Bystander influence of nematode exposure on subsequent herpesvirus infections *in vivo*

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Thesis Presented for the Degree of

DOCTOR OF PHILOSOPHY

Clinical Science and Immunology

in the Department of Pathology

Faculty of Health Sciences

UNIVERSITY OF CAPE TOWN

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July 2019

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Acknowledgements

'La vie n'est facile pour aucun de nous. Mais quoi, il faut avoir de la persévérance, et surtout de la confiance en soi. Il faut croire que l'on est doué pour quelque chose, et que, cette chose, il faut l'atteindre coûte que coûte.'

'Life is not easy for any of us. But what of that? We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing, at whatever cost, must be attained.'

… Marie Curie

Thank you to:

Associate Professor William Horsnell for his time, support and encouragement through this project and my academic career thus far. The lessons I have learned from you are priceless.

Dr Benjamin Dewals, Marion Rolot, Annette Dougall and colleagues at the Université de Liège for their warm welcome and guidance during my visits.

Prof Frank Brombacher and Munadia Ansarie for providing the IL-4Rα-deficient mice

The UCT Research Animal Facility (RAF) and Rodney Lucas for facilitating this project.

My scholarship and project funders for the financial assistance.

My mother and step-father for being a safety blanket in difficult times. My sister, Melissa, Aunt Saroj, Uncle Uge and cousin Gabi for their love and support.

My friends and colleagues for their support, especially Dr Matthew Darby, Dr Melissa Govender, Brunette Katsandegwaza and Dr Katie Smith for their help in and out of the lab.

And everyone else who have played a part in this process.

Stephen Vogts, I'm relieved to have met you. Thanks for your support and love.

This Thesis is dedicated to my niece, Baby Diya and my late Father, Monty Chetty.

Personal Funders:

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Abbreviations

4′,6-diamidino-2-phenylindole	DAPI
Alphaherpesvirus	α-HV
Alternately activated macrophages	AAMs; M2
Alveolar macrophages	AMs
Analysis of variance	ANOVA
Antibody-dependent cell-mediated cytotoxicity	ADCC
Antigen presenting cells	APCs
Baby Hamster Kidney	BHK
Betaherpesvirus	β-HV
Brefeldin A	BFA
Central T cell memory	T_{CM}
Chitinase and fizz family proteins	ChaFFs
Chronic Obstructive Pulmonary Disease	COPD
Classically activated macrophages	CAMs; M1
Conventional DCs	cDCs
Cytomegalovirus	CMV
Cytotoxic T cells	CTLs
Dendritic cells	DCs
Depo Provera®	DP
Dulbecco's Modified Eagle Medium	DMEM
Effector T cell memory	T_{EM}
Enzyme-linked immunosorbent assay	ELISA
Eosinophil cationic protein	ECP
Eosinophil-derived neurotoxin	EDN
Eosinophil peroxidase	EPO
Eosinophil secondary granule proteins	ESGPs
Epstein–Barr virus	EBV
Excretory/secretory	ES
Fas ligand	FasL
Female genital tract/tissue	FGT
Fetal calf serum	FCS
Forward-scatter	FSC
Gammaherpesvirus	γ-HV
Gastrointestinal	GI
Gastrointestinal tract	GIT
Granzyme b	GZb
Haematoxylin and eosin	H&E
Herpesviruses	HVs

Herpes simplex virus type I	HSV-1
Herpes simplex virus type II	HSV-2
Human immunodeficiency virus	HIV
Human Papillomavirus	HPV
Iliac lymph nodes	iLN
Immunohistochemistry	IHC
Interferon	IFN
Interferon stimulating genes	ISGs
Infectious mononucleosis	IM
Interleukin	IL
IL-4 receptor	IL-4R
IL-4 receptor alpha chain	IL-4Rα
Iscove's Modified Dulbecco's Medium	IMDM
Kaposi's sarcomas	KS
Kaposi's sarcoma-associated herpesvirus	KSHV
Langerhans cells	LCs
Lipopolysaccharides	LPS
Lower Limit of Quantification	LLOQ
Lymph nodes	LN
Major basic protein	MBP
Major histocompatibility complex	MHC
Matrix metalloproteinases	MMPs
Multiplicity of infection	MOI
Murine cytomegalovirus	MCMV
Murid gammaherpesvirus 4	MuHV-4
Murid gammaherpesvirus 4-luciferase	MuHV-4-LUC
Murine gammaherpesvirus 68	MHV-68
Murine norovirus	MNV
Mycobacterium tuberculosis	Mtb
Natural killer cells	NK cells
Neutrophil extracellular traps	NETs
Nippostrongylus brasiliensis	Nb
Nitric oxide	NO
Nitric oxide synthase	iNOS
Paraformaldehyde	PFA
Pathogen associated molecular patterns	PAMPs
Pattern recognition receptors	PRRs
Peripheral blood mononuclear cells	PBMCs
phorbol 12-myristate 13-acetate	PMA
Phosphate buffered saline	PBS

Piecemeal degranulation	PMD
Plasmacytoid DCs	pDCs
Plaque forming units	PFUs
Polymorphonuclear	PMN
Post infection	PI
Recombinant IL-4	rIL-4
Regulatory T cells	T_{regs}
Resident T cell memory	T_{RM}
Respiratory syncytial virus	RSV
Sexually transmitted infections	STIs
Side-scatter	SSC
Soil-transmitted helminths	STHs
Species	spp.
Spleen	SPL
Superficial cervical lymph nodes	scLN
T cell receptor	TCR
T helper 1	Th1
T helper 2	Th2
Thymic stromal lymphopoietin	TSLP
Toll-like receptors	TLRs
Transforming growth factor	TGF
Tumour necrosis factor	TNF
Type 2 innate lymphoid cells	ILC2s
Vaginal tissue	VT
Varicella zoster virus	VZV
Virtual T cell memory	T_{VM}
Wildtype	WT
World Health Organisation	WHO

Abstract

Parasitic worms have the ability to modulate the hosts immune response to promote host control of the infection and also parasite survival in the host. Helminth infections classically induce a potent Th2-biased and regulatory immune imprint. This immune response also influences unrelated inflammatory processes in the host. Studies have shown helminth infections have bystander influences on unrelated conditions such as allergy and autoimmunity. Additionally, helminth infections can alter susceptibility to other infections. In this thesis, we investigate the systemic influences of murine nematode *Nippostrongylus brasiliensis* infection on host immunity in colonized and non-colonized tissues, and the implications of these effects on susceptibility to subsequent herpesvirus infections *in vivo*.

We show that prior *N. brasiliensis* infection enhanced control of acute respiratory murid gammaherpesvirus (MuHV-4) infection, with an increase in viral-specific $CD8^+$ T cells in colonized lung tissue. Enhanced effector cytokine responses by cytotoxic T cells were also observed with prior helminth exposure. Conversely, despite enhanced primary control, prior helminth exposure was associated with earlier and heightened genital reactivation of MuHV-4. This demonstrates differences in local bystander and systemic effects of helminth exposure on the host, and on unrelated viral infections. We also show that *N. brasiliensis* infection, which transits the respiratory and gastrointestinal tracts, also systemically influences immunity in the female genital tract (FGT) *in vivo*. Here, helminth infection induced Th2-type immunity in the FGT, namely increased tissue IL-4, IL-5 and long-lasting eosinophilia. We further demonstrated that systemic influences of *N. brasiliensis* infection results in exacerbated genital pathology and inflammation, following subsequent intravaginal herpes simplex virus type II (HSV-2) infection. Increased HSV-2 pathology with prior helminth exposure was associated with diminished innate anti-viral immunity, increased IL-33, ILC2 and IL-5 responses, as well as significant eosinophilia. Interestingly, abolition of canonical Th2 immune signalling by the lack of IL-4Rα expression, enhanced innate anti-viral defences and provided protection from HSV-2 pathology. However, *N. brasiliensis*-induced exacerbation of HSV-2 illness was IL-4Rα-independent, associated with significant genital eosinophilia. Furthermore, antibody-depletion of eosinophils ameliorated nematode-exacerbated HSV-2 pathology, suggesting that nematode-induced genital eosinophilia mediates increased HSV-2 pathology in coinfected mice.

We have therefore shown that helminth infections can induce local and systemic bystander immunity to lymphoid and myeloid immune compartments, which alters susceptibility to subsequent herpesvirus infections.

Chapter 1: Thesis Introduction

In this thesis we will investigate bystander influences of soil-transmitted nematode infection on unrelated herpesvirus susceptibility. To set a basis for the studies to follow, some of the topics discussed in this introduction will include: a broad outline of the immune system and bystander immunity; protective type 1 responses against herpesvirus infections; type 2 host immunity against helminths; helminth-mediated immunomodulation and how helminth exposure may influence host immunity against viral infections.

1.1. The immune system: defence against foreign invaders

The body is exposed to numerous foreign agents which can compromise normal physiological function and survival. Defence against invading organisms is mounted by a complex immune system, made up of a network of cells and immune molecules. Foreign illness-causing organisms, referred to as pathogens, include viruses, bacteria and parasites. These are detected by the host's immune system, which sets of a multifaceted response. A successful immune defence results in the complete clearance of the pathogen and limits any damage caused. To establish an effective response, the immune system has several universal approaches to detect and destroy invading organisms, while launching specific defences against the pathogen. These strategies can be categorized into two lines of defence: innate and adaptive, and instruction and communication, within and between the two defences, is carried out by signalling molecules called cytokines [1-3].

1.1.1. Barrier defence and pathogen recognition

Anatomical sites that are frequently exposed to foreign organisms have barriers to protect against invasion. These primarily consist of the skin and mucosal membranes. The skin is made up of specialised epithelial and immune cells, creating the largest mechanical barrier defence in the body [4]. Mucosal membranes in the respiratory, gastrointestinal (GI) and genital tract compromise of a mucous lining, anti-microbial peptides, enzymes and complement molecules as well as specialised epithelial and innate immune cells, which provide a barrier defence and initiate inflammation in response to a pathogen [2]. Specialised cells at the barrier sense foreign organisms by cell surface receptors such as Pattern Recognition Receptors (PRRs) and Toll-Like Receptors (TLRs), which recognise highly conserved pathogen associated molecular patterns (PAMPs), including DNA, double stranded RNA, flagellin and lipopolysaccharides (LPS) [5]. PAMP recognition triggers intracellular signal transduction pathways, resulting in the production and release of various cytokines and chemokines which attract and activate immune cells.

1.1.2. Innate immunity

The innate immune response is the first line of non-specific defence against a broad range of pathogens. The innate arm is essential for detecting foreign invaders, within minutes or few hours following exposure, initiating immune defence. There is no classical immunological memory [2], however a growing body of literature has demonstrated the reprogramming of innate immune cells to have enhanced responsiveness upon pathogen re-exposure, termed "trained immunity" [6-8]. Importantly, rapid innate responses provides initial control, giving time and important information for the adaptive response to be appropriately mounted [2]. Innate immune cells which detect and defend against pathogens, are made up of phagocytic cells, granulocytes, innate lymphocytes and antigen presenting cells (APCs).

1.1.2.1. Neutrophils

Neutrophils detect and engulf, via phagocytosis, foreign organisms and cellular debris. Phagocytosis assists in destroying foreign invaders by fusion of the engulfed material in the phagosome, with acidic intracellular lysosomes, forming a phagolysosome where the intracellular break down of microorganisms takes place. Neutrophils also have cytoplasmic granules that are made up of anti-microbial factors. Degranulation and release of these molecules promotes inflammation and an anti-microbial environment at the site of exposure, which aids in killing extracellular pathogens [9, 10]. Neutrophils also extrude nucleic acids to form neutrophil extracellular traps (NETs) at the site of inflammation, which help contain and kill pathogens, with minimal damage to the host cells [11].

1.1.2.2. Macrophages

Macrophages develop from circulating monocytes and migrate to almost all tissues to guard against pathogens and remove dead cells by phagocytosis. Macrophages also are responsible for co-ordinating the innate immune response, by the production and release of chemokines, to recruit other immune cells to the site of infection [12, 13]. Tissue resident macrophages are specialised for the environment and role required within the given tissue. For example, lung macrophages, located in the alveoli, are adapted to initiate immunity and facilitate control to respiratory pathogens [14-16]. Splenic red pulp macrophages remove dead or dysfunctional red blood cells [17], while macrophages in the liver, known as Kupffer cells, play a role in hepatic tissue remodelling [18]. Macrophages can respond differently, suiting to the type of pathogen faced or signals received. Classically activated macrophages (CAMs; M1) are activated by LPS and interferon (IFN)-γ, in response to intracellular pathogens. CAMs are considered pro-inflammatory and express high levels of enzyme nitic oxide synthase (iNOS), which facilitates nitric oxide (NO)-mediated killing of intracellular pathogens [19-21].

Alternative activation of macrophages (AAMs; M2) are induced by interleukin (IL)-4, IL-13 and IL-10, following exposure to certain extracellular pathogens. They are considered immune regulatory, as they synthesize high levels of the enzyme arginase-1, which inhibits NO production [22]. AAMs play a role in immune regulation, tissue homeostasis and repair [23, 24] and are a key component during anti-helminth immune responses and allergic inflammation [25, 26].

1.1.2.3. Granulocytes

Granulocytes, named for their cytoplasmic granule content, include eosinophils, mast cells and basophils. Once recruited to the site of inflammation, granulocytes release cytotoxic enzymes and soluble inflammatory mediators to aid in destroying large extracellular pathogens. Granulocytes play a role in the immune response against helminths and other parasitic infections, not only by degranulation but also cytokine production, further promoting immune defences. In contrast, they may cause excessive inflammation and tissue damage during allergic disease, as they are recruited to the site of allergen encounter and release allergic mediators e.g. histamine [27, 28].

Eosinophils are classically identified by a bi-lobed nucleus and eosinophilic, granular cytoplasm [29]. Characteristic cytoplasmic granules contain cytotoxic cationic proteins, such as major basic protein (MBP) [30, 31], eosinophil peroxidase (EPO) [32], eosinophil cationic protein (ECP) and eosinophil-derived neurotoxin (EDN) [33], as well as cytokines [34-37], chemokines [38] and lipid inflammatory mediators [39]. At steady state, eosinophils express chemokine receptors CCR1 and CCR3, enabling rapid recruitment in response to eotaxin [40-42]. Once activated, eosinophils upregulate surface expression of chemokine receptors: CXCR4 [43]; CCR5; and CCR8 [44], which allows for their response to a broader range of chemokines. GM-CSF, IL-3 and IL-5 and chemokine eotaxin (CCL-11) are responsible for eosinophil development in the bone marrow, recruitment and survival in the tissue [40, 45-48]. Activated eosinophils commonly release cytoplasmic granules via a process called piecemeal degranulation (PMD), where small spherical and larger tubular vesicles bud from intracellular granules and migrate to and fuse with the cell membrane, releasing their cytotoxic and pro-inflammatory contents [49, 50]. Cytoplasmic granules can also be released by eosinophil cytolysis, where the cell membrane ruptures and intact granules are freed [51]. Interestingly, released granules can deposit within the tissue and function as a secretory organelle with membrane receptors, releasing contents when stimulated [52]. Eosinophil cytotoxicity can also be mediated by antibody [53, 54] and complement [55]. Eosinophils also have an antibacterial function, via the non-terminal release of mitochondrial DNA traps containing cytotoxic granule proteins [56].

Although eosinophils are commonly known for their cytotoxic ability, studies have demonstrated regulatory functions of eosinophils including tissue homeostasis and remodelling, via production of tissue repair mediators. Eosinophils are a major source of immunosuppressive transforming growth factor (TGF)-β1, which regulates fibrosis and angiogenesis during lung disease [57-61]. Eosinophils are also a source of IL-4 and IL-13 [62-65], important cytokines for the induction of AAMs and tissue remodelling [66]. In addition, eosinophil granule proteins and enzymes may contribute to debris clearance and tissue remodelling following injury [67-70]. For example, MBP and EDN have been shown to promote the proliferation of fibroblasts [71]. Eosinophils also express matrix metalloproteinases (MMPs) [72-76], which degrade tissue collagen allowing for infiltration of immune cells as well as tissue remodelling [77-79].

1.1.2.4. Natural killer cells

Natural killer (NK) cells are innate lymphocytes known for detecting and killing virally infected and cancer cells. NK cells are activated by IL-2, -12 and -18 and upon activation, release IFN-γ and tumour necrosis factor (TNF)-α, which can activate other immune cells (e.g. macrophages), potentiating the immune response [80]. Importantly, NK cells have the 'natural' ability to kill infected or diseased cells without priming. They patrol tissue, in constant contact with other cells and initiate cell killing in response to an imbalance of inhibitory and activating signals on cell surface receptors (Figure 1.1). Healthy, 'normal' cells express major histocompatibility complex class I (MHCI) molecules on their cell surface, coupled with endogenous peptides, known as self-antigen. MHCI are recognised by inhibitory receptors on NK cells and healthy cells are protected from NK cell killing. Alternatively, virally infected or tumour cells often downregulate MHCI expression and/or increase expression of signals recognized by activating receptors, resulting in reduced inhibition or strong activation of NK cells [81, 82].

The imbalance towards activation results in NK cell-mediated killing by the release of cytotoxic granules containing perforin and granzymes, which synergistically mediate osmotic cell lysis or apoptosis of target cells [83, 84]. Perforin is a glycoprotein which polymerizes and forms a pore in the target cell membrane, destroying the integrity of the cell [85, 86]. In addition, the pore allows for entry of granzymes into the cytoplasm of the target cell. Granzymes are proteases which cleave intracellular proteins, disrupting the production of viral proteins in virally-infected cells, and induce apoptosis of target cells [87]. To prevent bystander killing of surrounding healthy cells, cytotoxic granules converge and are released in the immune synapse between the NK and target cell [88]. Interestingly, how cytotoxic cells themselves are protected following degranulation is not fully understood.

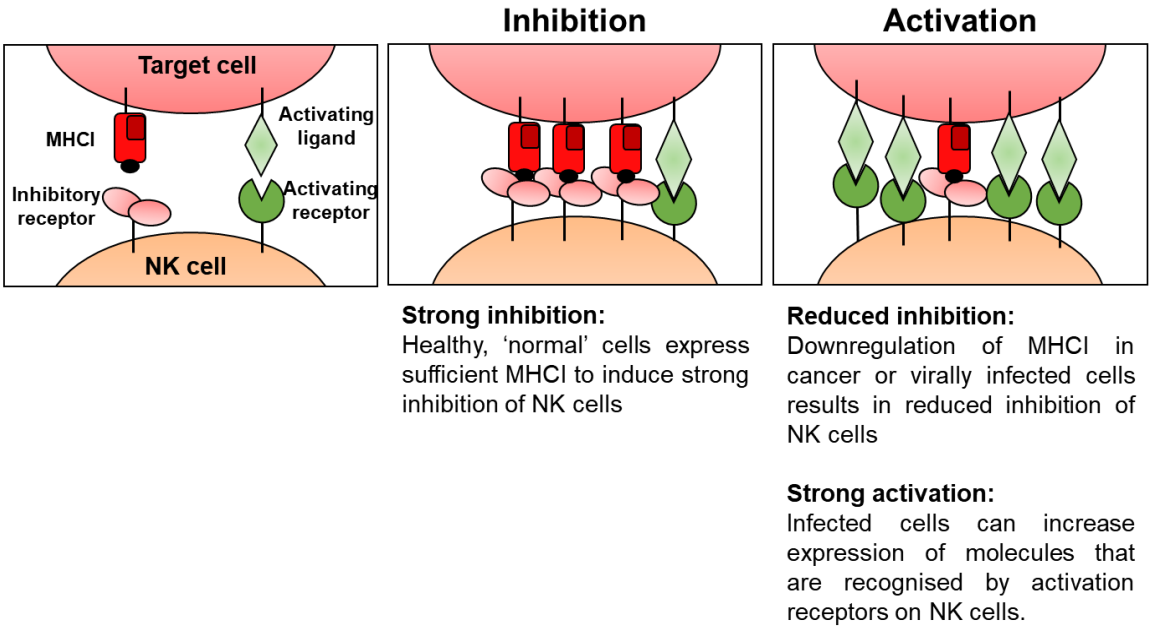


Figure 1.1. NK cell inhibition and activation: NK cells are able to differentiate stressed or infected cells from 'normal', healthy cells by inhibitory and activation receptors, which detect inhibiting and activating signals. Adapted from [89]

1.1.2.5. Antigen presentation and initiation of adaptive immunity

An important role of the innate response is to inform the adaptive arm of the immune system. Presentation of foreign peptides, by APCs (e.g. dendritic cells (DCs) and macrophages) to naïve lymphocytes is a method of communication between the innate and adaptive immunity. When a pathogen is encountered, immature DCs are activated into highly effective APCs, whose function is to carry information, in the form of antigen (Ag), to naïve lymphocytes in the associated secondary lymphoid tissue (e.g. lymph nodes). DCs engulf pathogens and break them down into small peptide fragments, known as pathogen antigen, which have a unique, 'non-self' sequence. As mentioned previously, antigen is presented on cell surface MHC molecules.

MHCI molecules present peptides derived from cytosolic proteins on all nucleated cells, while MHCII molecules present peptides derived from endocytosed proteins, but also self-antigen that travel via secretory pathways such as trans-membrane proteins. Pathogen antigens are coupled with MHC molecules and displayed on the DCs cell surface as an alert signal. Co-stimulatory molecules (e.g. CD80, CD86 and CD40), also expressed on APCs, interact with ligands CD28 and CD40L on naïve lymphocytes. Activated DCs also secrete cytokines which potentiate both innate and adaptive immunity. Presentation of antigen, along with co-stimulatory signals from APCs and cytokine production by innate cells, helps to direct an effective adaptive immune response [90-92].

1.1.3. Adaptive immunity

The swift response of the innate immune system is essential for initial defence against many pathogens. However, effectiveness is limited by innate cell PRRs recognising conserved PAMPs, which pathogens can evade by rapidly evolving or mutating [93-95]. The adaptive immune response, mediated by T and B lymphocytes, is pathogen-specific and forms immunological memory. Antigen presentation by APCs, activate pathogen-specific adaptive cells by their distinct antigen receptor. This signal results in lymphocyte differentiation and proliferation by clonal expansion, where by the lymphocyte progenitor gives rise to many lymphocytes that bear the same antigen-specificity. Clonal expansion results in a highly activated, short-lived effector cells, which mount the adaptive defence by activation of other immune cells, the release inflammatory cytokines and killing infected cells [96-98]. Importantly, the activation, differentiation and clonal expansion of T and B cells is followed by a contraction phase, once the pathogen is cleared. Majority of effector lymphocytes die off, while a small population persist as longer-lived, less active memory cells. Memory cells remain dormant until re-exposure to the pathogen, where they expand and respond more rapidly compared to primary exposure, improving immune control of the pathogen threat. Secondary expansion is followed by contraction and memory formation phases, further improving defences. The formation of classical memory is a hallmark of the adaptive immune response and results in an increasingly effective and rapid defence during subsequent pathogen exposure [99-102]. Classic memory of adaptive immunity has been manipulated for 'prime-boost' vaccine regimens, mimicking natural exposure and providing educated responses against pathogens during primary exposure [103, 104].

T cells, defined by their surface expression of CD3 molecules and αβ T cell receptors (TCR), are generated in the thymus and migrate to secondary immune organs such as lymph nodes (LN) and the spleen, where they await immune activation by pathogen-experienced APCs. T cells include $CD4^+$ T cells, which can differentiate into regulatory T cells (Tregs), type I (Th1) and type II (Th2) helper cells, as well as $CD8^+$ cytotoxic T cells [105].

1.1.3.1. $CD4^+$ T cell differentiation: T helper 1 vs T helper 2

Upon activation by APCs, naïve $CD4^+$ T cells (Th0) differentiate into distinct groups of effector cells, depending on type of pathogen and the cytokine environment, produced by APCs and other innate cells [106]. T cell differentiation is directed by the activation of TCRs, co-stimulatory ligands and cytokine receptors, initiating intracellular signalling pathways and selective gene expression [107]. The $CD4^+$ T cell differentiation includes T helper 1 (Th1), T helper 2 (Th2) and regulatory T cells (T_{reg}), and each response is associated with a certain cytokine and effector cell profile [2] (Figure 1.2).

Th1 is typically associated with cell-mediated immunity against viral and intracellular bacterial infections. Th1 cells differentiate by IL-12 signalling via STAT1/T-bet intracellular signalling pathway. Stimulation of the IL-12 receptor on naïve CD4+ T cells, activates STAT1/4, which results in the expression of transcription factor T-bet, initiating gene expression and production of Th1 cytokines IFN-γ, IL-2 and IL-12 by differentiated T cells. These cytokines act within a positive feedback loop, to potentiate the Th1 immune response [108-110]. Other hallmarks of Th1 immunity include classical activation of macrophages and activation of CD8+ T cells [107].

Th2 immunity is typically associated with allergy and extracellular parasitic infections. Th2 responses are prompted by the release of 'alarmin' IL-33 by epithelial cells, in response to epithelial damage or PRR signalling [111]. Innate lymphoid cells (ILCs), specifically ILC2s, respond via IL-33 receptor T1/ST2, and produce Th2 polarizing cytokine IL-4 [112, 113]. Briefly, IL-4 signalling on its respective receptor (IL-4Rα) on naïve T cells, results in the activation of STAT6 and expression of transcription factor GATA3, which mediates gene expression and production of Th2 cytokines, including IL-4, IL-5, and IL-13 [114-116]. In additional to Th2 cells, IL-4 can be produced by B cells [117], ILC2s [118], basophils [119] and eosinophils [65, 120]. Th2 immunity includes alternative activation of macrophages, eosinophil recruitment, goblet cell hyperplasia, mucous production and smooth muscle contraction [121]. Additionally, Th2 immunity is associated with humoral responses against extracellular pathogens, as IL-4 induce high-affinity antibody production by B cells [122] (discussed later).

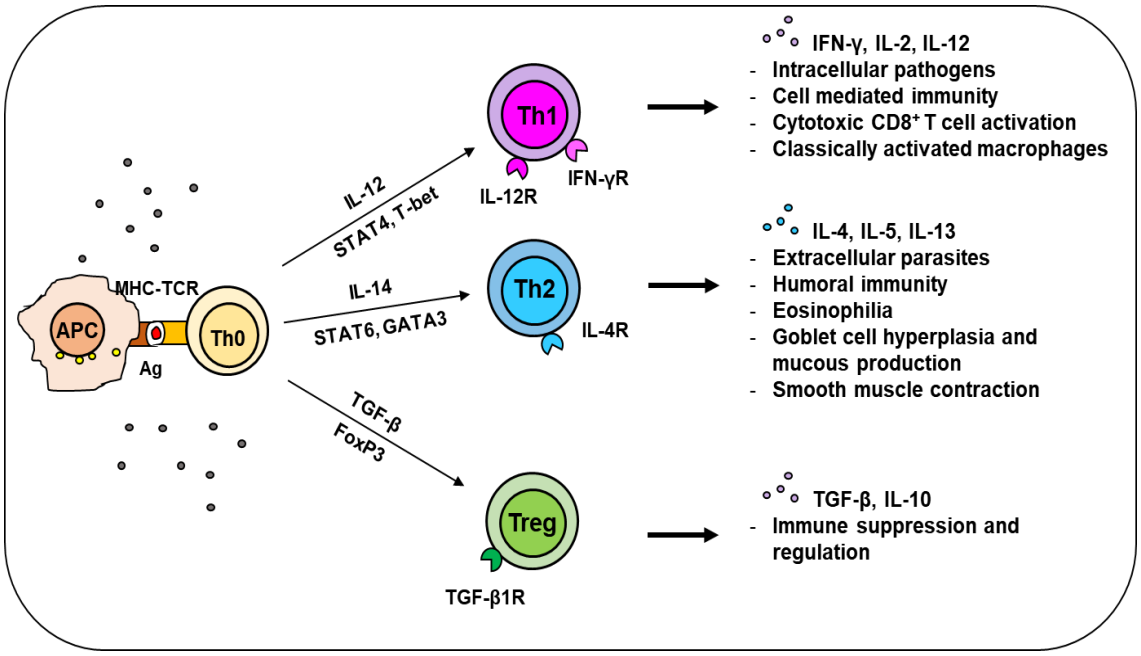


Figure 1.2. CD4⁺ T cell differentiation: Naïve CD4⁺ T cells (Th0) are presented foreign antigen by APCs that is recognised by antigen-specific TCR. TCR activation and cytokine stimulation leads to differentiation into Th subsets, determined by the signals received. Adapted from [123].

There are several other effector CD4+ T cell subsets, which function in co-ordination with Th1 and Th2 responses. An important subset are regulatory T cells, which mediate immune suppression and T cell homeostasis. TGF-β-mediated differentiation of T_{reg} cells is characterised by surface expression of TCR co-receptor CD4 and CD25, a high-affinity component of IL-2 receptor, along with the intracellular expression of nuclear transcription factor FoxP3 [124]. T_{reg} cells regulate immunity by the production of immunomodulatory cytokines IL-10 and TGF-β, that supress pro-inflammatory macrophage, DC, NK, B and T cell responses [125-127]. Additionally, T_{reg} cells sequester IL-2, by high expression of CD25, reducing IL-2-mediated activation of CD8+ T cells and NK cells [128, 129].

Immune regulation or control is important to prevent immune-mediated harm. Th1 and Th2 responses counterbalance each other, via intracellular inhibition effects e.g. Th1-induced IFN-γ and STAT1 supresses Th2 polarisation, and conversely, IL-10 and GATA3 inhibits Th1 differentiation [130-132]. A Th1/Th2 imbalance can have severe pathological effects on the host. Allergic inflammation results from exaggerated Th2 immunity to benign allergens [133-135], while some autoimmune diseases (e.g. type 1 diabetes and multiple sclerosis) are driven by inappropriate Th1 immune responses [136-138]. Studies have shown the role of T_{reg} cells in the prevention and alleviation of allergic inflammation, as well as autoimmunity. On-going research in the field includes the development of novel T_{reg}-based immunotherapy [139, 140].

1.1.3.2. CD8$^+$ T cells

Cytotoxic T cells (CTLs) are important for adaptive defence against intracellular pathogens (e.g. viruses and intracellular bacteria) and malignant cells [141, 142]. They are defined by the expression of dimeric TCR co-receptor, CD8. CD8$^+$ T cells recognise foreign antigen presented on MHCI molecules, with CD8 co-receptors binding a conserved region of MHCI during MHC-TCR activation [143-145]. Initial CD8$^+$ T cell activation is similar to CD4$^+$ T cells: APCs convey messages from infected tissues to secondary lymphoid organs, where naïve CD8$^+$ T cells reside. APCs present antigen to naïve CD8+ T cells via MHCI-TCR interaction, with or without CD4$^+$ T cell help [146-148]. Once activated by TCR, costimulatory and cytokine signals, CD8$^+$ T cells clonally expand into effector CTLs (Figure 1.3), which migrate to the site of infection [149]. Here, CTLs recognize infected or malignant cells by cognate antigen presented on surface MHCI, and release cytotoxic granules containing anti-microbial molecules perforin and granzymes. Activated CD8$^+$ T cells also express Fas ligand (FasL), which binds to cell death surface receptor Fas, on target cells, triggering caspase-mediated apoptosis. Additionally, effector CD8$^+$ T cells secrete pro-inflammatory cytokines TNF-α and IFN-γ, further promoting anti-viral or anti-cancer immunity [150-153]. Once the infection is cleared, a sub-population of antigen-specific CD8$^+$ T cell memory cells persist.

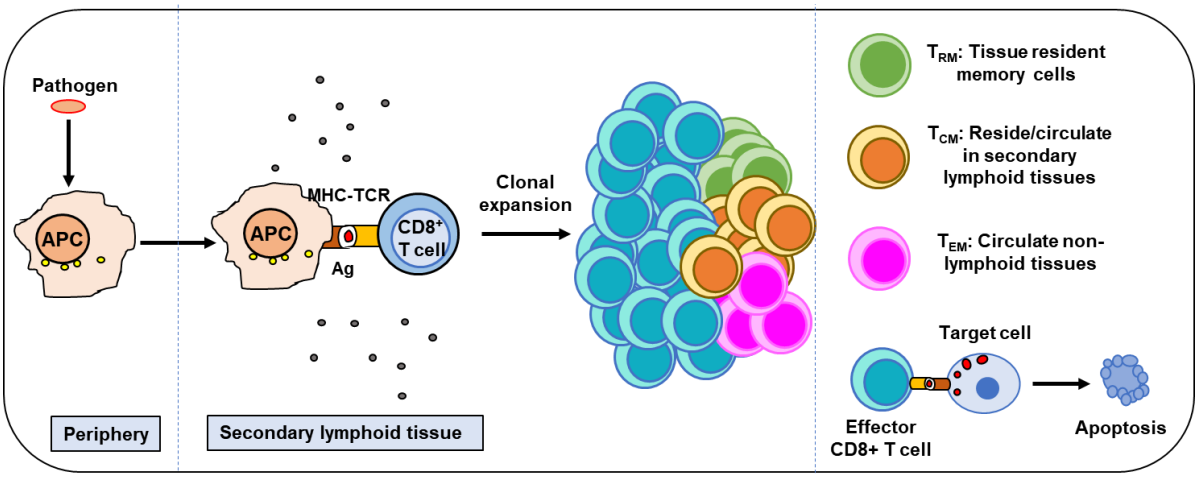


Figure 1.3. CD8⁺ T cell activation, expansion and memory formation. Adapted from [154].

Memory T cells were thought to exist as three subtypes: effector memory (T_{EM}), central memory (T_{CM}) and resident memory (T_{RM}), defined by where they reside, their proliferative and effector ability upon re-exposure (Figure 1.3) [102, 155-158]. A considerable body of evidence suggests another population of memory CD8⁺ T cells, called "virtual memory" (T_{VM}). These are antigen-inexperienced memory CD8⁺ T cells, which develop by homeostatic mechanisms and display enhanced functions [159-162]. The induction and significance of T_{VMs} during herpesvirus infection will be discussed later in this thesis.

1.1.3.3. B lymphocytes and antibodies

B cells are essential for the development of humoral immunity during the adaptive response. They can function as APCs, as they express MHCII, and produce chemokines and cytokines [163]. However, their main function is to produce soluble immunoglobulins (Ig), known as antibodies, against antigens. Mature naïve B cells, known as follicular B cells, circulate secondary lymphoid organs such as lymph nodes and the spleen. B cell receptors (BCR) can recognise soluble antigen directly, in a T-cell independent manner, as well as MHC-coupled antigen, by T-cell dependent activation. Direct antigen activation, results in proliferation and development of short-lived plasma cells, which produce low-affinity antibodies, like IgM, against the specific antigen. When activated with assistance from T cells, B cells migrate to the border of T cell zone, forming germinal centres (GCs). Within these specialised structures, B cells receive signals from follicular Th cells and DCs, proliferate and mature into high-affinity antibody producers. B cells also undergo class-switching within GCs, the process of gene recombination to produce high-affinity specialised antibody subtypes (e.g. IgG and IgE). Signals from follicular T cells and DCs determines the type of antibody subtypes produced. Importantly, a sub-population of memory B cells, with high-affinity BCRs, are maintained, resulting in a more efficient and effective humoral response upon secondary exposure [164-166].

1.2. Bystander immunity: How one disease influences another

There is increasing epidemiological evidence of pathogens altering immunity to unrelated infections or conditions, however, underlying mechanisms are poorly understood. In developing countries, multiple infections with parasites, bacteria and viruses is common. For example, studies have shown that *Mycobacterium tuberculosis* (Mtb) and Human immunodeficiency virus (HIV) coinfection is highly prevalent, with active Mtb infection associated with increased HIV pathogenesis and mortality [167-169]. *In vivo* models demonstrate Mtb-induced Th1 immunity alters susceptibility to subsequent *Plasmodium* spp. infection [170, 171], and the inverse model demonstrating *Plasmodium* impairs immunity against acute Mtb infection [172]. The geographical overlap of the prevalence of these pathogens, makes understanding coinfections and bystander immunity, clinically relevant. Bystander immunity may also arise from non-pathogenic conditions. For example, Benmerzoug *et al*., 2019 recently demonstrated how exposure to silica, an environmental hazard, induced sterile Th2-biased inflammation in the lung, which worsened subsequent Mtb infection *in vivo* [173].

In contrast to the mounting evidence on the negative impact of bystander immunity, there is evidence of beneficial bystander immune regulation. Chronic *in vivo* infection with protozoan parasite *Leishmania* spp. conferred protection and enhanced $CD8^+$ T cell immunity against bacterial *Listeria* spp. infection [174]. Latent murine cytomegalovirus (MCMV) infection, provided protection against subsequent bacterial infections, by increased macrophage activation and low-level IFN-γ [8]. Conversely, chronic human CMV-induced inflammation has been reported in association with reduced HIV immunity and increased HIV progression and mortality [175, 176]. This demonstrates the diverse effects of bystander immunity on unrelated diseases.

One of the most successful persistent pathogens are helminths i.e. parasitic worms, sophisticated in host immune modulation and evasion. In pursuit of survival, they may alter immunity and susceptibility to unrelated conditions or pathogens (detailed later) [162, 177-184]. This is of particular importance, considering the geographical overlap between helminth exposure and pathogens of great concern in developing regions e.g. Mtb and sexually transmitted infections (STIs) [185-189]. In this thesis, we will investigate bystander influences of prior helminth exposure, on immunity and susceptibility to herpesvirus infections, using *in vivo* murine models. Murine models are widely used in biomedical research as mice are easier to house and handle, and relatively inexpensive. In addition, mouse models have provided a wealth of preclinical knowledge. We have developed two nematode and herpesvirus coinfection models, to investigate local and systemic bystander effects of helminths on subsequent herpesvirus infections *in vivo*. First, we will discuss the clinical relevance of the pathogens modelled, detail the *in vivo* models used and protective host immunity against each pathogen.

1.3. Herpesviruses (HVs)

Herpesviridae is a family of more than 100 double-stranded DNA viruses, of which, 8 are known to infect humans (Table 1.1). Herpesviruses have a unique structure comprising a large linear DNA genome, enclosed in a capsid, which is encased by an outer glycoprotein and lipid bilayer envelope. A key feature of herpesvirus is the ability to establish latency in the host. The family can be subdivided into three groups based on distinct characteristics: alphaherpesviruses (α-HVs) have a broad range of hosts and short replication cycle; betaherpesviruses (β-HVs) have long replication cycles and limited hosts; and gammaherpesviruses (γ-HVs) also have a limited host range and infect lymphoid cells [190].

