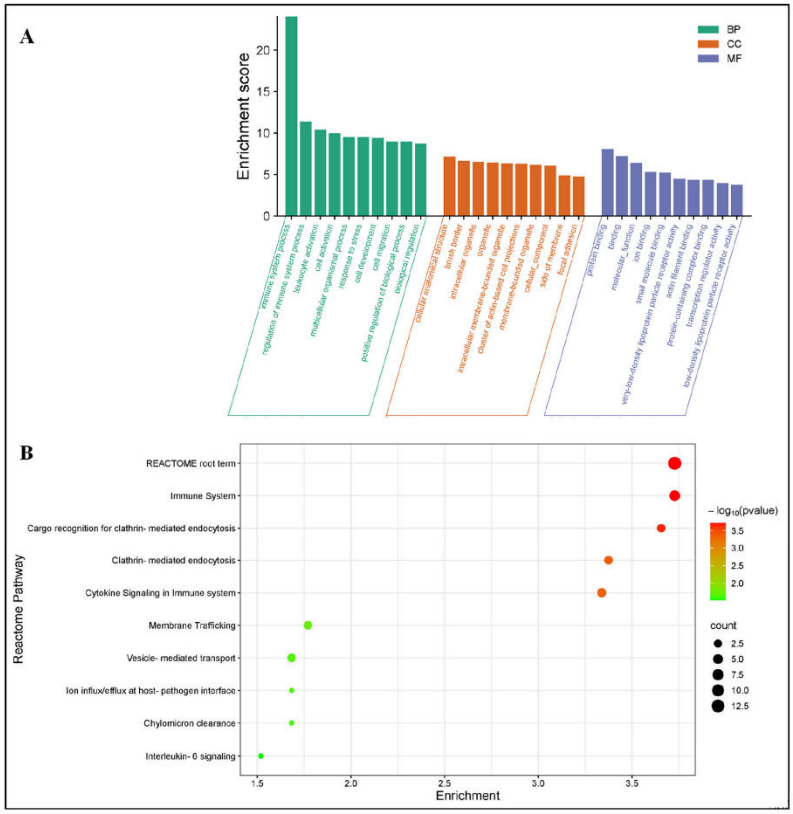

Figure 6. Immune-related differentially expressed genes (DEGs) in the uninfected versus HSV-2-infected FGT tissues: (**A**) GO enrichment analysis of immune-related DEGs and (**B**) REACTOME enrichment analysis of immune-related DEGs.

3.5. Protein–Protein Interaction (PPI) Network Analysis

To further explore PPI networks, hub genes, and key functional modules among all the DEGs identified in the group-wise comparisons, PPI network analysis was conducted using the STRING database. The resulting networks were visualized with Cytoscape software, and subsequently analysed for hub genes using the CytoHubba plug-in, and functional modules using the MCODE plug-in.

For the DEGs identified in the uninfected versus *Nb*-infected FGT tissue comparison, 291 nodes and 205 edges were obtained (PPI enrichment p-value < 1.0×10^{-16}; confidence score > 0.7) (Figure 7). Hub genes were identified from the generated data in PPI network analysis, and the top 10 hub genes were obtained (*Ptprc*, *Csf1r*, *Adgre1*, *Itgax*, *Cd68*, *Ccr2*, *Cx3cr1*, *Cdc6*, *Ctss*, and *C1qa*) (Figure 8A). Moreover, four functional modules [Module 1 (Figure 8B), Module 2 (Figure 8C), Module 3 (Figure 8D), and Module 4 (Figure 8E)] were established from the PPI network using the MCODE plug-in. GO enrichment analysis was performed on the resulting functional modules. In terms of GO BP, it was shown that genes in Module 1 (*Ptprc*, *Adgre1*, *Csf1r*, *Cd68*, *Itgax*, *Ccr2*, and *Cx3cr1*) were significantly enriched in "regulation of macrophage migration", "positive regulation of cell migration", "tumor necrosis factor superfamily cytokine production", "tumor necrosis factor production", and "positive regulation of hematopoietic stem cell migration". Module 2 genes (*Cdc6*,

Pold1, *Mcm3*, *Mcm6*, *Mcm10*, and *Clspn*) were mainly enriched in "DNA-templated DNA replication", "DNA replication initiation", "DNA replication", "DNA metabolic process", and "DNA damage response". Module 3 genes (*C1qc*, *Ctss*, *C1qa*, and *Cd74*) were enriched in "adaptive immune response", "synapse pruning", "antigen processing and presentation of exogenous peptide antigen via MHC class II", "antigen processing and presentation of peptide antigen via MHC class II", and "cell junction disassembly". Module 4 genes (*Vav1*, *Rac2*, and *Dock2*) were enriched in "chemotaxis", "taxis", "T cell activation", "neutrophil chemotaxis", and "granulocyte chemotaxis" (Supplementary File S3).

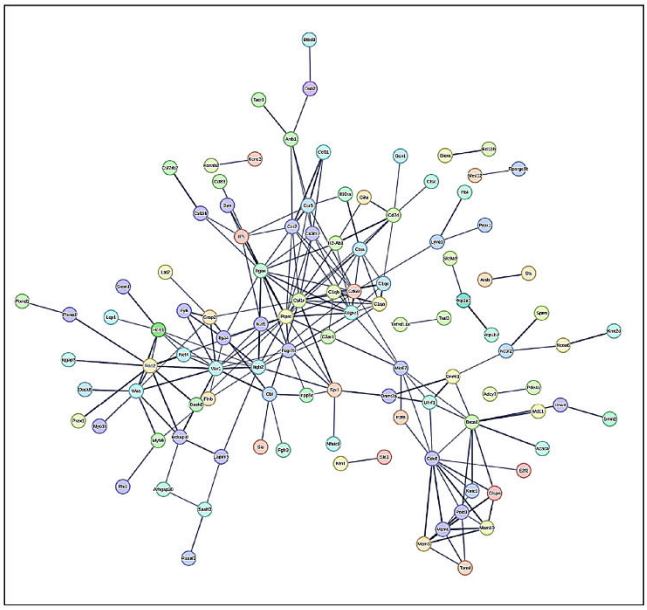

Figure 7. Differentially expressed genes (DEGs) analysed by protein–protein interaction (PPI) network. PPI network analysis was conducted on all DEGs in the uninfected versus *Nb*-infected FGT tissue comparison using STRING and the resulting network was visualized within Cytoscape. Colored nodes represent query proteins. Edges/lines represent functional associations. Solid lines indicate strong evidence or direct interactions. Edge thickness represents confidence score—thicker edges indicate higher confidence in interaction.

For the DEGs identified in the uninfected versus HSV-2 infected FGT tissue comparison, 103 nodes and 78 edges were obtained (PPI enrichment p-value $< 1.0 \times 10^{-16}$; confidence score > 0.7) (Figure 9). Hub genes were identified from the generated data in PPI network analysis, and the top 10 hub genes were identified (*Brca1*, *Cdc6*, *Clspn*, *Mcm10*, *Dtl*, *Uhrf1*, *Mki67*, *Mybl2*, *E2f8*, and *Cyp51*) (Figure 10A). Moreover, two functional modules [Module 1 (Figure 10B) and Module 2 (Figure 10C)] were established from the PPI network with the MCODE plug-in. In addition, GO enrichment analysis was performed on the resulting functional modules. In terms of GO BP, it was shown that genes in Module 1 (*Brca1*, *Cdc6*, *Clspn*, *Mcm10*, *Dtl*, *Uhrf1*, *Mki67*, *Mybl2*, *E2f8*, *Incenp*, *Pole*, and *Wdhd1*) were mainly enriched in "mitotic cell cycle", "mitotic cell cycle process", "cell cycle", "cell cycle process" and "DNA replication". Module 2 genes (*Cyp51*, *Lss*, *Srebf2*, *Hsd17b7*, *Stard4*, *Aacs*, *Sqle*, *Hmgcs1*, *Hmgcr*, and *Msmo1*) were mainly enriched in "cholesterol metabolic process", "sterol biosynthetic process", "sterol metabolic process", "secondary alcohol metabolic process", and "steroid biosynthetic process" (Supplementary File S4).

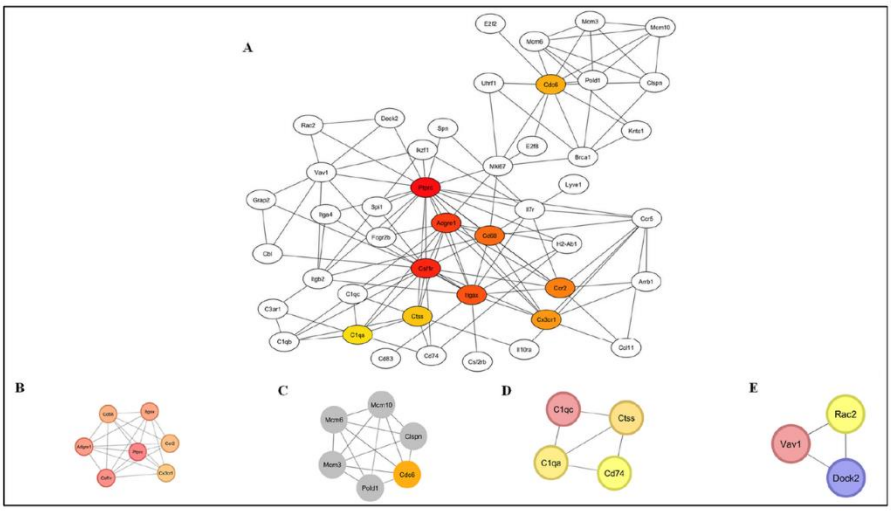

Figure 8. Differentially expressed genes (DEGs) analysed by protein–protein interaction (PPI) network. PPI network analysis was conducted on all DEGs in the uninfected versus *Nb*-infected FGT tissue comparison using STRING and the resulting network was visualized within Cytoscape. (**A**) Top 10 hub genes were identified using CytoHubba and (**B**–**E**) functional modules were identified using MCODE.

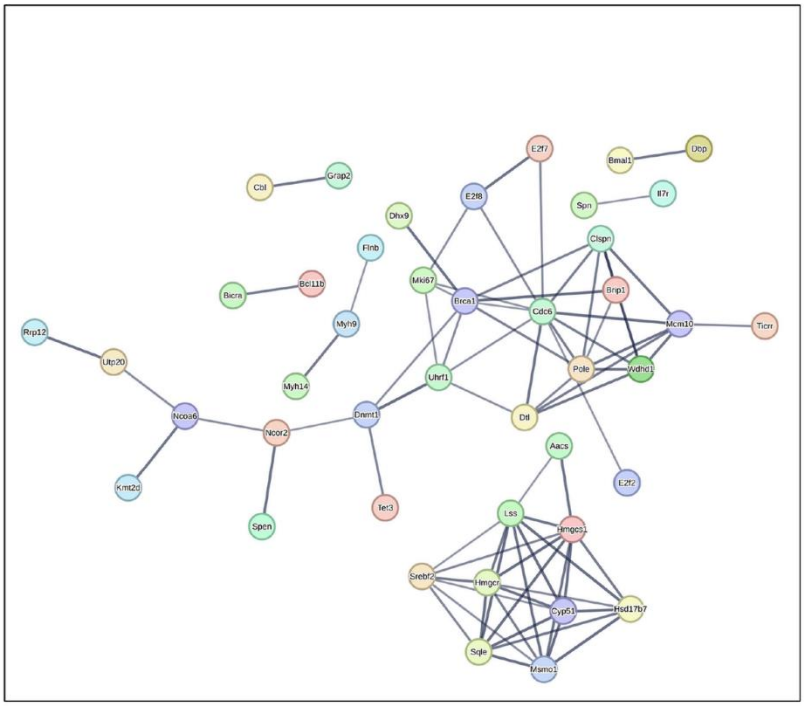

Figure 9. Differentially expressed genes (DEGs) analysed by protein–protein interaction (PPI) network. PPI network analysis was conducted on all DEGs in the uninfected versus HSV-2-infected FGT tissue

comparison using STRING and the resulting network was visualized within Cytoscape. Colored nodes represent query proteins. Edges/lines represent functional associations. Solid lines indicate strong evidence or direct interactions. Edge thickness represents confidence score—thicker edges indicate higher confidence in interaction.

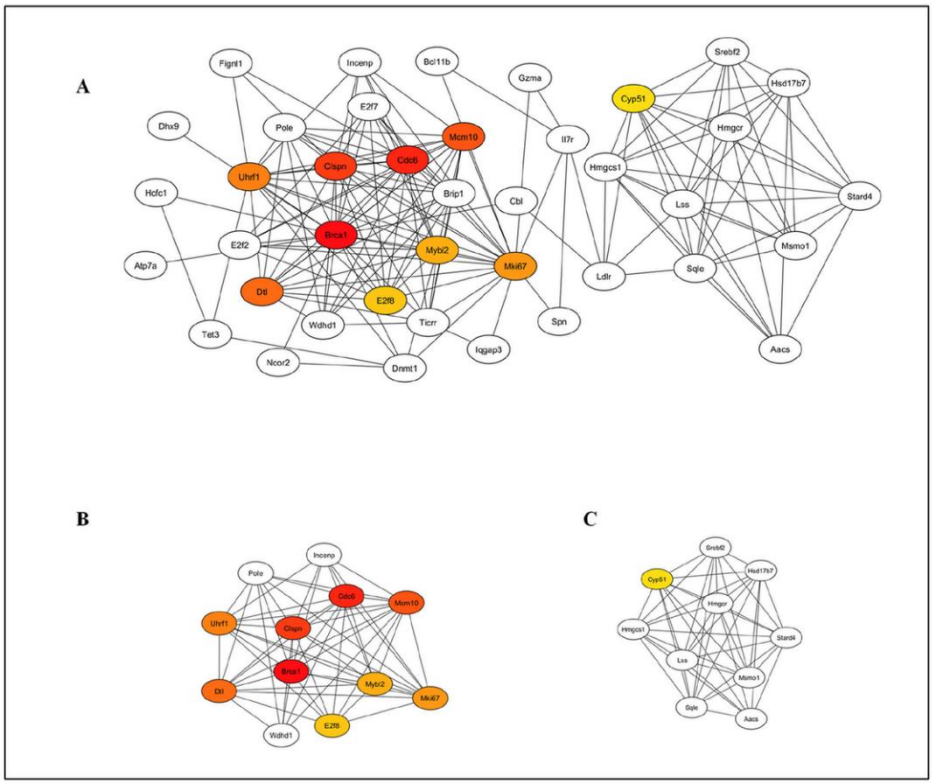

Figure 10. Differentially expressed genes (DEGs) analysed by protein–protein interaction (PPI) network. PPI network analysis was conducted on all DEGs in the uninfected versus HSV-2-infected FGT tissue comparison using STRING and the resulting network was visualized within Cytoscape. (**A**) Top 10 hub genes were identified using CytoHubba and (**B**,**C**) functional modules were identified using MCODE.

In summary, bioinformatic analyses predicted that the hub genes and key functional modules among all the DEGs identified in group-wise comparisons are associated with immune responses and host defense mechanisms against pathogens.

4. Discussion

In this preliminary study, we employed NGS and bioinformatics tools to identify DEGs and their associated immunological and biological functions in murine FGT tissues in the setting of *Nb* and HSV-2 single and co-infection. Our findings demonstrate the value of this approach in elucidating underlying molecular pathways potentially involved in host immune responses, while also revealing common and distinct features related to both pathogens.

4.1. Animal Infection

In this study, gene expression profiles in murine FGT tissues across four experimental groups were examined: (1) singly infected with *Nb*, (2) singly infected with HSV-2, (3) co-infected with both *Nb* and HSV-2, and (4) uninfected controls. For the duration of the study, body weight and clinical pathology scores were monitored to track infection-associated changes and ensure animal welfare. *Nb* infection dynamics are well-characterized and have been validated in previous studies; clinical symptoms are generally mild and brief, with the infection resolving within 10 days [17,19]. In *Nb*-infected mice, the transient weight loss observed around days 3–4 corresponds with the helminth's pulmonary migration phase and resolved as *Nb* migrated to the intestine, consistent with the parasite's natural lifecycle [19]. Similarly, following intravaginal infection of murine models with HSV-2, clinical symptoms are typically observed from day 3 onwards [17,20]. In our study, the absence of observable pathology at day 2 post-HSV-2 infection is consistent with previous findings, which indicate that early-stage HSV-2 infection in murine models causes minimal clinical symptoms [17]. Moreover, this early time point allowed us to capture transcriptional and immunological changes prior to the onset of overt pathology.

4.2. DEGs Identified in the Comparison of Uninfected Versus Nb-Infected FGT Tissues

Our comparative analysis of uninfected and *Nb*-infected FGT tissues identified 368 DEGs, comprising 356 upregulated and 12 downregulated genes. Among these, 89 DEGs were functionally annotated as immune-related based on MGI database comparisons, including key complement system components (e.g., *C3ar1*, *C1qa*, *C1qb*, *C1qc*). The complement system plays a vital role in innate immunity through opsonization, chemotaxis, and membrane attack complex formation, while also bridging innate and adaptive immune responses [21]. Consistent with these findings, GO enrichment analysis demonstrated significant representation of immune system processes among the DEGs, with 81 genes (e.g., *Rac2*, *Pik3ap1*, *Myo18a*, *Il7r*, *Laptm5*, *Csf1r*, *Ctsc*, *Dapk2*, *Inpp5d*, and *Myh9*) associated with immune-related processes. This finding was further supported by KEGG and Reactome pathway analyses, which revealed enrichment in critical immune pathways, including chemokine signaling and B-cell receptor signaling.

PPI network analysis identified ten hub genes central to immune function (*Ptprc*, *Csf1r*, *Adgre1*, *Itgax*, *Cd68*, *Ccr2*, *Cx3cr1*, *Cdc6*, *Ctss*, *C1qa*). Functional module analysis further confirmed immune-related roles for three key modules: (1) Module 1 (*Ptprc*, *Adgre1*, *Csf1r*, *Cd68*, *Itgax*, *Ccr2*, *Cx3cr1*), (2) Module 3 (*C1qc*, *Ctss*, *C1qa*, *Cd74*), and (3) Module 4 (*Vav1*, *Rac2*, *Dock2*).