Table 1.1. Classification of human herpesviruses [190-195]

Sub-family	Virus	Target cell	Site of latency	Clinical manifestations and spread
α-HV	Herpes Simplex virus type I (HSV-1)	Mucosal epithelial	Neurons	Common cause of oral herpes (cold sores). Virus is spread by contact or sexual transmission
	Herpes Simplex virus type II (HSV-2)			Main cause of genital herpes. Virus is spread by contact or sexual transmission. Causes neonatal herpes in babies born to infected mothers
	Varicella zoster virus (VZV)	Epithelial		Chicken pox and shingles. Transmitted by droplets in the air, close contact
β-HV	Cytomegalovirus (CMV)	Endothelial and epithelial cells, monocytes and other leukocytes	Endothelial and myeloid lineage cells	Clinical symptoms common with immune suppression/deficiency. Severe manifestations include retinitis, neurological damage with neonatal infections. Can be transmitted through saliva, urine and breast milk
	Human Herpes virus (HHV)-6 and HHV-7	Wide tropism but preferably infects $CD4^+$ T cells		Condition known as 'Exanthema subitem' with primary infection in infants, encephalitis upon reactivation. Transmitted from mother to child and through saliva
γ-HV	Epstein–Barr virus (EBV)	B cells and epithelial cells	B cells	Infectious mononucleosis, Burkitt's lymphoma. Transmission by saliva and transfusion
	Kaposi's sarcoma-associated herpesvirus (KSHV)	Lymphocytes, endothelial cells		Kaposi sarcoma in immunocompromised patients. Transmission by saliva and transfusion

1.3.1. α-HVs: Genital HSV-2

α-HVs primary infection occurs in the periphery e.g. mucosal epithelial, and thereafter the virus invades the neurons to establish lifelong latency in the peripheral nervous system [196]. During reactivation, infection is spread along the neurons back towards the periphery [197]. There are three commonly known human α-HV infections; HSV-1, a common cause of cold sores or oral herpes; HSV-2, the main cause of sexually transmitted genital herpes; and VZV, a very common infection in children known as chicken pox, with reactivation as shingles in adults [198].

Prevalent HSV-2 genital infections is of particular importance, as ulcerative infection is associated with increased risk of HIV acquisition [199]. It is estimated that over 500 million people worldwide are infected with HSV-2, with the highest prevalence in Africa [200, 201]. Infection is more prevalent in women than men, making HSV-2 a concern for female sexual and reproductive health. Initial symptomatic infection often results in genital ulcers and high viral shedding, as well as swollen lymph nodes and fever. Localised recurrent outbreaks are common, but shorter and less severe than initial genital herpes infection [202, 203]. Interestingly, HSV-2 reactivation is often asymptomatic, however, sexual transmission can still occur in the absence of visible genital lesions [204]. Infection is incurable, but symptoms can be treated with anti-viral acyclovir and valacyclovir. However, even at high doses, treatment does not reduce asymptomatic viral shedding and sexual transmission [205]. Vertical transmission from HSV-2-infected mother to the foetus may also occur, resulting in fatal herpes encephalitis in newborns [206]. As HSV-2 is a risk factor for HIV, transmission prevention is a public health priority, with research focused on the development of anti-microbicides and vaccines [207-212]. There is a significant geographical overlap between helminth and HSV-2 prevalence, with a high prevalence of both infections in Sub-Saharan Africa. It is not known whether underlying helminth infections may systemically influence the genital tract and susceptibility to HSV-2. In this thesis, we will investigate the systemic influence of helminth exposure of female genital immunity and susceptibility to HSV-2 infection *in vivo*.

HSV-2 infects vaginal epithelial cells by multiple viral envelop glycoproteins binding to their corresponding host cell surface receptors, resulting in membrane fusion and virus entry [213, 214]. Entry is followed by viral DNA integration and synthesis, transcription/translation of viral proteins and assembly of new virions, which bud from the infected cell, resulting in cell lysis [215, 216]. HSV-2 virions can spread to neighbouring epithelial cell and sensory neurons, leading to further lytic replication or the establishment of latency in ganglionic neurons [197]. Reactivation of HSV-2 from latency is associated with stimuli including stress, fluctuations in hormone levels and immune suppression. Reactivation events can result in genital ulcers or asymptomatic virus shedding [217].

Primary HSV-2 infections are difficult to investigate in humans as many are asymptomatic or undiagnosed [218]. The development of animal models has bettered our understanding of HSV virology, pathogenesis and immunity. HSV-2 modelling depends on the animal species, HSV-2 strain, infection route and dose used. Murine vaginal HSV-2 infections are fatal at high doses but fail to establish infection at low doses. HSV-2 latency is established in the dorsal root ganglia in both humans and mice. But unlike in humans, mice rarely have events of reactivation leading to viral shedding. Despite these limitations, intravaginal HSV-2 infection in mice allows for the analysis of innate immunity during acute infection as well as vaccine efficacy [219, 220].

1.3.1.1. *In vivo* intravaginal HSV-2

Female mice are used to model vaginal HSV-2 infection once they reach sexual maturity between 5-6 weeks old. BALB/c mice are preferable as they are easier to handle and more susceptible to HSV-2 infection. Following puberty, female mice experience a sex hormone cycle, divided into 4 stages; proestrus, estrous, metestrus, and diestrus. Pubescent female mice have varying susceptibility to HSV-2 infection and require exogenous progesterone pre-treatment to synchronize estrous cycles and reduce variability. Progesterone treatment alters vaginal epithelium thickness and HSV-2 entry receptors to promote viral uptake [219, 221-223]. Hormone-treated mice are inoculated intravaginally, under deep anaesthesia. The virus infects vaginal epithelial cells, causing genital inflammation and ulcers by day 2-3 post infection. Vaginal washes are performed to analyse virus shedding and immune responses. HSV-2 infection spreads to the vulva, urethra, bladder and rectum, manifesting in perianal swelling, urine retention and constipation. Disease progression results in purulent lesions, followed by hind limb paralysis and death [220].

1.3.1.2. Innate vaginal immunity

Innate immunity is essential for early host control following *in vivo* HSV-2 infection [224-226]. The vaginal mucosa is made up of stratified squamous epithelium, lined by mucous, commensal bacteria and anti-microbial molecules. In addition, the vaginal mucosa is surveyed by resident immune cells, for invading pathogens. Following infection, several immune cells are recruited to the mucosa to mount a rapid innate immune response. Essential components of the early anti-HSV-2 response include plasmacytoid DCs (pDCs), conventional DCs (cDCs) and skin resident DCs known as Langerhans cells (LCs), as well as TLR-mediated pathogen recognition, type I IFNs, inflammatory monocytes, NK cells and IFN-γ [227].

Studies have demonstrated that viral recognition by TLRs initiates immunity following HSV-2 infection. Tissue resident DCs constantly sample the mucosal environment and engulf HSV-2 virions upon genital infection. Endosomal TLR-9 detects viral DNA within DCs, while cell surface TLR-2 reportedly recognizes to viral glycoproteins [228-230]. In mice, vaginal administration of TLR-9 ligand oligonucleotides containing immunostimulatory CpG motifs, provides protection against genital HSV-2 [231, 232]. In addition, patients with TLR-2 polymorphisms have increased severity of genital HSV-2 ulcers and viral shedding [233].

TLR-mediated immunity is dependent on type I IFN production [232]. Lund *et al*., 2006 demonstrated that pDCs are recruited following HSV-2 infection and produce type I IFNs, in a TLR-9-dependent manner. The production of type I IFNs by pDCs were essential for early viral control [234, 235]. Increased viral load, genial ulcers and mortality have been observed in mice lacking type I IFN receptor signalling (IFNα/β$R^{-/-}$)[236, 237]. Type I IFNs act in an autocrine and paracrine manner, to induce an antiviral environment (Figure 1.4). Initial IFN-β production after 12 hours, followed by a second wave of IFN-β and IFN-α, 48 hours post HSV-2 infection [225, 232]. Following the initial wave, type I interferons induce the production of chemokine CCL2, which rapidly recruits inflammatory monocytes to the site of infection. It was recently reported that type I IFN-dependent IL-18 production from inflammatory monocytes, is important for early NK cell-mediated vaginal immunity [226].

NK cells are rapidly recruited and activated by IL-15 following HSV-2 infection, crucial for antiviral defence [230, 238]. Thapa *et al.*, 2007 demonstrated that impaired NK cell recruitment increased HSV-2 susceptibility [239]. Activated NK cells induce perforin- and granzyme-mediated apoptosis in virally infected cells as previously described (Figure 1.5). In addition, NK cells are the main source of IFN-γ during early HSV-2 infection. IFN-γ is an important antiviral mediator, with the absence of IFN-γ resulting in increased HSV-2 replication and mortality [224, 238, 239]. IFN-y-induced antiviral state is important for limiting replication in susceptible genital cells. IFN-γ induces the production of iNOS, catalysing the production of NO in infected and surrounding cells [240, 241]. Additionally, type I and II IFN-signalling results in the expression of interferon stimulating genes (ISGs), producing antiviral enzymes which inhibit or disrupt viral replication [242, 243]. Importantly, IFN-signalling regulates pDC maturation and enables adaptive responses by increasing MHCII expression and antigen presentation by APCs [244-247].

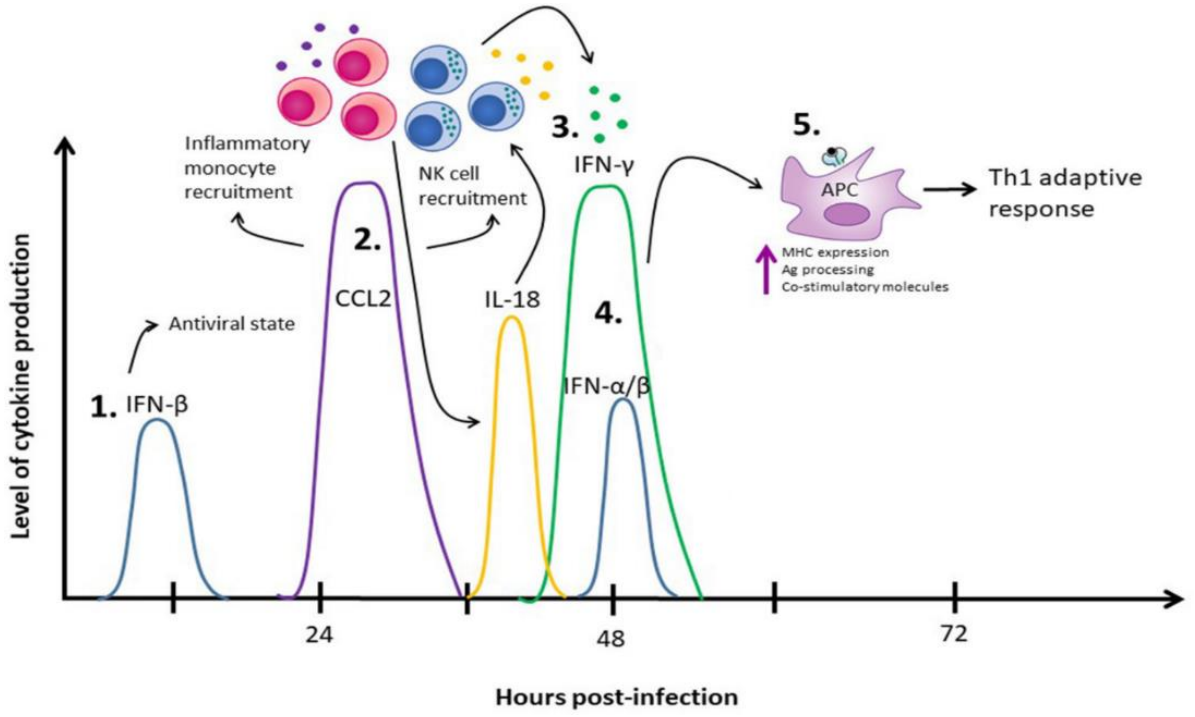


Figure 1.4. The importance of type I IFNs during early HSV-2 infection: (1) First wave of IFN-β production 12 hours post vaginal HSV-2 infection, inducing an antiviral environment by autocrine and paracrine signalling. **(2)** Type I IFN-induced CCL2 production between 24 and 36 hours post infection, results in inflammatory monocyte recruitment. **(3)** Inflammatory monocytes release IL-18, which signal NK cells along with IL-15, resulting in their activation and production of IFN-γ. **(4-5)** IFN-γ in cooperation with the second wave of type I IFNs, enhance antiviral immunity and facilitate adaptive responses. Adapted from [245]

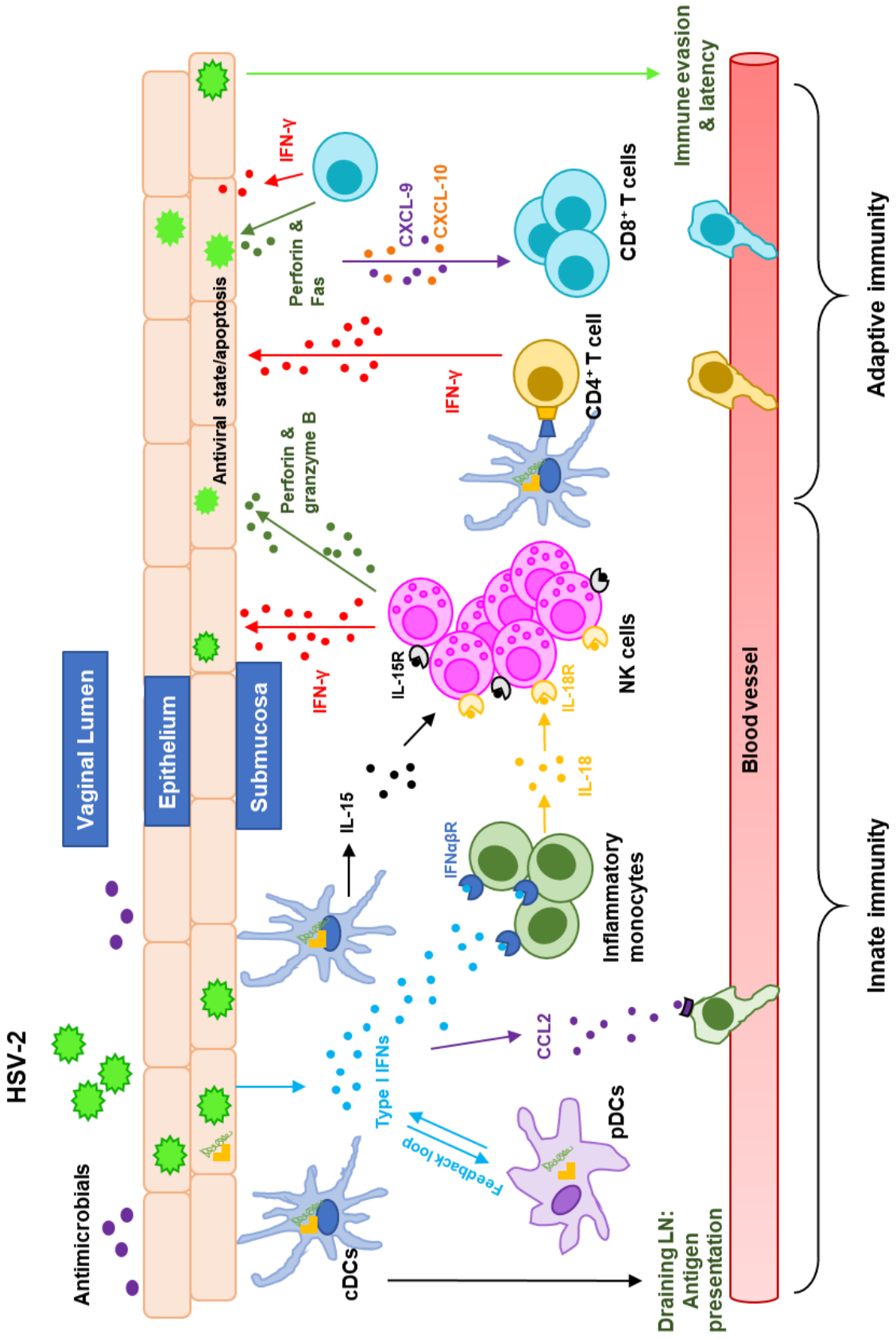


Figure 1.5. Innate and adaptive immunity against HSV-2: Protection against mucosal HSV-2 infection is mediated by both innate and adaptive immunity. Innate immunity is essential for early control while adaptive responses are required for viral clearance. Adapted from [226, 227]

1.3.1.3. Adaptive anti-HSV-2 immunity

Innate antiviral responses are important for early viral control and initiating HSV-2-specific adaptive immunity, which mediates viral clearance. DCs and innate cytokine and chemokine signals prime the adaptive immune response during vaginal HSV-2 infection. After pathogen recognition, DCs mature and migrate to the associated LNs to prime adaptive immune responses. Interesting, infected DCs are unable to present antigen to prime protective Th1 responses, as HSV-2 blocks migration, downregulates MHCI and MHCII expression, and induces apoptosis [248, 249]. Uninfected submucosal $CD11c^+$ and $CD11b^+$ cDCs take up viral antigen and drain into the associated LNs (i.e. iliac and inguinal LNs), where they present viral antigen to naïve $CD4^+$ T cells, while antigen-experienced $CD103^+$ and $CD8\alpha^+$ dermal cDCs prime naïve $CD8^+$ T cells [250-253]. HSV-2 antigen presentation and cytokine signals results in Th1 differentiation and migration to the site of infection. The local inflammatory signals induced during the innate response also recruits $CD4^+$ T cells to the vaginal tissue [254]. Several studies have investigated the importance of $CD4^+$ and $CD8^+$ T cells (Figure 1.5) as well as humoral immunity, during genital HSV-2 infection.

Cell-mediated adaptive immunity plays a pivotal role in viral clearance. Both $CD4^+$ and $CD8^+$ T cells can be found in genital ulcers of HSV-2 infected patients [255]. $CD4^+$ T cells can persist in genital skin, long after HSV-2 lesions have healed, where they can mount an appropriate response upon reactivation [256]. With primary infection, infiltrating virus-specific $CD4^+$ T cells are an important source of IFN-γ, producing the second antiviral wave during vaginal HSV-2 infection [224]. Milligan *et al*., 1998 demonstrated that HSV-2 immune protection was lost with depletion of $CD4^+$ T cells as well as neutralisation of IFN-γ [257]. Nakanishi *et al*., 2009 reported that $CD4^+$ T cells provide the necessary signals for the recruitment of effector $CD8^+$ T cells to the site of HSV-2 infection. $CD4^+$ T cell help was mediated by IFN-γ-inducible chemokines CXCL-9 and -10, which recruit $CD8^+$ T cells to the vagina, via their CXCR3 receptors [149].

Virus-specific $CD8^+$ T cells mediate control of mucosal HSV-2 infection, by detecting and killing virus-infected cells via perforin and granzymes release. $CD8^+$ T cell cytotoxicity is dependent on IFN-γ signalling [224, 258] and $CD8^+$ T cell-depletion increases viral pathogenesis in mice [259]. Additionally, in recurrent human HSV lesions, $CD8^+$ T cell infiltration is associated with viral clearance [255, 260] and tissue resident T cells provide immunosurveillance and readily respond during reactivation [261, 262].

Regulatory T cells are induced during HSV-2 infection, but their role is controversial. Soerens *et al*., 2016 reported that T_{reg} cells are required for DC migration to genital-associated LNs and $CD4^+$ T cell accumulation in the vaginal mucosal and viral control [263]. Conversely, T_{regs} were found to suppress protective T cell responses during adult and neonatal HSV-2 infection [264]. In recurrent human genital lesions, Milman *et al*., 2016 observed a correlation between increased T_{regs} and HSV-2 shedding, further suggesting an imbalanced T_{reg} response may impair anti-viral immunity [265].

B cell-mediated humoral immunity may be important for vaccine-mediated HSV-2 prevention. HSV-2-specific IgG and IgA are produced in both mice [219, 266] and humans [267-269] following vaginal infection. Neutralizing antibodies are specific to HSV-2 glycoproteins gD, gB and gC [270, 271]. Passive transfer of purified IgG from immunized mice to naïve recipients, provided protection against subsequent HSV-2 infection [272]. Additionally, immunized B cell-deficient mice failed to control viral replication and shedding compared to wildtype controls [273]. Conversely, Morrison *et al*., 2001 demonstrated that vaccine-induced antibody responses contribute to HSV-2 protection but is not essential for initial control [274]. Studies have also shown that B cells are not required for protection against lethal vaginal HSV-2 challenge [275, 276] and passive transfer of HSV-2-specific antibodies did not confer protection [266]. Despite conflicting reports, *in vivo* glycoprotein subunit vaccine studies show promising results [210-212].

1.3.2. γ-HVs: EBV and KSHV

γ-HVs are a public health concern due to the diseases caused by two human γ-HVs; EBV and KSHV. These oncogenic DNA viruses establish persistent infection in B lymphocytes and sporadic reactivation from latency results in spread to new cells and transmission through saliva to new hosts [277, 278]. Transmission through sexual contact has also been reported [279-281]. Primary infections are commonly asymptomatic however virus-associated cancers and other diseases can occur, especially in immunocompromised patients [282-284].

EBV is prevalent in the majority of the world's population [277]. EBV was isolated from Burkitt's lymphoma lymphoblasts in 1964, the first virus to be discovered in a human tumour [285]. EBV is commonly transmitted through saliva during the early childhood. EBV primarily infects B cells and epithelial cells and establishes life-long persistence in resting B cells [277, 286, 287]. Initial infection is commonly asymptomatic but may present as infectious mononucleosis (IM), typically in adolescents. IM manifests due to an exaggerated yet ineffective T cell response against EBV-infected cells. EBV is associated with multiple cancers including Burkitt's lymphoma, post-transplant lymphoproliferative disease, Hodgkin's lymphoma and gastric carcinoma [288-290]. Because of these associations, EBV is classified as a group I carcinogen in 1997 and remains a global health concern [291, 292].

While EBV has a global presence, KSHV is endemic to sub-Saharan Africa and regions near the Mediterranean Sea [293]. KSHV is also classified as a group I carcinogen by the World Health Organisation (WHO) as it is associated with 1% of all human cancers [294]. KSHV was originally discovered in Kaposi's sarcomas (KS) in older men of Mediterranean decent [295]. KS lesions can be described as vascular plaques or lesions on the skin or mucous membranes. Today, KS can be found in children and adults in Africa, as well as in immunocompromised patients e.g. HIV-infected patients and organ transplant recipients [296, 297]. KSHV is also associated with primary effusion lymphomas (PEL) and multicentric Castleman's disease (MCD) [298, 299]. Approximately 20% of MCD cases can progress to KSHV-associated non-Hodgkin's lymphoma [300]. With the prevalence of human γ-HV infections in helminth endemic regions, we aim to investigate the influence of helminth infections on susceptibility to γ-HV *in vivo*. Human γ-HVs EBV and KSHV have strict host tropism which makes *in vivo* investigation difficult [301]. To overcome this, we use a murine model for γ-HV infections, *murid gammaherpesvirus 4* (MuHV-4)[302].

1.3.2.1. MuHV-4: murine model of human γ-HV

KSHV and EBV strict host tropism limits the *in vivo* study of viral pathogenesis. Early primate models of EBV infection [303, 304] have limited experimental value as these animals are rare, endangered and expensive to house [302]. MuHV-4 currently provides an accessible and suitable *in vivo* model of human γ-HV pathogenesis [302, 305]. MuHV-4 is genetically similar to KSHV more so than EBV [306], and establishes latency in B cells like KSHV and EBV [307, 308]. The archetypal strain Murine gammaherpesvirus 68 (MHV-68) was first isolated in 1980 from *Myodes glareolus*; a small rodent known as a bank vole [309]. MuHV-4 was also identified in Slovakian yellow-necked mice (*Apodemus flavicollis*) thereafter [310]. It was later discovered that the natural host of MuHV-4 is the wood mouse; *Apodemus sylvaticus* [311]. MuHV-2 has not been isolated in house mice (*Mus musculus*), the natural relative of inbred laboratory mice, however the virus establishes non-pathogenic persistence in common laboratory strains e.g. BALB/c and C57BL/6 mice [312]. Sunil *et al*., 1994 demonstrated that long term MuHV-4 infection in BALB/c mice can result in the development of lymphomas in both lymphoid and non-lymphoid tissues, consistent with human γ-HVs EBV and KSHV [313]. It has since been reported that MuHV-4 infected mice may develop lymphoproliferative diseases, which is enhanced by immunodeficiency [314-316].

1.3.2.2. MuHV-4 lytic infection

The natural route of murine γ-HV infection is not known, however respiratory transmission is likely [307]. The experimental model of MuHV-4 infection typically involves intranasal inoculation under general anaesthesia and lytic respiratory infection [312], although the virus can establish productive infection by intraperitoneal injection [305]. Following intranasal administration, MuHV-4 primarily infects alveolar epithelial cells, resulting in lytic viral replication in the lung, which is typically controlled by day 9 post infection [312, 317, 318]. Lung infection is followed by relatively slow inflammation and bronchiolitis. Both innate and adaptive responses are involved in viral control during the acute phase of infection. The lung immune response involves an initial infiltration of macrophages, peaking at day 3 post infection. This is followed by an increase in lung $CD8^+$ T cells by day 7-10 post infection, which coincides with peak lytic viral replication [317].

1.3.2.3. MuHV-4 latency and reactivation

Despite control of lytic infection, viral latency is established [319], making it difficult for the host's immune system to clear the infection. Additionally, the virus hijacks host immune cells to migrate from lung tissue to secondary lymphoid tissues, where the cycle of latency, reactivation and productive infection can occur. During lytic respiratory infection, the virus establishes latency within DCs and B cells in the lung, which migrate to associated lymph nodes and then spleen [320]. Infected B cells clonally expand, increasing the number of latently infected B cells [321]. Splenic B cells, macrophages and DCs serve as new MuHV-4 latency reservoirs during latency expansion; local viral reactivation, replication and productive infection, increasing the degree of viral latency [322, 323]. This peaks at 2 weeks post infection and controlled by week 4, with persistence maintained by latently infected memory B cells [323]. Additionally, MuHV-4 infections reaches the genital tract by day 21 post nasal infection, following control of viral replication in the LN and spleen. Interestingly, MuHV-4 reactivation in the genital tract is sporadic, transient and recurrent, and is associated with virus shedding, allowing for sexual transmission [324].

1.3.2.4. MuHV-4 T cell-mediated protective immunity

$CD4^+$ and $CD8^+$ T cell cooperation mediates long-term viral control and protection during MuHV-4 infection. Stevenson *et al*., 1999a demonstrated that lethal viral infection was present in C57BL/6 mice lacking both $CD4^+$ and $CD8^+$ T cells, while delayed clearance was observed with single depletion of either T cell subset [325]. Virus-specific $CD8^+$ T cells eliminate MuHV-4-infected cells by classical perforin-, granzyme- and Fas-mediated cytotoxicity previously described [326, 327].

Ehtisham *et al*., 1993 demonstrated early on, the importance of $CD8^+$ T cell responses during lytic MuHV-4 infection. BALB/c mice depleted for $CD8^+$ T cells displayed severe respiratory infection and enhanced viral dissemination [328]. Antigen diversity of cytotoxic T cells have been characterised using MHC tetramer analysis. More than 30 MHCI-bound viral peptides have been described, during the different stages of MuHV-4 infection [329, 330]. Following respiratory infection, antigen-specific $CD8^+$ T cells can be detected in the lungs and draining LN. These cells are specific to viral lytic proteins present during acute infection [329, 330]. These data suggest that virus-specific CD8 responses are important for control of lytic MuHV-4 infection.

In addition to their role in the lung, conventional $CD8^+$ T cells control MuHV-4 infection in splenic B cells by perforin-mediated killing [331, 332]. Cush *et al*., 2007 reported the development of virus-specific memory $CD8^+$ T cells during early MuHV-4 infection, that are maintained throughout persistence, despite low viral load and antigen presence. Furthermore, Usherwood *et al*., 2000 demonstrated that $CD8^+$ T cells that recognize latency-associated epitopes, contribute to control during the establishment of latency [333]. Virus-specific $CD8^+$ T cells have long-term cytotoxic capability, without exhaustion [334, 335]. Interestingly, Braaten *et al*., 2006 reported a unique population of $CD8^+$ T cells, with the ability to produce anti-viral cytokines IFN-γ and TNF-α. These unconventional $CD8^+$ T cells adequately controlled chronic infection in the absence of conventional $CD8^+$ T cells [336, 337]. It should be noted that there are conflicting reports on the importance of $CD8^+$ T cells for MuHV-4 control, in different mouse strains. In the same study by Ehtisham *et al*., 1993, $CD8^+$ depletion in C57BL/6 did not impair viral resistance as in BALB/c mice [328]. Conversely, β2 microglobulin-deficient BALB/c mice, which lack MHCI expression and $CD8^+$ T cells [338], have worsened lymphoproliferative disease following MuHV-4 infection, but this was not observed in C57BL6 mice with the same deficiency [316]. Also, $CD4^+$ T cell-deficient C57BL/6 mice develop chronic wasting disease with MuHV-4 infection [339], although levels of functional virus-specific $CD8^+$ T cells are maintained, suggesting limited control by $CD8^+$ T cells alone [335, 340].

$CD4^+$ T cells and IFN-γ production are important for control of MuHV-4 persistence. Mice lacking $CD4^+$ T cells can resolve acute infection but develop progressive wasting and lethal respiratory reactivation [339, 340]. CD4+ T cells control persistence, even in the absence of $CD8^+$ T cells, mediated by IFN-γ [341, 342]. Tan *et al*., 2017 demonstrated that while $CD8^+$ T cells controlled acute epithelial infection, $CD4^+$ T cells were required for chronic control [343]. Following MuHV-4 infection, the majority of naive $CD4^+$ T cells differentiate into IFN-γ-producing Th1 effector cells, accompanied by the establishment of $CD4^+$ T cell memory. Virus-specific memory $CD4^+$ T cells proliferate and spontaneously produce IFN-γ throughout long-term latency [344-346]. IFN-γ can directly regulate MuHV-4 reactivation [347].

Although the mechanisms of IFN-γ-mediated control of reactivation is not fully understood, some epigenetic mechanisms have been reported. IFN-γ directly suppresses the transcription of viral transcription factor ORF50, a switch gene which triggers lytic viral replication [348]. Although $CD4^+$ T cell responses may not be essential for acute viral control, T cell help is needed for effective cytotoxic $CD8^+$ T cell activity against long-term MuHV-4 [339, 349, 350]. These findings suggest that $CD4^+$ T cells are important for immune control of viral latency and reactivation, mediated by IFN-γ.

1.3.2.5. *In vivo* imaging of MuHV-4 replication

Oxidative luciferase enzymes that produce bioluminescence, are commonly used in biotechnology [351]. Milho *et al*., 2009 developed the enzyme-based imaging technique of MuHV-4 lytic replication, by inserting the luciferase encoding sequence into the viral promoter M3 gene (MuHV-4-LUC). Thus, the luciferase enzyme is expressed during lytic viral replication. For *in vivo* imaging, MuHV-4-LUC-infected mice are injected intraperitoneally with the substrate luciferin, which is acted upon by expressed luciferase, producing bioluminescence. Bioluminescence signals are analysed by real-time live imaging systems [305]. In Chapter 2, we use luciferase-based *in vivo* imaging to measure MuHV-4 lytic replication and reactivation in mice (Figure 1.6).

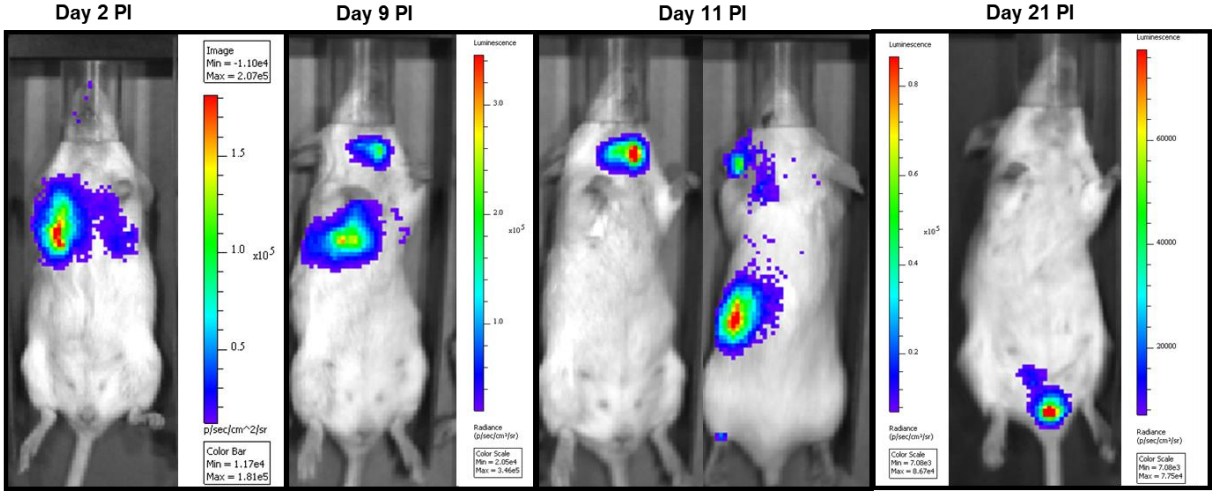


Figure 1.6. *In vivo* imaging of respiratory MuHV-4-LUC primary infection, and systemic reactivation: Mice are intranasally infected with MuHV-4-LUC under general anaesthesia and injected with luciferin and imaged for viral replication. Images are of a representative mouse at day 2, 9, 11 and 21 post infection (PI). Lytic respiratory infection is typically cleared by day 10 post infection, followed by spread and reactivation in the superficial cervical lymph nodes (day 9-11 PI), spleen (day 11 PI) and genital region (spontaneous and sporadic from day 15 PI onwards) [305].

1.4. Soil-transmitted helminths (STHs)

1.4.1. Burden of STH infection

Soil-transmitted helminths (STHs) are a global health concern, with more than 1.7 billion affected worldwide, particularly in tropical and subtropical regions [352]. STH was classed as a neglected tropical disease due to high prevalence in underdeveloped or developing countries, and the chronic illness associated with infection [353, 354]. Common human species (spp.) include: roundworm *Ascaris lumbricoides*; whipworm *Trichuris trichiura*; and hookworms *Necator americanus* and *Ancylostoma* duodenale. Hookworms affect both young adults and children, while *Ascaris* and *Trichuris* infection are prevalent mainly in children [354-356]. There is no direct person-to-person spread of parasitic worms. STHs are transmitted by eggs in the faeces of infected hosts, developing into infective larvae in the soil. Roundworm and whipworm larvae are transmitted to new hosts by ingestion of contaminating soil, water and food. Hookworm larvae in contaminated soil, actively penetrate the skin of new hosts [357]. Morbidities resulting from STHs is related to the intensity of infection: low intensity infections are often asymptomatic, while high intensity acute infections are associated with weakness, severe diarrhoea, bowel obstruction and abdominal pain. Severe or chronic infections can result in with anaemia and malnutrition, as well as impaired growth and development in children [358].

1.4.2. Helminth immunity

Host immunity to helminths has been studied in depth using mouse models reflective of human infection and immunity [359-362]. STHs induce a Th2-shewed immune response (Figure 1.7), associated with the production of canonical Th2 cytokines, alternatively activated macrophages, eosinophilia, and the 'weep and sweep' worm expulsion from the intestine.

1.4.2.1. Innate immune responses

Considering their large size, parasitic helminths can cause extensive damage to colonized tissue during transition through the host. In response to parasite-induced tissue damage, epithelial cells or specialised tuft cells in the colonized mucosa (e.g. lung or intestine) release 'alarmins' such as IL-33, IL-25 and thymic stromal lymphopoietin (TSLP) [363-367], which activate various innate immune cells, notably ILC2s [368-370]. Type 2 immune responses mediated by innate cells are sufficient for parasite clearance [36]. ILC2s are important initiators of protective type 2 immunity as early cytokine producers, aiding in directing both innate and adaptive immunity. ILC2s release type 2 cytokines IL-5, IL-9 and IL-13, which recruit and activate effector granulocytes and alternatively activate macrophages [368, 371-373]. Also, IL-13 activates mucous production by goblet cells [368, 374] and regulates migration of DCs during early infection [375].

Th2 polarisation is a hallmark of helminth immunity. As previously discussed, DCs play an important role during T cell differentiation, as they receive foreign antigen from the tissue microenvironment and drain into associated lymph nodes, where they instruct naïve T cells via MHC-TCR interactions [90, 106]. Studies have demonstrated that DCs pulsed with helminth antigen, preferentially promote Th2 differentiation [376-379] and DCs are able to respond directly to IL-33 and subsequently induce IL-5 and IL-13 production by T cells [380]. Additionally, Cruickshank *et al*., 2009, demonstrated that resistance to murine *Trichuris* infection was associated with increased DC chemotaxis and recruitment to the colon [381]. Even with their expansion and parasite-driven modulation following helminth infection [382], it has been reported that innate type 2 cells are able to induce Th2 responses independent of DCs [383]. In addition, mice genetically deficient for IL-4 or STAT6 have normal Th2 responses to *Nippostrongylus brasiliensis* infection [384]. Interestingly, parasite products may be potent inducers of Th2 differentiation following infection [363, 379, 385-388]. However, Th2-driving parasite products are not clearly defined, with studies suggesting helminths may express lipids and lipoproteins [389], glycans [390], glycoproteins [388, 391] and proteases, which may function as PAMPs to drive Th2 immune responses [392].

1.4.2.2. Th2 cytokines and effector cells

Th2 cells produce cytokines; IL-4, IL-13, IL-9 and IL-5, which activate effector cells and functions which aid in worm expulsion. The role of downstream effector mechanisms is well established (Figure 1.7). IL-4 and IL-13 stimulate smooth muscle contraction, goblet cell hyperplasia and mucous secretion, as well as changes to gut epithelial permeability, which collectively facilitate the 'weep and sweep' response and clearance of the parasite [393-397]. IL-4 and IL-13 effector mechanisms require signalling through type I and type II IL-4 receptor (IL-4R), resulting in JAK-dependent tyrosine phosphorylation of the IL-4R and activation of STAT6, promoting selective gene expression [36, 398]. Type I IL-4R, a heterodimer of IL-4R alpha chain (IL-4Rα) and common cytokine receptor γ chain (γc), is expressed on bone marrow-derived cells (e.g. macrophages, granulocytes, T and B lymphocytes) and binds IL-4 only [398, 399]. Both IL-4 and IL-13 signal through type II IL-4R, a heterodimer of IL-4Rα and IL-13Rα1, expressed on non-bone marrow-derived cells like fibroblasts, epithelial and endothelial cells, smooth muscle cells and also some haematopoietic cells excluding T cells [396, 399-401]. IL-4Rα is an essential component of both IL-4R receptors, and transgenic mice deficient of this chain are used to study the importance of Th2 immunity during helminth infection [396, 401-405].

Th2 cytokines IL-4 and IL-13 drive the alternate activation of macrophages, which aid in regulating inflammation, tissue repair and resistance to secondary infection [406-410]. AAMs are distinguishable by the production of high levels of arginase-1 and in turn, significantly lower levels of iNOS [411], as well as elevated expression of IL-4 receptor α-chain (IL-4Rα) and mannose receptor CD206 [410]. Protective immunity against tissue-dwelling murine nematode *H. polygyrus* was disrupted by macrophage depletion or inhibition of arginase-1 [410]. During filarial *Litomosoides sigmodontis* infection, AAMs downregulate inflammatory Th1 immune responses mediated by transforming growth factor-β (TGF-β) [406], which induce the development of regulatory T cells [412]. Herbert *et al*., 2004 demonstrated that AAMs downregulate acute tissue inflammation and are essential for survival following murine *Schistosomiasis* infection [407]. In addition, AAMs may also have more direct effects on parasites, by secreting proteins like chitinase and fizz family proteins (ChaFFs), which may enzymatically target helminth glycans, as well as promote type 2 immune responses [413].