Interestingly, we observed a predominant downregulation of immune-related DEGs in *Nb*-infected FGT tissues. This potentially reflects a complex interplay between various mechanisms, such as (1) FGT-specific factors related to hormonal and microbiota influences [22,23], and (2) immune modulation by the hookworm, downregulating pro-inflammatory/Th1 pathways in favour of a Th2/anti-inflammatory environment [11]. While beneficial for hookworm survival, this immune modulation may compromise critical FGT-specific mucosal defenses against secondary infections by impairing Th1 responses and epithelial protection mechanisms [4].

In addition, we identified Th2-associated chemokines (e.g., *Ccl11*) and receptors (e.g., *Ccr2* and *Cx3cr1*), which orchestrate leukocyte recruitment in normal and inflammatory conditions [24]. *Ccl11*, together with *Ccl24* and *Ccl26*, serves as a potent eosinophilic chemoattractant via *Ccr3* signaling [25]. These chemokines are induced by Th2 cytokines (IL-4 and IL-13) and work synergistically with IL-5 to drive eosinophilia [24], a hallmark of helminth infections [26]. Our observation of elevated *Ccl11* in *Nb*-infected FGT tissues aligns

with previous reports of eosinophil accumulation in the murine FGT post-*Nb* infection [17], and mirrors established gastrointestinal responses to helminth infections [27,28].

The chemokine receptor *Ccr2* interacts with multiple CC chemokines (*Ccl2*, *Ccl7*, *Ccl8*, *Ccl12*, *Ccl13*, and *Ccl16*), with the *Ccl2-Ccr2* axis being important for monocyte recruitment during inflammatory responses, and recruitment of lymphocytes, NK cells, and other leukocytes [24]. In helminth infections, this axis is crucial for Th2 responses, as was demonstrated in *Schistosoma mansoni*-infected *Ccr2*-deficient mice, which exhibited defective monocytes and macrophages, and reduced IL-4 production [29]. Elevated *Ccl2* levels have been linked to strong Th2 immune responses, as observed in STH infections including *Trichuris muris* [30], *Ascaris suum* [31], and *Nb* [32]. Notably, *Ccl2*-deficient mice failed to expel *Trichuris muris* due to weakened Th2 (decreased IL-4) and heightened Th1 (elevated IFN-γ/IL-12) immune responses [33].

We observed a downregulation of *Cx3cr1* in *Nb*-infected FGT tissues. *Cx3cr1*, a receptor for *Cx3cl1* (fractalkine), mediates monocyte adhesion and tissue migration, where they can differentiate into dendritic cells and macrophages [25]. In *Nb* infection, *Cx3cr1*-deficient mice exhibited reduced pathology and improved parasite control, suggesting that *Cx3cr1* impairs effective monocyte–helminth interactions [34]. *Cx3cr1* was also expressed on a subset of $CD4^+$ T cells that accumulated in helminth-infected tissues, indicating its role in Th2 immunity and tissue repair [35]. These findings underscore dual roles for *Cx3cr1* in monocyte and T cell responses during helminth infections.

Overall, our analyses demonstrate that *Nb* induces a robust immune response, with notable downregulation of immune-related genes in the FGT, a site distal to its primary site of infection. This is a significant finding given the hookworm's lifecycle, which involves penetration of the skin, a transient migratory phase through the lungs, and establishment in the intestine [19,36]. The gene expression signature observed in our study is consistent with a coordinated Th2 immune response, involving leukocyte activation, chemokine-driven recruitment, macrophage and T-cell regulation. These findings align with reported Th2 signatures in the FGTs of STH-infected mice [17] and women [14,16].

4.3. DEGs Identified in Comparison of Uninfected Versus HSV-2-Infected FGT Tissues

Our comparative analysis of uninfected and HSV-2-infected FGT tissues identified 140 DEGs, comprising 121 upregulated and 19 downregulated genes. Intersection with immune-related gene sets from the MGI database revealed 23 immune-associated DEGs. GO analysis demonstrated significant enrichment of these DEGs in immune system processes. For instance, 22 genes (e.g., *Ldlr*, *Camk1d*, *Lrp8*, *Epg5*, *Myo18a*, *Flnb*, *Lmo1*, *Cbl*, *Stk10*, *Chst3*) were assigned to this functional category. Reactome pathway analysis further confirmed the involvement of these DEGs in key immunological pathways, including immune system function and cytokine-mediated signaling.

Notably, we observed that HSV-2-infected FGT tissues exhibited predominant downregulation of immune-related DEGs at 2 days post-infection. This early transcriptional suppression is consistent with known HSV-2 immune evasion mechanisms, including host gene shutoff, mRNA degradation, and interferon signaling inhibition [37]. This observed suppression likely reflects viral disruption of both antigen presentation and innate immune pathway activation [38–40]. For example, molecular studies demonstrated that HSV-2 viral proteins actively suppress host immune responses: HSV-2 US1 inhibited IFN-β production by preventing IRF-3 from binding to the IRF-3 response element within the IFN-β promoter region [38], ICP22 significantly inhibited IFN-β induction and functioned as an E3 ubiquitin-protein ligase to block IFN-stimulated gene production [40], and ICP27 similarly suppressed IFN-β generation [39]. Furthermore, the observed transcriptional profile may reflect the temporal dynamics of HSV-2 infection, as murine models have demonstrated that

robust immune cell infiltration, particularly dendritic cells, macrophages, and T cells, and inflammatory gene expression typically peaked 3–5 days post-infection [41,42]. Therefore, the delay between initial viral replication and subsequent immune activation may account for the prevalence of downregulated genes in our early time point analysis.

PPI network analysis of hub genes and functional modules revealed no direct associations with immune responses or inflammation. Instead, the predominant functional annotations were related to mitotic cell cycle regulation, DNA replication initiation, and sterol biosynthesis.

The top hub gene, *Brca1*, is primarily recognized for its role in DNA repair and genomic stability [43]. While viral infections, including HCMV and HSV-1, have been shown to modulate *Brca1* expression [44], no such relationship has been described for HSV-2.

Notably, we identified multiple genes involved in (1) cell cycle (*Cdc6*, *Clspn*, *Mcm10*, *Dtl*, *Uhrf1*, *Mki67*, *Mybl2*, *E2f8*, *Incenp*, *Pole*, and *Wdhd1*), (2) DNA replication (*Cdc6*, *Mcm10*, *Pole* and *Wdhd1*), (3) cell cycle regulation (*Dtl*, *Mybl2*, *E2f8*), (4) DNA damage response (*Clspn*), (5) cytokinesis (*Incenp*), (6) chromatin organisation (*Uhrf1*), and (7) chromosome partition (*Mki67*) [45]. The downregulation of these genes in HSV-2-infected FGT tissues suggests viral manipulation of host cell cycle machinery. HSV-2 is known to hijack cellular replication pathways, particularly by promoting G1/S transition to access DNA synthesis machinery [46,47]. Recent evidence indicates that HSV-2 UL24 protein modulates cell cycle progression, while its N-terminal domain (UL24-N) specifically suppresses IFN-β production by inhibiting IRF-3 phosphorylation [48].

We identified several genes that regulate cholesterol biosynthesis and sterol metabolism (*Cyp51*, *Lss*, *Srebf2*, *Hsd17b7*, *Stard4*, *Aacs*, *Sqle*, *Hmgcs1*, *Hmgcr*, *Msmo1*). Cellular cholesterol plays a critical role in HSV infection, facilitating viral entry, nuclear transport of viral capsids, membrane fusion, and cell-to-cell spread [49,50]. Cholesterol depletion impaired viral protein expression, genome encapsidation, and virion release [50].

The sterol regulatory element-binding proteins (SREBPs), particularly SREBP2 encoded by *SREBF2*, are key regulators of lipid homeostasis [51]. Beyond lipid metabolism, SREBP2 influenced immune responses by activating the NLRP3 inflammasome in endothelial cells [52], regulated chemokine production through direct transcriptional activation of BHLHE40 and KLF6 [53], and antagonized type I IFN responses [54]. Notably, type I IFN signaling suppressed cholesterol synthesis and promoted fatty acid import, creating an antiviral state [54]. Taken together, this metabolic/immunological crosstalk suggests that HSV-2-mediated modulation of sterol metabolism contributes to both viral strategy for replication and host defense.

4.4. Limitations of the Current Study and Future Directions

A key objective of this study was to characterize FGT transcriptional changes in response to *Nb*/HSV-2 co-infection. Notably, our comparative analysis revealed no significant DEGs between uninfected and co-infected FGT tissues, which contrasts with the distinct transcriptional changes observed in single *Nb* and HSV-2 infections. It is plausible that this finding is biologically relevant, potentially reflecting immune antagonism, whereby *Nb*-induced Th2 and immunomodulatory responses downregulate early HSV-2-mediated Th1/pro-inflammatory responses [11], resulting in transcriptional equilibrium. We emphasize that while this finding requires validation in future studies, it underscores the need to study STH/HSV-2 co-infections further to elucidate complex immunological interactions. However, we also acknowledge that subtle transcriptional changes in the co-infected FGT tissues may not have been detected due to the small sample size (n = 6 per group). Given that this was a proof-of-concept study, the sample size was selected based on previously established methods [17], while adhering to the ethical principles governing animal re-

search [55]. We assert that future studies should (1) use larger cohort sizes to improve statistical power, (2) compare acute versus chronic infection stages, (3) examine diverse STH species to assess species-specific effects on HSV-2 co-infection, and (4) validate the functional roles of identified DEGs through mechanistic studies. Despite these limitations, our findings provide novel insights into FGT transcription changes during single *Nb* and HSV-2 infections, enabling an enhanced understanding of host–pathogen interactions. Our study highlights NGS as a powerful tool for elucidating the molecular mechanisms of *Nb* and HSV-2 infections in FGT tissues. The transcriptional profiles generated through RNA-sequencing provide a foundation for future investigations to develop novel diagnostic biomarkers distinguishing single versus co-infections and identify potential therapeutic targets for these clinically relevant infections.

5. Conclusions

Our study highlights the value of NGS technology in exploring complex immunological responses to *Nb*, HSV-2 and their co-infection. The data generated through NGS enabled detailed analyses of gene expression profiles and immune-related pathways in the murine FGT, revealing previously unappreciated immune-related genes and unique transcriptional profiles. The findings of our study lay a foundation for future investigations into the molecular mechanisms underlying STH and HSV-2 infections, with the goal of enhancing diagnostic tools and therapeutic interventions, which is especially relevant in co-endemic regions where both pathogens are highly prevalent.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/pathogens14080795/s1, Supplementary Figure S1A: Daily weights were assessed between the groups post infection; Supplementary Figure S1B: Pathology scoring was assessed between the groups post infection; Supplementary File S1: List of Significant Differentially Expressed Genes Identified in the uninfected versus Nb-infected comparison; Supplementary File S2: List of Significant Differentially Expressed Genes Identified in the uninfected versus HSV-2-infected comparison; Supplementary File S3: GO Analysis of Functional Modules: Uninfected FGT tissues versus Nb-infected FGT tissues; Supplementary File S4: GO Analysis of Functional Modules: Uninfected FGT tissues versus HSV-2-infected FGT tissues.

Author Contributions: R.P.: Conceptualization, funding acquisition, data curation, data analysis, investigation, methodology, writing—original draft, writing—review and editing; P.N.: Project administration, supervision, writing—review and editing; Z.L.M.-K.: Funding acquisition, project administration, supervision, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: Approval to conduct this study was granted by the Animal Ethics Committee of the University of Cape Town (approval number: FHS AEC REF NO: 021_012, 24 September 2021) and the Animal Research Ethics Committee of the University of KwaZulu Natal (approval number: AREC/00005911/2023, 9 November 2023).

Data Availability Statement: The data underlying the findings have been included in the article and Supplementary Material. Further inquiries may be directed to the corresponding author.

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Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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CHAPTER FIVE
SYNTHESIS, CONCLUSION AND FUTURE DIRECTIONS

5.1 Introduction

This synthesis chapter highlights the key findings of this thesis, which investigated how co-infection with the murine hookworm *Nippostrongylus brasiliensis (Nb)* modulates miRNA and mRNA expression in the female genital tract (FGT), and whether these transcriptomic changes influence (i) immune responses to herpes simplex virus type 2 (HSV-2) and (ii) molecular pathways implicated in cervical carcinogenesis. The rationale for this study stems from a significant gap in our understanding of how co-infections with soil-transmitted helminths (STHs) and HSV-2 influence mucosal immunity and disease progression within the FGT. Both STHs and HSV-2 are highly prevalent in sub-Saharan Africa (SSA) and other resource-poor regions, where their geographical overlap increases the likelihood of co-infections. Notably, STHs induce potent T-helper 2 (Th2) and immunomodulatory responses that can modulate host responses to bystander pathogens, including viral infections such as HSV-2 (Chetty et al., 2020). This is particularly concerning given the role of HSV-2 as a co-factor in cervical carcinogenesis (Sausen et al., 2023), and recent studies suggesting that chronic STH-induced immunomodulation may also contribute to tumor-promoting microenvironments (Chetty et al., 2020, Clark et al., 2021, Damane et al., 2025).

Despite these potential risks, the immunological and epidemiological consequences of STH/HSV-2 co-infection remain poorly understood, with only one pre-clinical study to date demonstrating the deleterious effects of *Nb* on HSV-2-associated pathology (Chetty et al., 2021). Moreover, the molecular mechanisms underpinning STH/HSV-2 interactions, particularly the roles of miRNA- and mRNA-mediated regulation in modulating host immunity and influencing disease progression, are unexplored. In the absence of such mechanistic insight, the potential for co-infections to alter immune regulation, enhance disease severity, and contribute to cervical cancer risk in women remains largely uncharacterized. This thesis therefore provides a novel contribution to an underexplored area of STH/HSV-2 co-infection with potentially significant implications for female reproductive health.

5.2 Summary of Key Findings

To address the significant gap in current knowledge regarding the immunological and epidemiological consequences of STH/HSV-2 co-infections, this thesis began with a comprehensive literature review. This review underscored the substantial epidemiological overlap between STHs and HSV-2, particularly in SSA, where co-infections are likely but under-studied. The review emphasized the biological plausibility that STH-induced Th2 immune responses and

immunomodulatory properties may influence host susceptibility to HSV-2 infection, as well as the course and severity of disease. It also highlighted a broader concern, that such immunological interactions may contribute to long-term pathological outcomes, including cervical carcinogenesis. On this basis, the literature review advocated for focused research into the epidemiological and immunological dynamics of STH/HSV-2 co-infections and their potential implications for sexual and reproductive health (Chapter 2).

In addition, the review explored the roles of host- and pathogen-derived miRNAs as critical regulators of immune responses in STHs and HSV-2 infections. This provided a conceptual framework for investigating how miRNA-mediated post-transcriptional regulation might modulate immune responses during co-infection and potentially contribute to disease progression (Chapter 2).

Building on this foundation, a key objective of the experimental work was to characterise miRNA expression profiles in the murine FGT following single and co-infection with *Nb* and HSV-2. Using miRNA-sequencing and a bioinformatics approach, differentially expressed (DE) miRNAs were identified in the singly infected groups, several of which are known to regulate immune pathways. Specifically, when compared to the uninfected controls, eight and nine DE miRNAs were identified in the *Nb*-infected and HSV-2-infected mice, respectively ($p < 0.05$, $|\log FC| \geq 1$). Selected DE miRNAs were validated using RT-qPCR and results were consistent with those observed through sequencing. In contrast, only one DE miRNA was observed in the co-infected group, likely reflecting a combination of the immunomodulatory influence of *Nb*, potential antagonism of opposing T-helper 1 (Th1) and Th2 immune responses, and technical limitations in detecting subtle changes in the co-infected group (Chapter 3).

Importantly, several of the DE miRNAs identified in this study have been previously implicated in female reproductive tract cancers, suggesting that infection-driven miRNA dysregulation may overlap with pathways relevant to cervical cancer. Ingenuity Pathway Analysis (IPA) further supported these observations, with DE miRNAs and their predicted mRNA targets enriched in key immune-related pathways: neutrophil degranulation, interleukin-4 and interleukin-13 signaling, natural killer cell signalling, interferon alpha/beta signalling, and ISGylation. Although IPA did not predict direct links to cervical cancer, several enriched canonical and disease-associated pathways were associated with infection, inflammation, and cancer, particularly in the co-infected group (Chapter 3).

To complement the miRNA findings, mRNA profiling was conducted using RNA-sequencing and various bioinformatics tools. Distinct transcriptional signatures were observed in the FGT following single infection with either *Nb* or HSV-2, with significant differentially expressed genes

(DEGs) relative to uninfected controls (adjusted $p < 0.05$, $|logFC| \geq 1$). In the *Nb*-infected group, 368 DEGs were identified (356 upregulated and 12 downregulated), including immune-associated genes such as *Ptprc*, *Ccl11*, *Ccr2*, and *Cx3cr1*. Pathway enrichment and network analyses revealed significant immune-related functions, and Th2-associated genes, characteristic of a *Nb*-induced Th2 immune signature within the FGT, an anatomically distal site from the parasite's intestinal niche. This finding demonstrates that *Nb* induces systemic immunomodulation, with transcriptional signatures in the FGT consistent with Th2 polarization and the suppression of pro-inflammatory immune-related genes (Chapter 4).

Similarly, HSV-2 infection induced 140 DEGs (121 upregulated, 19 downregulated), including immune-related transcripts such as *Ldlr*, *Camk1d*, *Lrp8*, and *Epg5*. Functional enrichment highlighted not only immune-related pathways but also processes related to the mitotic cell cycle, DNA replication, and sterol biosynthesis, features that have relevance in viral pathogenesis and host immunity (Chapter 4).

Notably, mirroring the miRNA findings, no significant DEGs were identified between uninfected and co-infected groups at the mRNA level. This reinforces the hypothesis that *Nb*-induced immunomodulation may suppress the host transcriptional response to HSV-2, potentially dampening antiviral immunity and influencing the trajectory of infection or disease progression (Chapter 4).

Taken together, the analyses of miRNA and mRNA data offer novel and complementary insights into how immune responses are modulated during single and co-infection with *Nb* and HSV-2. This study provides the first transcriptomic evidence that helminth-induced immunomodulation in the FGT may alter host responses to HSV-2 infection and dysregulate molecular pathways associated with inflammation and cancer. Given the oncogenic potential of HSV-2 and the immunomodulatory capacity of STHs, these findings underscore the need for deeper mechanistic investigations into co-infection dynamics and their implications for HSV-2 outcomes and cervical cancer risk in co-endemic regions.

5.3 Limitations of the study

This study has several limitations that should be acknowledged. Firstly, the relatively small sample size per experimental group may have reduced the statistical power to detect subtle but biologically relevant changes in miRNA and mRNA expression profiles, particularly for low-abundance or moderately regulated miRNAs and genes. It is acknowledged that in mouse experiments, $n = 6$ is frequently used. Moreover, for this study, the sample size was calculated as $n = 6$, based on a power of 80%, and effect size of 1.9, in line with previous data (Chetty et al., 2021). However, in the context of analysing large datasets, such as RNA sequencing datasets,

recent studies recommend a larger n (e.g. 8 - 12 mice per group) to maximize true discoveries and minimize false positives, especially for detecting more subtle or low-expression transcriptomic changes (Halasz et al., 2025). Considering this, the relatively small sample size is described as limitation in this study. In addition, a key objective of this study was to predict the functional significance of DE miRNAs by using bioinformatics tools to predict immune-related gene targets, regulatory interactions, and potential involvement in cervical cancer-associated molecular pathways. While several DE miRNAs were associated with cancers of the female reproductive tract, the core expression analysis did not specifically identify cervical cancer as a significantly enriched disease. Instead, the expression analysis identified cancer in general as significantly enriched, particularly in the co-infected group. This may reflect limitations in pathway annotation databases or the complexity of cervical carcinogenesis, which may not have been fully captured in the model of acute infection employed in this study. While this has been identified as a limitation in keeping with the study's objectives, it is also acknowledged that the enrichment of cervical cancer-specific pathways should not necessarily be expected, given the substantial overlap in cancer signalling networks across different cancers. Furthermore, due to resource constraints, only a subset of DE miRNAs was validated by RT-qPCR, limiting the validation of the entire dataset of DE miRNAs. An integrated analysis of the miRNA and mRNA sequencing datasets was not performed. Budgetary constraints precluded access to fully licensed bioinformatics software, thereby limiting the integrated multi-omics analysis of the single *Nb* and HSV-2 infection groups. The miRNA and mRNA expression profiling were also confined to a single time point post-infection, providing only a snapshot of the host response rather than capturing transcriptomic changes over time. In addition, although bioinformatic analyses predicted several relevant miRNA and mRNA interactions, functional validation was not performed to confirm direct regulatory relationships. Despite these limitations, the study offers novel insights into *Nb*/HSV-2 co-infection and its potential role in modulating immune- and cancer-related pathways in the FGT, providing a valuable foundation for future studies using human tissues or clinical cohorts.

5.4 Conclusion and Future Directions

This thesis presents an integrated investigation of *Nb* and HSV-2 single and co-infections in the FGT, highlighting a complex interplay between STH-induced immunomodulation and antiviral responses at a molecular level. The study addresses a critical gap in our understanding of STH/HSV-2 co-infection and its potential role in modulating immune responses and promoting disease-relevant pathways, including those implicated in cancer. By combining in-depth literature reviews, and next-generation sequencing and bioinformatics approaches to characterise miRNA and mRNA expression profiles, this work provides a foundation for future studies examining the

molecular mechanisms underpinning STH/HSV-2 co-infection outcomes. Looking ahead, mechanistic studies using *in vitro* and *in vivo* models, and miRNA mimic/inhibitor approaches will be essential to explore the contribution of these pathways to cervical carcinogenesis. Functional validation of candidate miRNAs and their targets, time-course analyses, and human studies in co-endemic regions to assess the true burden of STH/HSV-2 co-infections, are also critical next steps. Ultimately, this work underscores the importance of evaluating STH/HSV-2 co-infections in the context of host immunity and cancer risk, with implications for diagnostics, vaccine design, and integrated control strategies in regions where these infections are co-endemic.

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APPENDIX 1 A: ETHICAL APPROVAL TO CONDUCT ANIMAL RESEARCH

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

New Application	
Protocol Reference number:	AREC/00005911/2023
Project title:	**Soil-transmitted helminths and herpes simplex virus-2 co-infections: Impact on female genital tract microRNA and immune profiles, and potential role in cervical carcinogenesis**
Main Applicant and School:	R Pillay (203508752) School of Laboratory Medicine and Medical Sciences

09 November 2023

Dear Ms Pillay

Regarding your revised application received on 01 November 2023, the UKZN Animal Research Ethics Committee (AREC) has accepted the documents submitted, and **FULL APPROVAL** for the protocol was granted on the quorate committee meeting of 08 November 2023.

The protocol has been approved, subject to the following conditions:

1. All animal work to be conducted as per the requirements of the UCT FHS Animal Ethics Committee under reference number 021-012

About your approved protocol please note:

1. All correspondence with the AREC about this protocol must be accompanied by the full reference number.
2. Any alterations to the approved protocol (e.g. title, location, methods, research team) must be reviewed and approved through an official amendment application, via the RIG system, prior to its implementation.
3. Any Veterinary and Para-Veterinary procedures must be conducted by an SAVC registered veterinarian/para-veterinarian or SAVC authorized person, and their details must be logged with the AREC if it changes during the approval period.
4. The AREC may actively monitor research, and ask further questions, request addition information, require modification on welfare grounds. If animal welfare is compromised in any way the AREC may suspend the study/withdraw approval.
5. The research team must declare any adverse or serious adverse event(s) to the AREC, via the RIG system, as soon as possible, but no later than 48 hours after the event.
6. Research data should be securely stored in the discipline/school for a period of 5 years.
7. The approval is valid for a period of one year from the date of issue. If the project is continuing, a renewal application must be submitted to the AREC at least 30 days before the expiration date. If work is completed at the end of the approval period, a close-out report must be submitted. Renewals/close out reports are submitted via the RIG system.

I take this opportunity to wish you all the best with your study.

Yours faithfully

Dr Dalene Vosloo, PhD, Pr. Nat. Sci.
Chair: Animal Research Ethics Committee
/kr

cc Supervisors: Prof Z Mkhize-Kwitshana; Dr P Naidoo; Prof W Horsnell (UCT)

The UKZN AREC is registered with the SA National Health Research Council (Reg. No. AREC-200111-006; Expires on 31/12/2027) in accordance with the National Health Act (No 61 of 2006) and conforms to the guidelines of the Department of Health (DOH2015) and SANS10386:2021. The UKZN AREC has an active US NIH OLAW assurance (Reg. No. F18-00371; Expires on 31/12/2027).

Animal Research Ethics Committee (AREC)
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APPENDIX 1 B: ETHICAL APPROVAL TO CONDUCT ANIMAL RESEARCH RENEWAL

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

12 February 2024

Renewal
Protocol Reference number: AREC/00005911/2023 (00028554)
Project title: : Soil-transmitted helminths and herpes simplex virus-2 co-infections: Impact on female genital tract microRNA and immune profiles, and potential role in cervical carcinogenesis
Main Applicant and School: Pillay Roxanne, School Of Lab Med & Medical Sc

Dear Ms Pillay

Regarding your revised application received on 13 December 2024, the UKZN Animal Research Ethics Committee (AREC) has accepted the documents submitted, and **FULL APPROVAL** for the protocol was now granted.

The protocol has been approved, subject to the following conditions:

1. Renewal based on outstanding sample analyses and write-up.
2. Eight animals remaining of 72- can the researcher please explain how this came about as it seems that all animal work is completed?

About your approved protocol, please note:

1. All correspondence with the AREC about this protocol must be accompanied by the full reference number.
2. Any alterations to the approved protocol (e.g. title, location, methods, research team) must be reviewed and approved through an official amendment application, via the RIG system, prior to its implementation.
3. Any Veterinary and Para-Veterinary procedures must be conducted by a SAVC registered veterinarian/para-veterinarian or SAVC authorized person, and their details must be logged with the AREC if it changes during the approval period.
4. The AREC may actively monitor research, ask further questions, request additional information, or require modification on welfare grounds. If animal welfare is compromised in any way, the AREC may suspend the study/withdraw approval.
5. The research team must declare any adverse or serious adverse event(s) to the AREC via the RIG system as soon as possible but no later than **48 hours** after the event.
6. Research data should be securely stored in the discipline/school for a period of 5 years.
7. The approval is valid for one year from the date of issue. If the project is continuing, a renewal application must be submitted to the AREC at least **30 days before the expiration date if a timeline extension is applied for**, and **60-90 days ahead of the expiration date if amendments are included in the renewal application**. If work is completed at the end of the approval period, a close-out report must be submitted. Renewals/close-out reports are submitted via the RIG system.

I take this opportunity to wish you all the best with your study.

Yours faithfully

[redacted]

Dr Dalene Vosloo, PhD, Pr. Sci. Nat.
Chair: Animal Research Ethics Committee
/kr

cc Supervisor:
cc BRU Manager:

The UKZN AREC is registered with the SA National Health Research Council (Reg. No. AREC-200111-006; Expires on 31/12/2027) in accordance with the National Health Act (No 61 of 2006) and conforms to the guidelines of the Department of Health (DOH2015) and SANS10386:2021. The UKZN AREC has an active US NIH OLAW assurance (Reg. No. F18-00371; Expires on 31/12/2027).

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APPENDIX 2: SUPPLEMENTARY DATA FOR PUBLICATION 4

SUPPLEMENTARY DATA

RESEARCH ARTICLE

Characterisation of microRNA expression profiles of murine female genital tracts following *Nippostrongylus brasiliensis* and Herpes Simplex Virus type 2 co-infection

Roxanne Pillay [1,2,3], Pragalathan Naidoo [2,3] and Zilungile L. Mkhize-Kwitshana [3,4*]

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[2] Department of Medical Microbiology, College of Health Sciences, School of Laboratory Medicine & Medical Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban 4001, South Africa
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[4] Biomedical Sciences Department of Life and Consumer Sciences, College of Agriculture and Environmental Sciences, University of South Africa, Florida Campus, Johannesburg 1710, South Africa
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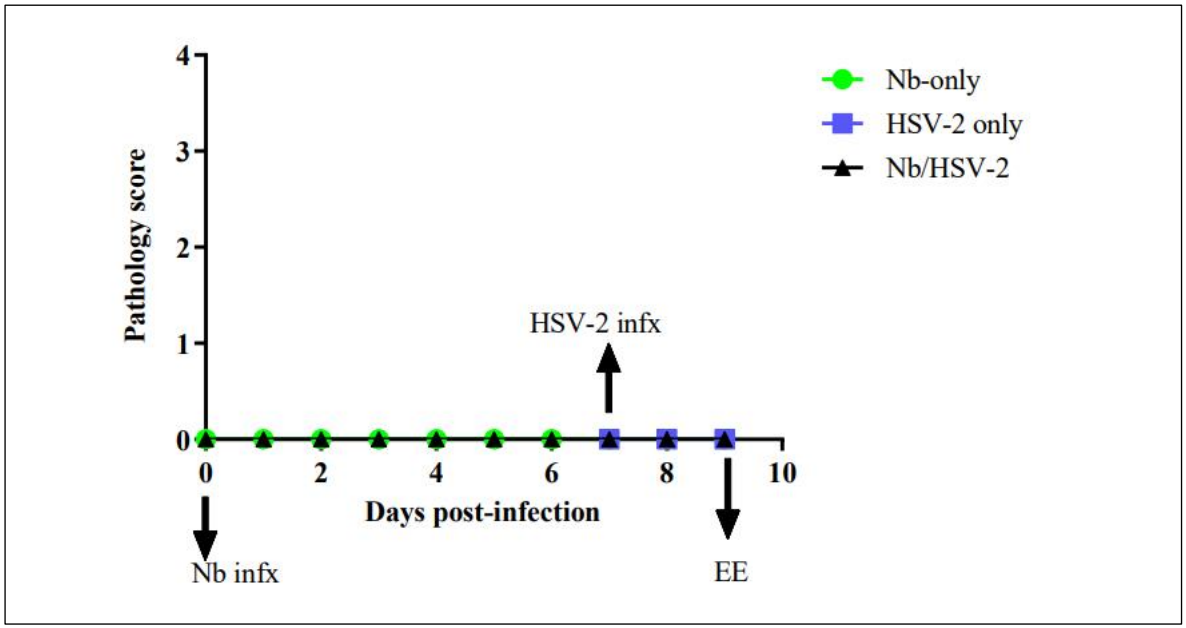
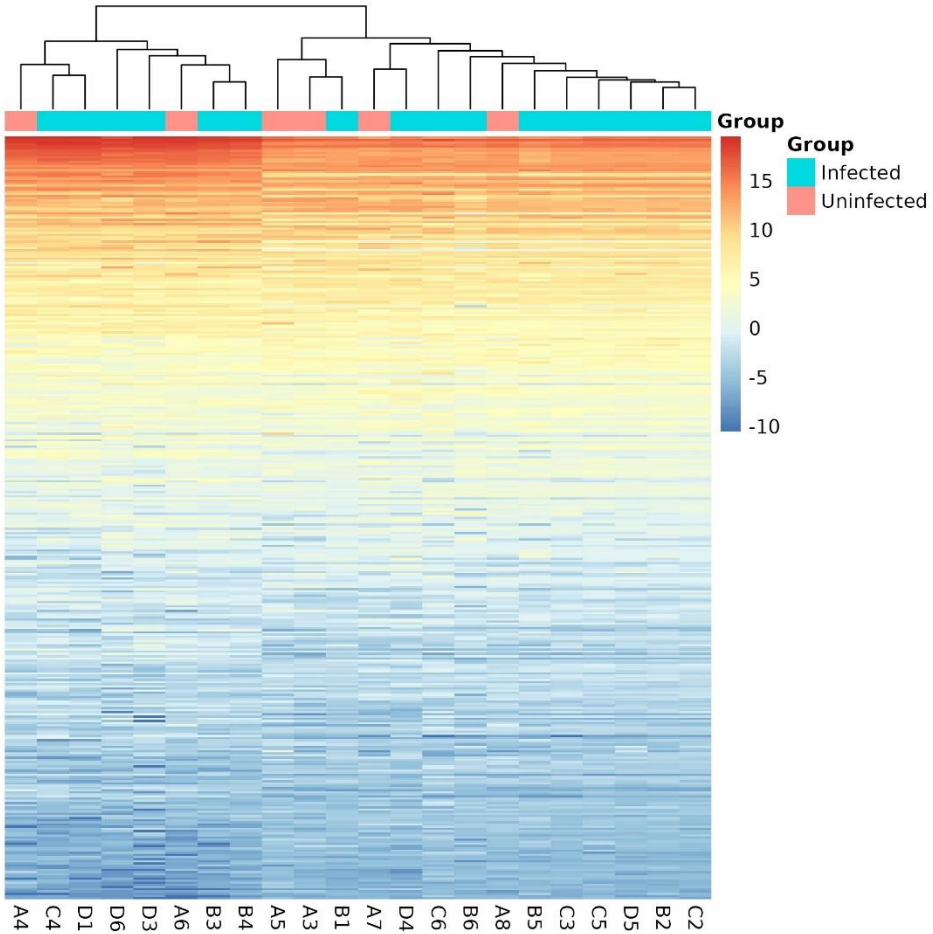


Supplementary Figure S1: Pathology scoring was assessed between the groups post-infection.