Induction of granulocytes is also a feature of Th2 helminth immunity. IgE-mediated mast cell and basophil activation and degranulation during parasite infection results in the release of soluble immune mediators and proteases, promoting epithelial permeability and smooth muscle contraction [414-417]. Following infection, granulocytes produce IL-4, suggesting they may play a role in early Th2 development or recruitment [418, 419]. Mast cells have been associated with protection against *Trichinella spiralis* [416, 420], however, they are not require for *N. brasiliensis* expulsion [403, 417]. In addition, mast cell ablation had no effect on *T. muris* infection [421].

IL-5-mediated eosinophilia is a key feature during parasite immunity however, their role is not fully understood. Following nematode infection, eosinophils migrate to the parasite and degranulate, releasing eosinophil secondary granule proteins (ESGPs) [422, 423]. Eosinophils may play a role in protective immunity [422, 424, 425], however, their disruption does not alter susceptibility to infection [421, 425]. Inhibition of IL-5, which is required for helminth-induced eosinophilia, did not alter resistance to *T. spiralis* infection [426]. The chemokine eotaxin recruits eosinophils to the site of infection by chemokine receptor 3 [427]. Blocking eosinophil chemotaxis by anti-CCR3 treatment, reduced protection against *Strongyloides stercoralis in vivo* [428]. Conversely, primary immunity in transgenic mice deficient of eosinophils, is unaltered following *N. brasiliensis* infection [429]. Eosinophils and ESGPs may aid in the clearance of cellular debris and tissue repair following parasite tissue invasion [423]. Eosinophils may also regulate Th2 immunity following helminth infection, by the production of cytokine IL-4 and IL-13 [64, 65], however, eosinophil depletion does not significantly impair the induction of Th2 immune responses during nematode infection [430].

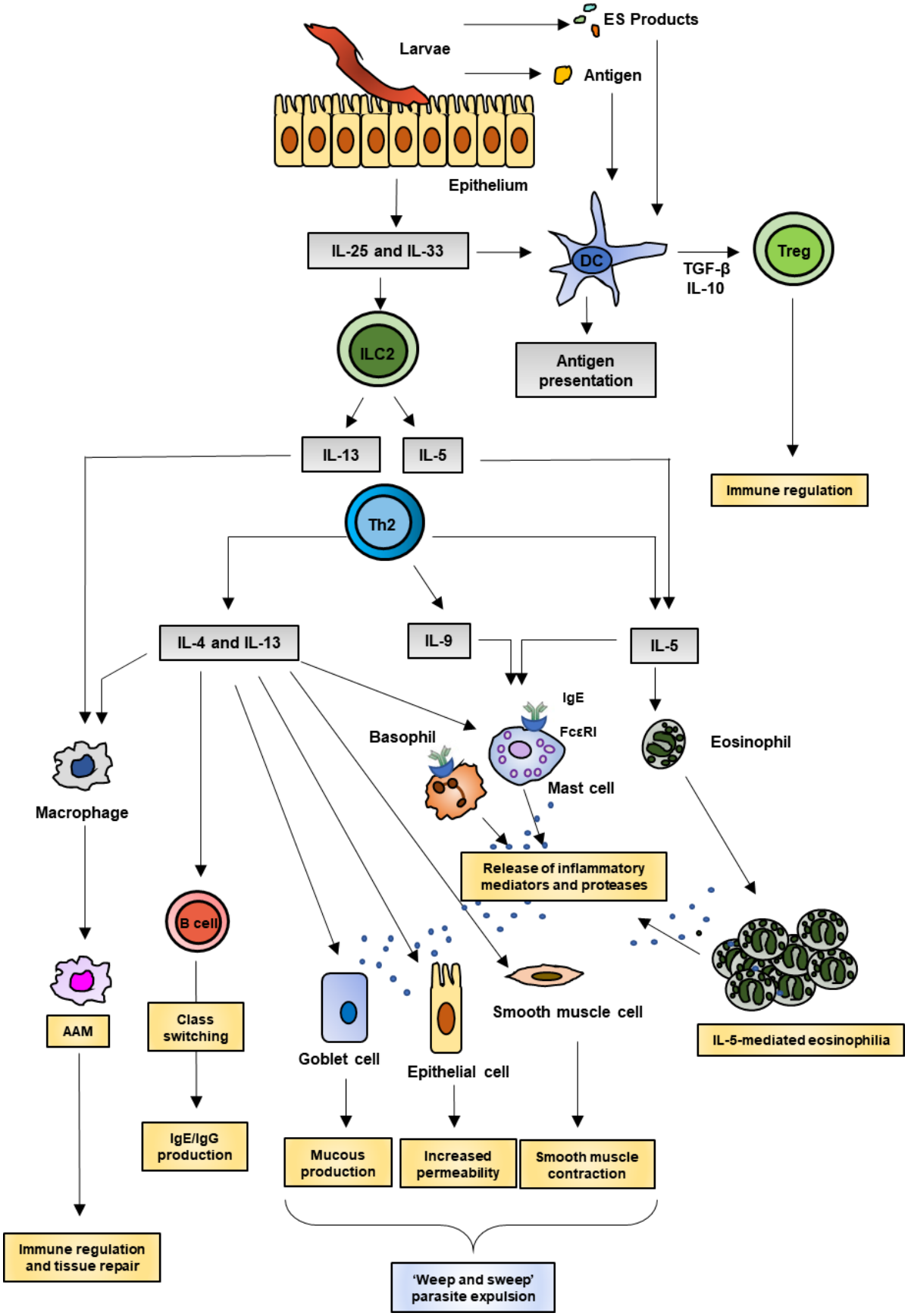


Figure 1.7. Typical type 2 immune response to STH infection: Overview of protective anti-helminth Th2 immunity, in mice. Adapted from [431, 432].

1.4.2.3. Humoral immunity

During Th2 immunity, IL-4/STAT6 signalling pathways mediate class switching and IgM, IgE and IgG1 (mice)/IgG4 (humans) production by B cells, although there is conflicting evidence on the importance of antibody for immune protection [394, 433-437]. Parasite-specific IgE coats tissue dwelling helminths, and infiltrating eosinophils, mast cells and basophils bind to IgE via IgE receptor (FcεR) on their cell surface. This initiates antibody-dependent cell-mediated cytotoxicity (ADCC), where antigen crosslinking of IgE bound to surface FcεRI triggers degranulation of granulocytes [414, 438-441]. However, IgE responses have shown to be beneficial [433, 436, 442-445], detrimental [446, 447] or non-essential [435, 437, 448-450] for host protection during helminth infection. Watanabe *et al*., 1988 demonstrated that eosinophilia, primary worm burden and secondary protection was similar in IgE-deficient mice infected with *N. brasiliensis* and *T. spiralis*, compared to IgE-producing controls [435]. Moreover, Liu *et al*., 2010 reported that B cell-deficient mice had unaffected Th2 responses and parasite expulsion following *N. brasiliensis* infection, as compared to wild type mice. However, reduced expulsion of *H. polygyrus* was observed in B cell-deficient mice [437]. Rajan *et al*., 2005, demonstrated the critical role of circulating IgM for filarial expulsion [434], while Ligas *et al*., 2003 and Kerepesi *et al*., 2004 demonstrated that helminth-specific IgG and IgM antibodies mediated ADCC-dependent and -independent protection against *S. stercoralis* larvae in mice [451, 452]. Additionally, Matsumoto *et al*., 2013 reported that IgG and IgE responses mediated rapid *S. venezuelensis*, but not *N. brasiliensis* expulsion, dependent on the presence of mast cells [436]. As the induction of humoral immunity is a hallmark of Th2 immune responses during parasite infection, research on the protective role of antibodies is important and may contribute to the development of helminth vaccines [450, 453].

1.4.2.4. Regulatory T cells

Helminth infection is associated with the induction of T_{regs} in mice [454-458] and humans [459-462]. Regulation of inflammation by T_{reg} cells is important to prevent exacerbated responses and bystander tissue damage [455, 456, 459]. T_{regs} suppress pro-inflammatory responses via IL-10 and TGF-β production [124, 126, 177, 412, 454, 458, 463] and helminths promote T_{reg} responses by the release of excretory/secretory (ES) products [177, 412, 464-467]. For example, Navarro *et al*., 2016 and Ferreira *et al*., 2017 demonstrated the induction of T_{regs} by hookworm recombinant anti-inflammatory protein-1 (AIP-1) and AIP-2 [465, 466]. Additionally, Johnston *et al*., 2017 identified a TGF-β mimic excreted/secreted by *H. polygyrus*, which potently induced T_{regs} [467]. Interestingly, there is a growing body of work suggesting intestinal helminths may induce immune regulation via interactions with gut microbiota [468-470]. Importantly, helminth-induced immunomodulation has bystander effects on unrelated conditions (discussed later).

1.4.3. Murine hookworm *N. brasiliensis*

N. brasiliensis (Nb) is a natural rodent parasite, closely related to human hookworms *N. americanus* and *A. duodenale*. *N. brasiliensis* is widely used to study Th2 immunity and host-parasite interactions *in vivo*. The parasite establishes acute infection in experimental mouse models [359]. The well-defined kinetics of murine Nb infection makes it a suitable model to study helminth-induced bystander influence on unrelated viral infections.

1.4.3.1. *N. brasiliensis* lifecycle

The lifecycle of Nb is shown in figure 1.8. Briefly, parasite eggs are passed through faeces of infected animals. After 24 hours, the eggs hatch and moult twice into third-stage filariform infective larvae (L3) after 3-5 days. These external non-parasitic stages require a favourable aerobic, humid environment, with temperatures around 26°C. Infective L3 larvae, typically 620-750 µm in length, penetrate the skin of the host, shortly after contact, and migrate to the lungs via the circulatory system within 24 hours following infection. The parasite moult into L4 lung-stage larvae at day 1-2 post infection, which are coughed up and swallowed into the intestine. The parasite undergoes a final moult, in the small intestine, into adult worms (L5) by day 5-6 post infection. Isolated adult worms are bright red in colour due to internalised haemoglobin. Adult males measure between 3 to 4.5 mm in length and have an identifiable umbrella-like external structure. Females are larger than males, measuring between 4 to 6 mm long with a hooked caudal end (Figure 1.8). By day 6 post infection, the adult worms have mated, and the females produce large quantities of ellipsoidal, thin shelled eggs, excreted in the faeces of infected animals. The parasite is naturally cleared from immunocompetent hosts within 2 weeks, when using a common infection dose of 500-700 L3 [359, 471].

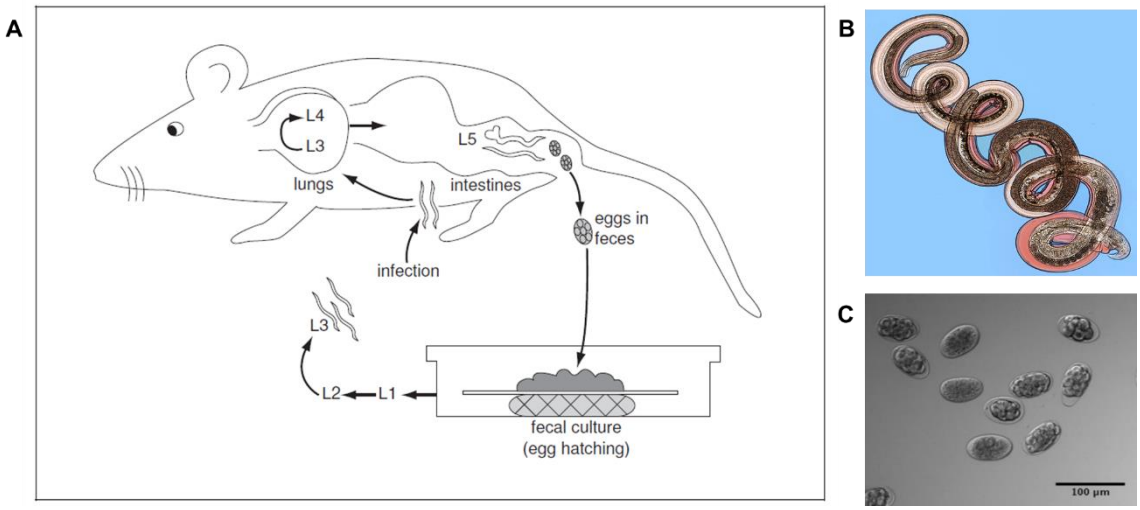


Figure 1.8. (A) Lifecycle of *N. brasiliensis*: Complete nematode lifecycle performed in our laboratory [359]. **(B) Adult female *N. brasiliensis*:** Characteristic hook end and single row of developing eggs [472]. **(C) Ellipsoidal *N. brasiliensis* eggs [473].**

1.4.3.2. *N. brasiliensis* immunity

Nb infection induces a potent Th2 immune response as discussed previously. Nematode transition through the lungs results in structural and immunological changes (e.g. airway sensitisation, granulocyte recruitment, alternatively activated macrophages and tissue reconstruction), present long after the parasite is cleared [474-476]. Initial colonisation of L3 larvae in the lungs is accompanied by lung haemorrhage, and infiltration of neutrophils and ILC2s. Neutrophil influx may contribute to lung damage [121], however, Pesce *et al*., 2008 reported that neutrophils phagocytose parasite-associated bacteria in the lung and ensures optimal type 2 responses [477]. Additionally, Chen *et al*., 2014 demonstrated an alternative induction of neutrophils, which induce long-lived AAMs that mediate rapid clearance of the nematode during secondary infection [476]. Recruited ILC2s respond to lung epithelial 'alarmins' such as IL-25 and IL-33, and initiate type 2 immunity via their production of IL-5 and IL-13 [368, 373, 375, 478, 479]. Moreover, ILC2s also mediate Th2 differentiation [113, 480] and co-operate with T cells to induce and maintain AAMs in the lung for long term protection [481]. Interestingly, Huang *et al*., 2015 described two populations of lung ILC2s: IL-25-responsive inflammatory ILC2s (iILC2s) which are induced early on; and IL-33-responsive natural ILC2s (nILC2s) which are longer-lived and present in the lung at later timepoints. This study hypothesised that recruited iILC2s are transient progenitors of nILC2s during Nb infection [482].

Recruited granulocytes, such as eosinophils and basophils, contribute to immune defence against tissue-dwelling Nb larvae via the release of cytotoxic molecules and type 2 cytokines [418, 429, 483-486]. Importantly, following larvae transition and migration to the gut, AAMs mediate tissue repair in the lungs [475, 487-490]. There is also the Th2 polarisation of resident and recruited $CD4^+$ T cells in the lung and draining mediastinal lymph nodes [481, 491]. However IL-4-responsive T cells are not essential for primary immune control, demonstrated by Mearns *et al*., 2008 who reported that IL-4-induced Th2 responses in the lung enhanced Nb-associated pathology, with reduced lung inflammation in $CD4^+$ T cell-specific IL-4R$\alpha^{-/-}$ mice, compared to immunocompetent controls, and no effect on intestinal worm burden [492]. Interestingly, non-haematopoietic cells can contribute to Th2 immunity in the lung. Horsnell *et al*., 2011 demonstrated smooth muscle cell contribution to Th2 immunity in the lung following Nb infection, dependent on IL-4Rα signalling. In this study, Nb-infected smooth muscle-specific IL-4R$\alpha^{-/-}$ mice had reduced airway mucus and type 2 cytokine responses, compared to immunocompetent controls [401].

Importantly, $CD4^+$ T cell, AAM and eosinophil levels in the lung peak after larvae transition and migration to the intestine, with long-lived resident populations contributing to secondary defences [429, 481, 491, 493].

Primary Nb infection is cleared in the intestine by an IL-13-, IL-4Rα- and STAT-6-dependent 'weep and sweep' response, which mediates rapid expulsion of the parasite from the intestine [403, 494-499]. Like in the lung, intestinal epithelial and tuft cells release 'alarmins' such as IL-25, IL-33 and TSLP in response to parasite disruption and protective type 2 immunity is launched by alarmin-responsive ILC2s [366, 367, 479, 500-504]. ILC2 production of type 2 cytokines such as IL-5 and IL-13 which induces protective Th2 effector responses [368, 478, 480, 504, 505]. Interestingly, ILC2-derived IL-13 promotes goblet and tuft cell expansion, creating a positive feedback loop and enhanced IL-25 response [501, 503].

Th2 activation of $CD4^+$ T cells occurs in the colonized intestine and associated mesenteric lymph nodes (MLNs) [506]. Once activated in the MLNs, effector Th2 cells home to the colonized intestine, however, studies demonstrate that T cell-derived type 2 cytokines are not essential for primary immune control of Nb [36, 507]. Schmidt *et al*., 2012 reported that while IL-4 responsive T cells were not required for parasite expulsion, T cell-specific IL-4Rα$^{-/-}$ mice displayed impaired intestinal hypercontractility during Nb infection, compared to immunocompetent controls [499]. Parasite expulsion is mediated by multiple physiological and immunological mechanisms previously discussed, i.e. type 2 cytokine-mediated goblet cell hyperplasia, mucous secretion and smooth muscle contraction [368, 396, 403, 497, 508], which together expel worms from the intestine. Host defences against Nb requires the meeting of the nervous and immune systems. Horsnell *et al*., 2007 demonstrated delayed parasite expulsion in smooth muscle-specific IL-4Rα$^{-/-}$ mice, associated with impaired goblet cell and type 2 cytokine responses, as well as reduced expression of M3 muscarinic receptor (M3R), a receptor for neurotransmitter acetylcholine [396]. Darby *et al*., 2015 studied the role of M3 muscarinic receptor (M3R), a receptor for neurotransmitter acetylcholine, on primary Nb immunity. Here, they demonstrated that M3R expression was required for primary control, with impaired smooth muscle contraction and parasite clearance in the intestines as well as reduced T cell responses in associated MLNs [509]. These studies demonstrate co-operation between the immune and nervous systems to clear Nb infection.

Following parasite clearance, repair and remodelling of disrupted tissue is mediated by type 2 cytokines, ILC2s and AAMs [489, 510, 511] however, long-term tissue impairment and an immunological footprint has been reported [474-476, 512]. Secondary Nb infection is controlled in the lung, driven by rapid ILC2, AAM and $CD4^+$ T cell responses which reduce larvae viability and migration [410, 481, 487, 491, 493]. Harvie *et al*., 2010 reported that priming of lung $CD4^+$ T cells during primary Nb infection, conferred protection during reinfection, dependent on IL-4/STAT6 signalling [491]. Moreover, Thawer *et al*., 2014 demonstrated that IL-4Rα-dependent Th2 polarisation of resident lung $CD4^+$ T cells were sufficient for secondary immune defence [493].

Bouchery *et al*., 2015 showed T cell-ILC2 cooperation and IL-13 maintaining AAMs in the lung, which mediate Nb killing during reinfection [481]. Additionally, the role of neutrophils [476], eosinophils [429, 513], basophils [514], as well as antibody-dependent [515] and -independent [405] B cells responses have been described during reinfection. Horsnell *et al*., 2013 demonstrated that MHCII antigen presentation by B cells was required for secondary Nb control, mediated by IL-4Rα signalling [405]. Co-operation between host nervous and immune systems is also required for control of Nb re-infection. Darby *et al*., 2015 reported impaired pulmonary memory responses during secondary Nb infection in $M3R^{-/-}$ mice, with reduced type 2 cytokine responses, goblet cell mucus production and AAMs [509]. This demonstrates the importance of cholinergic signalling on adaptive Th2 responses and secondary Nb control in the lung.

In this thesis, we use primary Nb infection to determine nematode-induced bystander immunity in colonized and uncolonized biological compartments, and the implications for subsequent herpesvirus infections. We hypothesize that helminth-induced local and systemic changes to host immunity would alter viral susceptibility.

1.5. Helminth-induced immune modulation

Co-evolution has resulted in sophisticated parasitic worms that are able to modulate and evade their host's immunity. Helminths achieve this by the release of a repertoire of excretory/secretory products, which can inhibit anti-parasite immune mechanisms or induce favourable immune regulation [516]. For example, Osbourne *et al*., 2017 described the ability of *H. polygyrus* Alarmin Release Inhibitor (HpARI) secreted protein to bind to and supress IL-33 activity, reducing ILC2 and eosinophil responses, and promoting parasite survival [517]. Also, Bancroft *et al*., 2019 recently identified an immunomodulatory molecule p43, a major secreted protein by murine whipworm *T. muris*, which binds to and inhibits IL-13 activity [518]. Helminth-driven immune hyporesponsiveness results in asymptomatic or chronic infections, beneficial for parasite survival, demonstrated in asymptomatic lymphatic filariasis patients who displayed skewed Th2 and T_{reg} cytokine profiles i.e. favourable IL-4 and TGF-β, over IFN-γ and IL-17 production by activated T cells [519-522]. Alternatively, symptomatic patients had dominant pro-inflammatory responses, resulting in immune-mediated damage to colonized tissues [523]. As discussed previously, T_{reg} cells are induced during helminth infections [460, 461, 524-526] . Helminths can induce differentiation of T_{regs} from naïve $CD4^+$ T cells and drive their expansion [412, 457]. Interestingly, helminth-induced immune modulation can have bystander effects on unrelated conditions such as allergies, autoimmune and inflammatory disorders (Supp table 1.1 and 1.2).

With allergy and autoimmunity, amelioration is typically associated with suppression of proinflammatory responses by regulatory cells and immune modulatory cytokines like TGF-β and IL-10 [463, 527-536]. McSorley *et al*., 2012 reported the suppression of type 2 allergic lung inflammation from treatment with *H. polygyrus* excretory secretory products (HES) [177], associated with TGF-β-like activity. Furthermore, Johnston *et al*., 2017 demonstrated the suppression of skin allograft rejection by treatment with a TGF-β mimic isolated from HES [467]. In support, Li *et al*., 2018 demonstrated suppression of allograft rejection with *H. polygyrus*-induced Th2 and T_{reg} bystander immunity [537, 538]. Recombinant hookworm anti-inflammatory proteins have been shown to reduce inflammation during experimental colitis [466] and asthma [465], associated with the induction of T_{regs}. With abrogation of IL-33 responses, helminth-derived HpARI protein also supressed allergen-induced inflammation *in vivo* [517]. Conversely, Jõgi *et al*., 2018 reported increased risk of allergy manifestations in Norwegian children with anti-*Toxocara canis* IgG4 seropositivity [183]. Helminth-induced bystander immunity has also been implicated with altered vaccine responses [539-542]. Interestingly, Zaiss *et al*., 2015 demonstrated that infection with GI *H. polygyrus*, results in changes to host intestinal microbiota and increased microbial-derived short chain fatty acids, which contributes to helminth-induced T_{reg} suppression of allergic lung inflammation [469]. Furthermore, Doonan *et al*., 2019 demonstrated that the subcutaneous administration of an immunomodulatory

helminth ES product, glycoprotein ES-62, protected against inflammatory joint disease, associated with 'normalisation' of the gut microbiome [543]. This demonstrates systemic modulation of host immunity and susceptibility in uncolonized compartments. In addition to helminth-induced bystander modulation of allergies and autoimmunity, Darby *et al*., 2019 recently demonstrated pre-conception maternal helminth exposure influences offspring immunity. Prior Nb infection imprinted Th2 immunity in mothers, which was transferred via breast milk and conferred protection against the parasite in the offspring. Protection was associated with maternally-derived Th2 primed $CD4^+$ T cells [544].

Due to the high prevalence of helminths in developing regions plagued with other microbial infections, coinfection between helminths and bacteria, viruses or other parasites is common [184-188, 545-549]. Helminth-induced bystander immunity may alter immunity and susceptibility to unrelated microbial infections. For example, Bobat *et al*., 2014 demonstrated impaired host immunity and vaccine efficacy against *Salmonella typhimurium* infection, by concurrent hookworm infection [181]. Tuberculosis patients coinfected with intestinal helminths, presented with more severe disease, associated with reduced IFN-γ and Th1 responses [550]. du Plessis *et al*., 2013 demonstrated reduced pulmonary *M. bovis* Bacille Calmette–Guérin (BCG) burden with concurrent Nb infection, mediated by AAMs [178]. Additionally, O'Shea *et al*., 2018 reported a negative association between hookworm infection and latent Mtb infection in Nepalese immigrants and improved *in vitro* control of mycobacterial growth by whole blood, which reversed following anti-helminth treatment. Enhanced mycobacterial *in vitro* control was associated with elevated eosinophil numbers and gene expression [184]. Coincidence of filarial infection was associated with moderate protective immunity during latent Mtb infection [551] and in a recent study, *S. stercoralis* infection in latent tuberculosis patients, was associated with down-regulated chemokine responses [549]. And recently, Lin *et al*., 2018 studied the IL-4-dependent induction of virtual memory $CD8^+$ T cells by GI *H. polygyrus* infection, conferring protection against subsequent bacterial infection of the peritoneal cavity [161].

In addition to altering host immunity to bacterial infections, recent studies have demonstrated that prior nematode infection can confer resistance to subsequent infection by a different nematode species. Yasuda *et al*., 2018 demonstrated that *S. venezuelensis* exposure mice had significantly increased pulmonary ILC2, IL-5, IL-13 and eosinophil responses, associated with enhanced *N. brasiliensis* resistance [552]. Interestingly, Filbey *et al*., 2019 reported that infection with *H. polygyrus*, which colonises the GI system, induced enhanced pulmonary immunity and resistance to subsequent *N. brasiliensis* infection, associated with IL-33-activation of $CD4^+$ T cells, enhanced IL-5 production and eosinophil recruitment [553]. This further demonstrates the systemic immunomodulation during helminth infection, which can alter immunity and pathogen susceptibility in an uncolonized biological site.

With bystander immunity influencing susceptibility to unrelated pathogens in co-endemic areas, it is important to understand immune modulation during helminth-microbial coinfections. In this thesis, we will be investigating helminth-viral coinfections.

1.6. Helminth and viral coinfections

The influence of helminths on concurrent viral infections has been investigated in both animal models and human studies (Table 1.2). With the sophisticated and multifaceted modulation of host immunity by parasitic worms, an interesting concept arises; helminth-induced bystander immunity in co-colonized tissue vs systemic changes to host immunity which may alter viral outcome in biological compartments not colonized by the parasite.

Osbourne *et al*., 2014 demonstrated that infection with GI nematodes *T. spiralis* and *H. polygyrus* impairs immunity to murine norovirus (MNV) in the co-colonized intestine [554]. Rolot *et al*., 2018 demonstrated bystander IL-4-mediated expansion of virtual memory $CD8^+$ T cells during helminth infection, which enhanced control of subsequent murine γ-HV respiratory infection. Direct lung colonized by *S. mansoni* eggs or Nb induced local $CD8^+$ T_{VMs}, reducing subsequent γ-HV replication with elevated numbers and functions of virus-specific $CD8^+$ T cells [162]. GI nematode infection also alters susceptibility to respiratory viruses, although the lung is not directly colonized by the parasites. McFarlane *et al.,* 2017 showed that infection with *H. polygyrus* alters gut microbiota, which resulted in a systemic increase in proinflammatory type I IFN, and protection against subsequent respiratory syncytial virus (RSV) in the lung [470]. Additionally, *in vivo* infection with *T. spiralis* reduced pathological inflammation of the airways following influenza A virus infection [555]. These studies demonstrate that helminth infection, confined to the gastrointestinal tract (GIT), can systemically alter immunity in an uncolonized mucosal site.

The genital mucosa is an important site of defence against common sexually transmitted microbes. Coinfection with helminths and sexually transmitted viruses is common in co-endemic areas. Urogenital schistosomiasis, caused by colonization of the urogenital tract with *S. haematobium* eggs and granuloma formation around embedded eggs, is associated with: haematuria (i.e. blood in the urine); fibrosis of the bladder, ureter and kidneys; genital nodules and fibrotic lesions, vaginal bleeding and genital tissue damage [556-560]. Genital tissue inflammation and damage induced by *Schistosoma* eggs increases the number of HIV target cells present in the genital mucosa, increasing the risk of HIV acquisition in infected men and women [561-564]. Recently, Yegorov *et al*., 2019 showed that *Schistosomiasis* treatment reduced *ex vivo* HIV entry into CD4+ T cells isolated from post-treatment blood and cervical fluid of HIV-uninfected Ugandan women [565].

Additionally, Dzhivhuho *et al*., 2018 demonstrated that *S. mansoni* infection impairs HIV vaccine-induced Th1 cellular and virus-specific antibody responses, associated with biased Th2 responses [566].

Helminth may also systemically alter viral susceptibility is uncolonized biological sites. Interestingly, Gravitt *et al*., 2016 reported increased Human Papillomavirus (HPV) prevalence with STH infections and a Th2 cytokine profile in the cervicovaginal fluid of women in a helminth-endemic region of Peru, even though there is no direct colonization of the genital tract by STH species. Other parasitic infections of women in non-STH-endemic regions, was not associated with HPV prevalence, suggesting STH exposure increased HPV risk [567]. Conversely, Jacobs *et al*., 2018 demonstrated decreased *in vitro* uptake of HPV 16 pseudovirus with *N. brasiliensis* antigen treatment of cervical cancer HeLa cells [182]. Despite conflicting results, these studies demonstrate the effect of STH infection or exposure, on HPV susceptibility, an important investigation considering the high prevalence of HPV infections in helminth-endemic regions.

Together, the evidence presented demonstrates helminth-induced bystander immunity in the colonized mucosa, and systemic immune modulation in uncolonized sites, both of which may alter susceptibility to concurrent or subsequent viral infections in the host.

Table 1.2. Influence of helminths on viral coinfections

Mouse models			
Strain	Helminth	Virus	Outcome
NIH	*T. spiralis*	Influenza A virus	Helminth infection reduced virus-induced cell infiltration and pathology of the airways (555)
C57BL/6	*T. spiralis/ H. polygyrus*	MNV	Impaired anti-viral immunity in coinfected mice (554)
C57BL/6	*H. polygyrus/ S. mansoni* egg treatment	γ-HV	Helminth exposure exacerbates γ-HV reactivation from latency, associated with IL-4/STAT6 inhibition of IFN-γ (568)
C57BL/6/ BALB/c	*S. mansoni*	Influenza/ Pneumonia virus of mice (PVM)	Chronic schistosomiasis enhanced anti-viral immunity and protected against secondary viral challenge (569)
C57BL/6	*L. sigmodontis*	Retrovirus	Reduced virus-specific antibody responses in coinfected mice (570)
C57BL/6/ BALB/c	*H. polygyrus*	RSV	Intestinal helminth infection alters lung immunity to RSV, provides protection by microbiota triggered type I IFN (470)
BALB/c	*S. mansoni*	HIV vaccine	*S. mansoni* infection impaired HIV vaccine-mediated Th1 immunity and antibody responses, with biased Th2 immunity induced by the parasite (566)
BALB/c	*S. mansoni* *N. brasiliensis*	γ-HV	Prior helminth infection induced $CD8^+$ T_{VMs}, which protected against subsequent γ-HV infection. Bystander $CD8^+$ T_{VM} expansion was mediated by IL-4 (162)
Human studies			
Location	Helminth	Virus	Outcome
Egypt	*S. mansoni*	Hepatitis C Virus (HCV)	Coinfected patients had increased levels of viral proteins at different stages of liver fibrosis (571)
Uganda	*N. americanus*	HIV	Hookworm infection was associated with reduced $CD4^+$ T cell counts in HIV infected adults (572)
Zimbabwe Tanzania	*S. haematobium*	HIV	Urogenital schistosomiasis in women is associated with increased HIV risk (561, 564)
Uganda	*S. mansoni*	HIV	Schistosomiasis was associated with systemic modulation of host immunity and *Chlamydia trachomatis* infection, which may contribute to increased HIV risk (573) Schistosomiasis treatment reduced HIV entry into $CD4^+$ T cells isolated from blood and cervical fluid, associated with increased genital type I IFNs (565)
Peruvian Amazon	STHs	HPV	STH+ women had higher HPV prevalence and Th2 cytokine signature in cervical fluid (567)

1.7. Study rationale

The influence of helminth-induced bystander immunity on viral coinfections is under studied, with the most insight given from *in vivo* models of infections. It is reported that chronic nematode infestation is associated with substantial host modulation *in vivo* [470, 554], however, it is not known whether acute nematode infection may have bystander effects on unrelated infections, during its relatively brief colonization of the host. Here, we investigated the influence of nematode exposure on subsequent herpesvirus infection, using two *in vivo* coinfection models. These coinfection models allowed us to determine bystander effects in co-colonized and uncolonized biological compartments of the host. Considering the geographical overlap between STH and herpesvirus prevalence [200, 293, 357], it is important to understand the bystander influence of helminth infection on susceptibility and immunity to herpesviruses.

1.8. Objectives

In this thesis, we aimed to investigate the local bystander influences of prior *N. brasiliensis* (Nb) infection, on subsequent MuHV-4 respiratory infection, as well as systemic viral reactivation. To follow this, we aimed to determine systemic influences of Nb exposure on female genital immunity and susceptibility to primary vaginal HSV-2 infection.

Objective 1a: Does prior nematode transition through the lung, influence subsequent control of MuHV-4 lytic replication?

Objective 1b: Does nematode exposure alter systemic reactivation of MuHV-4 in the lymph nodes, spleen and/or genital region?

Objective 2: How does nematode infection influence immunity in the female genital tract (FGT)?

Objective 3: How does nematode-induced bystander changes to FGT immunity alter HSV-2 susceptibility?

1.9. Supplementary Information

Supp table 1.1. Helminth-induced immune modulation of allergic disease

Mouse models		
Strain	Helminth species	Outcome
BALB/c, C57BL/6	*H. polygyrus*	• Suppression of allergy response to ovalbumin (BALB/c) and house dust mite allergen Der p 1 (C57BL/6) [454] • Infected BALB/c mice were protected against allergic airway inflammation but not atopic dermatitis [574] • Soluble excretory/secretory products supressed allergen-induced inflammation [177, 575] and parasite secreted product (HpARI) supressed IL-33 and reduced Th2 allergic responses [517] • *H. polygyrus*-induced changes to host microbiota contributes to regulation of lung allergic inflammation [469]
BALB/c, C57BL/6	*N. brasiliensis*	• Reduction of allergy-induced eosinophilia in the airway, mediated by IL-10 [527]
BALB/c	*Schistosoma mansoni*	• Prevention of airway hyperresponsiveness (AHR) and anaphylaxis in mice infected with *S. mansoni* worm only. Infection with egg-laying male and female worms worsened AHR [576] • Parasite antigen treatment down-modulated allergic inflammatory mediators [528]
C57BL/6	*T. spiralis*	• Reduced allergic airway inflammation in allergen-challenged infected mice [577]
C3H/HeJ	*H. polygyrus*	• Protection from dietary allergy [578]
Human studies		
Participants	Helminth species	Outcome
8-18 years old	*A. lumbricoides*	• Increased risk of asthma and sensitization to aeroallergens in infected children [579]
Children	*T. trichiura*	• High intensity infection in early childhood resulted in lower prevalence of reaction to allergen skin-test later in life [580]
1-4 years old	*Ascaris, Trichuris* and hookworm	• Wheezing less prevalent in *Ascaris*-infected children. No association with *Trichuris* and hookworm infection [581]
Children	STHs	• Increased prevalence of allergic sensitization after 1 year of mass drug (albendazole) administration [582]
Children	*A. lumbricoides* and *T. trichiura*	• Decreased frequency of atopy was associated with unhygienic living conditions and exposure to parasites [583]
Parents and children	*A. lumbricoides* and *T. canis*	• Increased risk of allergic manifestations in children tested positive for anti-*T. canis* IgG4. [183]

Supp table 1.2. Helminth infections and autoimmune disorders

Mouse models		
Strain	Helminth species	Outcome
Non-obese diabetic (NOD)	*S. mansoni*	• Ova treatment reduced spontaneous development of type I diabetes in infected mice (584) • Treatment with egg/adult worm extract prevented onset of type I diabetes. Protection associated with T_{reg} modulation of immune responses (529, 530)
IL-10 deficient C57BL/6	*H. polygyrus*	• Transfer of T cells from infected mice reduced colitis, via increased T_{reg} immune control and inhibition of Th1 inflammation (532)
BALB/c	*S. mansoni*	• Reduced TNBS-induced colitis with egg treatment, associated with lower IFN-γ and increased IL-10 expression in colon (531)
C57BL/6	*T. spiralis*	• Infection reduced colitis severity, associated with downregulation of Th1 and development of Th2 immune responses (533) • Treatment with excretory/secretory (ES) products up-regulated T_{reg} cells and reduced inflammatory colitis (463)
BALB/c C57BL/6	*H. polygyrus*	• TGF-β mimic (*Hp*-TGM) identified in HES reduced rejection of allogenic skin transplantation from BALB/c donor mice to C57BL/6 recipients treated with *Hp*-TGM (467)
DBA/1	*Acanthocheilonema viteae* (filarial nematode) ES product	• Subcutaneous treatment with immunomodulatory glycoprotein ES-62, protected against collagen-induced inflammatory arthritis, associated with 'normalisation' of the gut microbiota and maintenance of intestinal barrier integrity (543)
Human studies		
Disease	Helminth species	Outcome
Crohn's disease	*Trichuris suis*	• Live ova treatment relived symptoms of disease (585) • Viable 250-7500 ova treatment fortnightly over 12 weeks did not alleviate symptoms significantly compared to placebo group (586)
Active colitis	*T. suis*	• Ova treatment improved ulcerative colitis (587)
Multiple sclerosis	Intestinal helminths	• Helminth-infected MS patients had significantly reduced number of relapses associated with T_{reg} cells, with anti-helminth treatment increasing MS activities and IFN-γ, and reduced T_{reg} cells, TGF-β and IL-10 (534-536)

Chapter 2: Nematode-induced lung immunity enhances protection of subsequent γ-HVs infection

Some data presented in this chapter contributed to a larger publication: Rolot M, Dougall AM, **Chetty A**, Javaux J, Chen T, Xiao X, et al. Helminth-induced IL-4 expands bystander memory CD8(+) T cells for early control of viral infection. Nature communications. 2018;9(1):4516.

2.1. Introduction

Humans encounter multiple micro- and macro-pathogens in the natural environment, resulting in a high probability of coinfections. It has been shown that exposure to a pathogen can alter susceptibility to subsequent unrelated pathogen [555, 588-590]. In this chapter we determine the influence of prior nematode infection on subsequent gammaherpesviruses infection *in vivo*.

Gammaherpesviruses (γ-HVs), such as prevalent EBV and KSHV, are important pathogens in both humans and animals. Murid herpesvirus-4 (MuHV-4) is a natural rodent pathogen that has been used as a model to study γ-HVs pathogenesis as it is closely related to human γ-HVs. Like in most human cases of γ-HVs, MuHV-4 establishes asymptomatic persistence in mice [591]. Upon respiratory MuHV-4 infection, the virus infects primarily lung epithelial cells as well as alveolar macrophages [592]. The virus colonises its host via latently infected B cells which migrate to associated lymph nodes and later systemically. Acute MuHV-4 replication in the lung resolves after 7-10 days post infection [305, 318] and infection is mainly controlled by an effective antigen-specific $CD8^+$ T cell response [593]. Therefore, prior lung infection by an unrelated pathogen, may affect the host's antiviral immune response and control of primary lytic infection.