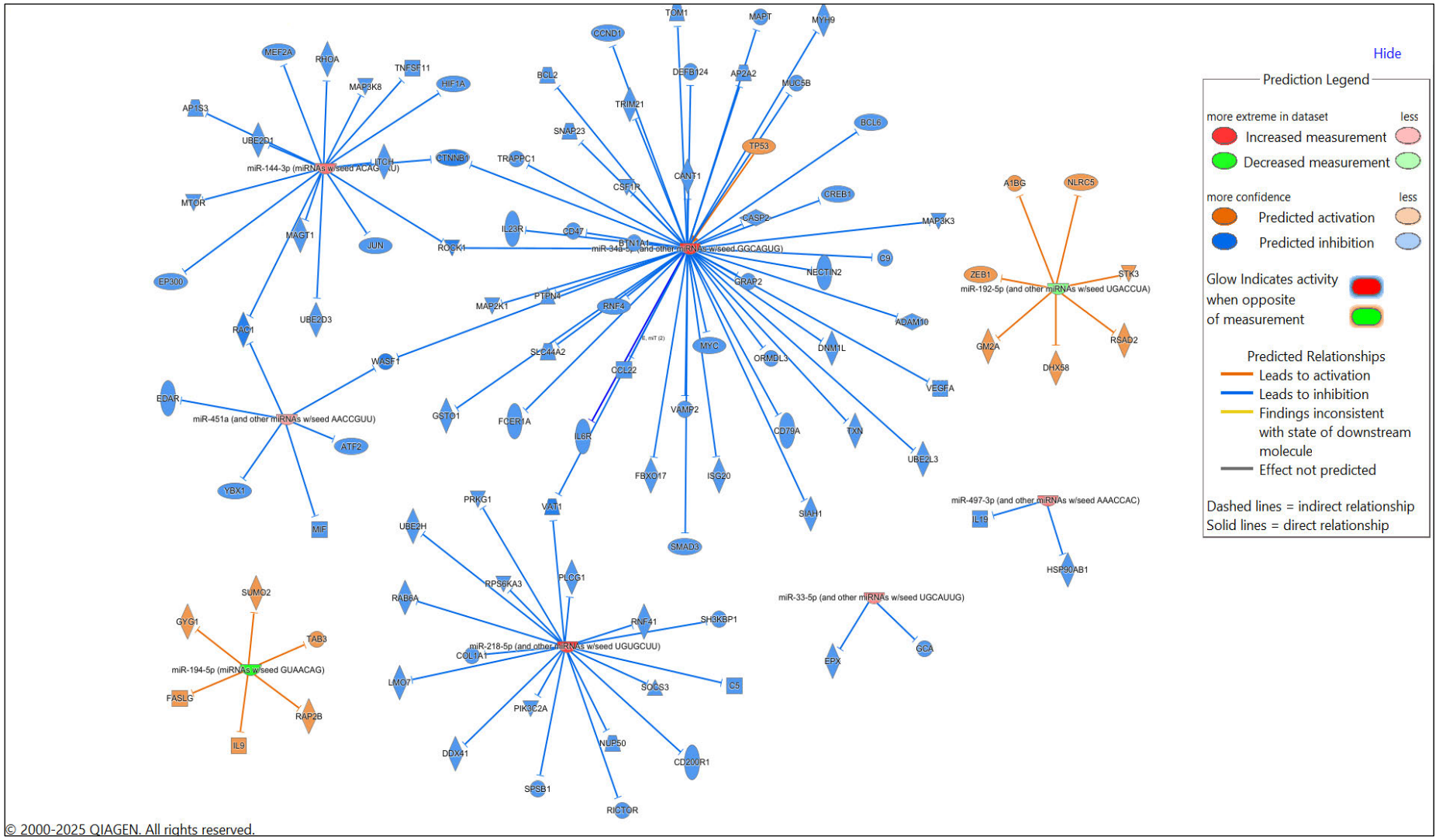
Footnote: *Nb*: *Nippostrongylus brasiliensis*, HSV-2: Herpes Simplex Virus type 2, Infx: Infection, EE: Experimental Endpoint

Supplementary Table S1: Summary of the numbers of reads, precursor and mature miRNAs, and the number of miRNAs reads with ≥5x coverage detected for each sample in groups: A (Uninfected controls), B (singly infected with *Nb*), C (singly infected with HSV-2), and D (*Nb*/HSV-2 co-infected)

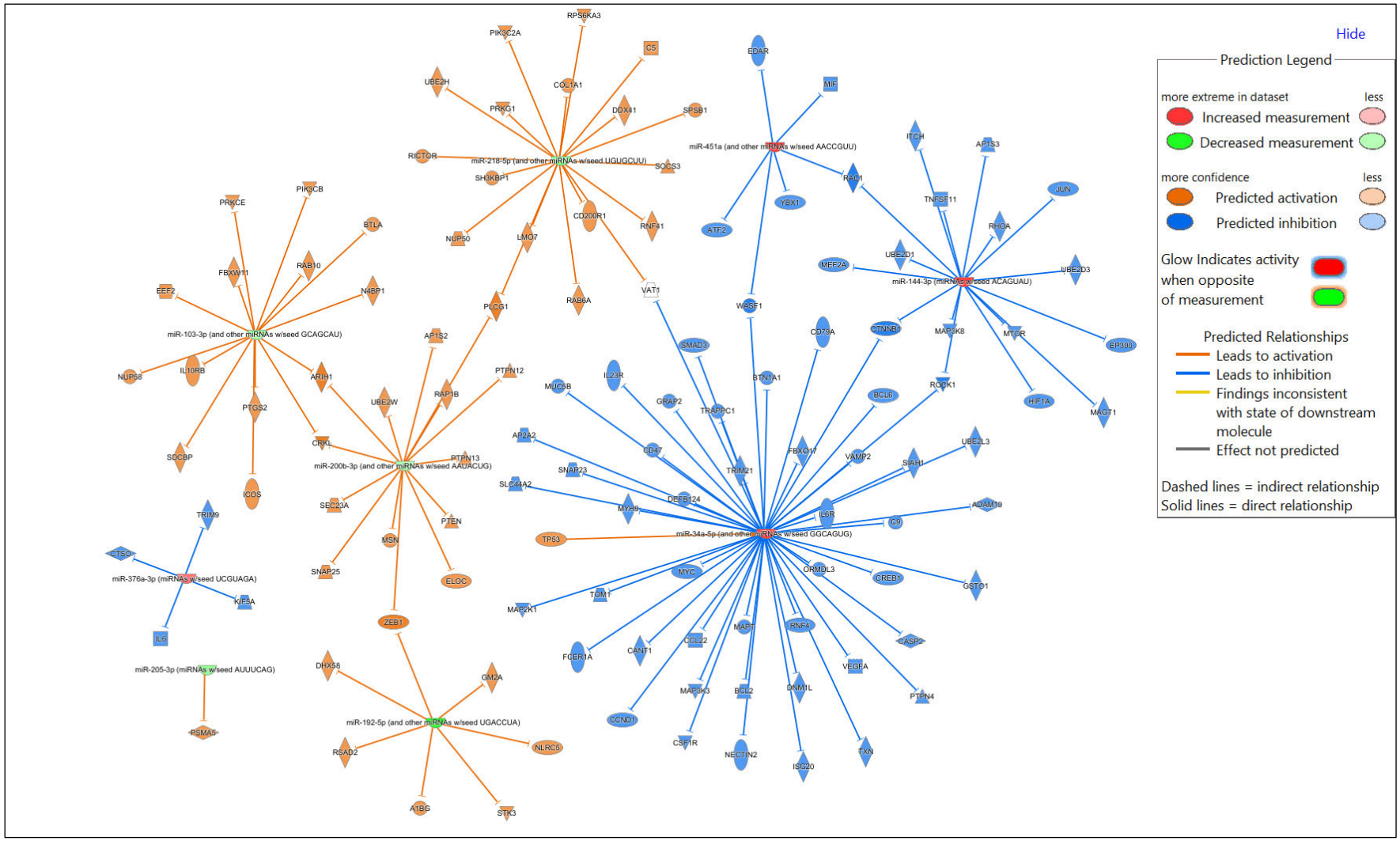
Sample	Reads sent to Aligner	Precursor miRNA Reads	Mature miRNA Reads	Known miRNA with ≥ 5x coverage
A3	20119581	16688	13507171	741
A4	22080150	4137	17162131	521
A5	43263780	28420	31877037	881
A6	22526233	6702	19214322	562
A7	14224394	8600	8186252	702
A8	4884676	2800	3063711	532
B1	19624390	13593	11968979	746
B2	17030994	11229	11707727	753
B3	11648074	4165	9199198	511
B4	11567618	3844	8804494	521
B5	13966191	8533	9236569	745
B6	12537759	11634	7241392	639
C2	17625373	12412	10987299	712
C3	15080880	11177	9437592	727
C4	19293332	4796	16064770	482
C5	13878779	8558	10324506	678
C6	19629940	10223	13664854	725
D1	18003779	3660	15570168	497
D3	20211339	7282	13381540	518
D4	14516633	13575	7602598	690
D5	13704205	9591	8545108	673
D6	7818418	3668	5984503	467



Supplementary Figure S2: Heat map illustrating the overall trend of miRNA expression changes across the four groups: Samples A3-8 (Uninfected control), B1-6 (*Nb*-infected), C2-6 (HSV-2-infected), and D1,3-6 (*Nb*/HSV-2 co-infected)



Supplementary Figure S3: Network showing predicted miRNA-target mRNA relationships in the comparison of *Nb*-infected versus uninfected controls.



Supplementary Figure S4: Network showing predicted miRNA-target mRNA relationships in the comparison of HSV-2-infected versus uninfected controls

APPENDIX 3: SUPPLEMENTARY DATA FILES FOR PUBLICATION 5

SUPPLEMENTARY DATA

RESEARCH ARTICLE

Exploring Gene Expression Changes in Murine Female Genital Tract Tissues Following Single and Co-Infection with *Nippostrongylus brasiliensis* and Herpes Simplex Virus Type 2

Roxanne Pillay [1,2,3], Pragalathan Naidoo [2,3] and Zilungile L. Mkhize-Kwitshana [3,4*]

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[2] Department of Medical Microbiology, College of Health Sciences, School of Laboratory Medicine & Medical Sciences, Nelson R. Mandela School of Medicine, University of KwaZulu-Natal, Durban 4001, South Africa
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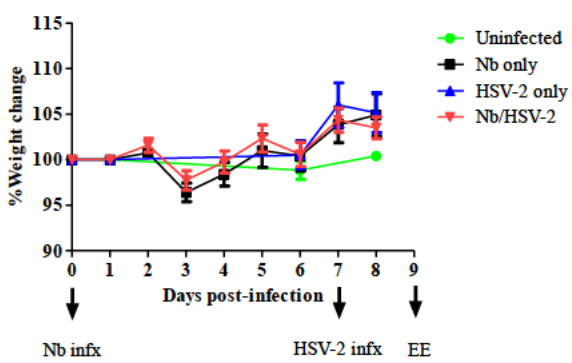
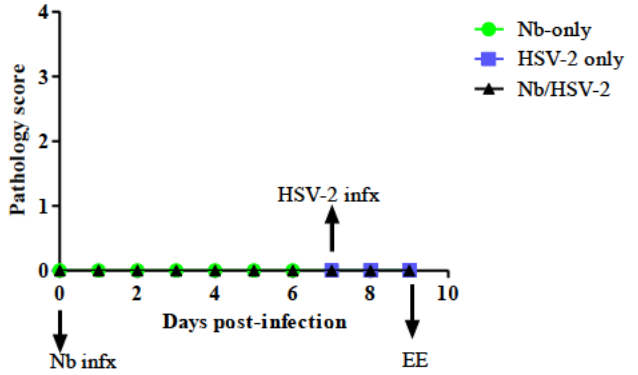
* Correspondence: thungaveloo.roxanne@mut.ac.za and mkhizzl@unisa.ac.za

SUPPLEMENTARY FIGURES S1A & 1B



Supplementary Figure S1A: Daily body weights were assessed between the groups post infection. Data is representative of 6 mice per group (mean +/-SEM)
Footnote: Nb: Nippostrongylus brasiliensis; HSV-2: Herpes simplex virus type 2; Infx: Infection; EE: Experimental endpoint



Supplementary Figure S1B: Pathology scoring was assessed between the groups post infection. Data is representative of 6 mice per group (mean +/-SEM)
Footnote: Nb: Nippostrongylus brasiliensis; HSV-2: Herpes simplex virus type 2; Infx: Infection; EE: Experimental endpoint

SUPPLEMENTARY FILE 1: LIST OF SIGNIFICANT DIFFERENTIALLY EXPRESSED GENES IDENTIFIED IN THE UNINFECTED FGT TISSUES VS *Nb*-INFECTED TISSUES

SUPPLEMENTARY FILE 1
LIST OF SIGNIFICANT DIFFERENTIALLY EXPRESSED GENES IDENTIFIED IN THE UNINFECTED VERSUS Nb-INFECTED COMPARISON

Significant Upregulated Genes: Uninfected versus *Nb*-infected comparison							
Gene.ID	**logFC**	**AveExpr**	**t**	**P.Value**	**adj.P.Val**	**B**	**Gene.name**
ENSMUSG00000033016.17	1.013089711	3.664685647	6.154894301	2.29749767372594e-6	0.015250564	4.703881857	Nfatc1
ENSMUSG00000033220.8	1.304447416	4.688733224	6.104444943	2.59984479866432e-6	0.015250564	4.716450009	Rac2
ENSMUSG00000025017.11	1.54781566	2.922492673	5.744703304	6.32453256771557e-6	0.015250564	3.541738043	Pik3ap1
ENSMUSG00000029098.18	1.023871125	4.420333053	5.59908408	9.0946582522597e-6	0.015250564	3.575129119	Acox3
ENSMUSG00000038644.15	1.022907448	3.965164184	5.365769625	1.63338601907815e-5	0.015250564	3.018449656	Pold1
ENSMUSG00000039637.16	1.054884706	4.246659925	5.343776996	1.7264271116346e-5	0.015250564	2.982887148	Coro7
ENSMUSG00000033083.17	1.108524773	3.371843705	5.316319452	1.85014519496723e-5	0.015250564	2.836357156	Tbc1d4
ENSMUSG00000089960.2	1.272976795	4.024021598	5.258580148	2.14033293475749e-5	0.015250564	2.778539108	Ugt1a1
ENSMUSG00000029547.11	1.157201635	4.557877934	5.258387741	2.14137309495913e-5	0.015250564	2.803313507	Ints1
ENSMUSG00000041638.19	1.035722094	5.492382335	5.186514451	2.56791376626582e-5	0.015250564	2.651097705	Gcn1
ENSMUSG00000000631.21	1.138992164	5.947895233	5.122427949	3.02014796890329e-5	0.015250564	2.501983015	Myo18a
ENSMUSG00000003882.6	1.536321094	1.265410989	5.07912911	3.37036819468236e-5	0.015250564	1.575354605	Il7r
ENSMUSG00000032536.13	1.084688279	6.36110277	5.078719128	3.37387286688088e-5	0.015250564	2.399534133	Trak1
ENSMUSG00000028581.18	1.677664503	5.54747713	4.974974823	4.38986573137294e-5	0.015250564	2.154645238	Laptm5
ENSMUSG00000004099.17	1.055600725	4.8381111	4.93037257	4.91655368519483e-5	0.015250564	2.049277305	Dnmt1
ENSMUSG00000034584.4	1.5641272	2.87341354	4.902573858	5.27651279906123e-5	0.015250564	1.86722767	Exph5
ENSMUSG00000031004.9	1.054339666	4.022290296	4.889864352	5.44979884638675e-5	0.015250564	1.943675707	Mki67
ENSMUSG00000024621.17	1.675040348	5.540656934	4.882520078	5.55252928226262e-5	0.015250564	1.938062396	Csf1r
ENSMUSG00000026116.12	1.098473223	5.470970569	4.862028017	5.84957669639351e-5	0.015250564	1.889975493	Tmem131
ENSMUSG00000030560.18	1.32256447	5.672706479	4.839088433	6.20108697718402e-5	0.015250564	1.835922495	Ctsc
ENSMUSG00000022946.11	1.119397113	4.473006066	4.764841335	7.49094666882017e-5	0.015250564	1.658546146	Dop1b
ENSMUSG00000032380.10	1.373980135	3.230321967	4.757750723	7.62741225781173e-5	0.015250564	1.56833606	Dapk2
ENSMUSG00000020357.4	1.48326546	2.645170668	4.756881031	7.64432111830697e-5	0.015250564	1.470248137	Flt4
ENSMUSG00000061607.16	1.085025079	3.567966114	4.731079983	8.16342795292979e-5	0.015250564	1.555126498	Mdc1
ENSMUSG00000028931.13	1.468432504	2.082988344	4.72775567	8.23283383296007e-5	0.015250564	1.236099568	Kcnab2
ENSMUSG00000120290.1	1.078152712	4.076971543	4.71994937	8.39815090464625e-5	0.015250564	1.547501228	Gm56959
ENSMUSG00000026288.15	1.669838918	3.176626304	4.694021699	8.97149123918216e-5	0.015250564	1.414253776	Inpp5d

ENSMUSG00000022443.18	1.286651528	7.241934628	4.665400481	9.6500606755868e-5	0.015250564	1.427001479	Myh9
ENSMUSG00000020100.16	1.158797164	3.722151821	4.663670373	9.69268974288624e-5	0.015250564	1.405607839	Slc29a3
ENSMUSG00000048865.17	1.503202611	2.718899232	4.657685629	9.84161124321008e-5	0.015250564	1.255935745	Arhgap30
ENSMUSG00000046179.18	1.271708202	1.732322758	4.613197596	1.10229e-4	0.015250564	1.013427555	E2f8
ENSMUSG00000033327.19	1.106180252	4.992535421999	4.61070066	1.10932e-4	0.015250564	1.300701406	Tnxb
ENSMUSG00000042606.10	1.115917798	3.117872731	4.605347569	1.12456e-4	0.015250564	1.231325385	Hirip3
ENSMUSG00000050107.3	1.222715262	1.101300789	4.602275513	1.1334e-4	0.015250564	0.670976346	Haspin
ENSMUSG00000014444.18	1.001815357	5.630577639	4.58546537	1.183e-4	0.015250564	1.237802505	Piezo1
ENSMUSG00000099708.2	1.216288651	1.848060376	4.577964023	1.20583e-4	0.015250564	0.895666946	Gm29361
ENSMUSG00000039087.18	1.144887364	4.944796078	4.576817466	1.20936e-4	0.015250564	1.220803614	Rreb1
ENSMUSG00000041762.17	1.092678772	1.782525611	4.572046695	1.22415e-4	0.015250564	0.857787926	Gpr155
ENSMUSG00000042129.9	1.299414319	2.822709225	4.557039147	1.27188e-4	0.015250564	1.070002057	Rassf4
ENSMUSG00000021998.17	1.26573811	5.741745422	4.550451173	1.29341e-4	0.015250564	1.15536805	Lcp1
ENSMUSG00000039218.18	1.04342192	6.813831376	4.547315201	1.30379e-4	0.015250564	1.147213154	Srrm2
ENSMUSG00000068876.17	1.034190071	5.054362833	4.534859197	1.34584e-4	0.015250564	1.121588833	Cgn
ENSMUSG00000047248.21	1.013187942	3.977059213	4.531771763	1.35647e-4	0.015250564	1.112458348	C2cd3
ENSMUSG00000050271.13	1.394939744	3.686874396	4.525565755	1.37809e-4	0.015332446	1.089109834	Prag1
ENSMUSG00000036995.8	1.039979794	3.632796848	4.520983387	1.39428e-4	0.015336257	1.073873319	Asap3
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ENSMUSG00000060012.10	1.085189321	4.412647236	4.507036353	1.44472e-4	0.01553798	1.059837255	Kif13b
ENSMUSG00000076435.4	1.538507932	4.940901669	4.487982057	1.5166e-4	0.015870179	1.013572331	Acsf2
ENSMUSG00000059323.15	1.213300274	1.949721711	4.476606056	1.5612e-4	0.015942755	0.757311149	Tonsl
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ENSMUSG00000040840.8	1.113384492	1.949529862	4.462955006	1.61646e-4	0.01617187	0.712478297	4930579G18Rik
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ENSMUSG00000020143.16	1.585539618	2.476818265	4.397383711	1.91034e-4	0.016914185	0.662119162	Dock2
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ENSMUSG00000034853.17	1.039490857	3.773761824	4.350754182	2.1512e-4	0.017039666	0.695738989	Acot11

ENSMUSG00000042700.17	1.00008232	5.151706565	4.346398081	2.17519e-4	0.017039666	0.678393632	Sipa1l1
ENSMUSG00000024130.17	1.15771989	6.685067291	4.3422151	2.19848e-4	0.017039666	0.663176145	Abca3
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ENSMUSG00000030789.10	1.943373327	2.420602926	4.332652731	2.25265e-4	0.017039666	0.516384809	Itgax
ENSMUSG00000022488.10	1.699310708	3.487719768	4.315633771	2.35238e-4	0.017039666	0.601393253	Nckap1l
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ENSMUSG00000027799.13	1.073596138	3.41398906	4.242817189	2.83115e-4	0.018144831	0.445120575	Nbea
ENSMUSG00000033066.18	1.352738071	2.509092713	4.236583336	2.87638e-4	0.018257001	0.356582932	Gas7
ENSMUSG00000018909.16	1.149243672	4.787669721	4.233781905	2.89694e-4	0.018327418	0.41958226	Arrb1
ENSMUSG00000105776.2	1.21712858	1.937031916	4.228614762	2.93525e-4	0.018424286	0.243320098	Gm43292
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ENSMUSG00000042489.16	1.237803941	1.26932963	4.206825309	3.10241e-4	0.018424286	0.069481625	Clspn
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ENSMUSG00000027324.2	1.052289289	1.904515633	4.177429584	3.34301e-4	0.018570087	0.129041944	Rpusd2
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ENSMUSG00000017499.16	1.177028444	1.556715521	4.165126017	3.44913e-4	0.018651321	0.074410652	Cdc6
ENSMUSG00000071714.8	1.204769332	2.421484434	4.154211251	3.54607e-4	0.018667081	0.170853117	Csf2rb2
ENSMUSG00000079227.11	1.99443968	2.507984668	4.151423683	3.57126e-4	0.018667081	0.134839807	Ccr5
ENSMUSG00000021275.17	1.053434064	3.071442386	4.148790707	3.59521e-4	0.018667081	0.221996719	Tecpr2
ENSMUSG00000115767.2	1.179531844	3.549477605	4.138991303	3.68577e-4	0.018667081	0.20887368	Gm10366
ENSMUSG00000022504.11	1.42387317	1.852112325	4.137020872	3.70425e-4	0.018667081	-0.023583597	Ciita
ENSMUSG00000026786.15	1.385106834	2.769501988	4.134599853	3.72709e-4	0.018667081	0.147870223	Apbb1ip
ENSMUSG00000029478.17	1.033327504	5.881456696	4.128382327	3.78637e-4	0.018667081	0.159647382	Ncor2
ENSMUSG00000020272.9	1.110775219	3.449727577	4.126006926	3.80926e-4	0.018667081	0.18151277	Stk10
ENSMUSG00000052336.9	2.004254327	4.044767997	4.125056497	3.81846e-4	0.018667081	0.180575298	Cx3cr1
ENSMUSG00000027475.10	1.027277448	4.59737121	4.123178352	3.83671e-4	0.018694898	0.164253798	Kif3b

ENSMUSG00000055805.16	1.623906219	3.214734735	4.105784166	4.00982e-4	0.018918235	0.113869824	Fmnl1
ENSMUSG00000036155.14	1.007815141	4.427396028	4.103959907	4.02842e-4	0.01892395	0.122556975	Mgat5
ENSMUSG00000057337.15	1.4343023	0.783728768	4.089572566	4.17813e-4	0.018980819	-0.354328716	Chst3
ENSMUSG00000022831.15	1.39769191	3.57790253	4.087442982	4.20075e-4	0.018980819	0.091755724	Hcls1
ENSMUSG00000030527.16	1.02131411	4.24997527	4.082048537	4.25861e-4	0.018980819	0.077602363	Crtc3
ENSMUSG00000018008.9	1.425439783	4.155087393	4.077560484	4.30734e-4	0.018980819	0.071676491	Cyth4
ENSMUSG00000121914.1	1.132975259	1.77896315	4.069058238	4.40118e-4	0.018991757	-0.16348524	Sts
ENSMUSG00000021097.16	1.15583526	6.053617707	4.064362157	4.45388e-4	0.018991757	0.00940604	Clmn
ENSMUSG00000061894.16	1.002563108	1.836719	4.062296604	4.47725e-4	0.018998101	-0.134627444	Zscan20
ENSMUSG00000030223.15	1.206069355	0.946536642	4.061907662	4.48167e-4	0.018998101	-0.441693792	Ptpro
ENSMUSG00000022372.15	1.357470091	2.412784078	4.060768989	4.49462e-4	0.018998101	-0.051815743	Sla
ENSMUSG00000025278.10	1.017856991	7.131820265	4.055132227	4.55928e-4	0.019197295	-0.008441351	Flnb
ENSMUSG00000085327.3	1.406960213	2.003717989	4.05461694	4.56523e-4	0.019197295	-0.129253925	Gm16104
ENSMUSG00000042759.13	2.276060233	2.518926602	4.047593762	4.64719e-4	0.019270699	-0.075159799	Apobr
ENSMUSG00000033871.15	1.210441765	2.566251825	4.047272533	4.65098e-4	0.019270699	-0.060521147	Ppargc1b
ENSMUSG00000012123.18	1.0794080130000	3.429285973	4.046006142	4.66592e-4	0.019270699	5.86192e-4	Crybg2
ENSMUSG00000102918.2	1.044746058	3.637436188	4.030495567	4.85287e-4	0.019602432	-0.033939108	Pcdhgc3
ENSMUSG00000073421.7	1.125060522	7.143112358	4.029172217	4.86916e-4	0.019602432	-0.070346485	H2-Ab1
ENSMUSG00000073600.6	1.057961497	3.240590105	4.020999072	4.97097e-4	0.019753576	-0.061113197	Prob1
ENSMUSG00000021624.10	1.65357796	1.235648141	4.0174993	5.0152e-4	0.019773794	-0.501692307	Cd180
ENSMUSG00000099874.2	1.71189618	0.503857828	4.003609909	5.19464e-4	0.01996432	-0.81014575	Gm29629
ENSMUSG00000034342.10	1.012939686	4.259549735	3.997972314	5.26927e-4	0.01996432	-0.117742169	Cbl
ENSMUSG00000031659.14	1.411183346	2.733062455	3.989280608	5.38642e-4	0.020130427	-0.159767984	Adcy7
ENSMUSG00000027188.9	1.213054012	4.256534129	3.985014188	5.44486e-4	0.020230344	-0.140241155	Pamr1
ENSMUSG00000074785.6	1.287997804	1.817402268	3.977206335	5.55343e-4	0.020400177	-0.312859296	Plxnc1
ENSMUSG00000030123.16	1.095206073	4.532825714	3.974285429	5.5946e-4	0.020430676	-0.176535992	Plxnd1
ENSMUSG00000042359.19	1.406274622	2.247313878	3.964903231	5.72888e-4	0.020829774	-0.242499072	Osbpl6
ENSMUSG00000034116.18	1.571920422	2.799276023	3.954583188	5.88027e-4	0.020836518	-0.24132051	Vav1
ENSMUSG00000021279.6	1.068435314	5.782848561	3.952125813	5.9169e-4	0.020836518	-0.252303302	Cdc42bpb
ENSMUSG00000103309.2	1.098596227	2.974697703	3.949371978	5.95821e-4	0.020889751	-0.223804047	BC037039
ENSMUSG00000017692.9	1.124253664	2.728614977	3.939896763	6.10255e-4	0.021098594	-0.269737752	Rhbdl3
ENSMUSG00000030737.18	1.056107628	3.437985339	3.932480937	6.21792e-4	0.021166016	-0.256213845	Slco2b1

ENSMUSG00000021277.17	1.150739626	3.956714055	3.932409205	6.21905e-4	0.021166016	-0.258033058	Traf3
ENSMUSG00000121608.1	1.301640065	0.080019177	3.928337618	6.28332e-4	0.021166016	-1.190195826	
ENSMUSG00000041773.9	1.09744876	3.18393367	3.926155174	6.31803e-4	0.021197716	-0.272333782	Enc1
ENSMUSG00000097440.3	1.047838854	2.067902608	3.920625466	6.40685e-4	0.021261154	-0.403287788	Gm6277
ENSMUSG00000085178.2	1.070882888	1.935855063	3.918215578	6.44594e-4	0.021261154	-0.421765224	Kdm6bos
ENSMUSG00000098912.9	1.473110664	0.176803193	3.91767999	6.45466e-4	0.021261154	-1.094296356	1500004A13Rik
ENSMUSG00000026417.14	1.60383693	8.634822925	3.917552303	6.45674e-4	0.021261154	-0.316217475	Pigr
ENSMUSG00000055061.6	1.59066266	0.642405178	3.912689331	6.53648e-4	0.021303693	-0.921040162	4931431C16Rik
ENSMUSG00000026177.12	1.422335442	2.694694119	3.903184553	6.69514e-4	0.021492548	-0.356806638	Slc11a1
ENSMUSG00000030583.17	1.141793286	5.255949578	3.902561811	6.70567e-4	0.021492548	-0.36203892	Sipa1l3
ENSMUSG00000026355.12	1.093686891	4.680894628	3.900850236	6.73468e-4	0.021525591	-0.353959901	Mcm6
ENSMUSG00000026604.18	1.140547288	4.852215713	3.897385018	6.79381e-4	0.021525591	-0.360493885	Ptpn14
ENSMUSG00000038267.16	1.104654095	4.799251517	3.89463159	6.84116e-4	0.021534625	-0.367633215	Slc22a23
ENSMUSG00000052085.8	1.226039534	4.430800413	3.884239686	7.0228e-4	0.021682137	-0.384720445	Dock8
ENSMUSG00000047810.10	1.189922925	3.574116574	3.869539129	7.28791e-4	0.021781229	-0.394812659	Ccdc88b
ENSMUSG00000021366.9	1.141610145	4.686431317	3.866986499	7.33494e-4	0.021781229	-0.428746286	Hivep1
ENSMUSG00000085009.2	1.177314741	2.608273081	3.860093683	7.46343e-4	0.021865672	-0.437101291	Gm12977
ENSMUSG00000034871.3	1.080982023	2.374965868	3.859656994	7.47165e-4	0.021865672	-0.457392737	Fam151a
ENSMUSG00000039852.18	1.188168906	5.794298812	3.855355814	7.55304e-4	0.021910191	-0.475524612	Rere
ENSMUSG00000096768.10	1.046798123	3.347334743	3.853786753	7.58295e-4	0.021910191	-0.431112198	Erdr1y
ENSMUSG00000048779.6	1.527188362	2.869004623	3.850153219	7.65266e-4	0.021952669	-0.460466167	P2ry6
ENSMUSG00000020661.17	1.032632125	4.230307733	3.845484337	7.74316e-4	0.022128091	-0.4652328	Dnmt3a
ENSMUSG00000046541.10	1.045704755	2.54391898	3.841267553	7.8258e-4	0.022128091	-0.482027335	Zfp526
ENSMUSG00000046805.11	1.670505288	5.617916623	3.840759651	7.83581e-4	0.022128091	-0.503542523	Mpeg1
ENSMUSG00000085582.8	1.71106463	-0.250661214	3.83538611	7.94252e-4	0.022128091	-1.531186356	3110099E03Rik
ENSMUSG00000041757.18	1.14788315	5.452481091	3.833446329	7.98139e-4	0.022128091	-0.523987387	Plekha6
ENSMUSG00000024301.11	1.200770165	1.312061945	3.82724443	8.10693e-4	0.022149601	-0.76092639	Kifc5b
ENSMUSG00000120949.1	1.021273216	2.293215417	3.826452816	8.12309e-4	0.022149601	-0.530256959	Gm56545
ENSMUSG00000070808.5	1.114083556	3.306537694	3.823075337	8.19241e-4	0.022149601	-0.498020771	Bicra
ENSMUSG00000098609.2	1.288432273	3.469216493	3.811959815	8.42468e-4	0.022257291	-0.524416334	Anxa11os
ENSMUSG00000007817.16	1.235733999	5.968828397	3.808668138	8.4947e-4	0.022257291	-0.586148404	Zmiz1
ENSMUSG00000038369.15	1.090470906	4.471311105	3.801266222	8.65426e-4	0.022365084	-0.573640982	Ncoa6

ENSMUSG00000024393.15	1.05579087	7.616684998	3.801081498	8.65827e-4	0.022365084	-0.596799727	Prrc2a
ENSMUSG00000036896.6	1.413346954	6.091366585	3.797058086	8.74628e-4	0.022478926	-0.611233152	C1qc
ENSMUSG00000039621.14	1.086972767	3.417481326	3.794501663	8.80265e-4	0.022486456	-0.562501172	Prex1
ENSMUSG00000035697.15	1.052884929	3.762802625	3.791269796	8.87442e-4	0.022575555	-0.570807133	Arhgap45
ENSMUSG00000026669.15	1.059083839	1.580190052	3.78478755	9.02013e-4	0.02272991	-0.679391026	Mcm10
ENSMUSG00000086618.2	1.924313585	0.730025187	3.781815214	9.08773e-4	0.022790228	-1.175457959	Gm13256
ENSMUSG00000036834.18	1.059925052	2.626889641	3.775401293	9.23529e-4	0.022790228	-0.613245654	Plch1
ENSMUSG00000031386.15	1.027689165	5.248219182	3.774676187	9.25212e-4	0.022790228	-0.658228093	Hcfc1
ENSMUSG00000097405.3	1.003002812	3.671030992	3.766656729	9.44028e-4	0.023002483	-0.628584425	D630044L22Rik
ENSMUSG00000103009.3	1.036818891	1.511449604	3.760401208	9.58967e-4	0.023119099	-0.779737349	Gm56350
ENSMUSG00000093390.2	1.319934591	-0.077372188	3.754366321	9.73598e-4	0.023211685	-1.468473922	Gm20652
ENSMUSG00000032528.6	1.421958098	5.155327885	3.752535454	9.7808e-4	0.023261251	-0.706241442	Vipr1
ENSMUSG00000029603.16	1.367539745	2.199813062	3.747769627	9.89843e-4	0.023483298	-0.74591143	Dtx1
ENSMUSG00000033792.13	1.199379189	2.395170983	3.746470383	9.93074e-4	0.023495116	-0.679564046	Atp7a
ENSMUSG00000070047.15	1.155852193	6.39083561	3.743466967	0.001000582	0.023536165	-0.737466365	Fat1
ENSMUSG00000092405.2	1.468725794	0.902588127	3.734982653	0.001022094	0.023570501	-1.091744241	Gm20402
ENSMUSG00000024300.18	1.50329347	3.28380062	3.733875858	0.001024934	0.023570501	-0.694915801	Myo1f
ENSMUSG00000041859.11	1.054726451	4.117765304	3.733809213	0.001025105	0.023570501	-0.717868605	Mcm3
ENSMUSG00000044788.11	1.441024312	1.248796313	3.72904282	0.001037424	0.023655477	-0.937872415	Fads6
ENSMUSG00000086717.2	1.067542812	2.173647519	3.719779298	0.001061785	0.023984945	-0.770931847	Gm15655
ENSMUSG00000092371.2	2.518732723	-0.621400421	3.717865377	0.001066888	0.023988247	-2.170824502	Gm20511
ENSMUSG00000039976.5	1.262734593	4.914255111	3.714961689	0.001074675	0.023989901	-0.782637676	Tbc1d16
ENSMUSG00000055013.17	1.064394099	5.4611834	3.709614632	0.001089162	0.024124818	-0.811225221	Agap1
ENSMUSG00000020901.14	1.617252445	1.369691049	3.70588383	0.001099384	0.02423507	-1.023004194	Pik3r5
ENSMUSG00000026483.14	1.414223101	6.566155801	3.698097574	0.00112102	0.024466544	-0.840532285	Niban1
ENSMUSG00000035557.10	1.482311138	6.691655117	3.691197975	0.001140541	0.02463742	-0.855797758	Krt17
ENSMUSG00000079487.12	1.132102052	3.917894704	3.689159655	0.001146372	0.024685893	-0.806831448	Med12
ENSMUSG00000043279.10	1.202933865	4.407800389	3.68806957	0.001149502	0.024698353	-0.82362355	Trim56
ENSMUSG00000046618.8	1.268175239	3.282409544	3.673483967	0.001192201	0.025114064	-0.830641239	Olfml2a
ENSMUSG00000042744.17	1.065352459	4.193645266	3.672727333	0.001194458	0.025120536	-0.85882933400	Hectd4
ENSMUSG00000097816.2	1.181031862	3.622040403	3.669805089	0.001203215	0.02520491	-0.839316047	Gm26811
ENSMUSG00000031103.13	1.090248568	3.277880403	3.669435739	0.001204326	0.02520491	-0.836639261	Elf4