Murine hookworm, *N. brasiliensis* (Nb), colonises the lung at day 2 post infection. This results in local tissue damage and inflammation [488]. However, the lung is also the site of protection during a secondary Nb infection, due to enhanced local immune control [405, 491]. Nematodes drive a strong type 2 mucosal immune response in the infected host [359, 492]. The bystander influence of nematode-induced immunity on a subsequent γ-HVs infection is not known. Generally, type 1 and type 2 are thought to be opposing immune responses. Therefore, an assumption is made that nematode infection may hinder immunity against an unrelated virus, where a typical type 1 immune response is required for protection [594]. Here, we demonstrate that prior Nb infection provides early and enhanced control of a subsequent respiratory MuHV-4 infection. This enhanced protection is associated with an increase in virus-specific $CD8^+$ effector T cells in the lung. We also give evidence which suggests prior Nb exposure results in earlier heightened genital reactivation of MuHV-4, which attests to the complexity in bystander effects during coinfections.

2.2. Methods

2.2.1. Cells

Baby Hamster Kidney (BHK)-21 [C13] fibroblasts were purchased from ATCC (ATCC® CCL-10™, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, 100mg/ml streptomycin and 2mM glutamine, at 37°C, 5% of CO_2.

2.2.2. Virus

Murid HVs-4 (MuHV-4), expressing the enzyme luciferase under the control of the M3 promoter (MuHV-4-Luc) [305], was propagated on BHK-21 cells. At 4 days post-infection, cells and supernatant were harvested and debris was removed by low-speed centrifugation at 1000 × g for 10 min at 4°C. Virus present in the supernatant was isolated by ultracentrifugation (100,000 × g, for 2 hours at 4°C) and then purified through a 30% w/v sucrose cushion (100,000 × g, 2 hours at 4°C). Following a wash step with phosphate buffered saline (PBS), the virus was stored in PBS at −80°C.

Viral titres were determined by plaque assay [595]. Briefly, BHK-21 cell monolayers were incubated with 10-fold dilution of viral stock at 37°C, 5% CO_2 for 3 hours to allow for absorption. The inoculum was then replaced by semi-solid medium containing 0.6% carboxymethylcellulose. Cells were further incubated for 4 days then fixed in 4% paraformaldehyde (PFA) and stained to determine viral plaque forming units (PFU).

2.2.3. Animals

Female wild-type BALB/c, aged 6-8 weeks old, were purchased from Envigo (Venray, Netherlands). Female, 6-8 week old BALB/b10 mice (C.B10-H2^b/LilMcdJ), that are BALB/c congenic for the C57BL/10-derived H-2b region, were maintained at the Scientific Institute of Public Health, Belgium and transferred to the University of Liege, Department of Infectious Diseases for experiments. Mice were randomly sorted into experimental groups, with 4-5 mice co-housed per cage. Food and water was provided *ad libitum*. All the animals were housed in the University of Liège, Department of Infectious Diseases. The study complied with the guidelines of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (CETS n° 123). The protocol was approved by the Committee on the Ethics of Animal Experiments of the University of Liège, Belgium (Permit Number: 1357, 1713 and 1849).

2.2.4. Parasite infection

N. brasiliensis was maintained in male Sprague-Dawley rats and L3 larvae were isolated from day 6-8 faecal cultures as previously described [359]. Mice were infected with 500x L3 larvae subcutaneously, 6 days prior to viral infection.

2.2.5. Viral infection and quantification by *in vivo* imaging

Mice were anesthetized with isoflurane and inoculated intranasally with 10^4 PFU of MuHV-4-Luc, in 30 µl of PBS. For viral quantification, mice were injected with D-luciferin (75 mg/kg, Perkin Elmer), intraperitoneally. Viral replication was measured by light emission, through *in vivo* bioluminescence imaging, using an IVIS Spectrum *In Vivo* Imaging System (Perkin Elmer). Living Image V4.1 (Perkin Elmer) was used to analyse a fixed-sized area of interest, to obtain total flux (photons/seconds).

2.2.6. Tissue processing and cell preparation

Prior to removal, lungs were perfused with 5ml of ice-cold PBS through the right ventricle. Lung tissue was dissociated with the gentleMACS dissociator (Miltenyi Biotec) in C-tubes (Miltenyi Biotec) and incubated in Hank's Balanced Salt Solution (HBSS; Gibco) supplemented with 5% fetal calf serum (FCS), 1mg/ml of collagenase D (Roche) and 0.1mg/ml of DNase I (Roche), under agitation for 30 min at 37°C. The tissue was dissociated once more on the gentleMACS dissociator and the resulting cell suspension was washed with cold PBS/2mM EDTA and filtered through a 100µm cell strainer (Falcon, BD). Erythrocytes were lysed in red cell lysis solution (155 mM NH_4CL, 0.12 mM EDTA, 10 mM $KHCO_3$). Cells were counted by trypan blue exclusion of live cells on a haemocytometer.

2.2.7. Flow cytometry

Incubations were performed in FACS buffer (PBS containing 0.5% BSA and 0.1% NaN_3) at 4°C. Approximately 1 x 10^6 cells per sample were first incubated with anti-mouse CD16/32 antibody (Ab; clone 93, BioLegend). A cocktail of fluorochrome-conjugated anti-mouse antibodies against cell-surface markers (Table 2.1) was then added. Cells were incubated in the dark for 20 min, at 4°C. Samples were analysed on a BD LSR Fortessa X-20 flow cytometer. Data was analysed using FlowJo V10. The gating strategies used to differentiate and identify cell populations in the lung, are shown below (Supp Figure 2.1 and 2.2).

Table 2.1: List of fluorochrome-conjugated antibodies and viability markers

Surface marker	Fluorochrome	Clone	Source
Myeloid panel			
CD11b	APC/Cy7	M1/70	eBioscience™
CD11c	Alexa Fluor® 700	N418	eBioscience™
CD103	Brilliant Violet (BV) 421™	2E7	eBioscience™
CD45	BV510™	30-F11	eBioscience™
F4/80	BV711™	BM8	eBioscience™
Fixable Viability Dye	eFluor ™ 520	-	BD Biosciences
Ly-6C	PE	HK1.4	eBioscience™
Ly-6G	BV605™	1A8	eBioscience™
MHC class II	PE/Cy7	M5/114.15.2	eBioscience™
Siglec-F	Alexa Fluor® 647	E50-2440	eBioscience™
Lymphoid panel			
4′,6-diamidino-2-phenylindole (DAPI)	-	-	ThermoFisher
B220	V500	RA3-6B2	eBioscience™
CD3	APC/Cy7	17A2	eBioscience™
CD4	PerCP/Cy5.5	RM4-5	eBioscience™
CD8	Alexa Fluor 488	53-6.7	BD Bioscience
CD49b	APC	DX5	eBioscience™
MHC class II	PE/Cy7	M5/114.15.2	eBioscience™

2.2.8. MHC-tetramer staining

Lungs were processed from BALB/b10 mice, as previously described. Prior to staining for cell-surface markers (Table 2.2), cells were incubated with BV421-conjugated tetramers H2-D^b-$ORF6^{487-495}$ (AGPHNDMEI) or H2-K^b-$ORF61^{521-531}$ (TSINFVKI) (Tetramer Core Facility) at a concentration of 90 and 45 mM, respectively, for 30min at room temperature (RT). Samples were analysed on a BD LSR Fortessa X-20 flow cytometer (BD Biosciences) and data was further analysed using FlowJo V10. The gating strategy to identify $tetramer^+$ (viral-specific) $CD8^+$ T cells is shown below (Supp Figure 2.3).

Table 2.2: List of fluorochrome-conjugated antibodies and tetramers

Surface marker	Fluorochrome	Clone	Source
CD3	APC	145-2C11	eBioscience
CD8	Alexa Fluor® 488	53-6.7	BD Biosciences
CD19	APC/Cy7	1D3	BD Biosciences
CD44	PE/Cy7	IM7	BioLegend
H2-D^b-ORF6$^{487-495}$	BV421™	-	NIH Tetramer Core Facility (Emory University Vaccine Center) [596]
H2-K^b-ORF61$^{521-531}$	BV421™	-	

2.2.9. *Ex vivo* restimulation and intracellular cytokine staining

Cytokine production by $CD8^+$ T cells were assessed by intracellular cytokine staining and flow cytometry analysis [162]. Briefly, cells were cultured Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FCS, at 37 °C for 4 hours in the presence of 10 µg/ml brefeldin A (eBioscience™) and peptides H-2b-restricted MuHV-4 ORF6$^{487–495}$ and ORF61$^{521-531}$ (1 µM). Following restimulation, cells were stained for viability and surface markers, fixed in 2% PFA and washed in Permeabilization buffer (eBioscience), before incubation with antibodies against IFN-γ (clone XMG1.2, PE, BioLegend) and TNF-α (clone MP6-XT22, BV711, BD Biosciences) in Permeabilization buffer for 20 minutes at 4°C. Samples were analysed on a BD LSR Fortessa X-20 flow cytometer (BD Biosciences) and data was further analysed using FlowJo© V10 (Treestar, Ashland, OR).

2.2.10. Anti-IL-4 treatment

Mice received three intraperitoneal injections of 25 µg anti-IL-4 Ab (BioLegend, Clone: 11B11, LEAF purified) at day 14, 16 and 18 post MuHV-4 infection.

2.2.11. Statistical analysis

Data is represented as group mean and standard error of the mean (mean±sem). Statistical analysis was performed either by analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test or by non-parametric Mann-Whitney test, as indicated. A p-value < 0.05 was considered significant. Statistical analyses were performed using GraphPad Prism V6 (La Jolla, CA).

2.3. Results

2.3.1. Prior nematode infection enhances control of primary MuHV-4 infection

To determine the influence of prior exposure to the nematode *N. brasiliensis* on a subsequent MuHV-4 primary infection, we infected female BALB/c mice with 500x L3 larvae subcutaneously, 6 days before intranasal infection with 10^4 PFU of MuHV-4-luc under general anaesthesia (Figure 2.1a). Control groups consisted of naïve (PBS treated), Nb only and MuHV-4 only mice. Following viral infection, mice were monitored for weight loss and viral progression by *in vivo* bioluminescence imaging. Prior exposure to the nematode protected against transient weight loss at day 8, typical of lytic respiratory infection (Figure 2.1b). We measured the level of infection in the thoracic cavity at day 2, 5, 7 and 9 by live imaging of light emission (total flux; p/s = photons per second). We observed peak lytic viral replication at day 7 post MuHV-4 infection in virus only mice [305]. Nematode exposure resulted in a significant reduction in respiratory infection at day 7 and 9 post viral infection (Figure 2.1c). Despite enhanced primary control, live imaging of the superficial cervical lymph nodes (scLN), showed viral colonisation of the host was not altered by prior exposure to Nb (Figure 2.1d).

2.3.2. Enhanced viral control is accompanied by elevated alveolar macrophages

Next, we assessed the cellular immune response in the lung at the peak of lytic viral replication, day 7 post MuHV-4 [305]. Mice infected with Nb 6 days prior to viral infection (Nb+MuHV-4) had significantly increased lung cellularity, compared to mice infected with virus only and parasite only (Figure 2.2a). Coinfected mice had increased numbers of neutrophils and eosinophils, compared to MuHV-4 only mice, however these numbers were equivalent to mice infected with Nb only (Figure 2.2b-c). Alveolar macrophages (AMs) are a prominent target for MuHV-4 during lytic respiratory infection, with a decrease in AMs infection due to cell death [597, 598]. This was evident in MuHV-4 only infected mice, with a significant reduction in AMs compared to the naïve group. Strikingly, prior Nb infection prevented this decrease (Figure 2.2d). This suggests that nematode pre-exposure may result in protection from productive lytic infection in AMs. It is well established that lung Nb infection induces AAMs (also known as M2 macrophages) via type-2 cytokines IL-4 and -13 [487]. Alternatively, reduced lung viral load and early control, by other immune mechanisms, may have a bystander effect on alveolar macrophages. Further investigation is required to investigate whether M2 macrophages are resistant to MuHV-4 infection.

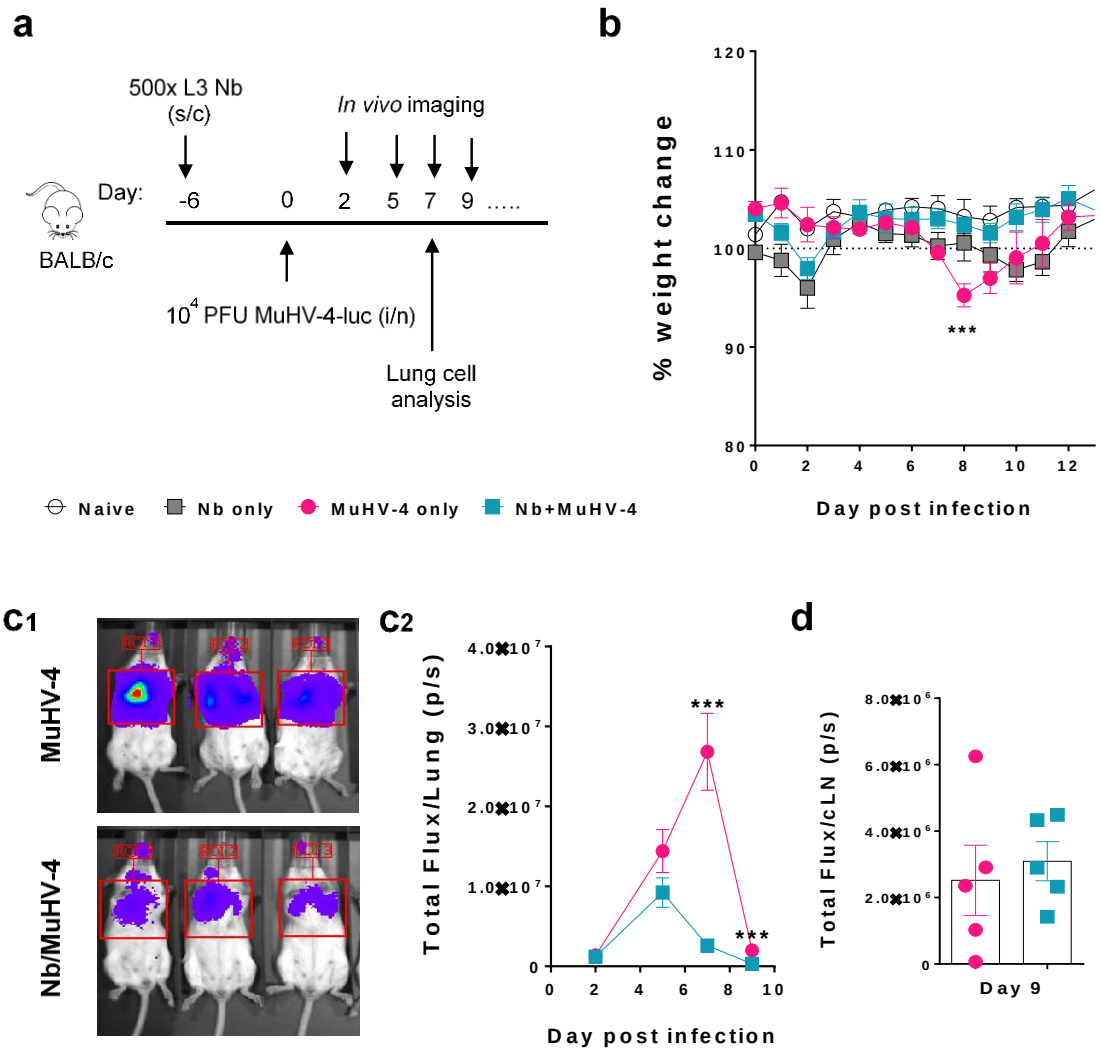


Figure 2.1. Parasite exposure enhances control of MuHV-4 in the lung: (a) Female BALB/c mice were infected with *N. brasiliensis* (Nb) 6 days prior to intranasal infection with MuHV-4-luc. Progression of primary viral infection was established by **(b)** weight change and **(c)** *in vivo* imaging of the thoracic cavity. **(c1)** Representative photographs of bioluminescence signals following D-luciferin injection at day 7 post MuHV-4-luc infection, 3 mice per group. **(c2)** Combined dorsal and ventral thoracic measurements by live imaging total light emission (p/s = photons per second) at day 2, 5, 7 and 9. **(d)** Live imaging of light emission from superficial cervical draining lymph node (scLN) at day 9 post MuHV-4-luc infection. Data is representative of two to three independent experiments with 5 mice per group (mean±sem). Statistical significance was calculated by two-way ANOVA with Bonferroni correction for multiple comparisons. ****p* ≤ 0.001

Along with the reduction in AMs, the recruitment of Ly-6C^{hi} regulatory monocytes into the lungs following MuHV-4 infection has been shown previously [598]. We observe elevated numbers of Ly-6C^{hi} monocytes in the lungs of coinfected mice, compared to all other experimental groups (Figure 2.2e). Further study is needed to determine the importance of these monocytes during respiratory MuHV-4 infection.

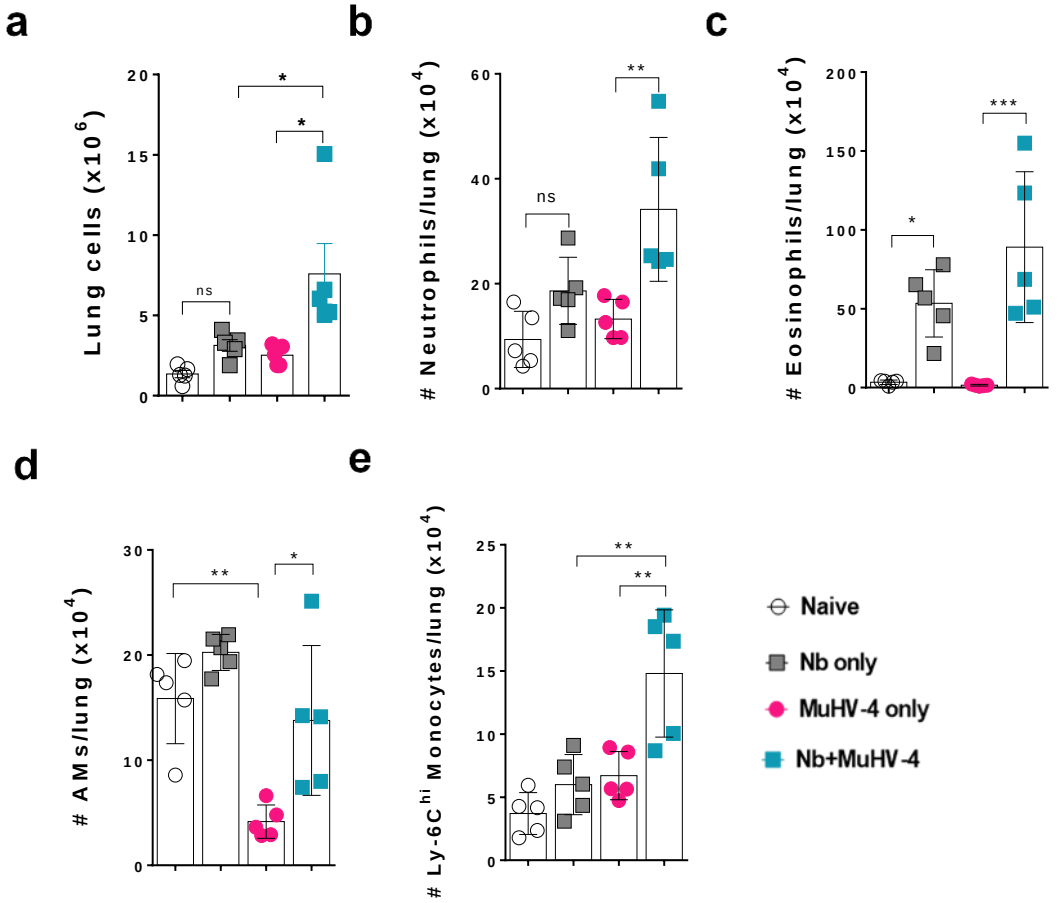


Figure 2.2. Prior parasite exposure enhances lung cellularity: Number of cells in the lungs at day 7 post MuHV-4 infection: **(a)** Total lung cells **(b)** Neutrophils: CD11b^{hi}Ly-6G^{+} **(c)** Eosinophils: CD11b^{+}Siglec-F^{+} **(d)** Alveolar macrophages: CD11b^{+}F480^{+}Siglec-F^{+} and **(e)** Monocytes: CD11b^{hi}Ly-6C^{hi}. Data is representative of one experiment with 5 mice per group (mean±sem). Statistical significance was calculated by one-way (a-e) and two-way ANOVA and Bonferroni's multiple comparisons test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ns: not significant

2.3.3. Nematode infection enhances anti-viral CD8⁺ T cell responses in the lung

MuHV-4 infection is regulated by both CD4⁺ and CD8⁺ T cells, with CD8⁺ T cells controlling acute lung infection and CD4⁺ T cells playing an essential role in immunity during chronic infection [343, 593]. Although equivalent in proportions, the number of CD4⁺ and CD8⁺ T cells in the lung were significantly elevated with coinfection (Figure 2.3a-c). Nb infected groups had equivalent CD4⁺ T cell numbers (Figure 2.3b). This suggests that prior nematode infection results in enhanced CD8⁺ T cells numbers during subsequent respiratory viral infection.

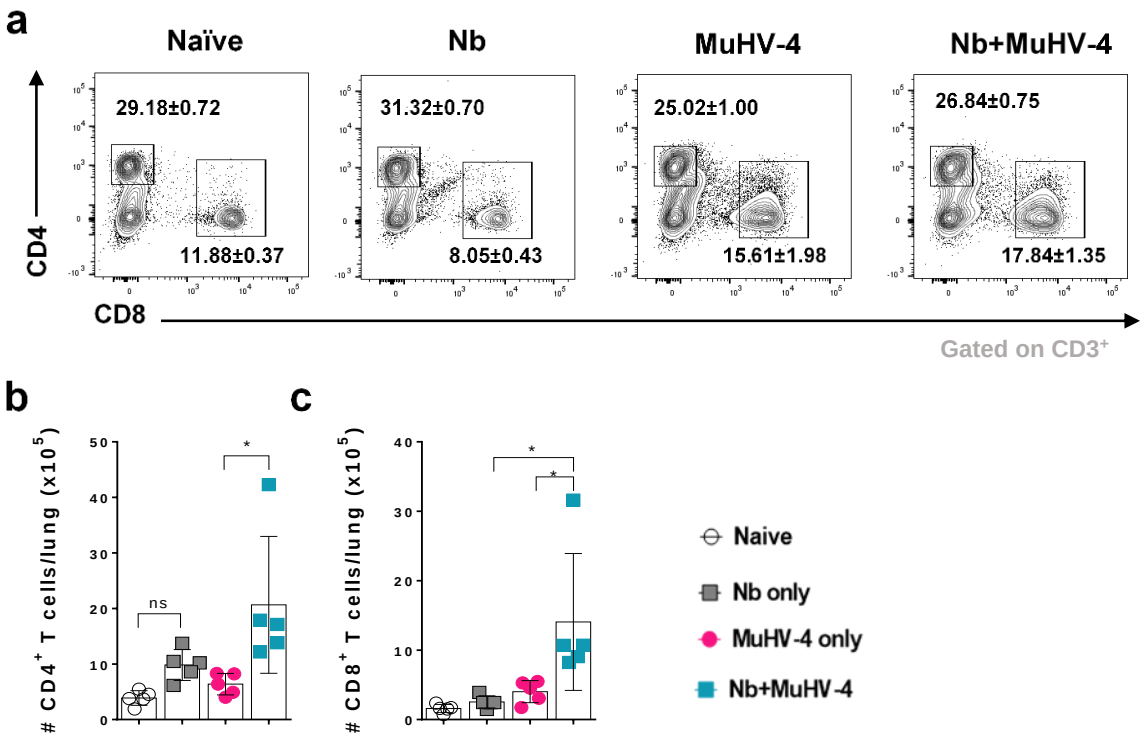


Figure 2.3. Enhanced viral control by is associated with increased anti-viral immunity in the lung: (a) Representative flow contour plots of gated live CD3⁺ cells, showing proportions of CD4⁺ and CD8⁺ T cells at day 7 post MuHV-4, in the lungs of mice infected with *N. brasiliensis* (Nb) 6 days prior to viral infection and naïve, Nb only and MuHV-4-only controls. Number of lung **(b)** CD4⁺ and **(c)** CD8⁺ T cells in mice at day 7 post MuHV-4 infection. Data is representative of one experiment with 5 mice per group (mean±sem). Statistical significance was calculated by one-way ANOVA and Bonferroni's multiple comparisons test. *$p \leq 0.05$, ns: not significant

The severity of primary lytic MuHV-4 infection is directly dependent on the development of effector antigen-specific $CD8^+$ T cells [325, 343]. An increase in lung virus-specific $CD8^+$ T cells could be associated with the enhanced control we observe in coinfected mice. Studies have shown that conditioning of T cells can result in a memory-like phenotype in the absence of cognate antigen. This TCR-independent priming can result in "virtual memory" T cells (T_{VM}) [159, 160]. Pre-conditioned T_{VMs} result in enhanced $CD8^+$ T cell-mediated immunity when cognate-antigen is experienced [159, 161, 162]. Additionally, recent studies have shown that helminth exposure results in T_{VM} conditioning [161, 162]. Here, we hypothesize that prior nematode exposure conditions $CD8^+$ T cells into a T_{VM} phenotype, which results in an increase in virus-specific cytotoxic T cells and enhanced control during subsequent MuHV-4 infection. To investigate this, we assessed the effect of prior Nb exposure on virus-specific $CD8^+$ T cell responses in the lung. We coinfected H-2^b congenic BALB/b10 mice in which the response against the well-established MuHV-4 immunodominant H-$2D^b$-restricted AGPHNDMEI (ORF$6^{487–495}$) and H-$2K^b$restricted SVYGFTGV (ORF$61^{524–531}$) epitopes could be measured [596].

Like previous experiments, we infected BALB/b10 mice with Nb, 6 days before intranasal infection with MuHV-4-Luc (Figure 2.4a). We assessed viral progression by *in vivo* bioluminescence imaging and measured viral-specific $CD8^+$ T cell responses by tetramer staining for ORF$6^{487-495}$ and ORF$61^{524-531}$ epitopes and flow cytometry analysis. Prior nematode infection resulted in a significant reduction in respiratory infection at day 6 and 7 post MuHV-4 infection and an increase in total lung $CD8^+$ T cells at day 7 post infection (Figure 2.4b-c), confirming a similar coinfection phenotype in both BALB/c and BALB/b10 mice. Tetramer staining revealed an increased proportion and number of $CD8^+$ T cells that express the ORF$6^{487-495}$ and ORF$61^{524-531}$ epitopes (Figure 2.4d-e). Additionally, lung $CD8^+$ T cells conditioned by prior Nb infection, show increased IFN-γ and TNF-α co-production when stimulated by ORF$6^{487-495}$ and ORF$61^{524-531}$ peptides (Figure 2.4f). This demonstrates that lung colonization with Nb larvae, results in an increase in viral-specific effective $CD8^+$ T cells during subsequent primary MuHV-4 infection, which suggests $CD8^+$ T cells priming in the lung.

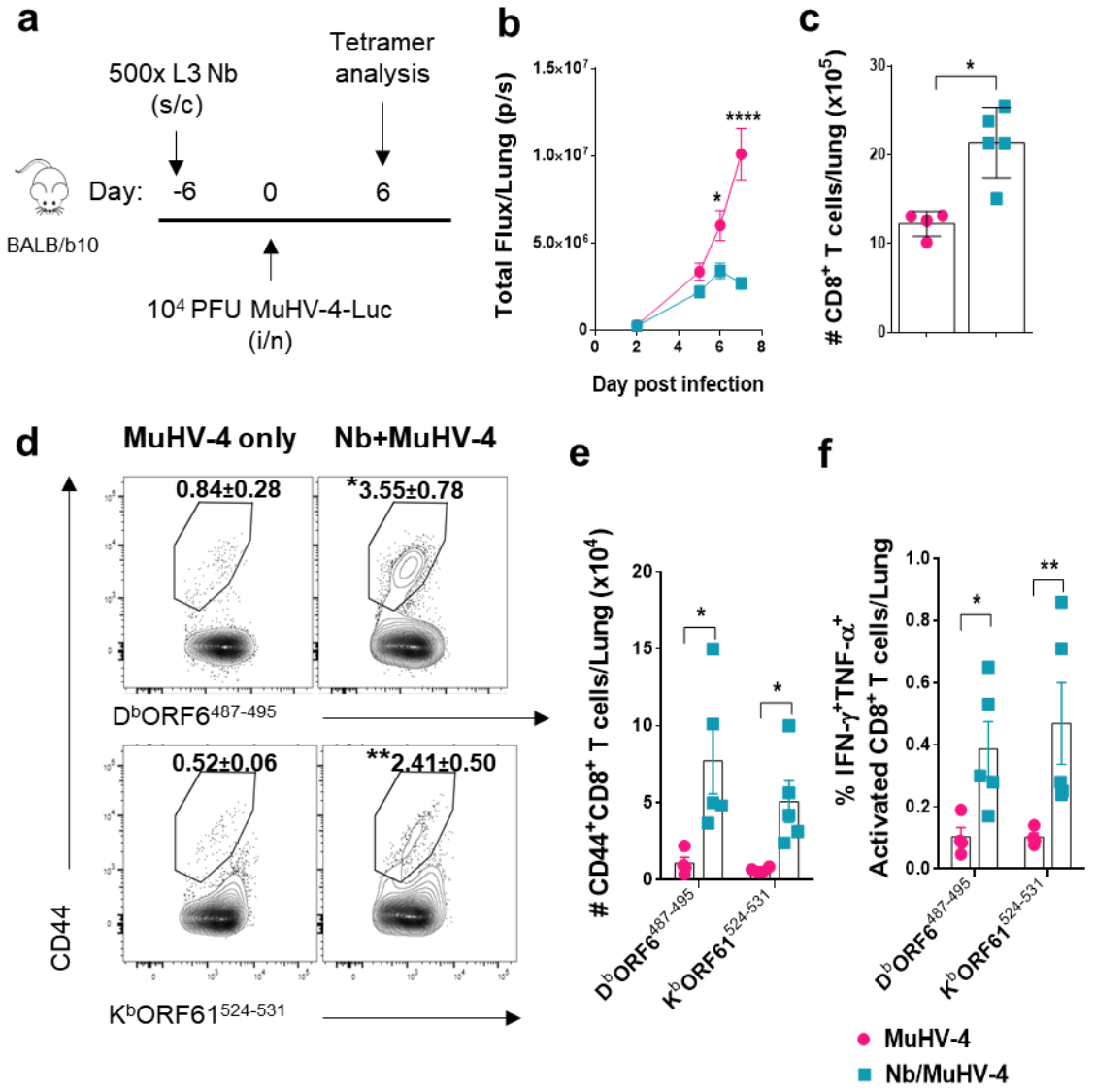


Figure 2.4. Prior *N. brasiliensis* infection enhances virus-specific CD8$^+$ T cell responses: **(a)** Female BALB/b10 mice were infected with *N. brasiliensis* (Nb) 6 days prior to intranasal infection with MuHV-4-Luc. **(b)** Progression of primary viral infection was established *in vivo* imaging of the thoracic cavity. Combined dorsal and ventral thoracic measurements by live imaging total light emission (p/s = photons per second) following D-luciferin injection. **(c)** Number of lung CD8$^+$ T cells in virus only and coinfected mice, at day 7 post viral infection. **(d)** Representative flow plots of D^{b}ORF6$^{487-495}$ and K^{b}ORF61$^{524-531}$ expression on CD44$^+$CD8$^+$ lung T cells (mean±sem shown). **(e)** Number of viral-specific CD44$^+$CD8$^+$ T cells in the lung at day 7 post viral infection. **(f)** Percentage of IFN-γ and TNF-α co-producing lung CD44$^+$CD8$^+$ T cells after ORF6$^{487-495}$ or ORF61$^{524-531}$ peptide restimulation and intracellular staining. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$, **$p \leq 0.01$, ****$p \leq 0.0001$

2.3.4. Nematode exposure alters viral reactivation in non-colonized biological compartments

MuHV-4, like other γ-HVs, persists preferentially in B cells. Once latency is established, infection is lifelong and can spread by proliferation and circulation of latently-infected B cells [322, 323, 599]. We have shown that despite enhanced control of respiratory infection, MuHV-4 established host colonization in coinfected mice (Figure 2.1d). It has been shown previously that helminth infection, via IL-4, results in increased reactivation of latent γ-HVs [568]. Using bioluminescence *in vivo* imaging, we examined the influence of prior nematode exposure and anti-IL-4 treatment on MuHV-4-luc reactivation (Figure 2.5a). At day 11 post MuHV-4 infection, we observe no significant difference in splenic viral reactivation (Figure 2.5b). From day 15 post MuHV-4 infection, we monitored viral reactivation expected in the genital tract [305, 324]. Genital MuHV-4 reactivation occurs spontaneously and sporadically, resulting in variation within experimental groups. Interestingly, despite this variation, we observe mice with prior Nb exposure, display earlier and overall heightened genital reactivation (Figure 2.5c). Preliminary IL-4 neutralization by anti-IL-4 Ab treatment did not reverse this effect (Figure 2.5d).

2.3.5. Increase in latency reservoirs during primary respiratory infection

The degree of viral reactivation may be influenced by latency reservoirs accessible during infection [600, 601]. We hypothesize that prior lung inflammation may result in an increase in viral reservoirs present during a subsequent MuHV-4 respiratory infection. This is evident by the increase in B cell numbers in the lungs of nematode infected mice, during peak lytic replication (Figure 2.5e). It has been shown that MuHV-4 infects dendritic cells (DCs), prior to establishment in B cells. Additionally, a significant proportion of latent virus in B cells have passed through DCs, suggesting the virus exploits DC- B cell interactions for host colonisation [602]. At day 7 post MuHV-4 infection, we find the number of total DCs ($CD11c^+MHCII^+$) in the lung increases with infection but equivalent in virus only and coinfected mice (Figure 2.5f). There are two major subsets of DCs in the lung: $CD11b^+CD103^-$ and $CD11b^-CD103^+$ conventional DCs (cDCs) [603, 604]. With inflammation, circulating monocytes, known for their plasticity, can migrate to the lung and differentiate into monocyte-derived DCs (moDCs) [605]. Further analysis revealed a significantly elevated numbers of $CD11b^+$ and $CD103^+$ cDCs in coinfected mice compared to MuHV-4 only. Ly-$6C^+$ monocyte-derived DCs (moDCs) were similar between these groups. The difference in MuHV-4 infectivity and latency within cDCs and moDCs is not known. Further investigation is needed to understand the mechanism behind the heightened genital reactivation we observed in mice previously infected with Nb.

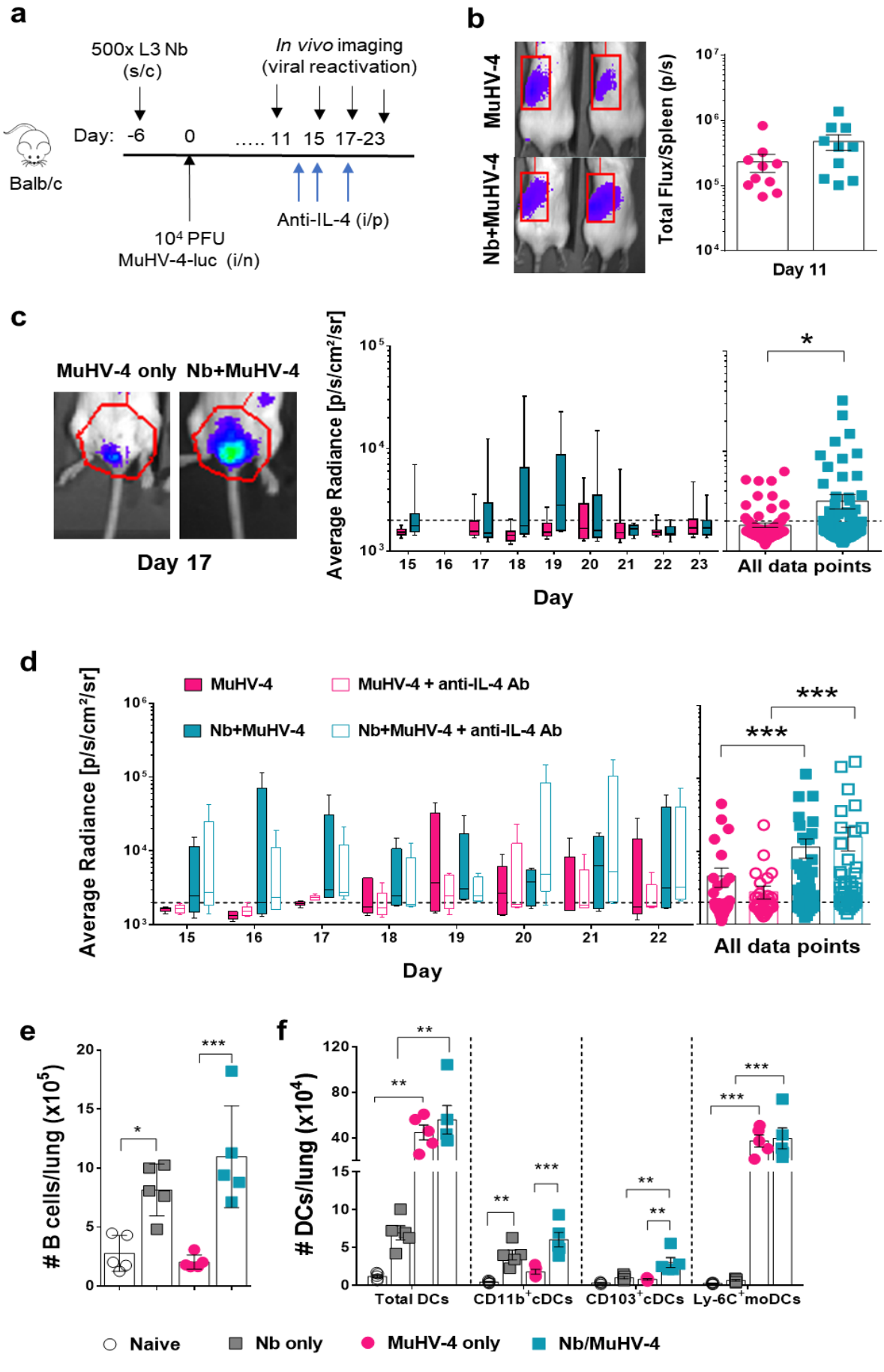

a
500x L3 Nb
(s/c)
In vivo imaging
(viral reactivation)
Day: -6 0 ..... 11 15 17-23
Balb/c
10⁴ PFU
MuHV-4-luc (i/n)
Anti-IL-4 (i/p)
b
MuHV-4
Nb+MuHV-4
Total Flux/Spleen (p/s)
Day 11
c
MuHV-4 only
Nb+MuHV-4
Day 17
Average Radiance [p/s/cm²/sr]
Day
All data points
d
MuHV-4
MuHV-4 + anti-IL-4 Ab
Nb+MuHV-4
Nb+MuHV-4 + anti-IL-4 Ab
Average Radiance [p/s/cm²/sr]
Day
All data points
e
B cells/lung (x10⁵)
f
DCs/lung (x10⁴)
Total DCs
CD11b⁺cDCs
CD103⁺cDCs
Ly-6C⁺moDCs
Naive
Nb only
MuHV-4 only
Nb/MuHV-4

Figure 2.5. Heightened viral reactivation in the genital tract with prior parasite infection: **(a)** Female BALB/c mice were infected with *N. brasiliensis* (Nb) 6 days prior to intranasal infection with MuHV-4-luc. Viral reactivation was established by *in vivo* imaging in the spleen at day 11 post MuHV-4 infection and the genital tract at day 15-23 post infection. **(b)** Representative dorsal photographs of bioluminescence signals following D-luciferin injection, 2 mice per group. Combined ventral and dorsal spleen measurements by live imaging total light emission (p/s = photons per second; mean±sem). **(c)** Representative ventral photographs of bioluminescence signals of the genital tract following D-luciferin injection; 1 mouse per group. Daily measured light emission (viral reactivation) of the genital tract by live imaging; average ventral and dorsal emission per measurement area combined and represented by box and whisker plots. Measured reactivation from all time points were combined in a summary graph. Dotted line – average radiance threshold. **(d)** Genital viral reactivation measured by bioluminescence light emission, in mice treated with anti-IL-4 Ab at day 14, 16 and 18 post MuHV-4 infection. Measured light emission from all time points were combined in a summary graph. Dotted line – average radiance threshold. **(e)** Numbers of B cells, **(f)** $CD11c^{+}MHCII^{+}$ total DCs, $CD11b^{+}$, $CD103^{+}$ conventional DCs (cDCs) and $Ly\text{-}6C^{+}$ monocyte-derived DCs (moDCs) in the lung at day 7 post MuHV-4 infection. Data is representative of one experiment with 5 mice per group. Statistical significance was calculated by one-way ANOVA and Bonferroni's multiple comparisons test and Mann Whitney t test. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ns: not significant

2.4. Discussion

Helminths are highly prevalent in developing regions and have the ability to modulate host immunity. STH endemic areas also have a high seroprevalence of γ-HVs EBV and KSHV [606]. This geographical overlap suggests that coinfections are likely. In this chapter, we report that prior infection with murine hookworm *N. brasiliensis* enhances lung responses and early control of subsequent murine γ-HVs respiratory infection. Prior parasite infection resulted in an amelioration of virus-associated weight loss, reduced viral replication and PFU in the lungs. Previous studies have shown that helminth exposure can enhance [470] and impair [554] immunity against an acute pulmonary virus infection. Notably, McFarlane *et al*., demonstrated protection against respiratory syncytial virus (RSV) by infection with a strictly enteric parasite, *H. polygyrus.* Protection was associated with the remote modulation of lung immunity, by induction of anti-viral type I interferon responses. Interestingly, Th2 immune responses were not essential. Conversely, Osbourne *et al*., revealed that enteric coinfection with *Trichinella spiralis* and murine norovirus resulted in impaired anti-viral $CD8^+$ T cell responses. Diminished anti-viral immunity with helminth infection, was partially associated with AAMs. Here we demonstrate that acute nematode infection, which transitions through the lung, protected against a subsequent respiratory γ-HVs infection and enhanced anti-viral immunity.

Machiels *et* al., 2017 showed that MuHV-4 respiratory infection results in a decline in alveolar macrophages in bronchoalveolar lavage (BAL) fluid, due to cell death [598]. Here we showed that enhanced respiratory control in coinfected mice was accompanied by increased alveolar macrophage numbers. Macrophage polarization to an alternatively activated or M2 phenotype is key feature of Nb infection [476, 487]. The effect of macrophage polarisation on γ-HVs infectivity is not fully understood. In contrast to our preliminary findings, Reese et al., 2014 reported MuHV-4 replicates more effectively in bone marrow-derived macrophages (BMDMs), isolated from naïve mice and polarised to M2 phenotype by *ex vivo* IL-4 treatment [568]. Poglitsch *et al*., 2012 and Bayer *et* al., 2013 suggest that M2 polarised human macrophages were more permissive to Human cytomegalovirus (HCMV) infection compared to M1 macrophages. Human M1 and M2 macrophages were derived from HCMV-seronegative and –seropositive peripheral blood mononuclear cells (PBMCs) cultured in selective media [607, 608]. Conversely, Lee & Ghiasi., 2018 described protection from HSV-1 latency, reactivation and disease with a shift towards M2 polarisation. In this study, peritoneal macrophages were derived from zymosan-treated mice and polarised by *ex vivo* IFN-γ and lipopolysaccharide (LPS; M1) or IL-4 (M2) stimulation [609]. In contrast to our preliminary findings, these studies did not investigate HVs infectivity in tissue-specific macrophages isolated following a M2-inducing infection.

Further investigation is needed to determine whether helminth-induced M2 alveolar macrophages have altered susceptibility to subsequent MuHV-4 infection and pathogenesis.

It is also important to note that elevated macrophage numbers in the lungs of coinfected mice, may be a result of early control and reduced lung viral load, by other immune mechanisms, which have a bystander effect on alveolar macrophages.

Protective immunity against lytic γ-HVs infection is dependent on the development of specific cytotoxic $CD8^+$ T cells. Icheva *et al*., 2013 detailed treatment by the adoptive transfer of antigen-specific T cells in EBV infected patients [610]. Additionally, the importance of an effective virus-specific $CD8^+$ T cells response is well described in *in vivo* murine γ-HVs studies [325, 326, 328, 593]. In contrast, $CD8^+$ cytotoxic T cells have no apparent role in anti-helminth immunity [611]. Our data suggests prior nematode infection conditions $CD8^+$ T cells, resulting in an increase in virus-specific effective cytotoxic T cells following subsequent MuHV-4 respiratory infection. Traditional memory or "true memory" $CD8^+$ T cells (T_{TM}) develop following interaction with their specific antigen, a feature of the adaptive immune response. Interestingly, T cells can adopt a memory-like phenotype in the absence of cognate antigen stimulation [160]. These "bystander" or "virtual memory" $CD8^+$ T cells (T_{VM}) develop in naïve mice and humans, in response to various stimuli including IFN-I, IL-5 and IL-4 [159, 160, 612-614]. Importantly, IL-4, a canonical Th2 cytokine induced by helminth infection, is the main driver of T_{VM} expansion in BALB/c mice [615]. Recently, Lin *et* al., 2018 showed that infection with *H. polygyrus*, expands $CD8^+$ T_{VM} cells. These cells, when transferred to naïve mice, provided non-cognate protection against bacteria *Listeria monocytogenes* [161]. Rolot *et al*., 2018 demonstrated infection with helminths *Schistosoma mansoni*, *H. polygyrus* and *N. brasiliensis* expands bystander T_{VM} cells via IL-4. Prior conditioning of $CD8^+$ T cells during Th2-dominating immune responses, provided earlier and enhanced control of subsequent MuHV-4 acute infection, with increased virus-specific cytotoxic T cell responses [162]. It remains unclear whether helminth-induced T_{VM} cells are accompanied by the development of effective T_{TM} $CD8^+$ T cells, which may provide long term memory against γ-HVs latency and reactivation.

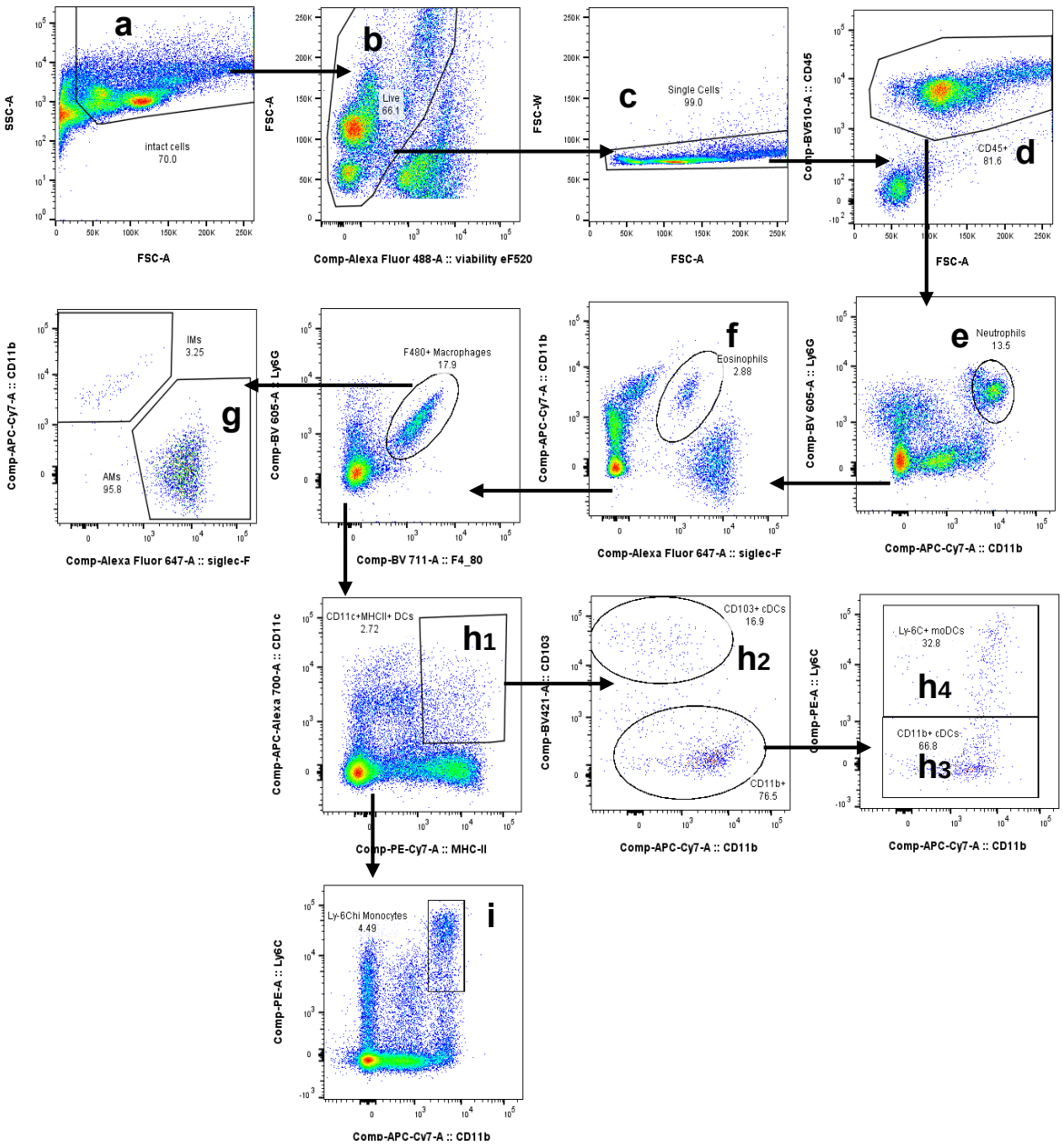
Despite ongoing immune assault, γ-HVs persists by evading $CD8^+$ T cell recognition and establishing latency [616]. Migration of latently-infected cells and lytic viral reactivation allows for spread to new hosts. This ability makes infection lifelong and difficult to control. With human γ-HVs infections, lifelong viral latency is associated with the development of lymphomas and other cancers [290, 617]. In contrast to enhanced primary control, our data suggests that prior nematode exposure was associated with earlier heightened reactivation of MuHV-4, specifically in the genital region. Tibbetts *et al*., 2003 showed the dose of initial murine γ-HVs infection does not substantially influence the degree of latency [600].

Earlier control of respiratory MuHV-4 and the reduced lung PFU we observe in coinfected mice does not foreshadow reduced viral latency and lytic reactivation. Studies have shown that IFN-γ controls γ-HV reactivation and replication *in vivo* [322, 347, 348]. Reese *et al*., demonstrated that exposure to *H. polygyrus* or *S. mansoni* eggs, enhanced murine γ-HVs reactivation. Helminth-induced IL-4 promoted viral replication in latently-infected cells and inhibited the anti-viral action of IFN-γ [568]. Preliminary blocking of IL-4 during viral latency, by anti-IL-4 treatment, did not alter MuHV-4 reactivation. However, additional confirmation is required to determine whether Nb-induced IL-4 is associated with elevated MuHV-4 genital reactivation.

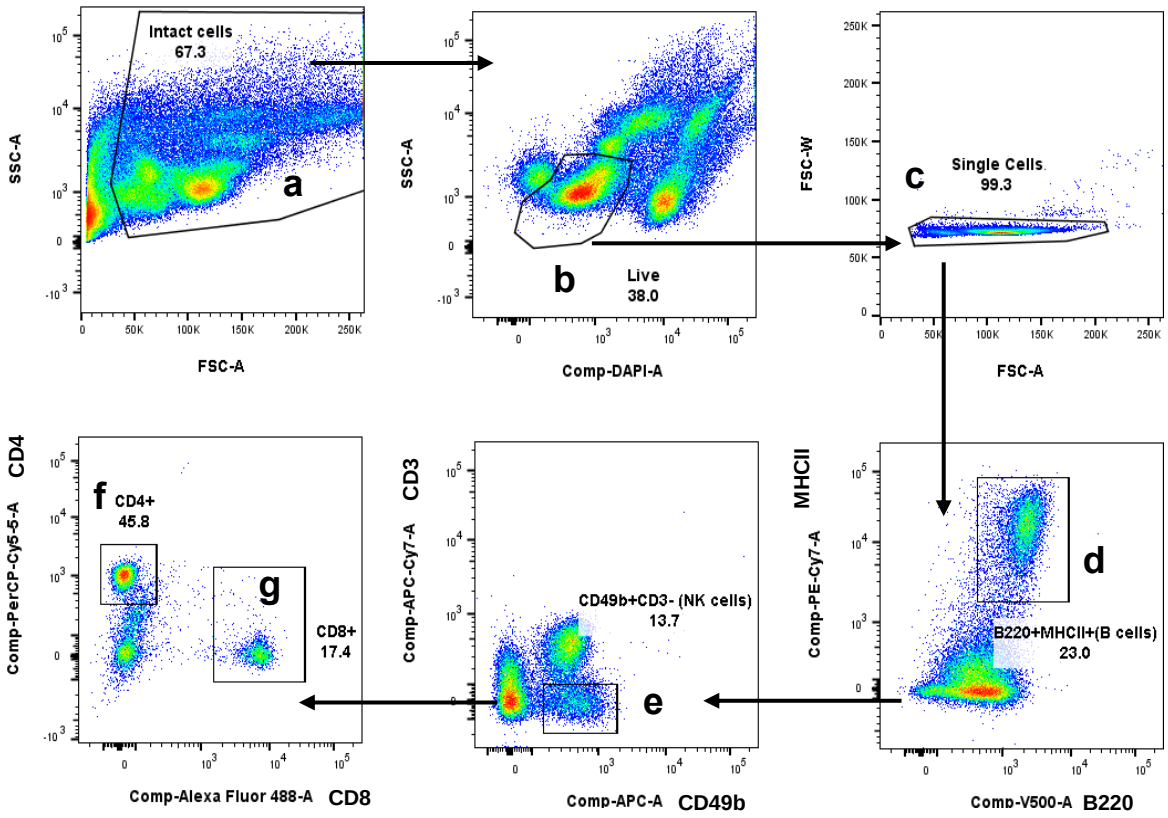
Flano *et al*., 2005 demonstrated that murine γ-HV establishes latency in lung B cells as early as 3 days post intranasal infection. Gasper *et* al., 2011 showed that virus transitions through DCs prior to infecting B cells. Additionally, the establishment of latency is independent of infection route, dose and degree of primary lytic infection [320, 600]. This suggests that proliferation and migration of latently infected cells is important for viral dissemination and reinforces the role of reservoirs during latency. Nematode transition through the lung results in an increase in B cells, which could serve as latency reservoirs during subsequent MuHV-4 respiratory infection. Coinfected mice also displayed elevated lung B cells during peak lytic infection (day 7 post MuHV-4). DCs are important for orchestrating anti-viral immunity in the lung and associated lymph nodes. Conversely, infected DCs serve as viral reservoirs, facilitating viral persistence. γ-HV infection increases total lung DCs, however, $CD11b^+$ and $CD103^+$ cDCs where elevated with prior nematode exposure. This was not seen for moDCs. Although it is established that subsets of lung DCs are phenotypically and functionally distinct [604, 605, 618-620], it is not known whether they differ in viral infectivity and latency. Further investigation is needed to determine whether an increase in lung B cells and cDCs, during peak viral infection, is associated with increased viral latency and reactivation. Importantly, heightened viral reactivation with prior hookworm exposure, despite enhanced primary control, demonstrates the complexity of nematode-induced bystander immunomodulation on subsequent viral infections in the host.

In conclusion, here we provide evidence that prior hookworm infection can boost immune control of a subsequent respiratory virus by possible conditioning of effector virus-specific $CD8^+$ T cell responses in the lung. Although the effects we observe in mice is significant, whether this will translate to humans is not known. If this translates, exposure to hookworms such as *Necator americanus* and *Ancylostoma duodenale* may alter immunity to human γ-HVs like EBV.

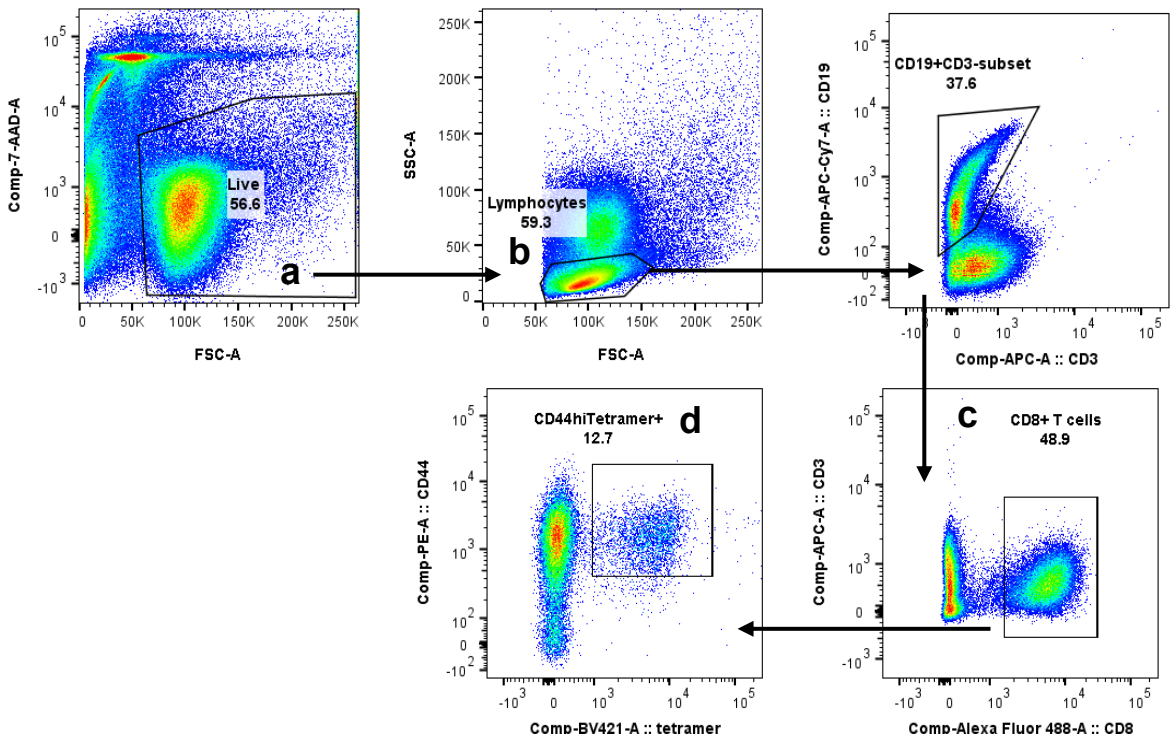
2.5. Supplementary Information



Supp Figure 2.1. Lung myeloid cell gating strategy: At day 7 post MuHV-4-luc infection, lungs were harvested and processed to a single cell suspension. Cells were stained with fluorochrome-conjugated antibodies and analysed by multi-colour flow cytometry. The following gating strategy was used to identify **(a)** intact cells, **(b)** live cells, **(c)** single cells, **(d)** $CD45^+$ cells **(e)** $CD11b^+Ly\text{-}6G^+$ Neutrophils, **(f)** $CD11b^+Siglec\text{-}F^+$ Eosinophils, **(g)** $CD11b^+F480^+Siglec\text{-}F^+$ alveolar macrophages, **(h1)** $CD11c^+MHCII^+$ total DCs, **(h2)** $CD103^+$ conventional DCs (cDCs), **(h3)** $CD11b^+$ cDCs, **(h4)** $Ly\text{-}6C^+$ monocyte-derived DCs and **(i)** $CD11b^{hi}Ly\text{-}6C^{hi}$ monocytes.



Supp Figure 2.2. Lung lymphoid cell gating strategy: At day 7 post MuHV-4-luc infection, lungs were harvested and processed to a single cell suspension. Cells were stained with fluorochrome-conjugated antibodies and analysed by multi-colour flow cytometry. The following gating strategy was used to identify **(a)** intact cells, **(b)** live cells, **(c)** single cells, **(d)** $B220^{+}MHCII^{+}$ B cells **(e)** $CD46b^{+}CD3^{-}$ Natural killer (NK) cells, **(f)** $CD3^{+}CD4^{+}$ and **(g)** $CD3^{+}CD8^{+}$ T cells.



Supp Figure 2.3. Lung tetramer gating strategy: At day 7 post MuHV-4-Luc infection, lungs were harvested from BALB/b10 mice and processed to a single cell suspension. Cells were stained with fluorochrome-conjugated antibodies and analysed by multi-colour flow cytometry. The following gating strategy was used to identify **(a)** live cells, **(b)** lymphocytes, **(c)** $CD3^{+}CD8^{+}$ T cells and **(d)** $CD44^{hi}Tetramer^{+}$ CD8 T cells.

Chapter 3: Nematode infection alters non-colonized genital immunity and exacerbates subsequent HSV-2 pathology

3.1. Introduction

In the previous chapter we demonstrated that prior nematode infection induced bystander immunity in colonized lung tissue, protecting against subsequent MuHV-4 infection. Additionally, we observed nematode exposed mice had earlier and heightened reactivation of murine γ-HV, in non-colonized genital tissue. In this chapter we investigated the systemic influence of Nb infection on female genital tract (FGT) immunity, and the implications on susceptibility to subsequent vaginal HSV-2 infection.

There is considerable geographical overlap between STH and sexually transmitted viral infections [200, 357]. Studies have previously shown that helminth infections can alter susceptibility to viral STIs. For example, infection with *S. haematobium* can result in the deposition of parasite eggs into female genital tissue, causing female genital schistosomiasis [564, 621]. This egg driven pathology and host inflammation in the genital tissue is associated with increased risk of HIV acquisition [561, 562, 565, 622]. If STH infections can cause systemic changes to the FGT, is not well understood. Blackwell *et al*., 2015 described associations between helminth infections and fecundity, while Gravitt *et al*., 2016 reported that women who tested positive for STHs, which did not directly colonise the genital tract, had increased HPV prevalence and a helminth-associated Th2 cytokine signature in genital fluids [567]. This suggests that STH infection can systemically alter physiology and immunity in the FGT.

As previously discussed, murine nematode Nb transits the lung and intestine during its in-host lifecycle. Nb infection induces canonical type 2 immunity such as 'alarmin'-mediated activation of ILC2s, Th2 polarisation of naïve $CD4^+$ T cells and type 2 cytokine production, IL-4/-13-induced alternate activation of macrophages and IL-5-mediated eosinophilia [359, 403, 481, 513]. Conversely, viral infections typically require Th1-mediated immune control. For example, control of *in vivo* vaginal HSV-2 infection is mediated by innate type 1 IFN responses, NK cell cytotoxicity and production of IFN-γ [224, 226, 239], followed by adaptive Th1 and cytotoxic $CD8^+$ T cell responses [250, 257, 259]. It has been reported that Th2 immune responses can counter-regulate Th1 immunity [623-625]. Reese *et al*., 2014 demonstrated that helminth-induced IL-4 hinders IFN-γ-mediated control of murine γ-HV [568]. In contrast, Rolot *et al*., 2018 reported IL-4 conditioning of $CD8^+$ T_{VM} cells by helminth infection, which enhanced control of subsequent murine γ-HV infection [162]. If nematode infection can alter immunity and viral susceptibility in the FGT is not known.

In support of possible helminth-mediated changes to HSV-2 susceptibility, Oh *et al*., 2016 demonstrated exacerbated HSV-2 pathology in antibiotic-treated mice, associated with elevated IL-33, ILC2s and eosinophilia, all hallmarks of anti-helminth Th2 immunity. Here, we investigate bystander changes to female genital immunity following Nb infection, and the impact on susceptibility to a subsequent vaginal HSV-2 infection.

3.2. Methods

3.2.1. Cells

African green monkey kidney (Vero) cells were obtained from ATCC (ATCC® CCL-81™, Manassas, VA, USA) and cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich) supplemented with 10% FCS, 100 U/ml penicillin, 100mg/ml streptomycin and 2mM glutamine, at 37°C, 5% CO_2.

3.2.2. Virus

Human herpesvirus 2 strain G (HSV-2, ATCC® VR-734™) was propagated in Vero cells [220, 626]. Confluent Vero cells were washed with serum-free media and the HSV-2 inoculum was added, at a multiplicity of infection (MOI) of 0.1. At 2-3 days post-infection, cells and supernatant was collected in autoclaved milk and sonicated with a water bath sonicator. Viral titres were determined by plaque assay. Briefly, confluent Vero cells were incubated with serial dilutions of viral stock, at 37°C, 5% CO_2, for 2 hours to allow for absorption. The inoculum was then replaced with supplemented DMEM and cells were incubated for 2 days, fixed with Methanol + 2% H_2O_2 and stained with Giemsa stain (Sigma Aldrich®) to determine plaque forming units (PFU). Viral aliquots were stored at -80°C until use.

3.2.3. Animals

Mice were bred and housed in specific pathogen-free conditions at the Research Animal Facility, University of Cape Town, South Africa. Food and water were provided *ad libitum*. All studies carried out are in accordance with ethical protocols 014/027 or 018/002, approved by the Faculty of Health Science Animal Ethics Committee from the University of Cape Town. Mice were randomly sorted into experimental groups. Female wildtype BALB/c and IL-4Rα$^{-/-}$ (BALB/c background) aged 6-8 weeks were injected subcutaneously with 2 mg Depo Provera® (Pfizer) in sterile PBS, 7 days prior to parasite infection, to synchronize estrous cycles and facilitate consistent intravaginal viral infection [219, 220].

3.2.4. Parasite infection

N. brasiliensis (Nb) was maintained in male Wistar rats (ethics protocol 014/042 or 018/037). Rats were anaesthetised by inhaled isoflurane and infected subcutaneously with 5000x L3 in sterile PBS. Faecal pellets were collected at day 6-8 post infection and cultured with activated charcoal. For experimental infections, L3 larvae were isolated from faecal cultures as previously described [(359)]. Mice were infected with 500 x Nb L3 larvae subcutaneously, 7 days prior to viral infection.

3.2.5. Intravaginal infection

Mice were anesthetized by Ketamine 100mg/kg + Xylazine 10mg/kg intraperitoneal injection and inoculated intravaginally with 5 x 10^5 PFU HSV-2 in 5 µl of sterile PBS. Virus-associated illness severity was determined by pathology scoring (Figure 3.1): 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 – Pre-moribund [(220)]. Vaginal lavages were performed by 10x flushing the vaginal vault with 50 µl sterile PBS. This was repeated three times. Viral shedding was quantified by plaque assay as described previously.

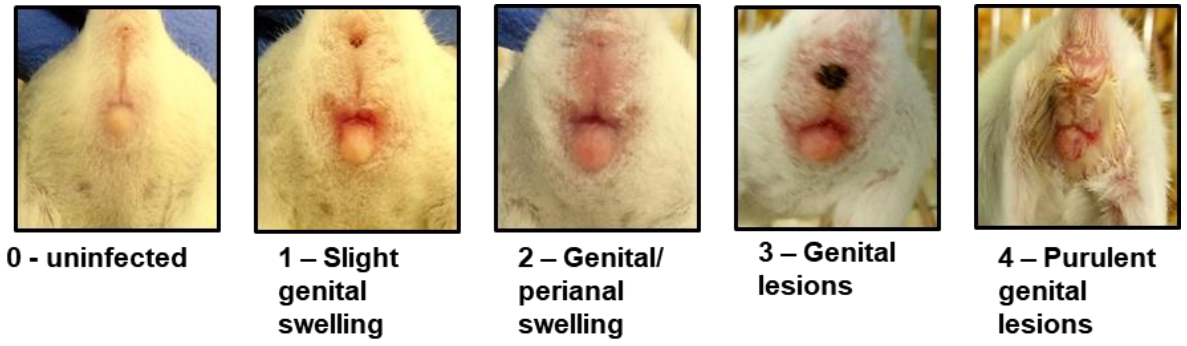


Figure 3.1. HSV-2 pathology scoring following intravaginal infection

3.2.6. Histology

Vaginal tissue was excised and fixed in phosphate-buffered formalin solution (Sigma Aldrich®) [(396)]. Following paraffin embedding, tissue were cut into 5 µm cross-sections and stained with haematoxylin and eosin (H&E) to visualize vaginal epithelial integrity and cell infiltration. All sections were viewed with Axioskop Microscope (Zeiss) and images were taken with a colour AxioCam HRc and AxioVision 4.7 supporting software. Epithelium integrity was measured using ImageJ Software (NIH) and percentage of ulcerated tissue was calculated: % ulcerated epithelium = $\frac{\text{Length of ulcerated epithelium}}{\text{Total epithelial length}}$ x 100

3.2.7. Tissue processing

Genital homogenates

Isolated female genital tissue (FGT, excluding ovaries) was snap frozen in liquid nitrogen and stored at -80°C until use. For cytokine analysis, tissue was homogenized in 500 µl RIPA lysis buffer containing protease inhibitor cocktail (Sigma Aldrich®), using a benchtop homogenizer (Kinematica Polytron™ PT 2500E homogenizer). Homogenates were centrifuged at 10 000 rpm for 10 minutes to isolate the supernatant. Protein concentrations was quantified by bicinchoninic acid (BCA) assay (Pierce™, Thermo Scientific) and all samples were standardised for cytokine analysis by Enzyme-linked immunosorbent assay (ELISA) [492, 627].

Preparation of single cell suspension

FGT was removed from individual mice, finely cut and digested in supplemented DMEM containing 1% HEPES and 20 µg/ml Liberase™ TL (Roche), for 1 hour at 37°C with gentle shaking. Digested tissue was passed through a 70 µm cell strainer and resuspended in fresh supplemented DMEM. Iliac lymph nodes (iLN) and spleens were excised and passed through a 40 or 70 µm cell strainer respectively. Splenocytes were subjected to red cell lysis by Ammonium-Chloride-Potassium (ACK) lysis buffer (Gibco™, Invitrogen). Cells were counted by trypan blue exclusion, using a haemocytometer slide and resuspended at a final concentration of 2 x 10^7 cells/ml for flow cytometry staining.

3.2.8. Flow cytometry

Approximately 2 x 10^6 cells per sample were stained in 96-well V-bottom plates (Corning®, NY, USA) with MACs buffer (PBS + 0.5% BSA and 2mM Ethylenediaminetetraacetic acid (EDTA)) containing 2% heat-inactivated rat serum, 1 µg anti-mouse CD16/32 antibody (clone 93, BioLegend) and fluorochrome-conjugated antibodies against cell-surface markers (Table 3.1) for 20 minutes at 4°C (dark). Cells were washed twice and resuspended in a final volume of 200 µl MACs buffer for acquisition or further processed for intracellular staining. Samples were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences) and data was analysed by FlowJo© V10 (Treestar, Ashland, OR). Appropriately stained compensation beads and unstained controls were run to compensate for spectral overlap between fluorochrome emissions. Gating strategies to identify myeloid cells, ILC2s, T cells and NK cells in the FGT, iLN and spleen are shown below (Supp Figure 3.1-3.4).

Table 3.1: List of fluorochrome-conjugated anti-mouse antibodies for cell surface staining

Surface marker	Fluorochrome	Clone	Source
CD45	Alexa Fluor® 700	30-F11	BioLegend
Myeloid panel			
CD11b	BV421™	M1/70	BioLegend
F4/80	BV605™	BM8	BioLegend
Ly-6C	FITC	HK1.4	BioLegend
Ly-6G	APC/Cy7	1A8	BioLegend
Siglec-F	PE	S17007L	BioLegend
ILC2 panel			
7-AAD	N/A	N/A	Novus Biological
Lineage cocktail	PE	CD3ε, clone 145-2C11; Ly-6G/Ly-6C, clone RB6-8C5; CD11b, clone M1/70; CD45R/B220, clone RA3-6B2; TER-119, clone Ter-119	BioLegend
IL-7Rα (CD127)	PE Cy7	A7R34	BioLegend
icos	APC	C398.4A	BioLegend
ST2 (IL-33Rα)	BV421	DIH9	BioLegend
NK cell panel			
CD3	FITC	17A	eBioscience
CD49b	APC	DX5	BioLegend
T cell panel			
CD3	Alexa Fluor® 700	17A	BioLegend
CD4	BV421™	GK1.5	BioLegend
CD8a	Alexa Fluor® 488	53-6.7	BioLegend

3.2.9. *Ex vivo* restimulation and intracellular staining

For intracellular cytokine analysis, cells were restimulated with supplemented DMEM containing mitogens phorbol 12-myristate 13-acetate (PMA, 20 ng/ml, Sigma Aldrich®) and ionomycin (1 µg/ml, Sigma Aldrich®), in the presence of Brefeldin A (BFA, 10 µg/ml, eBioscience) for 4 hours at 37°C, 5% CO_2. Following cell surface staining, cells were fixed in 2% PFA and permeabilized for 15 minutes in permeabilization buffer (eBioscience). Cells were then incubated with anti-mouse IFN-γ (clone XMG1.2, PE Cy7, BioLegend) and Granzyme b (clone QA16A02, PE, BioLegend) or CD206 MMR (clone C068C2, APC, BioLegend) in permeabilization buffer for 45 minutes at 4°C (dark). Cells were washed and resuspended in 200 µl MACs buffer for acquisition. Samples were acquired as previously detailed.

3.2.10. Enzyme-linked immunosorbent assay (ELISA)

Quantification of cytokines IL-4, IFN-γ and TNF-α was performed using ELISA MAX™ Standard kits (BioLegend®) following the manufacturer's instructions. IL-33 quantification was performed using Mouse IL-33 Duoset ELISA kit (R&D systems) as per manufacturer's instructions. Briefly, 96-well flat-bottom plates (Nunc Maxisorp, Thermo Fisher Scientific) were coated with 50 µl Capture Ab diluted in Coating Buffer, overnight at 4°C. Plates were washed three times with wash buffer and subsequently blocked with 200 µl assay buffer for 1 hour at RT with gentle shaking. Following 3x washing, samples and diluted standards were added to the appropriate wells and incubated overnight at 4°C. Plates were washed and subsequently incubated with 50 µl of biotinylated-Detection Ab for 2-3 hours at RT. After 3x washing, 50 µl of diluted Avidin-Horse radish peroxidase (HRP) was added for 1 hour at RT. Plates were washed four times, with 30 seconds soaking between each wash. The plates were developed with TMB microwell peroxidase substrate system (Thermo Fisher Scientific), and the reaction was stopped with 1M H_3PO_4. The plates were read at an absorbance of 450nm (Lm1) and 570 nm (Lm2; background) using a VersaMax microplate reader (Molecular Devices Corporation, CA, U.S.A).

3.2.11. Luminex

FGT homogenate and vaginal lavage cytokine levels were measured by Luminex xMap technology using the Invitrogen ProcartaPlex™ 36-plex mouse kit (Cat# EPX360-26092-901, Lot # 189084327), as per manufacturers. The Luminex plate was read using Bio-Rad Bio-Plex® 200 system and analysis software. Luminex was performed once, with samples from two independent experiments. Analyte levels below the lower detection limits were given an arbitrary value of half the Lower Limit of Quantification (LLOQ), defined by the manufacturer.

3.2.12. Anti-Siglec-F treatment

To deplete eosinophils, mice were treated with 20 µg mouse anti-Siglec-F (α-Siglec-F, monoclonal Rat IgG2A Clone # 238047, R&D Systems) antibody intraperitoneally on day 5, 7 and 9 post Nb infection. Control mice were treated with rat IgG2A isotype antibody.

3.2.13. Statistical analysis

Data is represented as group mean and standard error of the mean (mean±sem). Statistical analysis was performed either by analysis of variance (ANOVA) followed by the Bonferroni multiple comparison test or by non-parametric Mann-Whitney test, with a 95% confidence interval. A p-value < 0.05 was considered significant and are indicated by an asterisk (*). Statistical analyses were performed using GraphPad Prism V6.

3.3. Results

3.3.1. *N. brasiliensis* infection induces canonical type 2 immunity in the FGT

The FGT is unique to other mucosal sites, as significant physiological and immunological cyclic changes occur, influenced by fluctuations in sex hormones [628]. The mouse estrous cycle is divided into four stages; proestrus, estrous, metestrus and diestrus, with notable variations in the immune presence during each stage [222, 223, 629]. Additionally, mice are only susceptible to intravaginal HSV-2 infection during diestrus stage of the hormone cycle [219, 630, 631]. To determine the influence of nematode infection on FGT immunity, we treated female BALB/c mice with 2 mg Depo Provera® subcutaneously, to synchronise the estrous cycle to the progesterone-dominant diestrus stage. This eliminated cyclic variation and was a requirement for downstream intravaginal HSV-2 experiments [220].

We infected hormone-synchronised mice with 500x L3 Nb and examined immunology in the FGT, genital associated iliac lymph nodes; iLN and spleen (SPL), at day 9 post Nb infection (Figure 3.2.1a). Naïve or 'healthy' vaginal tissue in diestrus have a relatively thin stratified squamous epithelium and surveying leukocytes present in the submucosa, which often migrate through the epithelium into the vaginal lumen [223]. Histological analysis of vaginal tissue, at day 9 post Nb infection, suggested an increased presence of polymorphonuclear (PMN) cells, with eosinophilic cytoplasm, in the vaginal tissue of nematode infected mice, compared to naïve controls (Figure 3.2.1b). The composition and infiltration of immune cells in the vaginal tissue influences pathogen susceptibility as well as vaginal homeostasis [632, 633]. Our observations suggest that Nb infection results in increased infiltration of eosinophilic PMN cells into the vaginal tissue.

We confirmed the immune cell composition of the FGT by multicolour flow cytometry. We observed elevated proportions and numbers $CD11b^{+}$ cells in the FGT of Nb infected mice, compared to uninfected (Supp Figure 3.5). This suggests increased infiltration of myeloid cells into the FGT following Nb infection. Underlying this increase, we observed a significant increase in $CD11b^{+}$Siglec-F^{+}Side-$scatter^{high}$(SSC^{hi}) eosinophils in the FGT of Nb infected mice, compared to uninfected (Figure 3.2.2a), confirming previous histological observations. Significant eosinophilia was also observed in the iLN of Nb infected mice, compared to naïve controls (Figure 3.2.2d). This increase in genital eosinophils in Nb infected mice, mirrors systemic immunity observed in the spleen (Supp Figure 3.6). Nb-induced eosinophilia was long-lasting, as elevated levels of eosinophils were observed in the FGT and spleen at day 21 post Nb infection, compared to uninfected mice (Supp Figure 3.7). This data suggests that nematode infection systemically induces long-lasting eosinophilia in uncolonized genital tissue, which may have implications on vaginal tissue homeostasis.