ENSMUSG00000055629.6	1.412281953	1.508674009	3.663981076	0.001220854	0.025256065	-1.045961716	B4galnt4
ENSMUSG00000030091.18	1.337699845	2.723581526	3.663394591	0.001222644	0.025256065	-0.850330108	Nup210
ENSMUSG00000054252.19	1.08459865	6.305141481	3.660005911	0.001233039	0.02528644	-0.930495578	Fgfr3
ENSMUSG00000106896.2	1.357795381	2.666329858	3.656853061	0.001242788	0.02537189	-0.871374208	G630022F23Rik
ENSMUSG00000041000.9	1.112052757	4.144323536	3.654773001	0.001249261	0.02537189	-0.891668041	Trim62
ENSMUSG00000113184.2	1.607744172	0.593690062	3.652901157	0.001255115	0.02537189	-1.398017868	Gm49654
ENSMUSG00000040829.15	1.024164976	4.360795739	3.652760991	0.001255554	0.02537189	-0.905021541	Zmynd15
ENSMUSG00000002020.16	1.187931204	4.853827695	3.647047432	0.001273594	0.025496904	-0.929103138	Ltbp2
ENSMUSG00000052160.8	1.390831386	4.106199507	3.645475122	0.001278603	0.025570736	-0.903496374	Pld4
ENSMUSG00000121235.1	1.303460681	1.635376234	3.643228829	0.001285792	0.025618507	-1.044592665	Gm57252
ENSMUSG00000030657.12	1.23488607	1.33959734	3.643075361	0.001286285	0.025618507	-1.059127485	Xylt1
ENSMUSG00000032788.16	1.1151955	6.437426454	3.642011507	0.001289705	0.02562199	-0.97165603	Pdxk
ENSMUSG00000048251.16	1.002775827	3.396481705	3.629205135	0.00133158	0.02603844	-0.926905989	Bcl11b
ENSMUSG00000071984.13	1.268306623	2.868423114	3.62531436	0.001344564	0.026206313	-0.934728932	Fndc1
ENSMUSG00000052942.14	1.184075761	2.516220099	3.622267238	0.001354819	0.026237502	-0.946435229	Glis3
ENSMUSG00000104011.3	1.440279888	0.770563259	3.621346801	0.001357932	0.026237502	-1.329809124	Gm32391
ENSMUSG00000111375.6	1.079800419	0.340090699	3.621328717	0.001357993	0.026237502	-1.450599496	Btbd8
ENSMUSG00000030650.19	1.690121517	3.652552523	3.619678926	0.00136359	0.026237502	-0.947483814	Tmc5
ENSMUSG00000028068.15	1.172278643	2.212752629	3.619577233	0.001363936	0.026237502	-0.955922104	Iqgap3
ENSMUSG00000112652.2	1.049061559	5.835824291	3.619413309	0.001364494	0.026237502	-1.021203305	4921516A02Rik
ENSMUSG00000092134.2	1.47069444	0.329340421	3.615161653	0.001379032	0.026373078	-1.522682242	Gm17089
ENSMUSG00000092482.2	1.072186959	1.662815592	3.613921823	0.0013833	0.026403371	-1.058683612	Gm20531
ENSMUSG00000027009.19	1.410851518	1.713022979	3.611681229	0.001391046	0.026479108	-1.067742568	Itga4
ENSMUSG00000040761.17	1.174615122	4.404462501	3.60770776	0.001404887	0.026587996	-1.010339954	Spen
ENSMUSG00000074305.10	1.141041801	3.322564207	3.603293632	0.001420421	0.026726391	-0.983338082	Peak1
ENSMUSG00000036905.9	1.37913558	6.167447001	3.601833789	0.001425595	0.026742341	-1.061371639	C1qb
ENSMUSG00000001095.6	1.5668388	2.682364546	3.597443742	0.001441267	0.026931935	-0.996381638	Slc13a2
ENSMUSG00000104600.5	1.566864504	1.20971237	3.594424189	0.001452144	0.027082903	-1.199735795	Dmrt1i
ENSMUSG00000037003.17	1.066208668	5.567120447	3.593497459	0.001455498	0.027119337	-1.076566607	Tns2
ENSMUSG00000044393.16	1.149096594	4.69384543	3.591857825	0.001461452	0.027129234	-1.059708097	Dsg2
ENSMUSG00000010175.14	1.601018342	0.613434456	3.591440794	0.00146297	0.027129234	-1.436467455	Prox1
ENSMUSG00000039262.17	1.088550471	6.650196374	3.589435856	0.001470289	0.027180585	-1.090977051	Prrc2b

ENSMUSG00000032177.18	1.245577002	2.116371318	3.588468778	0.001473832	0.027180585	-1.038366598	Pde4a
ENSMUSG00000035299.18	1.003971796	1.658431591	3.582812608	0.001494725	0.027313422	-1.093281813	Mid1
ENSMUSG00000038642.11	1.531607575	6.137525938	3.574898226	0.001524445	0.027581052000000	-1.122552209	Ctss
ENSMUSG00000108255.2	1.054292017	0.643205058	3.566599289	0.00155623	0.027799553	-1.425388782	Gm16499
ENSMUSG00000016028.12	1.120564592	6.134628165	3.565773858	0.001559426	0.027799553	-1.146082906	Celsr1
ENSMUSG00000026321.9	1.219205352	3.673144929	3.562217384	0.001573273	0.027817566	-1.08794093	Tnfrsf11a
ENSMUSG00000041642.19	1.210687045	3.00470179	3.554459315	0.001603896	0.028082379	-1.084426885	Kif21b
ENSMUSG00000018774.14	1.301117274	4.654687152	3.550513692	0.001619693	0.028197519	-1.14017172000	Cd68
ENSMUSG00000073529.10	1.175491009	0.089997964	3.543742003	0.00164716	0.028467003	-1.569950316	F830208F22Rik
ENSMUSG00000031165.7	1.293348957	2.021360327	3.539093901	0.001666277	0.028673288	-1.173690725	Was
ENSMUSG00000005442.14	1.024348362	5.832413444	3.536325953	0.001677763	0.0287747	-1.212088441	Cic
ENSMUSG00000031709.16	1.261175214	3.519097478	3.534691542	0.001684582	0.028834503	-1.135807659	Tbc1d9
ENSMUSG00000085819.8	1.094450844	2.120985321	3.531016953	0.001700012	0.028945051	-1.165552828	Ube4bos1
ENSMUSG00000114934.2	1.022533737	3.962091505	3.521638113	0.001740022	0.029342401	-1.200093824	Gm48342
ENSMUSG00000026395.17	1.287709529	3.486568761	3.518036705	0.001755628	0.029508935	-1.168376822	Ptprc
ENSMUSG00000004730.16	1.250381278	3.696543446	3.516375481	0.001762873	0.029589743	-1.176137546	Adgre1
ENSMUSG00000048154.18	1.090313874	5.131341801	3.516149705	0.00176386	0.029589743	-1.245633715	Kmt2d
ENSMUSG00000116010.2	1.416658525	4.702900675	3.514363092	0.001771688	0.029629358	-1.236176553	Gm36026
ENSMUSG00000020261.16	1.231271587	3.922373479	3.493908504	0.001863768	0.030421579	-1.241665885	Slc36a1
ENSMUSG00000049103.15	1.520623909	4.576899409	3.491303003	0.001875827	0.030437725	-1.273062292	Ccr2
ENSMUSG00000042699.12	1.011592253	5.478899098	3.489903381	0.001882336	0.030493851	-1.314629851	Dhx9
ENSMUSG00000042082.8	1.060510496	4.452217714	3.478973944	0.00193393	0.030875892	-1.301255981	Arsb
ENSMUSG00000040751.13	1.161199135	2.296244075	3.478548167	0.001935968	0.030875892	-1.258949146	Lat2
ENSMUSG00000040552.9	1.568260373	3.078496028	3.476130374	0.001947578	0.030969484	-1.251743209	C3ar1
ENSMUSG00000068747.15	1.016558085	7.01114609	3.473991585	0.001957905	0.030978706	-1.350912102	Sort1
ENSMUSG00000020422.15	1.040096251	5.910566267	3.462599393	0.002013818	0.031341621	-1.380128424	Tns3
ENSMUSG00000042351.10	1.341210982	0.274380764	3.456665785	0.002043553	0.031460842	-1.710177953	Grap2
ENSMUSG00000056427.11	1.316280709	3.479642293	3.456582606	0.002043973	0.031460842	-1.305326394	Slit3
ENSMUSG00000042807.16	1.098299062	1.453432281	3.449100282	0.002082087	0.031714129	-1.361192902	Hecw2
ENSMUSG00000022949.10	1.605501441	4.404578915	3.446490027	0.002095546	0.03178801	-1.370504711	Clic6
ENSMUSG00000026656.16	1.231965117	4.108726471	3.446397558	0.002096024	0.03178801	-1.3532146	Fcgr2b
ENSMUSG00000020363.7	1.199558013	2.752364433	3.441716739	0.002120377	0.03201386	-1.325094033	Gfpt2

ENSMUSG00000108321.2	1.56015297	1.843282327	3.439026925	0.002134495	0.032106985	-1.361182197	Gm44649
ENSMUSG00000103570.2	1.007048416	0.685235169	3.438688093	0.00213628	0.032108886	-1.646754234	C630004M23Rik
ENSMUSG00000090761.2	1.348159652	2.400172365	3.43811817	0.002139286	0.032110505	-1.345908327	Gm17201
ENSMUSG00000030055.17	1.088540875	1.147218574	3.433720982	0.002162614	0.03227914	-1.495640275	Rab43
ENSMUSG00000022148.17	1.344313746	2.662725788	3.420983883	0.002231594	0.032644333	-1.373422573	Fyb
ENSMUSG00000068959.7	1.019685997	0.519168722	3.41453781	0.002267316	0.03281066	-1.734558849	Zfp619
ENSMUSG00000018983.10	1.259937315	2.144668834	3.413343924	0.002273993	0.032827905	-1.386429975	E2f2
ENSMUSG00000032089.17	1.184625703	2.644222285	3.405264435	0.002319682	0.033165973	-1.40519629	Il10ra
ENSMUSG00000026437.13	1.027281725	4.967223582	3.402355594	0.002336349	0.033263097	-1.495565779	Cdk18
ENSMUSG00000026525.10	1.124919696	0.805091677	3.401682789	0.002340221	0.033263097	-1.7238172	Opn3
ENSMUSG00000078945.11	1.49618885	2.267823599	3.390511063	0.002405427	0.033686572	-1.448426705	Naip2
ENSMUSG00002076948.1	1.142407199	0.202639241	3.38914637	0.002413512	0.033710812	-1.857085642	Gm55327
ENSMUSG00000015396.5	1.589652062	1.730347512	3.388507883	0.002417304	0.033715125	-1.533196128	Cd83
ENSMUSG00000086935.2	1.031229405	0.167627187	3.385931674	0.002432663	0.033756489	-1.877710781	Gm15956
ENSMUSG00000039477.17	1.06690733	6.009429985	3.383615703	0.002446551	0.033756489	-1.558767293	Tnrc18
ENSMUSG00000086898.2	2.089644438	0.608210142	3.383382586	0.002447953	0.033756489	-1.733166777	Itpr3os
ENSMUSG00000097712.2	1.528251576	0.45603043	3.376070066	0.002492336	0.03401571	-1.817203949	Gm10532
ENSMUSG00000046574.9	1.089293357	4.885870105	3.373010765	0.002511136	0.034076085	-1.556493269	Prr12
ENSMUSG00000071715.13	1.539866991	2.927369711	3.363538533	0.00257022	0.034498269	-1.488769324	Ncf4
ENSMUSG00000106663.2	2.056373259	-0.940581488	3.361075765	0.002585802	0.03457081	-2.493549475	Gm42839
ENSMUSG00000031732.13	1.005071084	2.590696377	3.360364387	0.002590319	0.034597403	-1.495300288	Phlpp2
ENSMUSG00000097368.3	1.028445865	1.304357782	3.341936614	0.002710059	0.035689811	-1.630312725	Gm10390
ENSMUSG00000073434.13	1.05638329	2.274312574	3.337631957	0.002738794	0.035756262	-1.546385328	Wdr90
ENSMUSG00000036304.15	1.037880396	1.960085795	3.334464923	0.002760123	0.035896092	-1.566206709	Zdhhc23
ENSMUSG00000043705.8	1.146957476	2.356466089	3.333743771	0.002765002	0.035896092	-1.556244447	Capn13
ENSMUSG00000032122.16	1.976773387	3.591397357	3.331407547	0.002780867	0.036034006	-1.575969973	Slc37a2
ENSMUSG00000049625.7	1.324977377	3.099275299	3.328534058	0.002800501	0.036191521	-1.5656966	Tifab
ENSMUSG00000040712.17	1.012265473	4.405498478	3.327167808	0.002809883	0.036264355	-1.636831756	Camta2
ENSMUSG00000089701.2	1.088368866	2.300002631	3.325430075	0.002821861	0.036297841	-1.572177387	Gm15978
ENSMUSG00000030787.5	1.194685509	3.088255274	3.325360635	0.002822341	0.036297841	-1.571330146	Lyve1
ENSMUSG00000097491.2	1.842791105	-0.948242248	3.313061926	0.002908552	0.036845164	-2.339367804	Gm2366
ENSMUSG00000054641.11	1.296989202	2.892792574	3.312271094	0.002914182	0.036845164	-1.596267332	Mmrn1

ENSMUSG00000086680.3	1.093118239	1.050322523	3.306360107	0.002956599	0.037046468	-1.751333232	Gm11592
ENSMUSG00000078719.3	1.085236048	2.427932059	3.305080283	0.002965861	0.037068981	-1.610789498	Msmp
ENSMUSG00000084892.2	1.269511055	0.936593516	3.30475129	0.002968247	0.037072443	-1.763118722	Gm14471
ENSMUSG00000022439.10	1.4477847	1.581543716	3.303860137	0.002974718	0.037129311	-1.690028152	Parvg
ENSMUSG00000020183.12	1.200021919	3.93778533	3.297874329	0.00301854	0.037354554	-1.67679809	Cpm
ENSMUSG00000108720.2	1.399597783	-0.27988337	3.29170117	0.003064388	0.037617937	-2.140976757	Gm44672
ENSMUSG00000097139.4	1.124261268	2.525018118	3.276494162	0.003180226	0.038356376	-1.669127449	Gm26626
ENSMUSG00000030084.13	1.006690504	5.998279535	3.274695789	0.003194203	0.038389636	-1.801846379	Plxna1
ENSMUSG00000047150.9	1.087023203	1.884889496	3.273761338	0.003201488	0.038389636	-1.690664033	1700001C19Rik
ENSMUSG00000026576.13	1.239611694	7.76341335	3.273618054	0.003202607	0.038389636	-1.789392831	Atp1b1
ENSMUSG00000081788.3	1.065736892	1.253277344	3.272038067	0.003214966	0.03845414	-1.783724722	Gm5898
ENSMUSG00000002111.10	1.158147393	4.126908256	3.267015944	0.003254559	0.038724646	-1.737431243	Spi1
ENSMUSG00000024330.18	1.051835712	0.816775838	3.264086861	0.003277868	0.038753531	-1.941869486	Col11a2
ENSMUSG00000086016.2	1.110983717	2.005254967	3.263926565	0.003279148	0.038753531	-1.704328345	1700084C06Rik
ENSMUSG00000039982.9	1.003485224	3.675803493	3.261991886	0.003294638	0.038819781	-1.732392423	Dtx4
ENSMUSG00000017146.13	1.028007117	1.524493107	3.259051188	0.003318319	0.038932929	-1.720841787	Brca1
ENSMUSG00000086589.2	2.287308954	-1.313878901	3.256855602	0.003336107	0.039023375	-2.723057281	Gm12915
ENSMUSG00000040907.16	1.765438238	1.860073373	3.251968114	0.003376035	0.039276836	-1.756770033	Atp1a3
ENSMUSG00000024053.12	1.064419974	3.81880121	3.247831899	0.003410186	0.039336429	-1.768524082	Emilin2
ENSMUSG00000018654.19	1.004237745	2.028786626	3.246373982	0.003422303	0.039336429	-1.739907238	Ikzf1
ENSMUSG00000032589.15	1.002841244	3.231710771	3.241599319	0.003462278	0.039590216	-1.754939603	Bsn
ENSMUSG00000038855.11	1.011475017	5.274188246	3.23939758	0.003480863	0.039662171	-1.868807015	Itpkb
ENSMUSG00000052142.16	1.490358814	1.13005493	3.235103318	0.00351739	0.039890537	-1.895585164	Rasal3
ENSMUSG00000071713.7	1.195299091	3.165126782	3.22683552	0.00358876	0.040368864	-1.78027275	Csf2rb
ENSMUSG00000121185.1	1.161072646	0.532716155	3.222119627	0.003630094	0.040613961	-2.004854427	Gm56740
ENSMUSG00000020902.13	1.029042326	3.465650139	3.221736071	0.003633476	0.040613961	-1.814489588	Ntn1
ENSMUSG00000029414.12	1.12852356	1.616506584	3.219852949	0.003650125	0.040704356	-1.803102542	Kntc1
ENSMUSG00000025784.6	1.664102884	5.296667136	3.211797291	0.003722178	0.041087688	-1.911171553	Clec3b
ENSMUSG00000049493.14	1.213858538	3.185232313	3.209778353	0.003740451	0.041119471	-1.832064265	Pls1
ENSMUSG00000020427.12	1.106729357	6.179597856	3.208930203	0.003748153	0.041157397	-1.948409367	Igfbp3
ENSMUSG00000022802.3	1.02069044	0.979115325	3.207688418	0.003759457	0.041211399	-1.901727573	Lmln
ENSMUSG00000062785.15	1.46943495	0.808429283	3.202251616	0.003809338	0.041462117	-2.008614446	Kcnc3