During tissue injury or infection, circulating monocytes and neutrophils infiltrate the site of inflammation [476, 634]. Ly-6C^{hi} monocytes have pro-inflammatory functions such as cytokine and chemokine production [12, 635]. At 9 days post Nb infection, we observed significantly increased proportions and numbers of CD11b^{+}Ly-6C^{hi} monocytes in the FGT and iLN, compared to uninfected mice (Figure 3.2.2b, d). Additionally, we observed significantly elevated numbers of CD11b^{+}Ly-6G^{+} neutrophils in the iLN of Nb infected mice, compared to uninfected (Figure 3.2.2d). This suggests an underlying inflammatory response in uncolonized genital tissue following nematode infection.

Infiltrating monocytes may also differentiate into macrophages in various tissues during inflammation [635-637]. Monocyte-derived and tissue-resident macrophages are activated by the cytokine milieu in the inflamed tissue. AAMs induced by IL-4 and/or -13, is a key feature of helminth infection and contributes to tissue repair following parasite transition through host tissue [638]. AAMs can be defined by increased expression of mannose receptor CD206 [639]. Proportions and numbers of FGT and iLN macrophages (CD11b^{+}F4/80^{+}) did not significantly differ in Nb infected and uninfected mice however, we observed an increase in the percentage of CD206^{+} macrophages in the FGT following parasite infection, compared to naïve controls (Figure 3.2.2c, d). This reflected a systemic increase in CD206^{+} macrophages observed in the spleen at day 9 post Nb infection, compared to uninfected mice (Supp Figure 3.6). These results suggest an M2-like macrophage phenotype is present in the FGT following nematode infection, further demonstrating the Nb infection induces Th2 immunity in uncolonized genital tissue.

Type 2 cytokines are key regulators of effector cell recruitment and activation during helminth infection. Previously, Gravitt *et al*., 2016 reported a type 2 cytokine signature in the vaginal fluids of STH-infected women [567]. Here, ELISA and Luminex analysis revealed Nb infected mice had elevated levels of canonical type 2 cytokines, IL-4 and IL-5, in the genital tissue, compared to uninfected controls (Figure 3.2.3a, b). Additionally, we found reduced levels of type 1 cytokines IFN-γ and TNF-α, in the genital tissue of Nb infected, compared to naïve mice (Figure 3.2.3b). This suggests that Nb infection induces a type 2 cytokine signature in the FGT. Increased levels of IL-4 and IL-5 are likely associated with elevated Th2 effector cells in the FGT. Together, type 2 cytokine FGT signature and eosinophilia, suggests that nematode infection induces canonical Th2 immunity in the FGT, a biological compartment not directly colonized by the parasite.

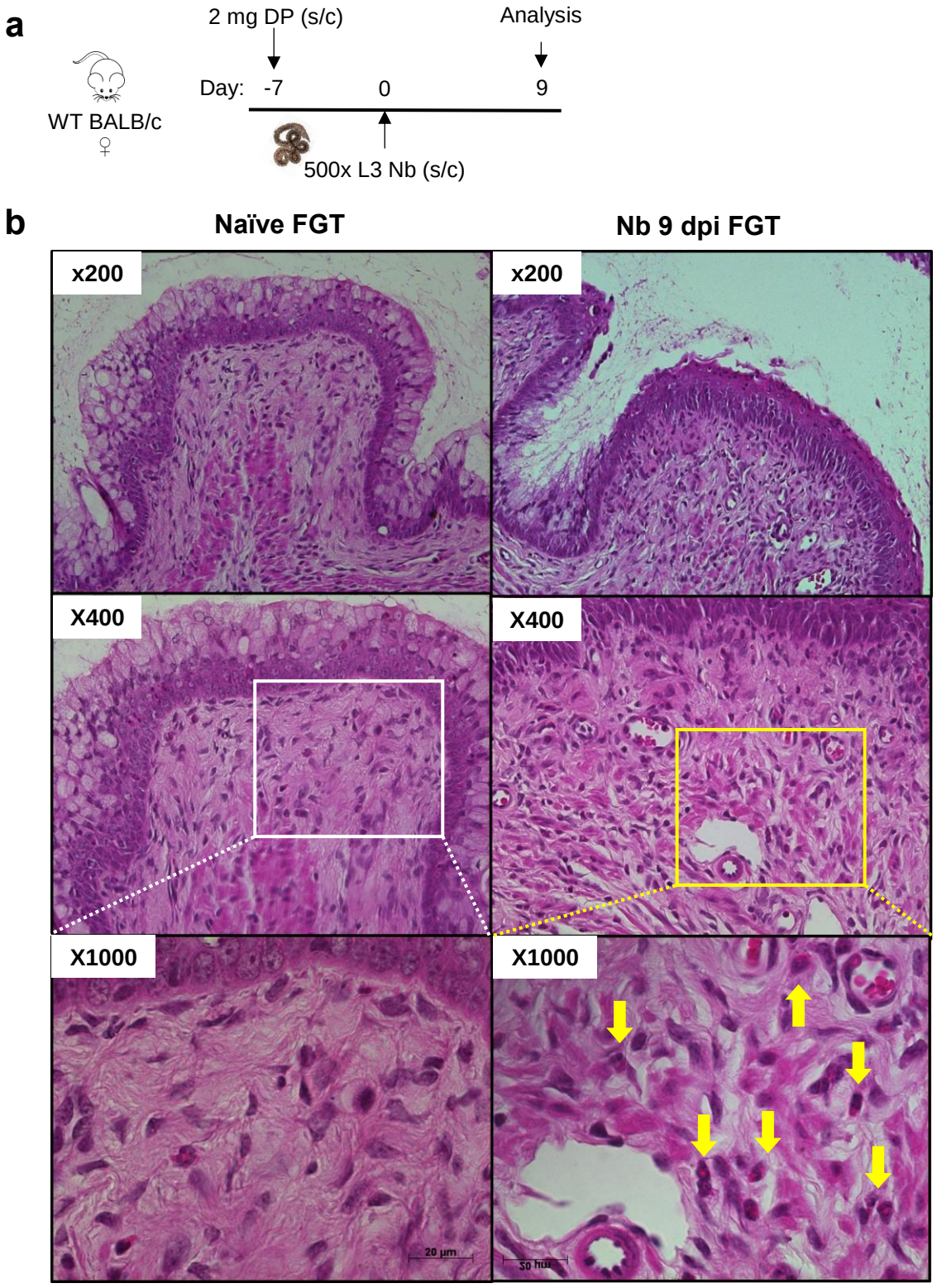


Figure 3.2.1. Influence of *N. brasiliensis* exposure on FGT immunity: (a) Female BALB/c mice were treated with 2 mg Depo Provera® (DP), 7 days prior to subcutaneous infection with Nb. Naïve controls were also hormone-synchronised. **(b)** At day 9 post Nb infection, vaginal tissue integrity and submucosal inflammation was determined by H&E histological analysis, compared to naïve controls. Images were taken at x200, x400 and x1000 magnification. Yellow arrows indicate infiltrating polymorphonuclear cells, elevated in Nb infected mice, compared to uninfected. Data is representative of two independent experiments with 4 mice per group.

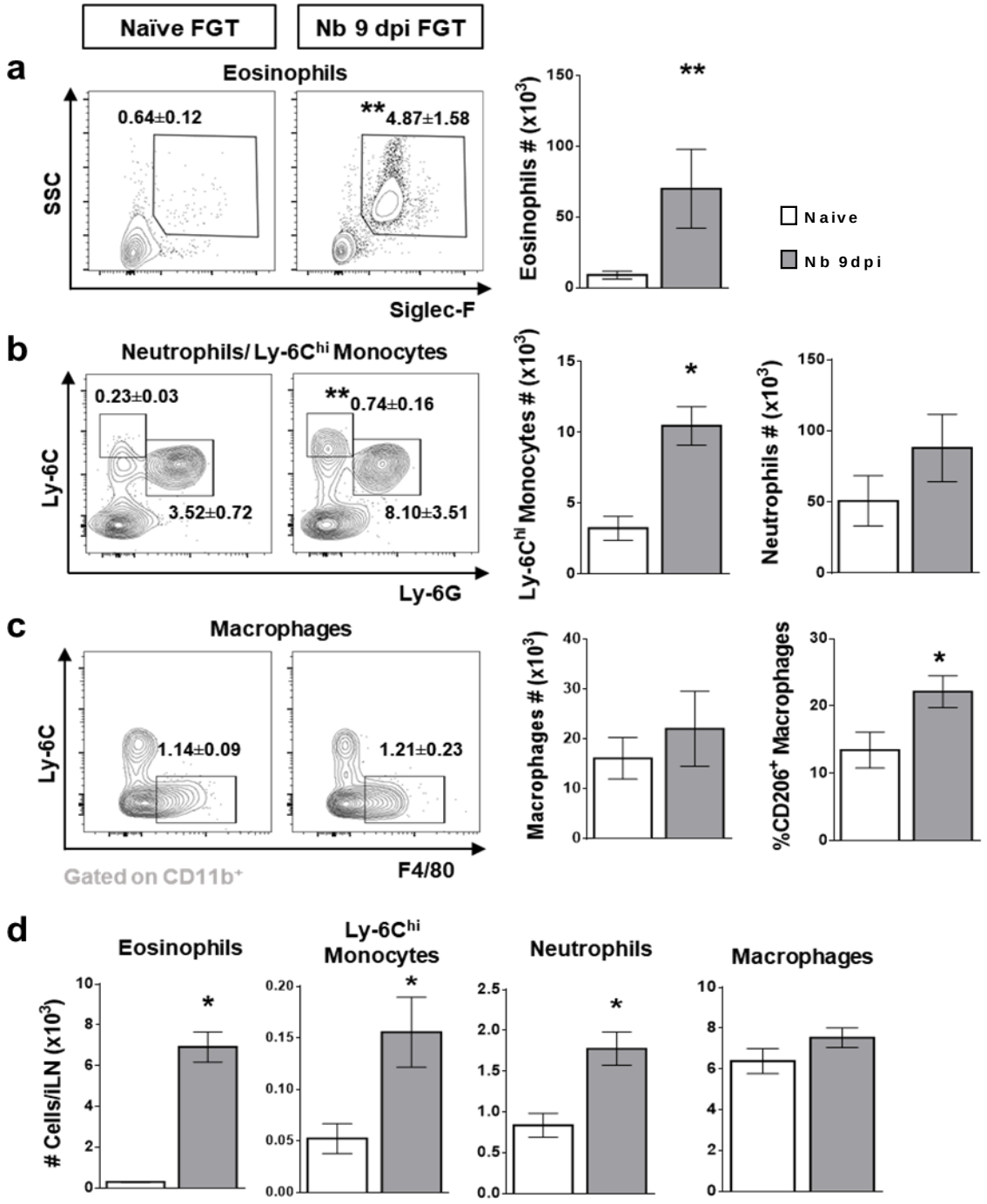


Figure 3.2.2. Increase in FGT eosinophils and Ly-6C^{hi} Monocytes following Nb exposure: At day 9 post Nb infection, FGT tissue was isolated and myeloid cell populations were analysed by flow cytometry; representative flow plots showing cell proportions (mean±sem) and numbers of FGT **(a)** eosinophils (CD11b^{+}Siglec-F^{+}SSC^{hi}), **(b)** Ly-6C^{hi} monocytes (CD11b^{+}Ly-6C^{hi}), neutrophils (CD11b^{+}Ly-6G^{+}), **(c)** macrophages (CD11b^{+}F480^{+}), and CD206+ (M2-like) macrophages in naïve and Nb infected mice. Cell proportions were calculated out of single cells acquired. **(d)** Number of myeloid cells in the iLN at day 9 post Nb infection, compared to naïve controls. FGT homogenates were assessed for levels of Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$. **$p \leq 0.01$

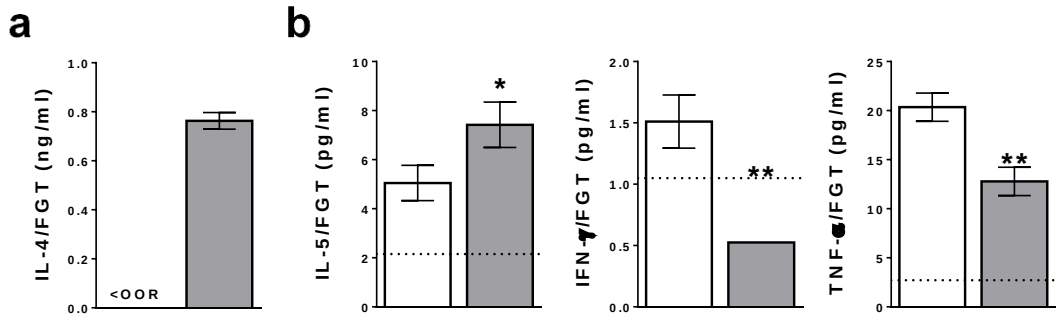


Figure 3.2.3. Increased type 2 and reduced type 1 cytokines in genital tissue following nematode infection: At day 9 post Nb infection, FGT was isolated and processed for cytokine analysis. FGT homogenates were analysed for **(a)** IL-4 by ELISA; <OOR: optical density readings below detection limit, and **(b)** IL-5, IFN-γ and TNF-α, by Luminex. Dotted line represents Lower Limit of Quantification (LLOQ) for each cytokine. Samples below detection limits were given an arbitrary value of half the LLOQ. Data is representative of two independent experiments with 4 mice per group (mean±sem). Statistical significance was calculated by Mann Whitney t test. *p ≤ 0.05, **p ≤ 0.01

3.3.2. 'Death' of non-haematopoietic FGT cells and systemic upregulation in GZb following Nb infection

Parasite transition through the host can cause significant local tissue damage and enhanced epithelial apoptosis, in colonized compartments [475, 479, 640, 641]. In addition, infiltrating eosinophils can release cytoplasmic granules which contain a variety of potent cytotoxic proteins, including major basic protein (MBP) and eosinophil peroxidase (EPO), which may cause bystander epithelial damage [642-645]. At day 9 post Nb infection, we investigated the viability of non-haematopoietic FGT cells, which includes epithelial, endothelial, stromal and smooth muscle cells [646, 647]. Cell viability was determined using fluorescent DNA intercalator 7-AAD, that exclusively penetrates cells with compromised cell membrane integrity. Preliminary analysis revealed significantly elevated proportions and numbers of 7-AAD$^+$ non-haematopoietic (CD45$^-$) cells in the FGT at day 9 post Nb infection, compared to uninfected mice (Figure 3.3a, b). No difference in cell 'death' was observed in associated iLN between experimental groups. This suggests that nematode infection is associated with increased non-haematopoietic cell 'death' in non-colonized genital tissue.

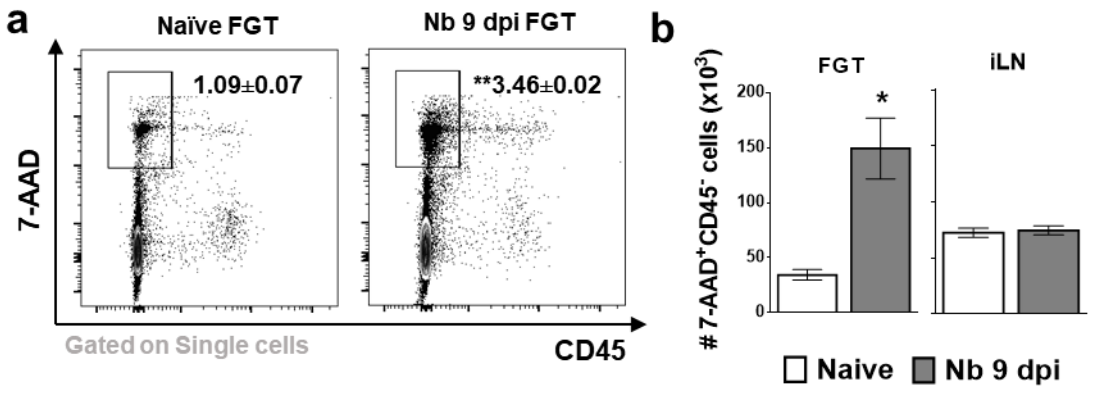


Figure 3.3. Increased 'death' of non-haematopoietic cells in the FGT following Nb infection: At day 9 post Nb infection, the viability of non-haematopoietic cells in the FGT and iLN was analysed by flow cytometry, compared to naïve controls. **(a)** Proportions (mean±sem) and **(b)** numbers of 7-AAD$^+$CD45$^-$ cells in the FGT and iLN of naïve and Nb 9 dpi mice. Data is representative of one experiment with 4 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired. Statistical significance was calculated by Mann Whitney t test. *p ≤ 0.05, **p ≤ 0.01

Fujiwara *et al*., 2004 reported upregulation of cytotoxic serine protease; granzyme b (GZb) during rodent nematode infection and its association with intestinal epithelial damage [640]. NK cells are an innate source of GZb in inflamed and/or infected tissue [88, 238, 648, 649], however the role of NK cells during Nb infections remains unclear. Unlike in the spleen, we found no significant difference in the proportion or number of iLN NK cells (CD3$^-$CD49b$^+$) between Nb infected and naïve mice (Figure 3.4a-b). However, we observed significantly elevated levels of intracellular GZb in splenic and iLN NK cells in Nb infected, compared to naïve mice (Figure 3.4c). This suggests a systemic increase in NK cell GZb potential following Nb infection, evident in uncolonized biological compartments.

We also assessed the number and function of CD4$^+$ and CD8$^+$ T cells in the iLN and spleen following Nb infection. Nb infection induces Th2 polarization of CD4$^+$ T cells, defined by enhanced IL-4 and reduced IFN-γ production [403, 508], whereas CD8$^+$ T cells have no reported protective function during nematode infections. Unlike the spleen, the number of CD4$^+$ and CD8$^+$ T cells in the iLN were not significantly altered at 9 days post Nb infection, compared to uninfected mice (Figure 3.5a-c). Overall, the production of GZb by CD8$^+$ T cells was less compared to NK cells, which has been reported previously [650-652]. However, we observed significantly increased GZb production by iLN CD8$^+$ T cells in Nb infected mice, compared to uninfected controls (Figure 3.5e). Collectively, these results suggest that Nb-induced upregulation of GZb potential in cytotoxic cells is not restricted to colonized tissue, which further demonstrates the systemic effects of nematode responses in non-colonized FGT.

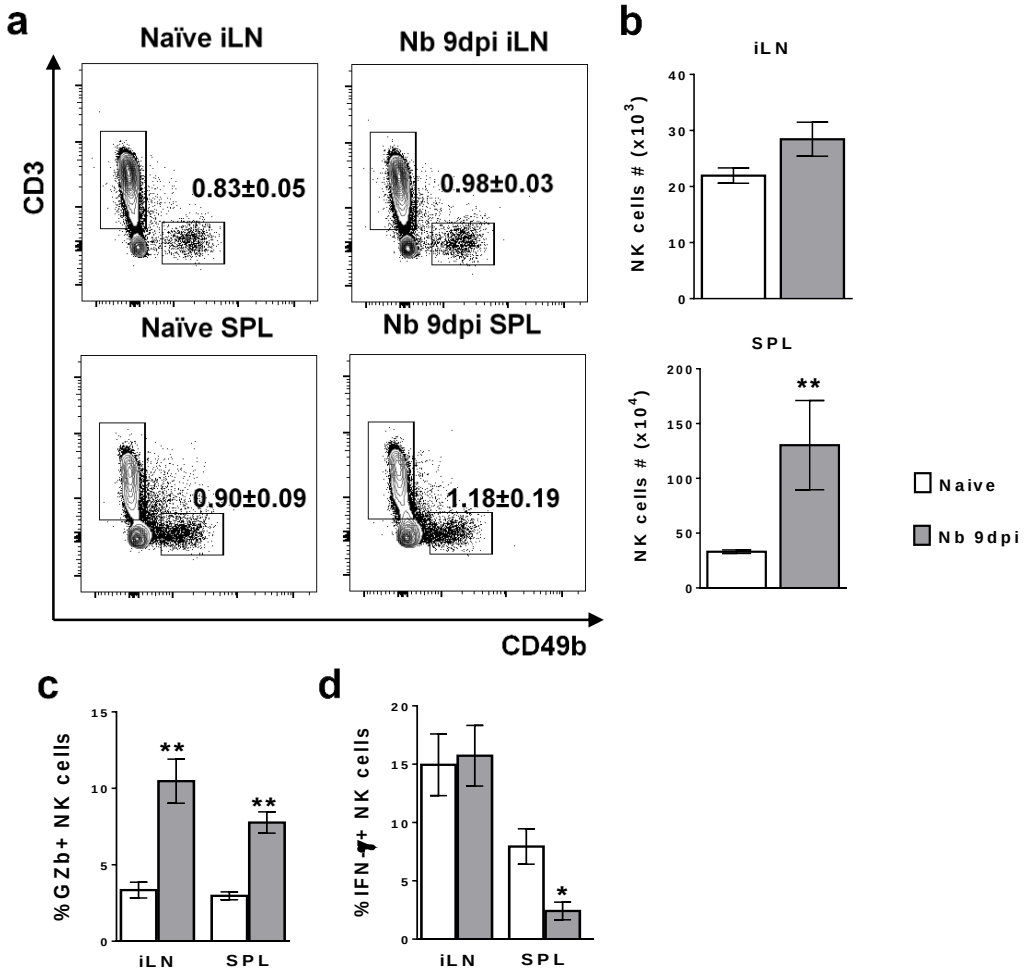


Figure 3.4. Systemic increase in NK cell Granzyme b production with Nb exposure: At day 9 post Nb infection, iliac lymph nodes (iLN) and spleen cells were mitogen-stimulated and analysed by flow cytometry. **(a)** Representative flow plots showing proportions (mean±sem) of iLN and spleen NK cells ($CD3^{-}CD49b^{+}$). **(b)** Numbers of iLN and spleen NK cells in Naïve and Nb infected mice. Percentage of **(c)** GZb+ and **(d)** IFN-γ^{+} NK cells in the iLN and spleen of naïve and Nb 9 dpi mice. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired (a) or the parent population (c, d). Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$, **$p \leq 0.01$

Earlier, we reported reduced type 1 cytokines in genital tissue of Nb infected mice, compared to naïve controls (Figure 3.2.3b). An important role of NK and $CD4^{+}$ T cells is to produce type 1 cytokine IFN-γ, during viral infections [226, 653, 654]. NK cells in the spleen of Nb infected mice had significantly reduced intracellular IFN-γ levels compared to uninfected mice, however we did not observe this decrease in iLN NK cells (Figure 3.4d). No significant differences in intracellular IFN-γ levels of $CD4^{+}$ T cells were observed (Figure 3.5d). Further investigation is needed to determine whether Nb infection supresses IFN-γ production by genital cells.

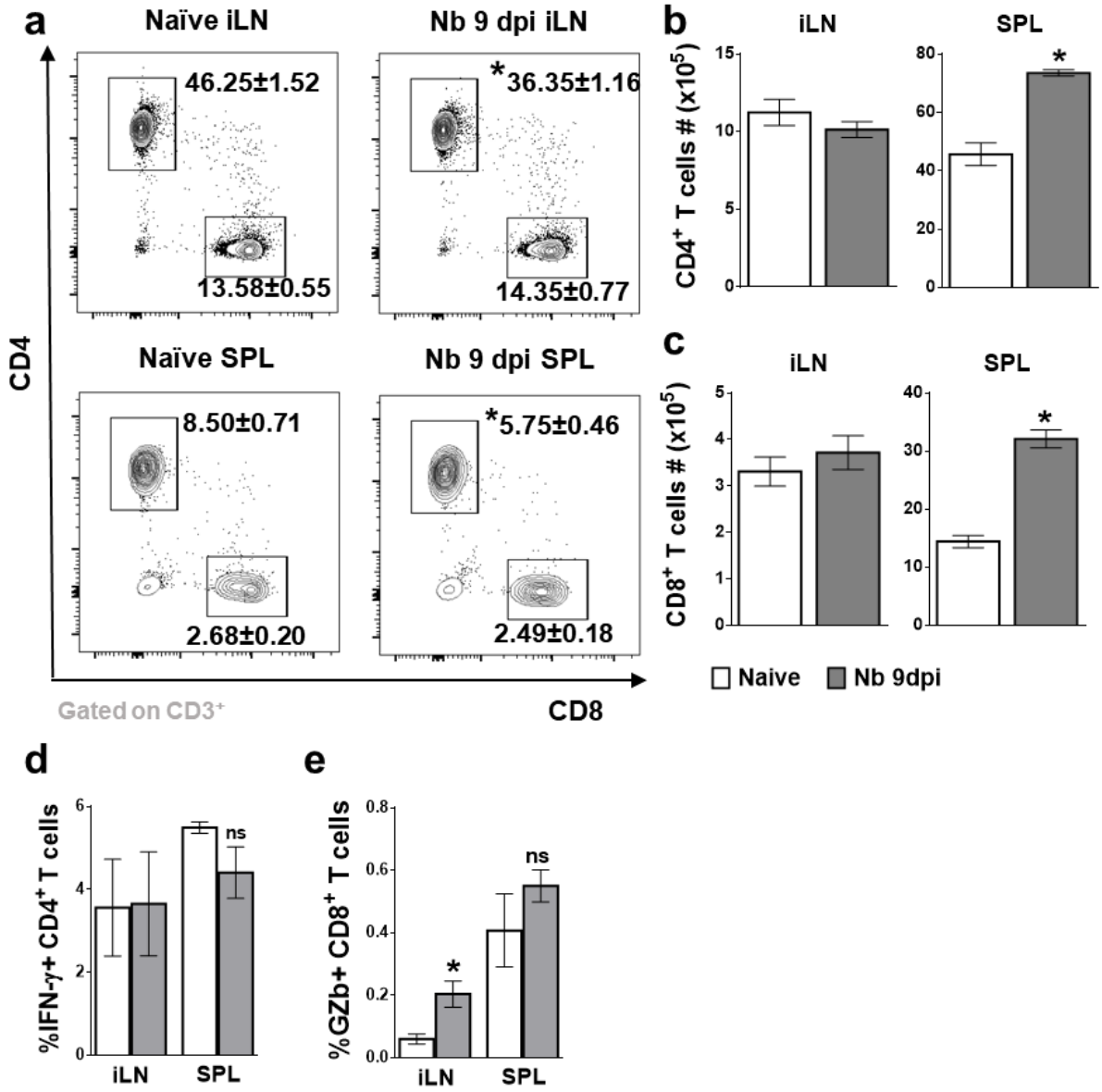


Figure 3.5. Nematode infection does not significantly alter genital T cell numbers: At day 9 post Nb infection, iliac lymph nodes (iLN) and spleen (SPL) cells were mitogen-stimulated and analysed by flow cytometry. **(a)** Representative flow plots and proportions (mean±sem) of iLN and spleen $CD4^+$ and $CD8^+$ T cells. Numbers of **(b)** $CD4^+$ and **(c)** $CD8^+$ T cells in the iLN and spleen of naïve and Nb infected mice. Percentage of **(d)** IFN-γ-producing $CD4^+$ T cells and **(e)** GZb^+ $CD8^+$ T cells in the iLN and spleen. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired (a) or the parent population (d, e). Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$, ns – not significant

3.3.3. Prior Nb exposure exacerbates subsequent HSV-2 genital pathology

The genital eosinophilia and underlying tissue damage we observed following nematode exposure, may have bystander effects on an unrelated vaginal infection. To investigate how prior nematode exposure influenced subsequent genital herpes virus infection, we infected hormone-synchronized mice with Nb, as performed previously. At day 7 post Nb infection, we inoculated mice intravaginally with $5x10^5$ PFU HSV-2 strain G, under deep anaesthesia (Figure 3.6.1a). Viral illness progression and severity was determined by daily genital pathology scoring. In mice previously infected with Nb, we observed earlier HSV-2-associated perianal inflammation and significantly heightened pathology from day 4 post HSV-2 infection, compared to mice infected with virus only, (Figure 3.6.1b). In contrast, no significant difference in viral shedding at day 1-3 post HSV-2 infection, was observed (Figure 3.6.1.c). However, previous studies have also reported no correlation between increased HSV-2 pathology and early viral shedding [225, 226].

HSV-2 primarily infects vaginal epithelial cells and causes cell lysis following viral replication. Epithelial cell lysis manifests as ulcerated vaginal epithelium [655, 656]. Histological analysis of vaginal tissue at day 6 post HSV-2 infection suggests a trend for increased epithelium ulceration (i.e. loss of stratified squamous epithelium and mucous-producing cells lining the vaginal tissue) in coinfected mice, compared to virus only controls (Figure 3.6.2). Furthermore, we noted elevated infiltration of PMN cells, with eosinophilic cytoplasm, in inflamed vaginal tissue of Nb+HSV-2 mice, compared to HSV-2 only. Together, these results suggest that prior Nb infection worsens subsequent HSV-2-induced vaginal inflammation and ulceration.

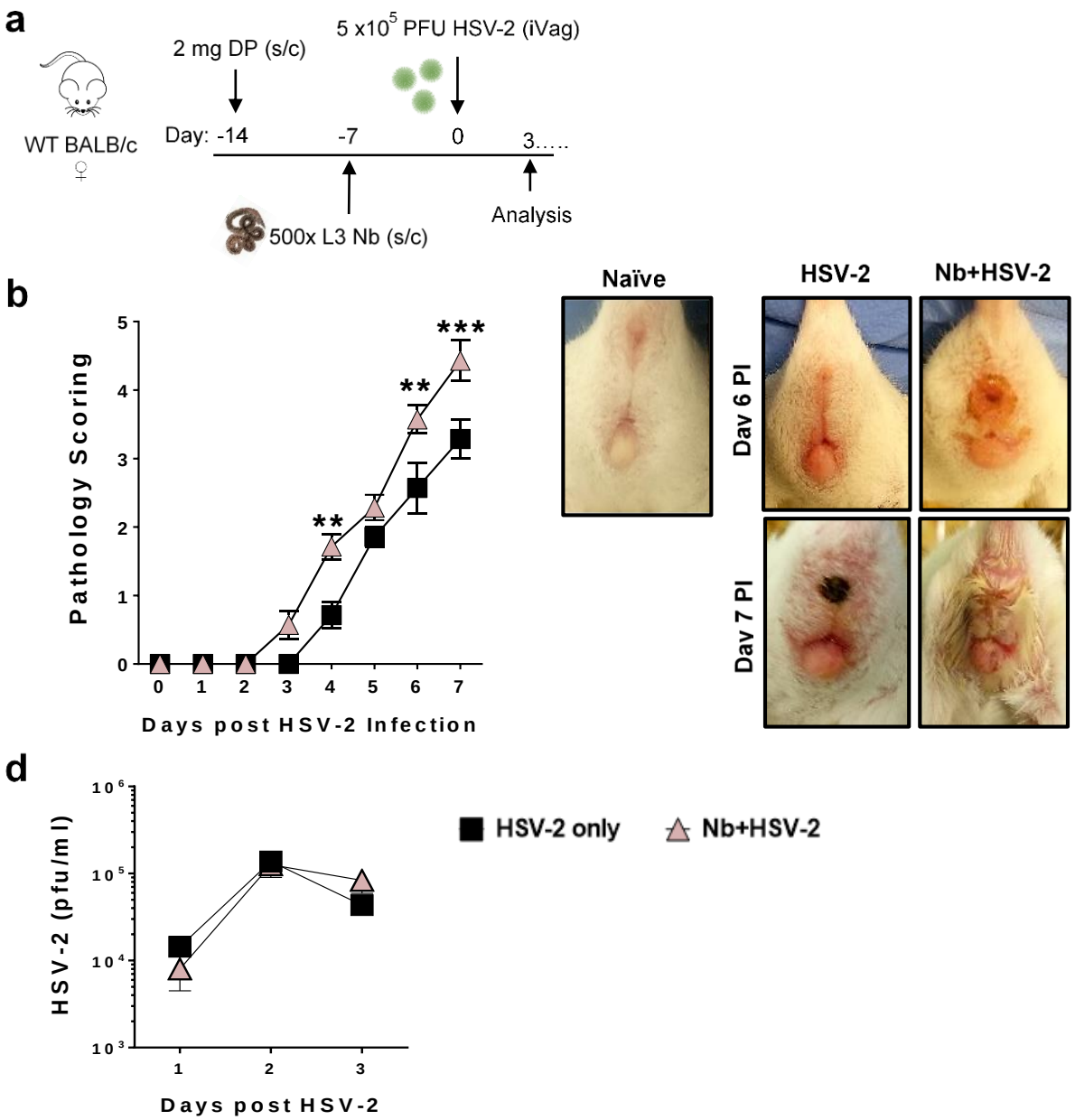


Figure 3.6.1. Prior Nb exposure results in earlier inflammation and exacerbated HSV-2 pathology: (a) Female BALB/c mice were treated with 2 mg Depo Provera® (DP) 7 days prior to subcutaneous infection with Nb. Seven days later, mice were infected intravaginally with $5x10^5$ PFU HSV-2 (strain G). **(b)** Illness progression was determined by daily pathology scoring following viral infection. **(c)** Viral shedding (PFU/ml) was determined by plaque assay of vaginal lavages at day 1-3 post infection. Data is representative of two independent experiments with 5-6 mice per group (mean±sem). Statistical significance was calculated by two-way ANOVA with Bonferroni correction for multiple comparisons. ***$p \leq 0.001$

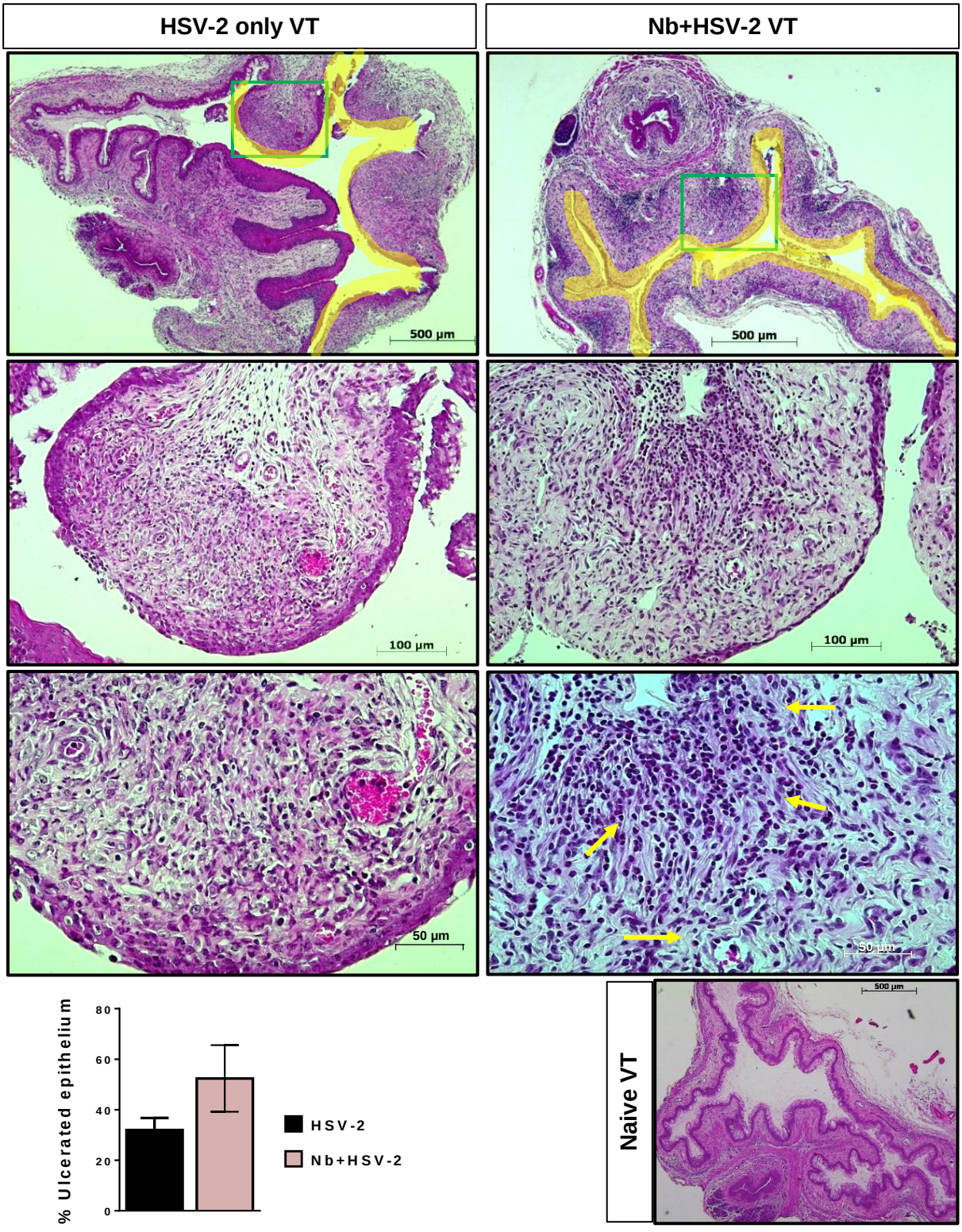


Figure 3.6.2. Histological analysis of vaginal tissue at day 6 post HSV-2 infection: Hormone-synchronised female BALB/c mice were infected with HSV-2, after Nb exposure as previously described. At day 6 post HSV-2 infection, vaginal tissue (VT) was isolated, formalin-fixed, embedded and sectioned for H&E staining. Images were taken at x50, x200 and x400 magnification. HSV-2 ulcerated epithelium is highlighted in yellow and quantified as percentage (%) of ulcerated epithelium. The green boxes indicate the section of tissue magnified. Yellow arrows indicate eosinophilic cell infiltration. Data is representative of two independent experiments with 3-4 mice per group.

3.3.4. Exacerbated HSV-2 pathology with prior Nb infection is associate with increased vaginal IL-33 and genital eosinophilia

Earlier exacerbation of HSV-2 pathology in Nb+HSV-2 mice, compared to mice infected with HSV-2 only, suggests that prior Nb infection impairs initial viral control in the FGT. Interestingly, genital eosinophils, along with 'alarmin' IL-33 responses, have been associated with exacerbated HSV-2 vaginal pathology [225]. Flow cytometry analysis of the FGT and iLN at day 3 post HSV-2 infection, revealed significantly increased proportions and numbers of genital eosinophils in coinfected mice, compared to mice infected with HSV-2 only (Figure 3.7a, e). Furthermore, we found elevated levels of IL-33 in vaginal washes of coinfected, compared to virus only mice, at day 2 post HSV-2 infection (Figure 3.7b). These results suggest that Nb-exacerbated HSV-2 inflammation and ulceration is associated with genital eosinophilia and elevated vaginal IL-33.

We also observed elevated proportions and numbers of inflammatory cells; Ly-6C^{hi} monocytes and neutrophils in the FGT and iLN of coinfected mice, compared to mice infected with virus alone (Figure 3.7c, e). We do not observe a significant difference in the FGT macrophages at day 3 post HSV-2 infection, however numbers of iLN macrophages were increased in coinfected mice, compared to virus only controls (Figure 3.7d, e). Systemically, we observed higher proportions and numbers of myeloid cells in the spleen of coinfected mice, compared to mice infected with virus alone (Supp Figure 3.8). These results suggest elevated inflammation of genital tissue in mice exposed to Nb prior to vaginal HSV-2 infection.

During nematode infection, Th2 immunity is mounted by epithelial 'alarmin' responses activating ILC2s, which release IL-5, promoting eosinophil recruitment [373, 479]. To investigate the induction of genital eosinophilia, we assessed ILC2 levels in the FGT and iLN at day 3 post HSV-2 infection. ILC2s were defined by their lack of cell surface lineage markers and expression of IL-7R, icos and IL-33R (Lin-IL-7Rα^{+}icos^{+}ST2^{+}) [657]. As reported previously, genital ILC2s constitute a very small proportion of total FGT cells [225], however preliminary analysis revealed significantly elevated proportions and numbers of ILC2s in the FGT and iLN of coinfected mice, compared to mice infected with virus only (Figure 3.8a, b). Additionally, Luminex analysis of vaginal lavages revealed increased IL-5 levels in Nb+HSV-2 mice, compared to virus only at day 2 post HSV-2 infection (Figure 3.8c). Collectively, this suggests that prior Nb exposure results in elevated levels of genital ILC2s during subsequent HSV-2 infection, which may promote genital eosinophilia through IL-5 production. Additional confirmation is needed to determine whether genital ILC2s mediate genital eosinophilia during nematode exposure.

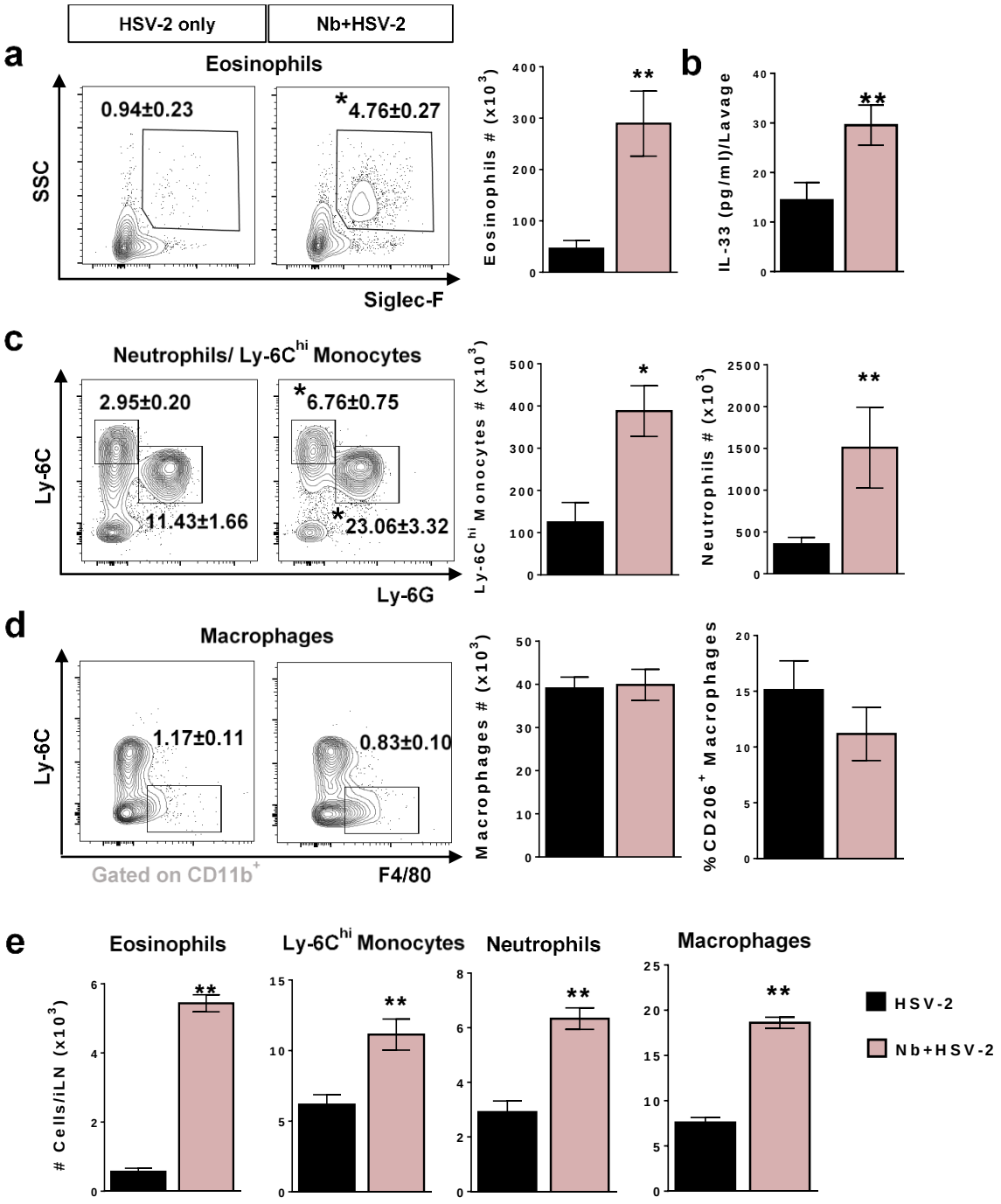


Figure 3.7. Prior Nb exposure increases myeloid cell inflammation in genital tissue, following HSV-2 infection: At day 3 post HSV-2 infection, FGT myeloid cell populations were analysed by flow cytometry. Representative flow plots show mean±sem of cell proportions. **(a)** Proportion and number of FGT eosinophils ($CD11b^+Siglec\text{-}F^+SSC^{hi}$). **(b)** Vaginal levels of 'alarmin' IL-33, measured in day 2 lavages by ELISA. Proportion and number of **(c)** Ly-$6C^{hi}$ monocytes ($CD11b^+Ly\text{-}6C^{hi}$), neutrophils ($CD11b^+Ly\text{-}6G^+Ly\text{-}6C^+$), **(d)** macrophage proportions and numbers ($CD11b^+F480^+$), and percentage of $CD206^+$ (M2-like) macrophages in the FGT of virus only and coinfected mice at day 3 post HSV-2 infection. **(e)** Number of myeloid cells in the iLN of coinfected or virus only mice at day 3 post HSV-2 infection. Data is representative of two independent experiments with 5-6 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$. **$p \leq 0.01$

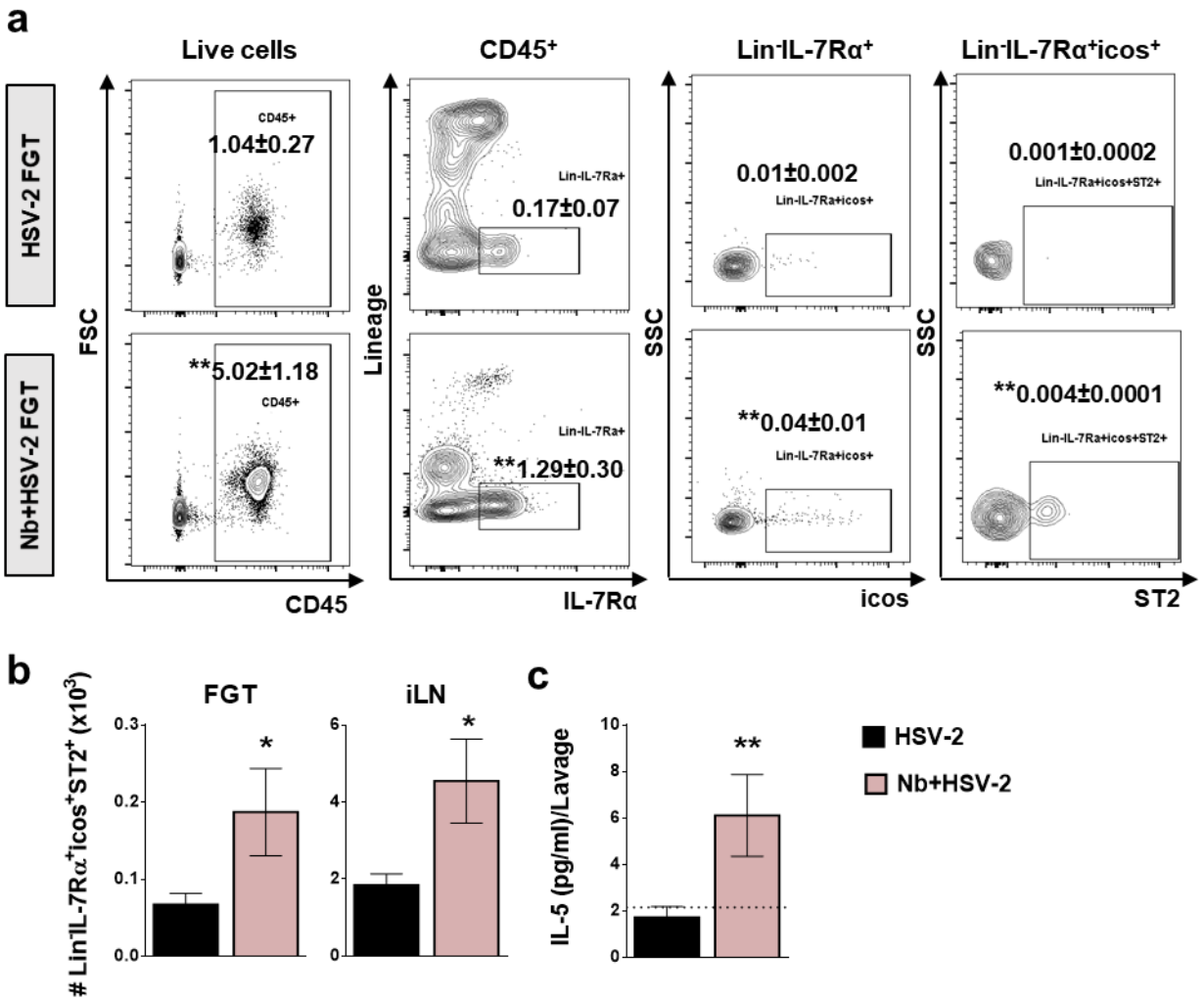


Figure 3.8. Increased ILC2s in genital tissue with prior nematode infection: At day 3 post HSV-2 infection, innate lymphoid cells were analysed in the FGT and iLN of virus only and coinfected mice, by flow cytometry. **(a)** Proportion (mean±sem) of $CD45^+$ cells, Lineage $(Lin)^-$ $IL\text{-}7R\alpha^+$, $Lin\text{-}IL\text{-}7R\alpha^+icos^+$ and $Lin\text{-}IL\text{-}7R\alpha^+icos^+ST2^+$ cells in the FGT of HSV-2 only and Nb+HSV-2 infected mice. **(b)** Number of $Lin\text{-}IL\text{-}7R\alpha^+icos^+ST2^+$ cells (ILC2s) in the FGT and iLN of virus only and coinfected mice. **(c)** IL-5 levels in vaginal washes of HSV-2 only and Nb+HSV-2 mice, determined by Luminex. Dotted line represents the LLOQ for the cytokine. ILC2 data is representative one experiment with 4-5 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired. Luminex data is representative of two independent experiments with 4-5 mice per group (mean±sem), run on one Luminex plate. Statistical significance was calculated by Mann Whitney t test. *p ≤ 0.05. **p ≤ 0.01

3.3.5. Exacerbation in HSV-2 genital pathology is associated with diminished early anti-viral immunity

Innate immune responses against vaginal HSV-2, namely NK cell cytotoxicity and IFN-γ production, are important for initial viral control [224, 226, 238]. Despite elevated levels of genital Ly-6C^{hi} monocytes (figure 3.7d, f), which have been described as initiators of early anti-HSV-2 immunity [226], coinfected mice displayed a significant reduction in vaginal IFN-γ levels at day 2 post HSV-2 infection, compared to mice infected with virus alone (Figure 3.9a). Accompanying this decline, we observed reduced proportions and numbers of FGT NK cells in Nb+HSV-2 mice, compared to HSV-2 only, at day 3 post viral infection (Figure 3.9b). Reduced early anti-viral immunity in the FGT may contribute to exacerbated HSV-2 pathology in coinfected mice. There were no significant differences in the proportions and numbers of iLN and splenic NK cells, between experimental groups (Figure 3.9c-d). As in mice at 9 days post Nb infection, we also found a significant reduction in IFN-γ and a trend for increased granzyme b production by splenic NK cells in coinfected mice at day 3 post HSV-2, compared to HSV-2 only mice (Figure 3.9e-f). However, this is not evident in the iLN. This data suggests that prior Nb exposure supresses protective IFN-γ responses in the vagina, during early HSV-2 infection. However, further investigation is needed to determine how nematode exposure may alter IFN-γ production in uncolonized genital tissue during HSV-2 infection.

Another important function of the innate response during vaginal HSV-2 infection, is to prime the T-cell mediated adaptive immunity that follows. Impaired innate protection may hinder adaptive responses [224-226]. We did not observe significant differences in CD4$^+$ and CD8$^+$ T cell numbers in the iLN and spleen at day 3 post HSV-2 infection between experimental groups (Supp figure 3.9a-c). As previously observed during Nb infection alone, production of IFN-γ by splenic T cells were reduced, while GZb potential was increased in coinfected mice, compared to mice infected with virus only (Supp figure 3.9d-e). No significant differences in iLN T cells were observed between experimental groups. This was expected as adaptive anti-viral T cell responses are typically evident at day 5-6 post HSV-2 infection [224, 225, 258].

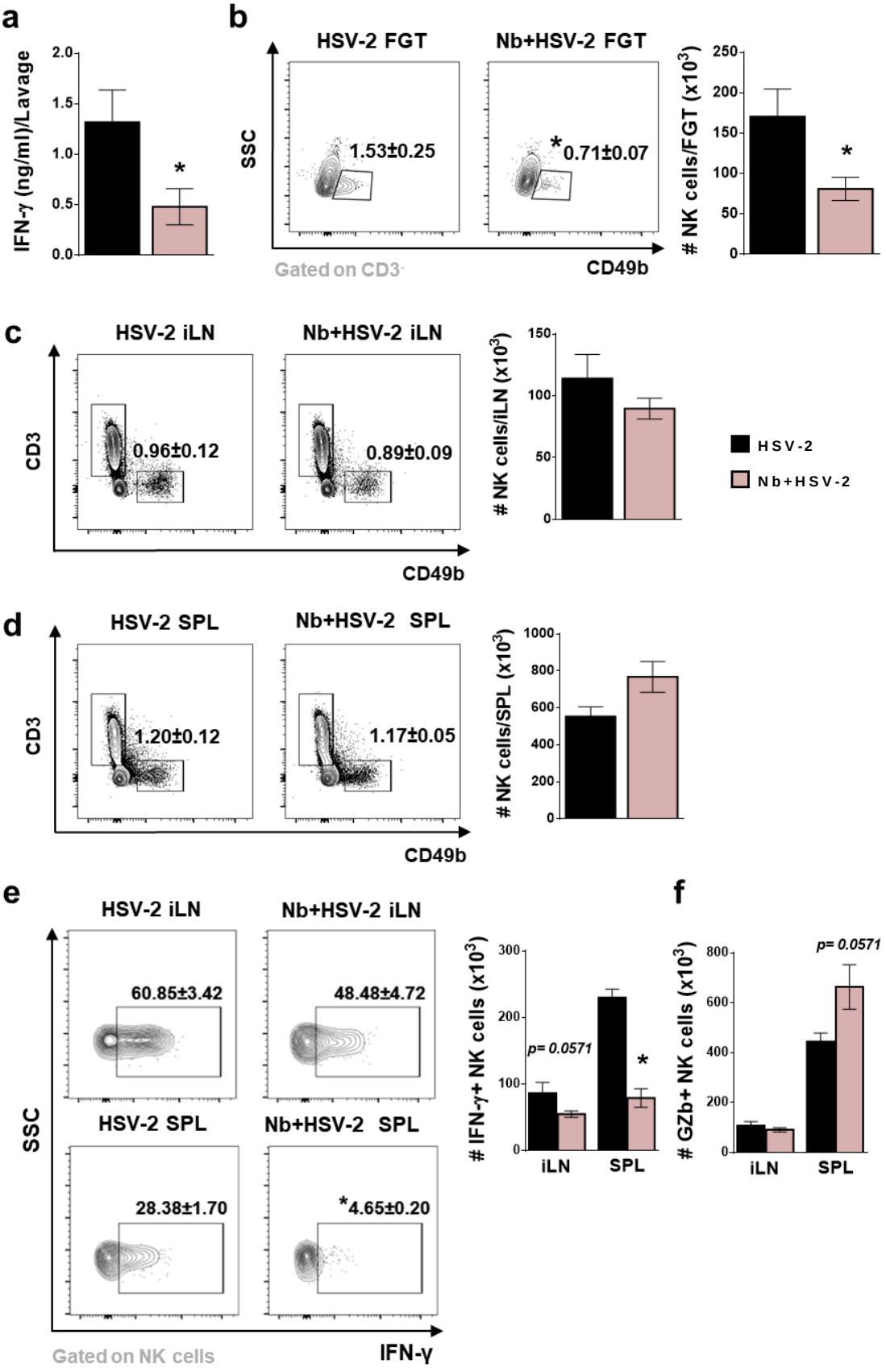

a
IFN-γ (ng/ml)/Lavage
2.0
1.5
1.0
0.5
0.0
*
b
HSV-2 FGT
Nb+HSV-2 FGT
SSC
1.53±0.25
*0.71±0.07
Gated on CD3-
CD49b
NK cells/FGT (x10³)
250
200
150
100
50
0
*
c
HSV-2 iLN
Nb+HSV-2 iLN
CD3
0.96±0.12
0.89±0.09
CD49b
NK cells/iLN (x10³)
150
100
50
0
HSV-2
Nb+HSV-2
d
HSV-2 SPL
Nb+HSV-2 SPL
CD3
1.20±0.12
1.17±0.05
CD49b
NK cells/SPL (x10³)
1000
800
600
400
200
0
e
HSV-2 iLN
Nb+HSV-2 iLN
60.85±3.42
48.48±4.72
HSV-2 SPL
Nb+HSV-2 SPL
28.38±1.70
*4.65±0.20
SSC
Gated on NK cells
IFN-γ
IFN-γ+ NK cells (x10³)
300
200
100
0
p= 0.0571
*
iLN
SPL
f
GZb+ NK cells (x10³)
800
600
400
200
0
p= 0.0571
iLN
SPL

Figure 3.9. Prior parasite exposure associated with reduced early IFN-γ responses during subsequent HSV-2 infection: (a) Lavage IFN-γ levels were determined by ELISA, at day 2 post HSV-2 infection. At day 3 post HSV-2 infection, FGT, iLN and spleen (SPL) cells were mitogen-stimulated and analysed by flow cytometry. Representative flow plots present proportions (mean±sem) of $CD45^{+}CD3^{-}CD49b^{+}$ NK cells. Proportion and number of **(b)** FGT, **(c)** iLN and **(d)** SPL NK cells, following HSV-2 only or Nb+HSV-2 infection. **(e)** Percentage and number of IFN-γ^{+} NK cells in the iLN and SPL. **(f)** Number of GZb^{+} NK cells. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired (b-d) or the parent population (e). Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$, ns – not significant

3.3.6. Nematode-induced exacerbation of genital HSV-2 is associated with eosinophilia in an IL-4Rα-independent manner

Anti-helminth Th2 immunity is mediated through IL-4/13 signalling via IL-4Rα, with delayed Nb expulsion observed in IL-4Rα$^{-/-}$ mice [396, 401, 403, 492]. To determine whether Nb-induced exacerbation of HSV-2 pathology and reduced anti-viral immunity is dependent on IL-4Rα signalling, we infected hormone-synchronised WT and IL-4Rα$^{-/-}$ mice with HSV-2, a week after Nb infection. We monitored daily illness progression and like previously, we observed exacerbated viral pathology in WT coinfected mice, compared to WT virus-only controls. Surprisingly, IL-4Rα$^{-/-}$ HSV-2 mice displayed significantly reduced pathology compared to all other experimental groups (Figure 3.10.1a). The significant protection we observed in IL-4Rα$^{-/-}$ mice infected with HSV-2 will be elaborated in Chapter 4. Interestingly, we observed similar genital pathology in IL-4Rα$^{-/-}$ coinfected mice, as in WT counterparts. Histological analysis of vaginal tissue at day 6 post HSV-2 infection confirms increased epithelial ulceration with prior Nb infection, in WT and IL-4Rα$^{-/-}$ mice, compared to HSV-2 only controls (Figure 3.10.1b). This suggests that protection in the absence of IL-4Rα signalling is lost with prior Nb infection and Nb-exacerbated HSV-2 pathology and epithelial ulceration is IL-4Rα-independent.

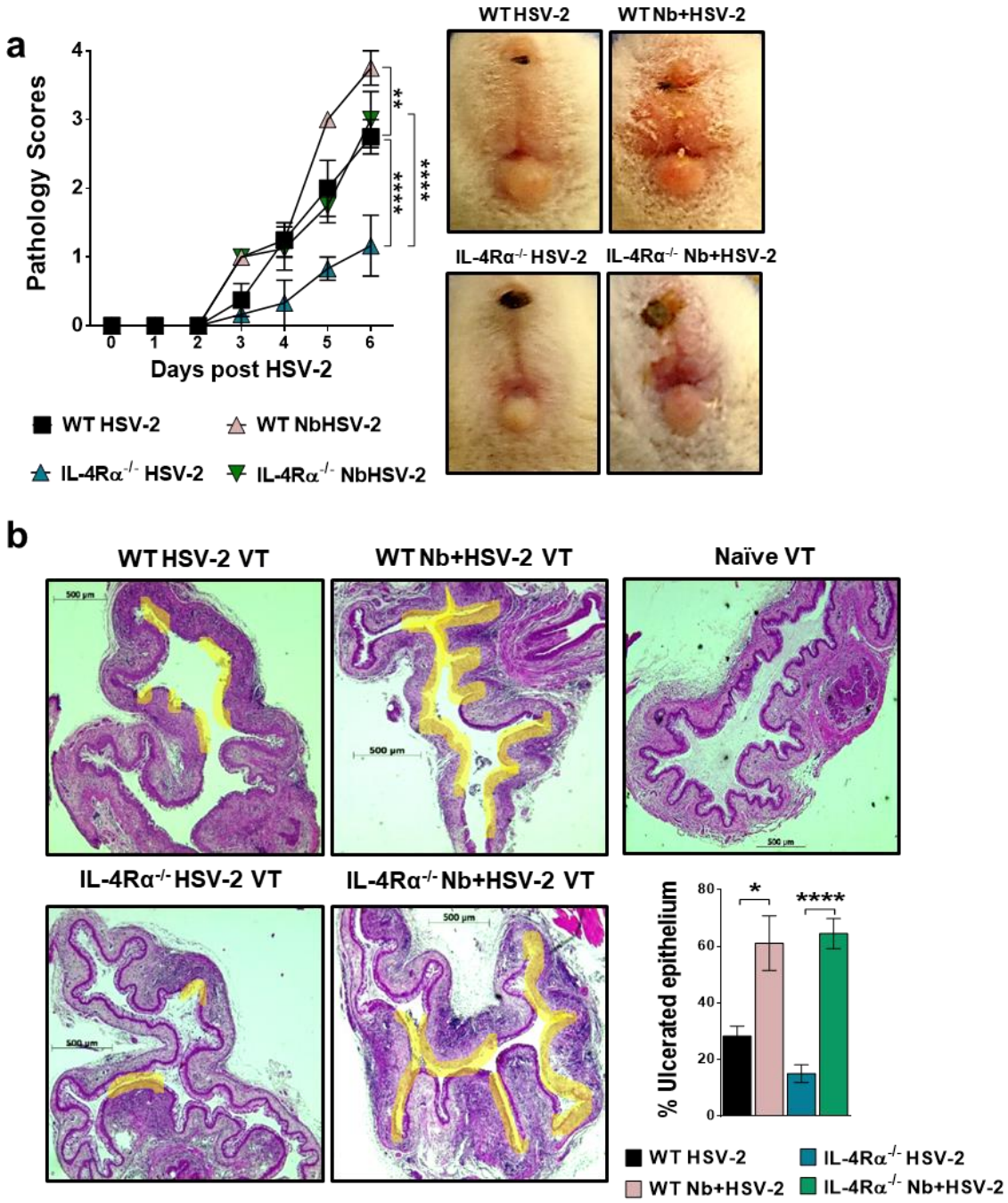


Figure 3.10.1. Nb-exacerbated HSV-2 pathology is IL-4Rα-independent: Hormone-synchronised WT and IL-4Rα$^{-/-}$ mice were intravaginally infected with HSV-2 following Nb exposure as previously described. **(a)** HSV-2 progression was determined by daily pathology scoring following viral infection. At day 6 post infection, vaginal tissue (VT) was isolated. Samples were formalin-fixed, embedded and sectioned for histological analysis. **(b)** Representative H&E stained sections, displaying ulceration and inflammation of vaginal tissue in WT and IL-4Rα$^{-/-}$ mice. Images were taken at x50 magnification. HSV-2 ulcerated vaginal epithelium is highlighted in yellow and quantified as percentage (%) of ulcerated epithelium. Data is representative of two independent experiments with 3-6 mice per group (mean±sem). Statistical significance was calculated by two-way ANOVA with Bonferroni correction for multiple comparisons and Mann Whitney t test. $^{**}p \leq 0.01$, $^{****}p \leq 0.0001$.

Further histological analysis of vaginal tissue at day 6 post HSV-2 infection, suggests increased infiltration of PMN cells, with eosinophilic cytoplasm, into the vaginal submucosa of coinfected WT and IL-4Rα$^{-/-}$ mice, compared to virus only counterparts (Figure 3.10.2a). As previously discussed, IL-5 is an important regulator of eosinophil development, activation and survival [426, 658-660]. During Th2 immunity, increased levels IL-4 potentiates IL-5 production and enhances chemotaxis of eosinophils [661-663]. Previous studies have demonstrated reduced lung eosinophilia in the absence of IL-4Rα signalling, during allergic airway inflammation [664, 665], respiratory viral [666, 667] and parasite [492, 668] infection. In contrast, flow cytometry analysis of genital tissue at day 3 post HSV-2 infection, confirmed that heightened HSV-2 pathology in coinfected WT and IL-4Rα$^{-/-}$ mice, was accompanied by significant FGT eosinophilia, compared to virus only counterparts (Figure 3.10.2b). Moreover, cytokine analysis by Luminex at day 2 post HSV-2 infection, revealed increased levels of genital IL-5 in coinfected WT and IL-4Rα$^{-/-}$ mice, compared to virus-only counterparts (Figure 3.10.2c). This suggests that elevated genital IL-5 and eosinophilia with prior Nb exposure, is IL-4Rα-independent. Additionally, we found significantly reduced vaginal levels of IFN-γ in WT and IL-4Rα$^{-/-}$ coinfected mice, compared to virus only controls at day 2 post infection (Figure 3.10.2d). However, IL-4Rα$^{-/-}$ coinfected mice had equivalent numbers of genital NK cells as virus only counterparts (Figure 3.10.2e). These results suggest that impaired HSV-2 immunity with prior Nb exposure, is not mediated through IL-4Rα signalling, but associated with FGT eosinophilia.

We previously observed increased vaginal ‘alarmin’ IL-33, ILC2s and IL-5 in WT coinfected mice, compared to HSV-2 only controls (Figure 3.7b, 3.8b). We hypothesize that the FGT eosinophilia observed with prior Nb infection, in both WT and IL-4Rα-/- mice, is downstream of IL-33 signalling. Oh et al., 2017 reported the induction of Th2 immunity in the genital tract by intravaginal administration of protease allergen papain, which drives vaginal epithelial IL-33 responses [669]. To confirm whether eosinophilia is induced by IL-33, in the absence of IL-4Rα signalling, we intravaginally treated WT and IL-4Rα$^{-/-}$ mice with papain and assessed eosinophil infiltration into the FGT by flow cytometry. Following papain treatment, we observed significant eosinophilia in the FGT of both WT and IL-4Rα$^{-/-}$ mice, compared to untreated controls (Figure 3.10.2f). This demonstrates that IL-33-induced eosinophilia in the FGT is independent of IL-4Rα signalling.

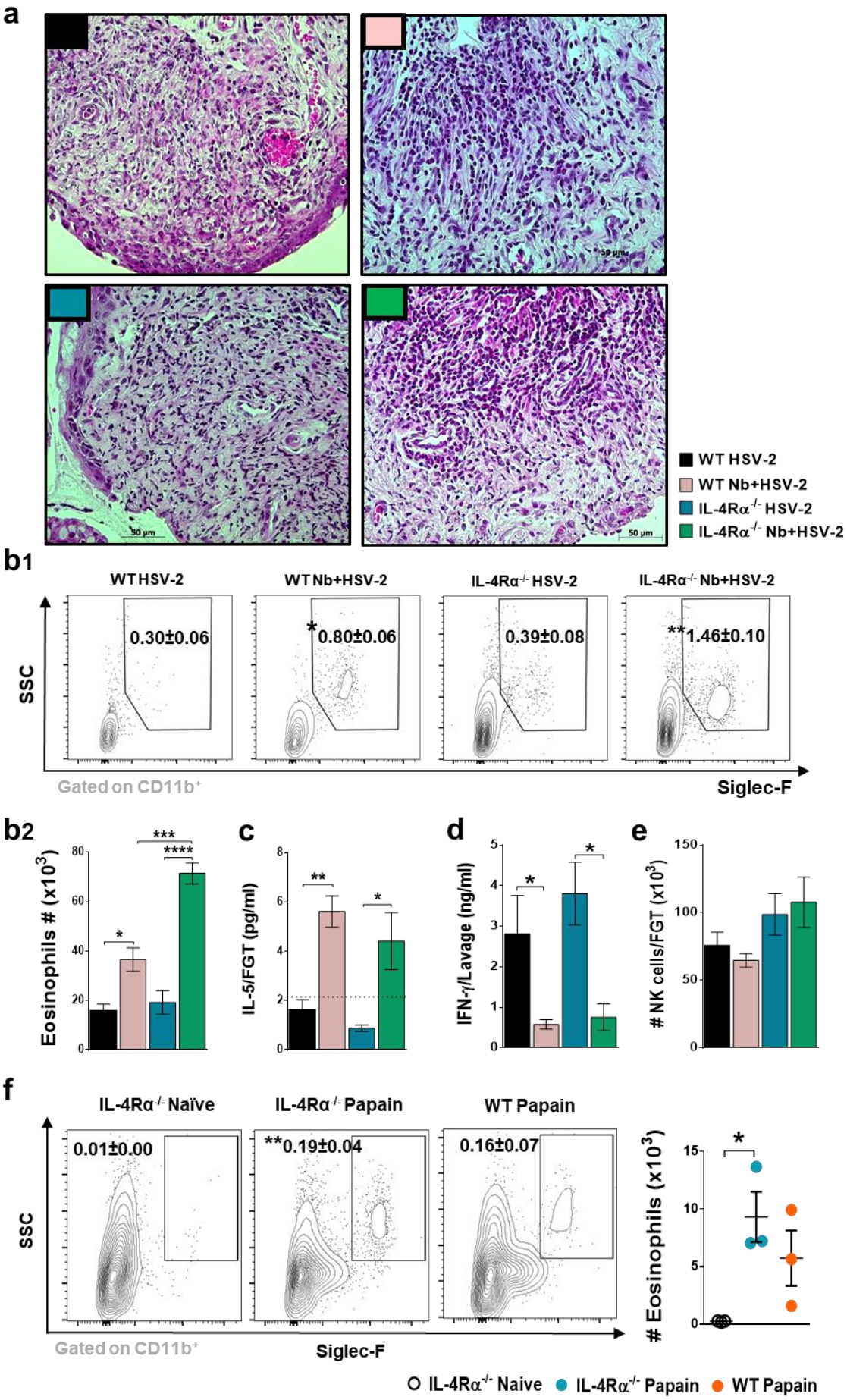

a
WT HSV-2
WT Nb+HSV-2
IL-4Rα-/- HSV-2
IL-4Rα-/- Nb+HSV-2
50 µm
50 µm
b1
WT HSV-2
WT Nb+HSV-2
IL-4Rα-/- HSV-2
IL-4Rα-/- Nb+HSV-2
0.30±0.06
*0.80±0.06
0.39±0.08
**1.46±0.10
SSC
Gated on CD11b+
Siglec-F
b2
Eosinophils # (x10^3)
c
IL-5/FGT (pg/ml)
d
IFN-γ/Lavage (ng/ml)
e
NK cells/FGT (x10^3)
f
IL-4Rα-/- Naïve
IL-4Rα-/- Papain
WT Papain
0.01±0.00
**0.19±0.04
0.16±0.07
SSC
Gated on CD11b+
Siglec-F
Eosinophils (x10^3)
IL-4Rα-/- Naive
IL-4Rα-/- Papain
WT Papain

Figure 3.10.2. IL-4Rα-independent eosinophilia in vaginal tissue of coinfected mice: (a) Representative H&E stained sections of ulcerated vaginal tissue at day 6 post HSV-2 infection. Images were taken at x400 magnification. Images are representative of two independent experiments with 3-4 mice per group. At day 3 post HSV-2 infection, FGT cell populations in WT and IL-4Rα$^{-/-}$ mice, were analysed by flow cytometry. **(b1)** Proportions and **(b2)** numbers of eosinophils (CD11b^{+}Siglec-F^{+}SSC^{hi}) in the FGT of WT and IL-4Rα$^{-/-}$ coinfected mice, compared to HSV-2 only controls. **(c)** Levels of IL-5 in FGT homogenates, determined by Luminex. Dotted line represents the LLOQ for the cytokine. **(d)** Vaginal lavage levels of IFN-γ at day 2 post HSV-2 infection, were determined by ELISA. **(e)** FGT NK cell numbers at day 3 post HSV-2 infection. Data is representative of one experiment with 3-6 mice per group (mean±sem). **(f)** Protease-induced FGT eosinophilia, in the absence of IL-4Rα signalling: Hormone-synchronised WT and IL-4Rα$^{-/-}$ mice were inoculated with 20 µg papain for three days, under deep anaesthesia. On the fourth day, mice were killed and the FGT was analysed by flow cytometry. Proportions and numbers of eosinophils, defined as CD11b^{+}Siglec-F^{+}SSC^{hi} are presented. Data is representative of one experiment with 3 mice per group (mean±sem). Cell proportions presented on flow plots were out of single cells acquired. Statistical significance was calculated by two-way ANOVA with Bonferroni correction for multiple comparisons and Mann Whitney t test. *$p \leq 0.05$, ***$p \leq 0.001$, ****$p \leq 0.0001$

3.3.7. Eosinophil depletion alleviates Nb-exacerbated HSV-2 pathology

To determine whether genital eosinophils drive exacerbated HSV-2 pathology we observed with prior Nb infection, we depleted eosinophils by intraperitoneal treatment with α-Siglec-F antibody (Figure 3.11a). α-Siglec-F treatment resulted in a significant reduction of eosinophils in the FGT, iLN, spleen and lung, compared to mice treated with the isotype control (Figure 3.11c-d). This demonstrates that α-Siglec-F treatment successfully depletes nematode-induced eosinophils, systemically. Interestingly, preliminary analysis revealed a trend for reduced vaginal IL-33 levels, numbers of Ly-6C^{hi} monocytes and neutrophils in Nb infected mice treated with α-Siglec-F, compared to isotype control (Figure 3.11b).

During HSV-2 infection, α-Siglec-F depletion of eosinophils in coinfected mice resulted in significantly reduced HSV-2 pathology from day 4 post infection, compared to isotype treated controls (Figure 3.12a, b). Histological analysis of vaginal tissue at day 6 post HSV-2 infection, suggests reduced inflammation and ulcerated epithelium in eosinophil-depleted coinfected mice, compared to isotype controls (Figure 3.12c). These results suggest that exacerbation of HSV-2 pathology with prior Nb infection, is mediated by Nb-induced genital eosinophils. Interestingly, α-Siglec-F treated coinfected mice, had significantly reduced IL-33 and elevated IFN-γ levels in vaginal lavages at day 2 post infection, compared to isotype controls (Figure 3.12d-e). Reduced IL-33 with eosinophil depletion suggests that Nb-induced eosinophils may perpetuate IL-33 release from vaginal epithelium, likely by mediating genital tissue damage. Further investigation is needed to determine how nematode-induced genital eosinophils are exacerbating subsequent HSV-2 pathology.

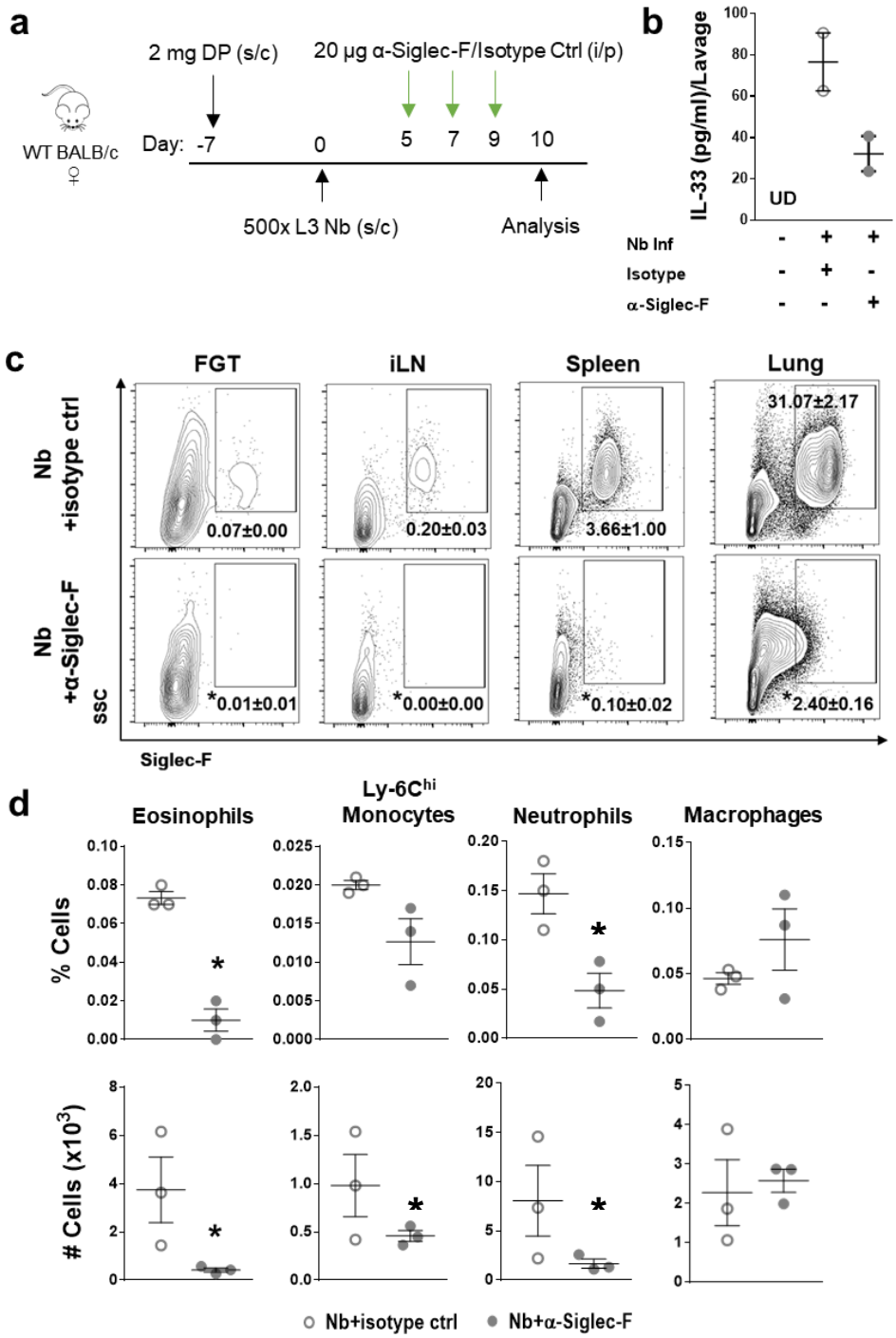


Figure 3.8. Anti-Siglec-F antibody administration depletes tissue resident eosinophils: **(a)** Hormone synchronised mice were infected with Nb as previously, then injected with 20 µg α-Siglec-F or isotype control, on day 5, 7 and 9 post infection. **(b)** Levels of alarmin IL-33 in vaginal lavages in Nb infected mice, treated with anti-Siglec-F or isotype control. **(c)** Representative flow plots showing frequency (mean±sem) of $CD11b^{+}Siglec\text{-}F^{+}$ eosinophils in the FGT, iLN, spleen and lung, following antibody depletion. **(d)** Frequencies and numbers of FGT myeloid cells after eosinophil depletion. Data is representative of one experiment with 3 mice per group (mean±sem).