ENSMUSG00000038147.15	1.441174657	2.641260804	3.202161377	0.003810171	0.041462117	-1.820653442	Cd84
ENSMUSG00000031101.7	1.192411673	2.102204588	3.198981964	0.003839643	0.041549272	-1.83933976	Sash3
ENSMUSG00000051339.11	1.028160608	6.228087921	3.184557791	0.003976133	0.042270161	-2.002539233	2900026A02Rik
ENSMUSG00000115923.2	1.288249688	1.170777576	3.177824668	0.004041437	0.042636609	-1.943762886	Gm49521
ENSMUSG00000090054.2	1.824389585	0.414969807	3.17454303	0.004073639	0.042813014	-2.238501715	Gm15629
ENSMUSG00000056411.3	1.484574418	0.27074505	3.173665081	0.004082296	0.042825232	-2.243722694	Gm12500
ENSMUSG00000120788.1	1.075088812	1.974783404	3.164549338	0.004173235	0.043389265	-1.901861608	Gm36393
ENSMUSG00000105645.2	1.151521516	1.391430469	3.163876997	0.004180019	0.043389265	-1.938799134	Gm6649
ENSMUSG00000042097.18	1.073686143	0.861432175	3.161491298	0.004204177	0.043490823	-2.006899173	Zfp239
ENSMUSG00000103793.2	1.182919673	0.123486998	3.159531923	0.004224118	0.043589926	-2.340903001	Pcdhga6
ENSMUSG00000055415.9	1.017052656	4.596176384	3.152238276	0.004299153	0.044129005	-2.031735568	Atp10b
ENSMUSG00000038453.18	1.503192679	0.957249753	3.146619016	0.004357836	0.044425144	-2.018679681	Srcin1
ENSMUSG00000056602.14	1.060144499	4.995235347	3.144640423	0.004378682	0.044590691	-2.075553237	Fry
ENSMUSG00000020532.19	1.054696234	4.813624746	3.141963295	0.00440704	0.044789397	-2.063781283	Acaca
ENSMUSG00000044708.6	1.736987168	1.128278871	3.14192485	0.004407449	0.044789397	-2.079204648	Kcnj10
ENSMUSG00000036887.6	1.132275209	6.790699802	3.141155092	0.004415636	0.044825537	-2.096156198	C1qa
ENSMUSG00000073530.12	1.753812313	1.569571354	3.137931974	0.004450077	0.045080608	-1.980490511	Pappa2
ENSMUSG00000086529.2	1.713710968	0.086004766	3.127990069	0.004557949	0.045789998	-2.393379968	Acss2os
ENSMUSG00000040345.11	1.00088935	2.482083274	3.12686946	0.004570264	0.045820004	-1.972183522	Arhgap9
ENSMUSG00000020437.13	1.43211775	1.714668805	3.124886834	0.004592131	0.045946265	-2.010895511	Myo1g
ENSMUSG00000024140.11	1.046362011	5.094350945	3.124801632	0.004593073	0.045946265	-2.116635982	Epas1
ENSMUSG00000037731.6	1.1784758340000	2.715249593	3.119627856	0.004650624	0.046264865	-1.98695362	Themis2
ENSMUSG00000030043.12	1.358776337	-0.601557809	3.109711531	0.004762872	0.046851852	-2.547083329	Tacr1
ENSMUSG00000091412.2	1.20633863	1.628087476	3.096920244	0.004911515	0.047646353	-2.050238309	Gm2895
ENSMUSG00000082791.4	1.216316188	1.069258202	3.095168859	0.004932211	0.047789122	-2.121594423	Gm4875
ENSMUSG00000022150.17	1.023793891	4.181861004	3.093020264	0.004957715	0.047892415	-2.134375678	Dab2
ENSMUSG00000070574.4	1.347847168	0.169590663	3.092608035	0.004962622	0.047902263	-2.216315313	2310016G11Rik
ENSMUSG00000015850.12	1.06788843	3.399413048	3.089885417	0.004995153	0.0480485	-2.073682451	Adamtsl4
ENSMUSG00000027068.7	1.040319703	5.211299247	3.089717034	0.004997172	0.0480485	-2.19820594	Dhrs9
ENSMUSG00000037341.15	1.010347802	1.148294125	3.088306599	0.005014111	0.048088991	-2.098111398	Slc9a7
ENSMUSG00000097124.3	1.027135968	1.51375011	3.087999308	0.005017809	0.048088991	-2.074362882	A530020G20Rik
ENSMUSG00000051379.13	1.079991865	1.861176406	3.084265149	0.005062955	0.048236317	-2.0621035	Flrt3

ENSMUSG00000047496.7	1.044075136	3.066516298	3.078614912	0.005132008	0.048725132	-2.08872362	Rnf152
ENSMUSG00000056553.15	1.694637441	3.904265221	3.076461488	0.005158563	0.048809688	-2.150972839	Ptprn2
ENSMUSG00000112510.2	1.33617082	0.252806722	3.075638812000(	0.005168743	0.048851566	-2.3903607	Gm47566
ENSMUSG00000050424.10	2.141368161	-2.006092478	3.073472096	0.005195645	0.049016813	-3.037724474	Pnma5
ENSMUSG00000055134.6	1.247483198	0.158178012	3.068806656	0.00525403	0.049327241	-2.374323041	9130017K11Rik
ENSMUSG00000049988.6	1.216724589	2.364310248	3.066859806	0.005278579	0.049437842	-2.091244782	Lrrc25
ENSMUSG00000073733.5	1.485420229	-0.721983025	3.064799642	0.005304677	0.049610265	-2.641566993	Cplane2
ENSMUSG00000041930.8	1.466462424	-0.550668769	3.06190473	0.005341559	0.049757931	-2.658228036	Fam222a
ENSMUSG00000055485.7	1.140952027	2.249558723	3.060950161	0.005353774	0.049780842	-2.106545629	Soga1

Significant Downregulated Genes: Uninfected versus *Nb*-infected comparison							
Gene.ID	**logFC**	**AveExpr**	**t**	**P.Value**	**adj.P.Val**	**B**	**Gene.name**
ENSMUSG00000056643.6	-2.15110314	-1.096599064	-3.692028457	0.001138174	0.02463742	-1.984940089	Chst13
ENSMUSG00000018211.14	-1.190193423	4.73367902	-3.569161785	0.001546347	0.027726197	-1.103870332	Wfdc15b
ENSMUSG00000094344.2	-2.552674892	3.360527062	-3.42453271	0.002212163	0.032601668	-1.360772336	Gm11942
ENSMUSG00000050830.18	-1.889606837	-0.779028501	-3.399569458	0.002352423	0.033346574	-2.406951711	Vwc2
ENSMUSG00000074479.4	-1.961924458	3.234877262	-3.339122933	0.002728808	0.035742209	-1.539597004	Rtraf-ps
ENSMUSG00000119584.1	-2.246803605	14.70495704	-3.249392472	0.003397262	0.039334683	-1.761611281	Rn18s-rs5
ENSMUSG00000035202.9	-2.392132879	10.75636954	-3.284832932	0.003116191	0.037974948	-1.726509014	Lars2
ENSMUSG00000026051.9	-1.950703072	1.907100012	-3.218600856	0.003661235	0.040781269	-1.796147173	Ecrg4
ENSMUSG00000022132.16	-1.250827689	6.445046578	-3.205456152	0.003779861	0.041331458	-1.95684013	Cldn10
ENSMUSG00000120777.1	-2.69317676	1.699449047	-3.179123012	0.004028764	0.042588863	-1.98679971	Gm56626
ENSMUSG00000020676.3	-1.105283086	4.624211954	-3.174809022	0.004071019	0.042808726	-1.984998432	Ccl11
ENSMUSG00000052854.16	-2.038427492	-1.363934571	-3.168107432	0.004137509	0.043202824	-2.585655539	Nrk

SUPPLEMENTARY FILE 2: LIST OF SIGNIFICANT DIFFERENTIALLY EXPRESSED GENES IDENTIFIED IN THE UNINFECTED FGT TISSUES VS *Nb*-INFECTED TISSUES

SUPPLEMENTARY FILE 2
LIST OF SIGNIFICANT DIFFERENTIALLY EXPRESSED GENES IDENTIFIED IN THE UNINFECTED VERSUS HSV-2-INFECTED COMPARISON

Significant Upregulated Genes: Uninfected versus HSV-2 only comparison							
Gene.ID	**logFC**	**AveExpr**	**t**	**P.Value**	**adj.P.Val**	**B**	**Gene.name**
ENSMUSG00000032193.10	2.273651352	5.2991549	6.498844886	9.96350091179391e-7	0.019288341	5.609495112	Ldlr
ENSMUSG00000031004.9	1.244664436	4.022290296	5.778440368	5.81559228262878e-6	0.028460275	3.970912854	Mki67
ENSMUSG00000039145.17	1.142267618	3.798487421	5.635061894	8.31259542604377e-6	0.028460275	3.625647958	Camk1d
ENSMUSG00000004356.9	1.042981607	3.990114585	5.586274198	9.3907697476069e-6	0.028460275	3.539908411	Utp20
ENSMUSG00000046179.18	1.531561837	1.732322758	5.461969613	1.28239138971764e-5	0.028460275	2.688100751	E2f8
ENSMUSG00000024251.11	1.020859582	3.021734512	5.416032326	1.43931849419615e-5	0.028460275	3.035200674	Thada
ENSMUSG00000028613.16	1.279514142	2.161814135	5.23746342	2.25759071452657e-5	0.028460275	2.405678076	Lrp8
ENSMUSG00000055116.9	1.267731426	3.721224667	5.199083767	2.48756796254348e-5	0.028460275	2.639341603	Bmal1
ENSMUSG00000024660.10	1.010115352	3.642394113	5.191197794	2.53767524484193e-5	0.028460275	2.613858309	Incenp
ENSMUSG00000114934.2	1.522774056	3.962091505	5.081218667	3.35256286515523e-5	0.028460275	2.38125796	Gm48342
ENSMUSG00000024378.10	1.254047897	4.384736455	5.078377191	3.37679866540538e-5	0.028460275	2.38841356	Stard4
ENSMUSG00000093930.3	1.182887638	6.338085072	4.992809595	4.19549899125082e-5	0.028460275	2.197642403	Hmgcs1
ENSMUSG00000004099.17	1.036665275	4.8381111	4.914476579	5.11925177177952e-5	0.028460275	2.01321934	Dnmt1
ENSMUSG00000073529.10	1.803448635	0.089997964	4.857811581	5.91265045745062e-5	0.028460275	0.197669442	F830208F22Rik
ENSMUSG00000022351.15	1.42465471	5.330415558	4.824493091	6.43570244286938e-5	0.028460275	1.801497657	Sqle
ENSMUSG00000039835.17	1.071786344	3.820639499	4.815232772	6.58916037365675e-5	0.028460275	1.769510288	Nhsl1
ENSMUSG00000039840.9	1.107871237	4.074311134	4.792723562	6.97766777285431e-5	0.028460275	1.722903646	Epg5
ENSMUSG00000000631.21	1.056080421	5.947895233	4.774585162	7.30740418999828e-5	0.028460275	1.682225948	Myo18a
ENSMUSG00000030739.20	1.22646257	6.147730554	4.772501358	7.34627325664685e-5	0.028460275	1.677437856	Myh14
ENSMUSG00000021670.15	1.051621475	5.046634673	4.763977127	7.50744721141202e-5	0.028460275	1.659126917	Hmgcr
ENSMUSG00000035049.5	1.133583775	3.982076517	4.739169045	7.99698337783263e-5	0.028460275	1.595911168	Rrp12
ENSMUSG00000039087.18	1.167165115	4.944796078	4.726785397000	8.25320277995678e-5	0.028460275	1.571836984	Rreb1
ENSMUSG00000022463.9	1.018670129	7.07716138	4.716478468	8.47272058145735e-5	0.028460275	1.546057427	Srebf2
ENSMUSG00000013629.17	1.015811774	5.06277077	4.702326018	8.78370978303716e-5	0.028460275	1.513439505	Cad
ENSMUSG00000001228.15	1.175143639	4.089848579	4.678833846	9.32539666810413e-5	0.028460275	1.4599916	Uhrf1
ENSMUSG00000046591.11	1.431848015	1.218975164	4.677667221	9.35315289401603e-5	0.028460275	0.927083313	Ticrr
ENSMUSG00000025278.10	1.169784323	7.131820265	4.659638966	9.79275509215575e-5	0.028460275	1.412295959	Flnb

ENSMUSG00000040721.10	1.224303522	2.658092723	4.658926695	9.81054193940886e-5	0.028460275	1.30917583	Zfhx2
ENSMUSG00000034584.4	1.415939249	2.87341354	4.647246326	1.01069e-4	0.028460275	1.337976732	Exph5
ENSMUSG00000038241.17	1.198017017	3.673519111	4.574213931	1.21741e-4	0.028460275	1.206278914	Cep250
ENSMUSG00000034342.10	1.149893562	4.259549735	4.564137337	1.24908e-4	0.028460275	1.192679351	Cbl
ENSMUSG00000001467.6	1.213577005	5.439031523	4.562818738	1.25328e-4	0.028460275	1.183416002	Cyp51
ENSMUSG00000070808.5	1.339036544	3.306537694	4.556167974	1.2747e-4	0.028460275	1.142509437	Bicra
ENSMUSG00000007080.15	1.287895327	2.3821429	4.553648527	1.28292e-4	0.028460275	1.068935131	Pole
ENSMUSG00000020272.9	1.207302263	3.449727577	4.529214315	1.36534e-4	0.028460275	1.099166202	Stk10
ENSMUSG00000037572.18	1.262667819	1.839197221	4.518249896	1.40402e-4	0.028460275	0.873357026	Wdhd1
ENSMUSG00000025429.10	1.000959921	3.460451975	4.477326308	1.55834e-4	0.028460275	0.977728371	Pstpip2
ENSMUSG00000056602.14	1.532430449	4.995235347	4.472186287	1.57888e-4	0.028460275	0.973841324	Fry
ENSMUSG00000099708.2	1.130494348	1.848060376	4.471289047	1.5825e-4	0.028460275	0.752885705	Gm29361
ENSMUSG00000057337.15	1.568561055	0.783728768	4.469998923	1.58771e-4	0.028460275	0.350417624	Chst3
ENSMUSG00000033105.13	1.205974702	4.942236521	4.463913808	1.61252e-4	0.028460275	0.954187686	Lss
ENSMUSG00000017499.16	1.240844286	1.556715521	4.457462835	1.63924e-4	0.028460275	0.692995116	Cdc6
ENSMUSG00000056271.14	2.333380121	0.382782033	4.449888363	1.67118e-4	0.028460275	-0.454803141	Lman1l
ENSMUSG00000076435.4	1.497052964	4.940901669	4.447716212	1.68046e-4	0.028460275	0.915766396	Acsf2
ENSMUSG00000026669.15	1.224151264	1.580190052	4.372356223	2.03607e-4	0.031272871	0.520733025	Mcm10
ENSMUSG00000026675.13	1.445105189	3.027437339	4.334267363	2.24341e-4	0.031945249	0.637178565	Hsd17b7
ENSMUSG00000048251.16	1.18747509	3.396481705	4.314507006	2.35914e-4	0.032621853	0.605128749	Bcl11b
ENSMUSG00000003882.6	1.162454873	1.265410989	4.307458043	2.40185e-4	0.032644592	0.325436076	Il7r
ENSMUSG00000028068.15	1.412312059	2.212752629	4.253977954	2.75192e-4	0.034020632	0.384290969	Iqgap3
ENSMUSG00000070047.15	1.30904837	6.39083561	4.242691238	2.83205e-4	0.034020632	0.425307713	Fat1
ENSMUSG00000030583.17	1.232719544	5.255949578	4.238848398	2.85986e-4	0.034020632	0.419687912	Sipa1l3
ENSMUSG00000012123.18	1.101215431	3.429285973	4.219543952	3.00372e-4	0.034020632	0.394866624	Crybg2
ENSMUSG00000025885.19	1.068417312	5.429886552	4.216744821	3.02517e-4	0.034020632	0.365575183	Myo5b
ENSMUSG00000042489.16	1.195436985	1.26932963	4.215593302	3.03404e-4	0.034020632	0.142849038	Clspn
ENSMUSG00000029478.17	1.040550635	5.881456696	4.175398449	3.3603e-4	0.034626849	0.265741646	Ncor2
ENSMUSG00000017146.13	1.366618434	1.524493107	4.150868379	3.5763e-4	0.035143917	0.047336672	Brca1
ENSMUSG00000108282.2	1.021881499	2.646373331	4.141595203	3.66149e-4	0.035619497	0.183987163	Gm44317
ENSMUSG00000035299.18	1.158137118	1.658431591	4.134288373	3.73004e-4	0.036104886	0.022378807	Mid1
ENSMUSG00000051457.8	1.107206731	1.60936905	4.127158307	3.79815e-4	0.036141902	0.017102546	Spn

ENSMUSG00000026924.18	1.003336496	6.071548239	4.11243518	3.94273e-4	0.036141902	0.117291835	Sec16a
ENSMUSG00000029482.5	1.110488745	5.158625283	4.101156314	4.05717e-4	0.036141902	0.097845145	Aacs
ENSMUSG00000070574.4	2.043901333	0.169590663	4.096721331	4.10306e-4	0.03626997	-0.776298362	2310016G11Rik
ENSMUSG00000086241.3	1.548386095	1.459663427	4.06708843	4.42321e-4	0.037151538	-0.209700329	4930483K19Rik
ENSMUSG00000033792.13	1.269614063	2.395170983	4.017046853	5.02095e-4	0.037714307	-0.090523176	Atp7a
ENSMUSG00000034329.17	1.000660702	1.281732462	4.005140398	5.17456e-4	0.037764271	-0.333110616	Brip1
ENSMUSG00000052928.10	1.084814195	3.596785958	3.999124746	5.25393e-4	0.037764271	-0.106453089	Ctif
ENSMUSG00000037692.15	1.088208754	4.812919122	3.992028408	5.34911e-4	0.037764271	-0.149658417	Ahdc1
ENSMUSG00000085327.3	1.30200526	2.003717989	3.985322343	5.44061e-4	0.037764271	-0.220545793	Gm16104
ENSMUSG00000074646.12	1.146057624	1.828787597	3.97671576	5.56033e-4	0.037764271	-0.273234446	6430550D23Rik
ENSMUSG00000035455.13	1.149105603	2.090825546	3.971541506	5.63355e-4	0.037764271	-0.230192963	Fignl1
ENSMUSG00000002057.5	1.079460158	2.438760358	3.970057615	5.65472e-4	0.037764271	-0.214756698	Foxn1
ENSMUSG00000007817.16	1.283276816	5.968828397	3.969598957	5.66128e-4	0.037764271	-0.216819222	Zmiz1
ENSMUSG00000068966.11	1.005892269	2.781494623	3.96291786	5.75771e-4	0.037764271	-0.195755148	Zbtb34
ENSMUSG00000120949.1	1.02589966	2.293215417	3.957797832	5.8327e-4	0.037764271	-0.235990756	Gm56545
ENSMUSG00000121608.1	1.246823195	0.080019177	3.917137059	6.46352e-4	0.038509943	-1.100769808	
ENSMUSG00000047150.9	1.287462402	1.884889496	3.866140857	7.35059e-4	0.040898209	-0.494893331	1700001C19Rik
ENSMUSG00000089968.2	1.617819795	0.672000038	3.84573849	7.7382e-4	0.041432039	-0.97931483	Nckap5los
ENSMUSG00000042699.12	1.109612316	5.478899098	3.845261195	7.74751e-4	0.041432039	-0.504772207	Dhx9
ENSMUSG00000020185.17	1.38751915	0.867105736	3.825801938	8.1364e-4	0.041908061	-0.834404591	E2f7
ENSMUSG00000074647.5	1.040359606	4.564034617	3.824590662	8.16124e-4	0.041908061	-0.530098704	Fam83c
ENSMUSG00000097491.2	2.271099883	-0.948242248	3.818035204	8.29694e-4	0.042019638	-1.822696219	Gm2366
ENSMUSG00000074136.10	1.026493399	0.860475025	3.813137171	8.39978e-4	0.042127274	-0.823703762	4930513N10Rik
ENSMUSG00000034832.16	1.079500843	5.137990412	3.810351281	8.45883e-4	0.042313813	-0.577945237	Tet3
ENSMUSG00000048373.9	1.203238627	5.071772887	3.803653956	8.60247e-4	0.042565344	-0.58915568	Fgfbp1
ENSMUSG00000031604.11	1.132694374	5.965656169	3.800112427	8.67939e-4	0.042565344	-0.612448089	Msmo1
ENSMUSG00000038369.15	1.064771347	4.471311105	3.792865099	8.83892e-4	0.042565344	-0.60271129	Ncoa6
ENSMUSG00000032528.6	1.41861901	5.155327885	3.788682822	8.93229e-4	0.042629024	-0.631510876	Vipr1
ENSMUSG00000108720.2	1.637260625	-0.27988337	3.785307241	9.00836e-4	0.042848376	-1.435089041	Gm44672
ENSMUSG00000109959.2	1.367377233	0.185890499	3.781786016	9.08839e-4	0.042975711	-1.042810449	Gm45355
ENSMUSG00000017861.12	1.055359528	1.786216107	3.777675187	9.18271e-4	0.042975711	-0.65985938	Mybl2
ENSMUSG00000037474.14	1.102561182	2.579167883	3.775180662	9.24041e-4	0.042975711	-0.607372983	Dtl

ENSMUSG00000022443.18	1.043490329	7.241934628	3.770270876	9.35502e-4	0.04311998	-0.673330897	Myh9
ENSMUSG00000105776.2	1.005099192	1.937031916	3.759518142	9.61094e-4	0.0431615	-0.674903226	Gm43292
ENSMUSG00000085830.2	1.043833913	2.502172605	3.754537248	9.73181e-4	0.043171878	-0.662658758	Grin1os
ENSMUSG00000048445.7	1.001820126	1.772635633	3.742998967	0.001001757	0.043253808	-0.758980553	Ccdc57
ENSMUSG00000031386.15	1.007932544	5.248219182	3.74271612	0.001002468	0.043253808	-0.740619734	Hcfc1
ENSMUSG00000097305.4	1.437566606	2.511625106	3.729992473	0.001034958	0.044034634	-0.722605709	Gm17276
ENSMUSG00000022565.16	1.063549695	7.548847216	3.706223968	0.001098448	0.045202959	-0.819723925	Plec
ENSMUSG00000040761.17	1.186510074	4.404462501	3.704211534	0.001103996	0.045280194	-0.80036195	Spen
ENSMUSG00000048154.18	1.13780634	5.131341801	3.700467169	0.001114391	0.045417902	-0.832967974	Kmt2d
ENSMUSG00000042744.17	1.048389103	4.193645266	3.678746078	0.00117662	0.046693599	-0.85400514	Hectd4
ENSMUSG00000018983.10	1.349545269	2.144668834	3.676270781	0.001183924	0.046693599	-0.832178412	E2f2
ENSMUSG00000084892.2	1.398797331	0.936593516	3.676041661	0.001184603	0.046693599	-1.074701132	Gm14471
ENSMUSG00000021366.9	1.057040842	4.686431317	3.668595999	0.001206856	0.046908573	-0.89622917	Hivep1
ENSMUSG00000050271.13	1.062238793	3.686874396	3.667356434	0.0012106	0.046908573	-0.863537352	Prag1
ENSMUSG00000039158.12	1.162384112	2.35441814	3.665474601	0.001216306	0.046908573	-0.850778958	Akna
ENSMUSG00000086016.2	1.224467972	2.005254967	3.663386111	0.00122267	0.046908573	-0.886443723	1700084C06Rik
ENSMUSG00000071714.8	1.002608938	2.421484434	3.66325125	0.001223082	0.046908573	-0.854237525	Csf2rb2
ENSMUSG00000055485.7	1.381572771	2.249558723	3.645971905	0.001277018	0.047171946	-0.900587982	Soga1
ENSMUSG00000031298.16	1.044824576	2.798142605	3.643454838	0.001285067	0.047225912	-0.892759979	Adgrg2
ENSMUSG00000049691.9	3.381464165	-3.009706024	3.635018884	0.001312407	0.047757315	-3.163362767	Nkx3-2
ENSMUSG00000086935.2	1.068306001	0.167627187	3.612269233	0.001389009	0.04835257	-1.462737145	Gm15956
ENSMUSG00000087265.2	1.189432929	1.861654459	3.608739335	0.00140128	0.04835257	-1.014882166	Gm12349
ENSMUSG00000098609.2	1.167414712	3.469216493	3.599643222	0.001433394	0.04894017	-1.00690574	Anxa11os
ENSMUSG00000026828.12	1.309411815	2.675378639	3.595625826	0.001447806	0.049107983	-0.996117094	Galnt5
ENSMUSG00000031391.19	1.103531529	5.589850281	3.583958178	0.00149047	0.049611433	-1.106321046	L1cam
ENSMUSG00000106663.2	2.133344221	-0.940581488	3.583029197	0.001493919	0.049611433	-2.162322498	Gm42839
ENSMUSG00000026321.9	1.194270597	3.673144929	3.582644133	0.001495351	0.049611433	-1.052163568	Tnfrsf11a
ENSMUSG00000085132.3	1.017380744	1.514047884	3.58081329	0.001502178	0.049611433	-1.109143735	Gm12265
ENSMUSG00000025019.18	1.069704239	2.102286223	3.567068541	0.001554415	0.049959006	-1.07110908	Lcor
ENSMUSG00000042351.10	1.36367429	0.274380764	3.564364672	0.001564898	0.049959006	-1.50166914	Grap2

Significant Downregulated Genes: Uninfected versus HSV-2 only comparison

Gene.ID	logFC	AveExpr	t	P.Value	adj.P.Val	B	Gene.name
ENSMUSG00000115783.3	-1.927955183	6.199053226	-4.657943845	9.83513886803736e-5	0.028460275	1.407657281	Bc1
ENSMUSG00000036111.9	-1.396789118	4.084735383	-4.629072326	1.05859e-4	0.028460275	1.342260114	Lmo1
ENSMUSG00000047965.10	-1.170846531	0.970530876	-4.624983607	1.06968e-4	0.028460275	0.605469719	Rpl9-ps7
ENSMUSG00000082691.7	-1.109601405	1.131815626	-4.395406822	1.91998e-4	0.030466346	0.342383718	Dynlt1-ps1
ENSMUSG00000117822.2	-1.064536497	3.540506033	-4.23211642	2.90924e-4	0.034020632	0.422670264	Eef1a1-ps1
ENSMUSG00000083992.2	-1.042498643	4.493339295	-4.208302401	3.09079e-4	0.034020632	0.361883298	Gm11478
ENSMUSG00000055497.6	-1.313662028	-0.156694487	-4.1730294	3.38058e-4	0.034626849	-0.856987113	Gm9974
ENSMUSG00000036305.8	-1.030129642	2.405780205	-4.12352901	3.83329e-4	0.036141902	0.114715326	Rpl39-ps
ENSMUSG00000059824.13	-1.318927006	6.226267261	-4.004175892	5.18721e-4	0.037764271	-0.135470339	Dbp
ENSMUSG00000094344.2	-2.97388583	3.360527062	-4.08306211	4.24768e-4	0.036546999	0.062923188	Gm11942
ENSMUSG00000074479.4	-2.240746178	3.234877262	-3.923755375	6.35643e-4	0.038509943	-0.293258363	Rtraf-ps
ENSMUSG00000048489.13	-2.043702968	3.031548622	-3.873864996	7.20889e-4	0.040759941	-0.386786887	Depp1
ENSMUSG00000091449.4	-1.211230366	0.342794849	-3.865184881	7.36831e-4	0.040898209	-1.068794527	Gm10269
ENSMUSG00000059776.8	-1.332748122	0.935327224	-3.683769962	0.001161931	0.046693599	-1.169223051	Rpl13-ps6
ENSMUSG00000110057.2	-1.002137831	1.181217421	-3.748516725	9.8799e-4	0.043171878	-0.887426404	Gm2225
ENSMUSG00000023132.9	-1.862563092	0.251895944	-3.815185908	8.35661e-4	0.042019638	-1.217655594	Gzma
ENSMUSG00000083481.5	-1.556336304	0.172487475	-3.639476805	0.001297889	0.047586815	-1.672707233	Rps8-ps2
ENSMUSG00000096696.2	-1.068005013	1.397312669	-3.591492175	0.001462782	0.049248706	-1.120194849	Zfp960
ENSMUSG00000060938.15	-2.09663685	8.509842863	-3.574479697	0.001526032	0.049781185	-1.110293228	Rpl26

SUPPLEMENTARY FILE 3: GO ANALYSIS OF FUNCTIONAL MODULES: UNINFECTED FGT TISSUES VERSUS *Nb*-INFECTED TISSUES

UNINFECTED FGT TISSUES VERSUS NB-INFECTED FGT TISSUES

TOP 5 GO: BIOLOGICAL PROCESSES FOR FUNCTIONAL MODULE 1

SOURCE	TERM NAME	TERM ID	ADJUSTED P VALUE	NEGATIVE LOG10 OF ADJUS	TERM S	QUERY S	INTERSECTION S	EFFECTIVE DOMA	INTERSECTIONS
GO:BP	regulation of macrophage migration	GO:1905521	0.00001476945403234096	4.830635558521773	45	7	3	26963	CCR2,CX3CR1,CSF1R
GO:BP	positive regulation of cell migration	GO:0030335	0.00001476945403234096	4.830635558521773	582	7	5	26963	ITGAX,PTPRC,CCR2,CX3CR1,CSF1R
GO:BP	tumor necrosis factor superfamily cytokine productio	GO:0071706	0.00001476945403234096	4.830635558521773	211	7	4	26963	PTPRC,CCR2,CX3CR1,CSF1R
GO:BP	tumor necrosis factor production	GO:0032640	0.00001476945403234096	4.830635558521773	208	7	4	26963	PTPRC,CCR2,CX3CR1,CSF1R
GO:BP	positive regulation of hematopoietic stem cell migrat	GO:2000473	0.00001476945403234096	4.830635558521773	2	7	2	26963	PTPRC,CCR2

TOP 5 GO: BIOLOGICAL PROCESSES FOR FUNCTIONAL MODULE 2

SOURCE	TERM NAME	TERM ID	ADJUSTED P VALUE	NEGATIVE LOG10 OF ADJUS	TERM S	QUERY S	INTERSECTION S	EFFECTIVE DOMA	INTERSECTIONS
GO:BP	DNA-templated DNA replication	GO:0006261	2.4915699468849 49e-9	8.603526916191196	150	6	5	26963	MCM3,MCM6,MCM10,CDC6,POLD1
GO:BP	DNA replication initiation	GO:0006270	2.4915699468849 49e-9	8.603526916191196	34	6	4	26963	MCM3,MCM6,MCM10,CDC6
GO:BP	DNA replication	GO:0006260	3.0414351024723747e-8	7.5169214459900875	270	6	5	26963	MCM3,MCM6,MCM10,CDC6,POLD1
GO:BP	DNA metabolic process	GO:0006259	8.025926798139674e-8	7.095504805642422	962	6	6	26963	MCM3,MCM6,MCM10,CLSPN,CDC6,POLD1
GO:BP	DNA damage response	GO:0006974	0.00000652998237311320 05	5.18508799104805	874	6	5	26963	MCM3,MCM6,MCM10,CLSPN,POLD1

TOP 5 GO: BIOLOGICAL PROCESSES FOR FUNCTIONAL MODULE 3

SOURCE	TERM NAME	TERM ID	ADJUSTED P VALUE	NEGATIVE LOG10 OF ADJUS	TERM S	QUERY S	INTERSECTION S	EFFECTIVE DOMA	INTERSECTIONS
GO:BP	adaptive immune response	GO:0002250	0.00016404449203245684	3.78503834693028 55	623	4	4	26963	C1QC,CTSS,CD74,C1QA
GO:BP	synapse pruning	GO:0098883	0.00037382245360767 66	3.427334616319824	13	4	2	26963	C1QC,C1QA
GO:BP	antigen processing and presentation of exogenous pe	GO:0019886	0.00041055009879318 59	3.386633839027240 4	21	4	2	26963	CTSS,CD74
GO:BP	antigen processing and presentation of peptide antige	GO:0002495	0.00041055009879318 59	3.386633839027240 4	25	4	2	26963	CTSS,CD74
GO:BP	cell junction disassembly	GO:0150146	0.00041055009879318 59	3.386633839027240 4	25	4	2	26963	C1QC,C1QA

TOP 5 GO: BIOLOGICAL PROCESSES FOR FUNCTIONAL MODULE 4

SOURCE	TERM NAME	TERM ID	ADJUSTED P VALUE	NEGATIVE LOG10 OF ADJUS	TERM S	QUERY S	INTERSECTION S	EFFECTIVE DOMA	INTERSECTIONS
GO:BP	chemotaxis	GO:0006935	0.00072806914576687 15	3.137827373160897	455	3	3	26963	RAC2,DOCK2,VAV1
GO:BP	taxis	GO:0042330	0.00072806914576687 15	3.137827373160897	457	3	3	26963	RAC2,DOCK2,VAV1
GO:BP	T cell activation	GO:0042110	0.00116764653742227 25	2.932688604209093	612	3	3	26963	RAC2,DOCK2,VAV1
GO:BP	neutrophil chemotaxis	GO:0030593	0.00177305270876069 53	2.751278353635594 3	103	3	2	26963	RAC2,VAV1
GO:BP	granulocyte chemotaxis	GO:0071621	0.00177305270876069 53	2.751278353635594 3	126	3	2	26963	RAC2,VAV1

SUPPLEMENTARY FILE 4: GO ANALYSIS OF FUNCTIONAL MODULES: UNINFECTED FGT TISSUES VS HSV-2-INFECTED TISSUES

UNINFECTED FGT TISSUES VERSUS HSV-2-INFECTED FGT TISSUES

TOP 5 GO: BIOLOGICAL PROCESSES FOR FUNCTIONAL MODULE 1

SOURCE	TERM NAME	TERM ID	ADJUSTED P VALUE	NEGATIVE LOG10 OF ADJUSTED P VALUE	TERM SIZE	QUERY SIZE	INTERSECTION SIZE	EFFECTIVE DOMAIN SIZE	INTERSECTIONS
GO:BP	mitotic cell cycle	GO:0000278	3.1389468707753456e-11	10.503216035024508	854	12	10	26963	POLE,INCENP,UHRF1,DTL,WDHD1,MKI67,CDC6,CLSPN,BRCA1,MYBL2
GO:BP	mitotic cell cycle process	GO:1903047	2.4203263720944134e-10	9.616126067059835	721	12	9	26963	POLE,INCENP,UHRF1,DTL,MKI67,CDC6,CLSPN,BRCA1,MYBL2
GO:BP	cell cycle	GO:0007049	2.4203263720944134e-10	9.616126067059835	1813	12	11	26963	POLE,INCENP,UHRF1,DTL,WDHD1,E2F2,MKI67,CDC6,CLSPN,BRCA1,MYBL2
GO:BP	cell cycle process	GO:0022402	2.649457021541311e-8	7.576843121048251	1262	12	9	26963	POLE,INCENP,UHRF1,DTL,MKI67,CDC6,CLSPN,BRCA1,MYBL2
GO:BP	DNA replication	GO:0006260	8.748556972589071e-8	7.058063575609031	270	12	6	26963	POLE,DTL,WDHD1,MCM10,CDC6,BRCA1

TOP 5 GO: BIOLOGICAL PROCESSES FOR FUNCTIONAL MODULE 2

SOURCE	TERM NAME	TERM ID	ADJUSTED P VALUE	NEGATIVE LOG10 OF ADJUSTED P VALUE	TERM SIZE	QUERY SIZE	INTERSECTION SIZE	EFFECTIVE DOMAIN SIZE	INTERSECTIONS
GO:BP	cholesterol metabolic process	GO:0008203	5.908648636155506e-18	17.228511835018814	142	10	9	26963	SREBF2,HSD17B7,STARD4,MSMO1,SQLE,LSS,HMGCR,HMGCS1,CYP51
GO:BP	sterol biosynthetic process	GO:0016126	5.908648636155506e-18	17.228511835018814	63	10	8	26963	SREBF2,HSD17B7,MSMO1,SQLE,LSS,HMGCR,HMGCS1,CYP51
GO:BP	sterol metabolic process	GO:0016125	5.980827362280482e-18	17.223238733398803	154	10	9	26963	SREBF2,HSD17B7,STARD4,MSMO1,SQLE,LSS,HMGCR,HMGCS1,CYP51
GO:BP	secondary alcohol metabolic process	GO:1902652	5.980827362280482e-18	17.223238733398803	153	10	9	26963	SREBF2,HSD17B7,STARD4,MSMO1,SQLE,LSS,HMGCR,HMGCS1,CYP51
GO:BP	steroid biosynthetic process	GO:0006694	1.6390944116566163e-17	16.785396030395084	176	10	9	26963	SREBF2,HSD17B7,STARD4,MSMO1,SQLE,LSS,HMGCR,HMGCS1,CYP51