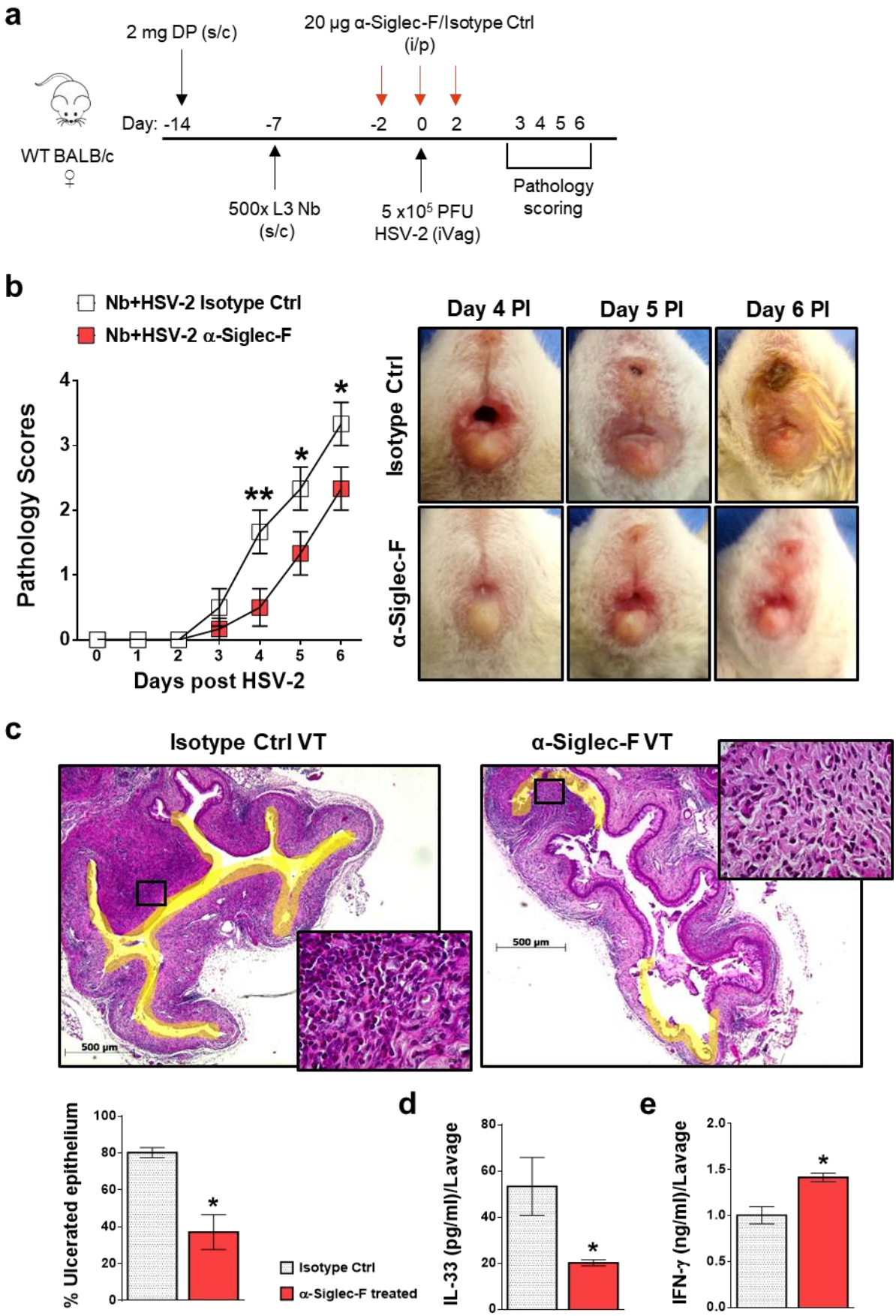

a
2 mg DP (s/c)
20 µg α-Siglec-F/Isotype Ctrl (i/p)
Day: -14 -7 -2 0 2 3 4 5 6
WT BALB/c ♀
500x L3 Nb (s/c)
5 x10⁵ PFU HSV-2 (iVag)
Pathology scoring
b
Nb+HSV-2 Isotype Ctrl
Nb+HSV-2 α-Siglec-F
Pathology Scores
Days post HSV-2
Day 4 PI
Day 5 PI
Day 6 PI
Isotype Ctrl
α-Siglec-F
c
Isotype Ctrl VT
α-Siglec-F VT
500 µm
% Ulcerated epithelium
Isotype Ctrl
α-Siglec-F treated
d
IL-33 (pg/ml)/Lavage
e
IFN-γ (ng/ml)/Lavage

Figure 3.12. Depletion of eosinophils in rescues HSV-2 pathology in coinfected mice: (a) Depo Provera (DP)-treated WT mice were infected with 500x L3 Nb subcutaneously and treated intraperitoneally (i/p) with 20 µg α-Siglec-F or isotype Ctrl antibody at day 5, 7 and 9 post Nb infection. One week following Nb infection, mice were intravaginally infected with $5x10^5$ PFU HSV-2. **(b)** Viral progression was determined by daily pathology scoring **(c)** Representative sections of H&E stained vaginal tissue (VT) at day 6 post HSV-2 infection. Images were taken at x50 and x1000 magnification. Black boxes indicate magnified sections. Ulcerated vaginal epithelium is highlighted in yellow and quantified as percentage (%) of ulcerated epithelium. At day 2 post HSV-2 infection, vaginal lavage levels of **(d)** IL-33 and **(e)** IFN-γ were determined by ELISA. Data is representative of one experiment with 3 mice per group (mean±sem). Statistical significance was calculated by two-way ANOVA with Bonferroni correction for multiple comparisons and Mann Whitney t test. *$p \leq 0.05$, **$p \leq 0.01$

3.4. Discussion

STH endemic regions have a high prevalence of sexually transmitted viruses, such as HSV-2, which makes coinfection likely [200, 357, 561, 567]. In this chapter, we demonstrate the systemic influence of nematode infection in non-colonized female genital tissue *in vivo*. Gravitt *et al*., 2016 previously described a Th2 cytokine profile in cervical fluids of STH-infected women [567]. In support, we show that Nb infection induced canonical Th2 immunity in the FGT, namely elevated tissue IL-4 and IL-5, as well as significant, long-lasting genital eosinophilia. It is known that genital schistosomiasis results in parasite eggs encased by eosinophil-rich granulomas in the cervix and/or vagina of afflicted women [564, 621, 622], however, to the best of our knowledge, nematode-associated genital eosinophilia has not been reported previously. In colonized tissue, eosinophilia is mediated by type 2 cytokine IL-5, produced by ILC2s and Th2 cells [373, 425, 426, 429, 659, 660]. Similarly, elevated genital IL-5 likely mediates eosinophilia in the FGT. Further study is needed to determine how Nb infection alone induces type 2 immunity in uncolonized genital tissue.

Migration of nematode larvae through the respiratory and gastrointestinal systems causes significant local tissue damage [670-672]. In addition, Kuroda *et al*., reported increased apoptosis of intestinal epithelial cells in the presence of Nb excretory-secretory products *in vitro* [671], suggesting that Nb releases cytotoxic molecules which may contribute to host tissue disruption. Interestingly, we found increased 'death' of non-haematopoietic cells in uncolonized genital tissue following nematode infection. Moreover, we observed elevated genital inflammatory cells following Nb infection. This suggests bystander tissue damage and inflammation in the FGT following nematode infection, despite no direct parasite colonisation. In colonized tissue, recruited eosinophils degranulate upon activation, releasing toxic molecules directed against tissue-resident parasites [424, 425, 428, 429]. Additionally, eosinophil-derived cytokines, such as IL-4, can potentiate anti-helminth immunity [37, 65, 484]. Yet, studies have reported that eosinophils are not essential for protection against nematode infection [425, 426, 430]. Importantly, unregulated eosinophilia, in response to parasite colonisation, can cause disease e.g. pulmonary eosinophilia and Loeffler's syndrome [673-676]. Thus, nematode-induced genital eosinophilia may have bystander effects on FGT integrity. Activated eosinophils can cause bystander tissue damage by releasing molecules such as cytotoxic ECP, EPO which generates oxidative stress [423, 677], lipid inflammatory mediators [678] and DNA-granule extracellular traps [679-682]. Eosinophil-mediated tissue disruption is associated with several conditions including atopic dermatitis [683-685], allergic airway inflammation [686-689] and lung disease [690, 691], as well as eosinophilic oesophagitis and gastroenteritis [692, 693]. Further investigation is required to determine whether nematode-associated tissue disruption is mediated by genital eosinophils.

An important feature of Th2 immunity is the induction of tissue repair following helminth-induced damage. Chen *et al*., 2012 reported the induction of AAMs and enhanced expression of IL-10, by IL-4R signalling, which contributed to repair of colonized tissue following Nb infection [488, 489]. Bouchery *et al*., 2015 demonstrated that the AAM population is maintained by IL-4/-13 producing ILC2s and $CD4^+$ T cells [481]. Here we show an increase in CD206-expressing M2-like macrophages in the FGT following nematode infection, which suggests an IL-4/-13-induction of AAMs in the genital tissue, a biological site not colonized by the parasite. Further study is needed to investigate the induction of AAMs in the FGT following nematode infection and whether it relates to Nb-associated genital tissue damage.

A surprising result was the systemic upregulation of granzyme b expression by cytotoxic cells, following Nb infection. While cytotoxic responses are important during intracellular infections, the role of cytotoxic cells and granzyme b during Th2 immunity is unclear. We hypothesise that upregulation in granzyme b expression during Nb infection, may be associated with nematode-induced tissue damage. Fujiwara *et al*., 2004 was the first to describe increased granzyme b expression, associated with intestinal epithelial damage during Nb infection [640]. Importantly, bystander cytotoxicity may damage uninfected cells, inducing pathogenic tissue injury [694]. Upregulation of granzyme b has been documented in several diseases associated with tissue damage, such as allergic Th2 inflammation [649, 695-697], Chronic Obstructive Pulmonary Disease (COPD) [698], cutaneous leishmaniasis [699], toxic shock syndrome [700, 701] and allograft rejection [702, 703]. It remains unclear whether Nb-associated upregulation of cytotoxic granzyme b is related to nematode-induced tissue damage. However, increased granzyme b expression by cytotoxic genital cells following Nb infection, further demonstrates that nematode-induced immune responses are present in uncolonized biological compartments.

Having identified nematode-associated disruption of uncolonized genital tissue, we studied the influence of prior Nb infection on subsequent vaginal HSV-2 susceptibility. As previously discussed, HSV-2 is an ulcerative virus and *in vivo* vaginal infection manifests as perianal and genital swelling, with progression to purulent lesions [220]. We observed earlier and significantly exacerbated HSV-2 pathology in mice previously infected with the parasite. Systemic influences of helminth exposure on unrelated infections has been described previously: Bobat *et al*., 2014 demonstrated impaired humoral immunity against *S. Typhimurium* with Nb coinfection [181]; and McFarlane *et al*., 2017 reported remote protection against respiratory viral infection by prior enteric *H. polygyrus* infection, mediated by microbiota-dependent systemic increases in type I IFNs [470]. Importantly, Gravitt *et al*., 2016 reported higher HPV prevalence in STH-positive women [567], the first study suggesting gastrointestinal nematodes systemically influence genital susceptibility to a viral infection. Our results demonstrate that nematode infection can systemically alter susceptibility to a genital viral infection.

With increased HSV-2 pathology in coinfected mice, we observed elevated vaginal IL-33 levels, which correlates with increased vaginal epithelial ulceration. Epithelial barrier cells release 'alarmin' IL-33 in response to tissue damage [704], which signals through ST2 receptors, inherently expressed on tissue-resident ILC2s [705]. During nematode infection, epithelial IL-33 responses drive ILC2 production of type 2 cytokines; IL-4, IL-5 and IL-13, promoting Th2 immunity [118, 368, 479]. Yasuda *et al*., 2012 reported the role of IL-33-activated ILC2s in mediating eosinophilic inflammation, via IL-5 and IL-13 production following nematode infection [706]. Here, elevated vaginal IL-33 in coinfected mice was accompanied by increased genital ILC2s, IL-5 and eosinophilia. Gasteiger *et al*., 2015 demonstrated the local expansion of tissue-resident ILC2s during acute Nb infection, followed by recruitment during chronic inflammation [707]. Additionally, Stier *et al*., 2017 reported that IL-33 responses to tissue disruption, promotes the migration of ILC2s from the bone marrow, to repopulate peripheral tissue [708]. Our results suggest that Nb infection results in the expansion of ILC2s in uncolonized genital tissue, likely in response to increased vaginal IL-33. Expansion of genital ILC2s may sustain eosinophilia through IL-5/-13 production [373, 709].

Here, we demonstrated that antibody-mediated depletion of eosinophils during coinfection was accompanied by reduced vaginal IL-33 levels, suggesting genital eosinophils may perpetuate vaginal IL-33 responses, likely by exacerbated genital tissue damage as hypothesised previously. Vicetti Miguel *et al*., 2017 showed that IL-4-producing eosinophils promoted endometrial repair during *Chlamydia trachomatis* murine infection, preventing *Chlamydia*-induced destruction of the upper genital tract [710]. In contrast, we found an IL-4Rα-independent increase in genital IL-5 and eosinophilia, associated with increased vaginal epithelial ulceration in coinfected mice. Similarly, Oh *et al*., 2016 demonstrated antibiotic-mediated vaginal dysbiosis and increased vaginal IL-33, ILC2s, IL-5 and eosinophils, associated with heightened HSV-2 pathology. Our results suggest that nematode-induced genital eosinophilia may mediate vaginal tissue disruption, exacerbating subsequent HSV-2 ulceration. Exacerbated viral pathology in coinfected mice, was accompanied by reduced IFN-γ in vaginal fluids and decreased genital NK cells, which are important mediators of early anti-viral control [238]. Few studies report the activation of NK cells during helminth infection [711-713]. Notably, McDermott *et al*., 2005 and Hepworth & Grencis., 2009 reported auxiliary IL-13 production by NK cells during nematode infection in immunodeficient mice [714, 715]. However, the role of NK cells during nematode infection in immunocompetent mice, remains unclear. Kiniwa *et al*., 2016 described IL-4-induced NK cells, with distinctive characteristics and enhanced IFN-γ production, compared to conventional NK cells [716]. In contrast, with prior IL-4-inducing Nb exposure, we found decreased numbers of NK cells in the FGT and an IL-4Rα-independent reduction in early vaginal IFN-γ responses during subsequent HSV-2 infection.

Our results suggests that prior nematode infection hinders early anti-viral immunity in the FGT, which may contribute to exacerbated HSV-2 pathology in coinfected mice. In addition to possible eosinophil-mediated genital tissue damage discussed previously, we hypothesize that nematode-induced genital eosinophilia may impair subsequent anti-viral IFN-γ responses and mediate increased vaginal HSV-2 pathology. Arnold *et al*., 2018 demonstrated the suppression of Th1 responses against *Helicobacter pylori* by gastrointestinal eosinophils, with reduced bacterial load and elevated IFN-γ responses in mice genetically-deficient or antibody-depleted for eosinophils. Interestingly, suppression of Th1 immunity was mediated by IFN-γ conditioning of eosinophils following *H. pylori* infection [717]. Here, we found increased vaginal IFN-γ, following antibody-mediated eosinophil depletion in coinfected mice, suggesting that genital eosinophils may impair early anti-viral responses. Alternatively, Oh *et al*., 2016 reported that increased vaginal IL-33, induced by oral antibiotic treatment, was associated with impaired effector T cell recruitment to vaginal tissue and reduced vaginal IFN-γ levels at day 6 post HSV-2 infection [225]. Here, we found elevated IL-33 and reduced IFN-γ levels in vaginal fluids of coinfected mice at day 2 post infection, during the initial wave of IFN-γ, mainly produced by vaginal NK cells [224, 226]. Also, reduced IL-33 correlated with elevated IFN-γ levels in eosinophil-depleted coinfected mice, at day 2 post HSV-2 infection. Whether IL-33 can impair NK cell-derived IFN-γ responses during early HSV-2 infection is unclear. Further investigation is needed to determine whether nematode-induced genital eosinophilia and/or elevated IL-33 contributes to impaired early anti-viral immunity.

In conclusion, in this chapter we have shown that nematode infection systemically induced canonical type 2 immunity in uncolonized genital tissue. Parasite-induced genital eosinophilia was associated with vaginal tissue disruption, elevated IL-33 responses and expansion of genital ILC2s and IL-5 levels, during subsequent HSV-2 infection (Figure 3.13). Additionally, we hypothesise that genital eosinophils and elevated IL-33 responses may impair early anti-viral IFN-γ responses, and together with eosinophil-mediated bystander tissue damage, exacerbates subsequent HSV-2 pathology. Additional study is needed to determine how nematode-induced genital eosinophilia impairs vaginal defences and worsens HSV-2 infection. Importantly, we demonstrate systemic influences of gastrointestinal nematode infections on genital susceptibility to viral infection. Further investigation is needed to determine whether the significant systemic effects on genital tissue we observed in mice, translates to human infections. In support, Gravitt *et al*., 2016 reported an association between STH infection and increased HPV prevalence in women, accompanied by a type 2 cytokine signature in their vaginal fluids. If STH exposure alters genital immunity and susceptibility to STIs, this would have significant clinical implications in co-endemic regions.

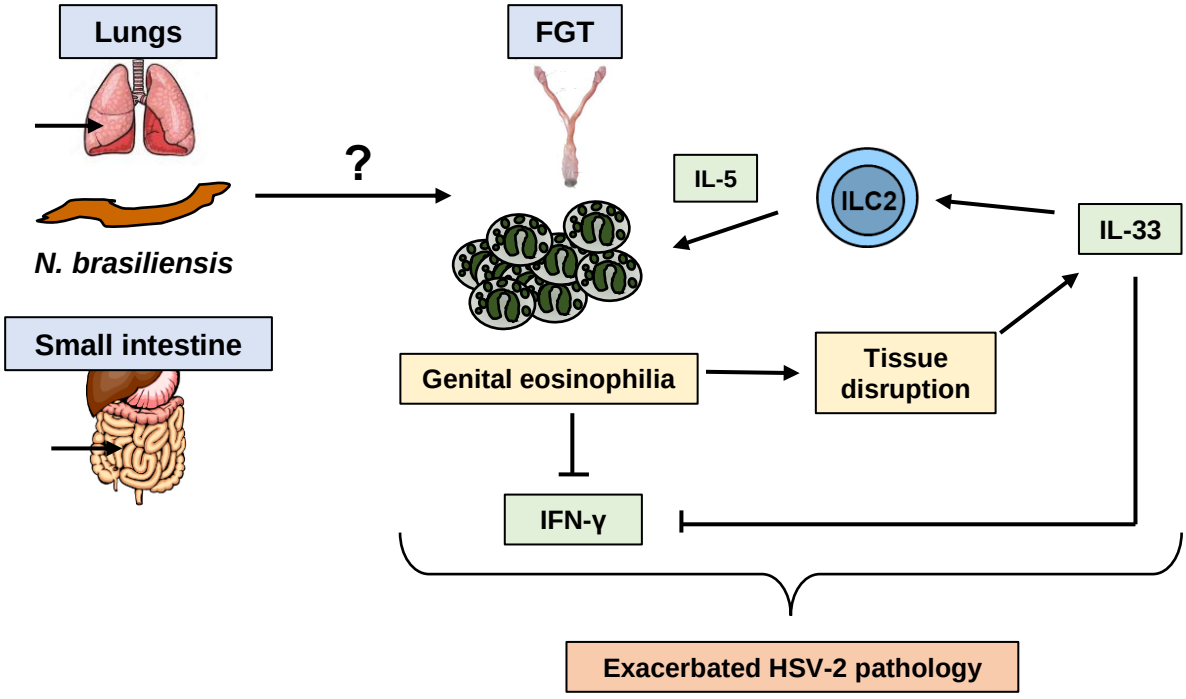
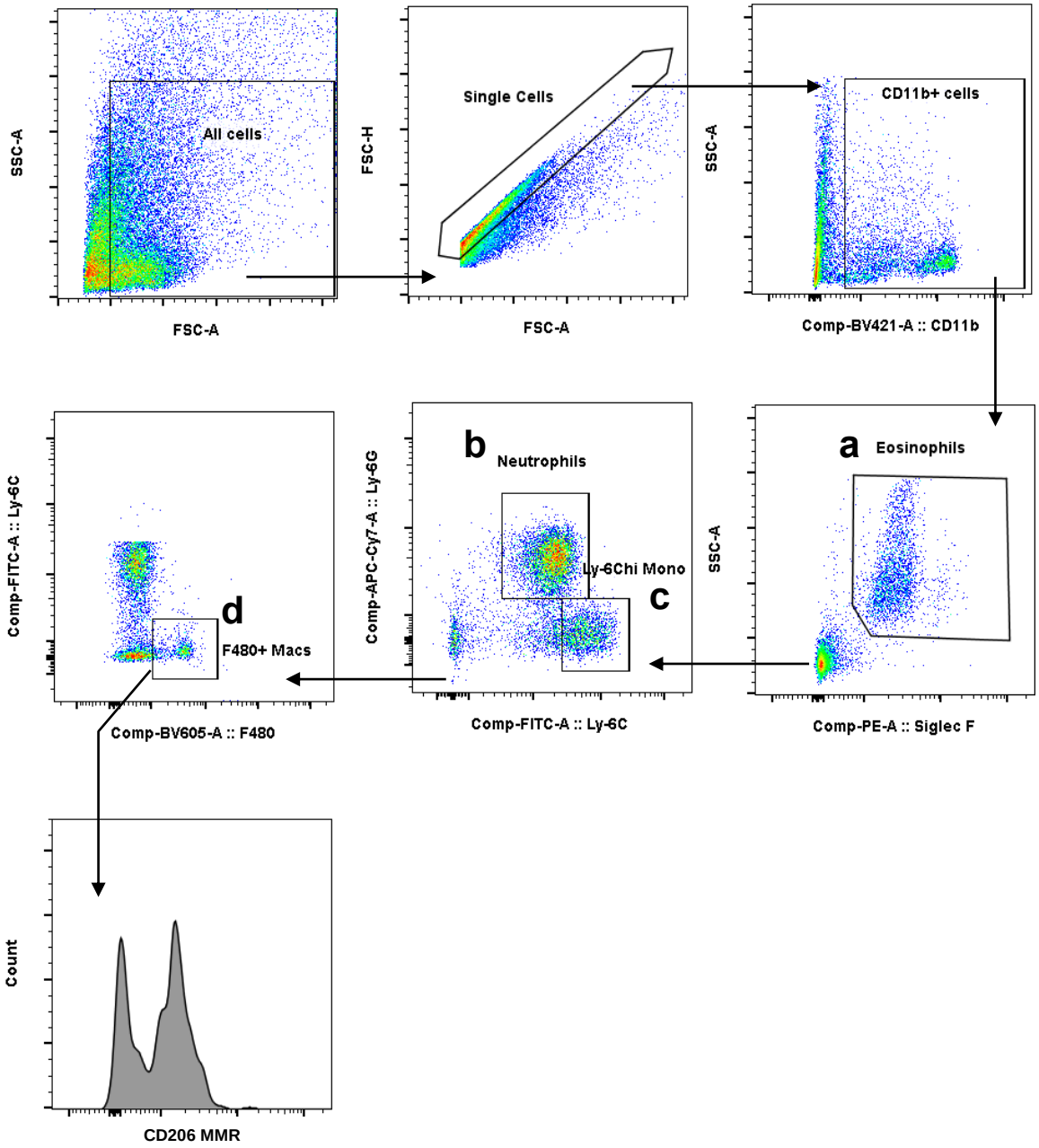
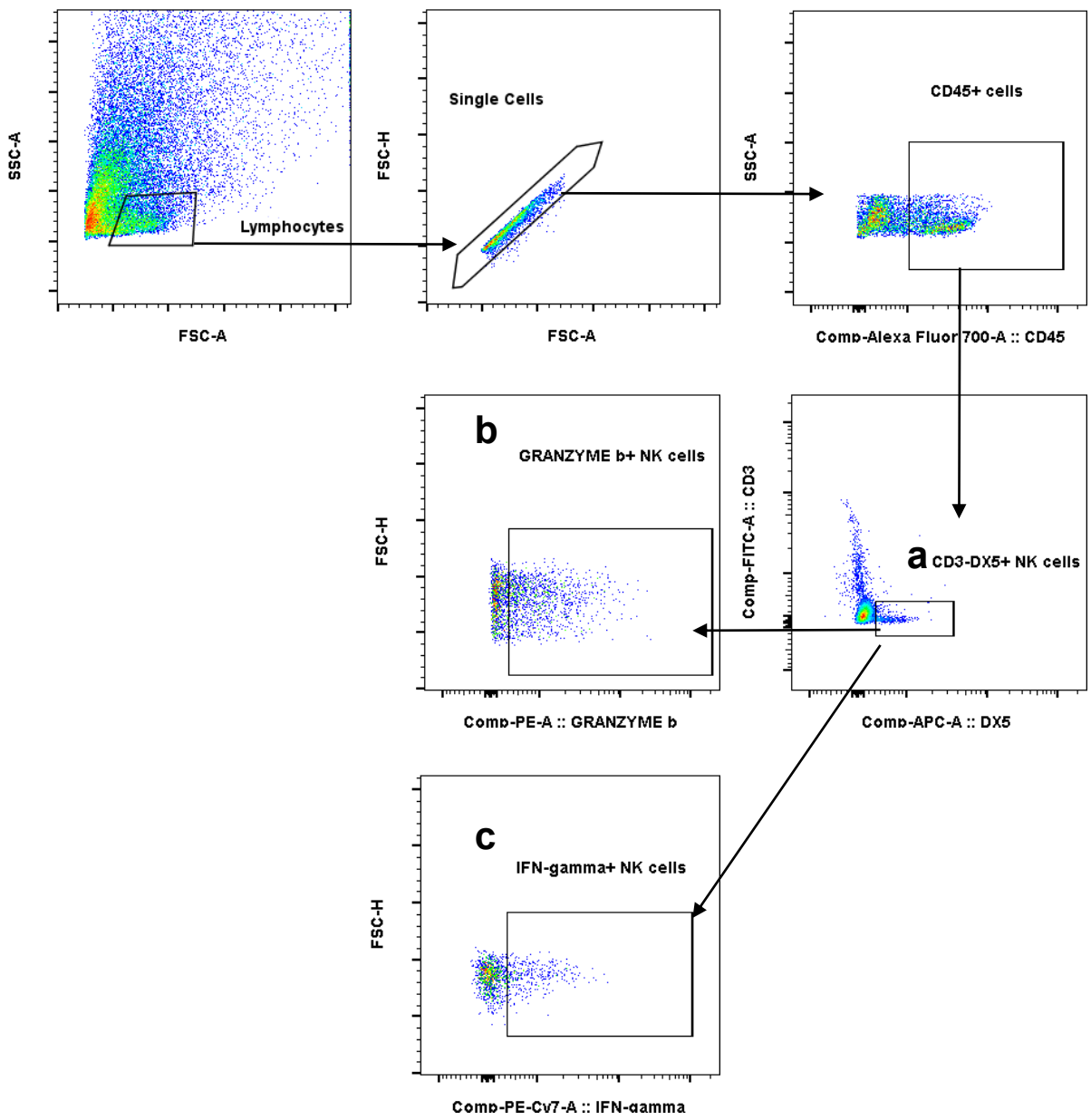


Figure 3.13. Hypothesised eosinophil-mediated tissue damage and exacerbated viral pathology during Nb+HSV-2 coinfection: Nb, which colonises the respiratory and gastrointestinal tracts, systemically induces Th2 immunity in the FGT. Nb-induced genital eosinophilia was associated with vaginal tissue disruption, elevated IL-33 responses and expansion of genital ILC2s during subsequent HSV-2 infection. We hypothesise that genital eosinophilia mediates bystander tissue damage, worsening subsequent HSV-2 pathology. Also, we hypothesise that genital eosinophils and elevated IL-33 responses may impair early anti-viral IFN-γ responses, contributing to exacerbated HSV-2 infection in the FGT.

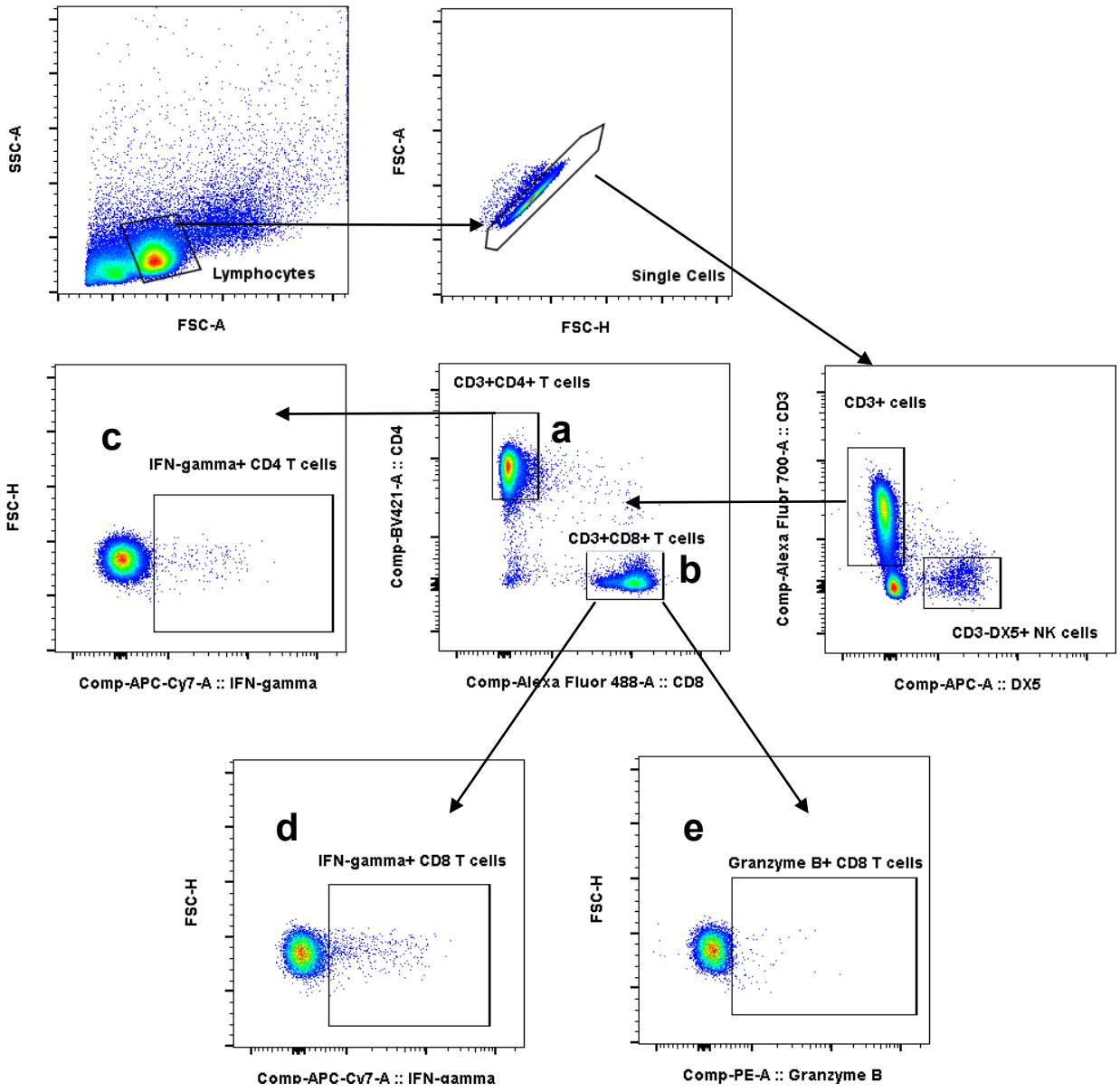
3.5. Supplementary Information



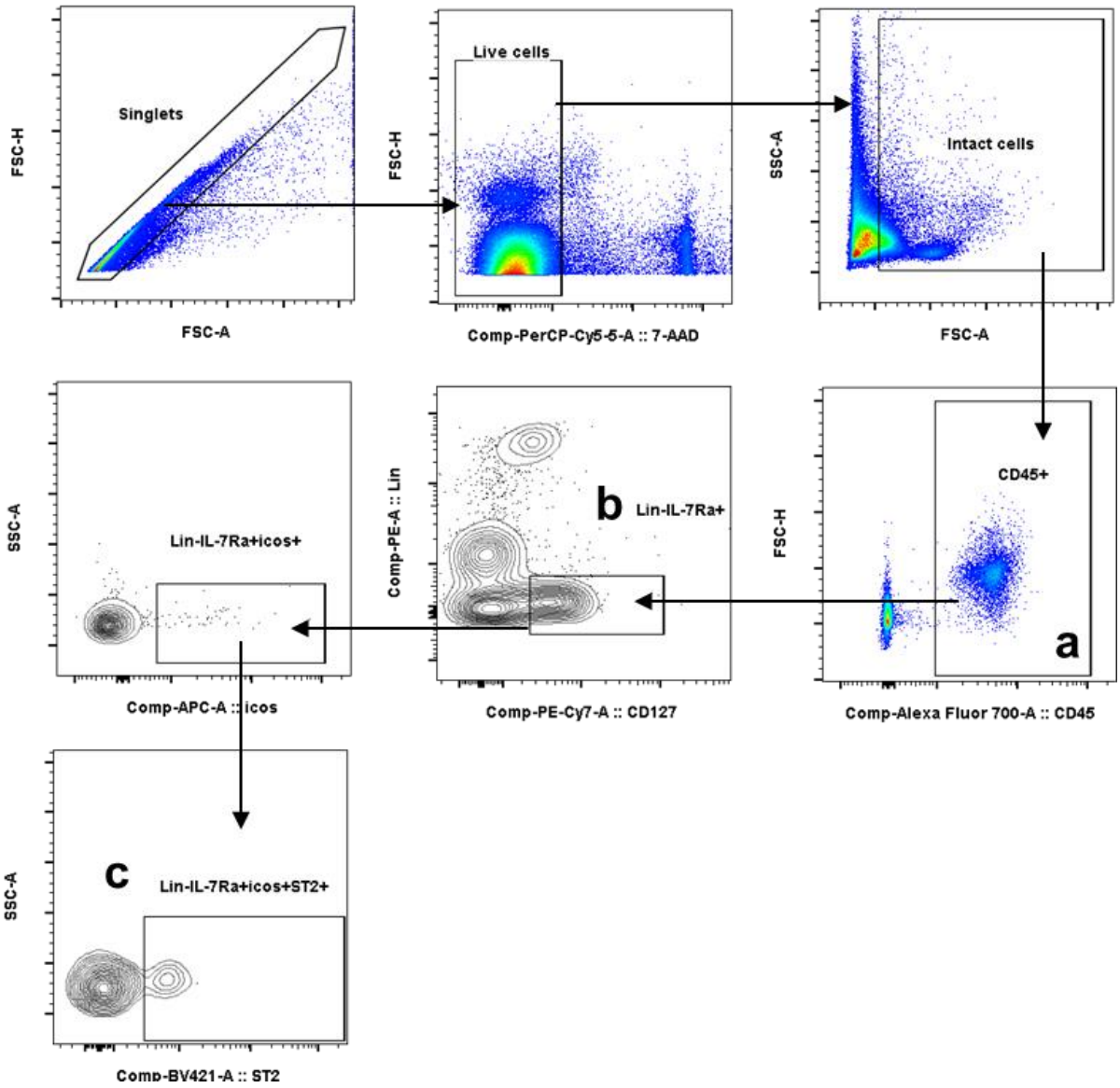
Supp Figure 3.1. FGT and Spleen myeloid cell gating strategy: At day 3 post HSV-2 infection, FGT and spleens were harvested and processed to a single cell suspension. Cells were stained with fluorochrome-conjugated antibodies and analysed by multi-colour flow cytometry. The following gating strategy was used to identify **(a)** $CD11b^{+}SigLec\text{-}F^{+}$ eosinophils, **(b)** $CD11b^{+}Ly\text{-}6G^{+}$ neutrophils, **(c)** $CD11b^{+}Ly\text{-}6C^{hi}$ monocytes and **(d)** $CD11b^{+}F4/80^{+}$ macrophages.



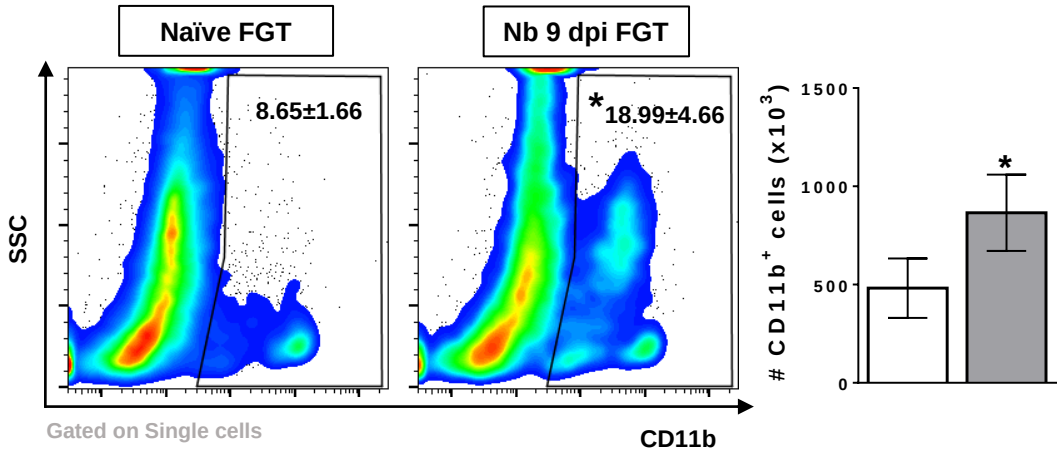
Supp Figure 3.2. FGT, iLN and Spleen NK cell gating strategy: At day 3 post HSV-2 infection, FGT, iLN and spleen were harvested and processed to a single cell suspension. Cells were stained with fluorochrome-conjugated antibodies and analysed by multi-colour flow cytometry. The following gating strategy was used to identify **(a)** $CD45^{+}CD3^{-}CD49b^{+}$ Total NK cells, **(b)** granzyme b^{+} and **(c)** IFN-γ^{+} NK cells. Positive intracellular gating was determined using 'fluorescent minus one' (FMO) controls.



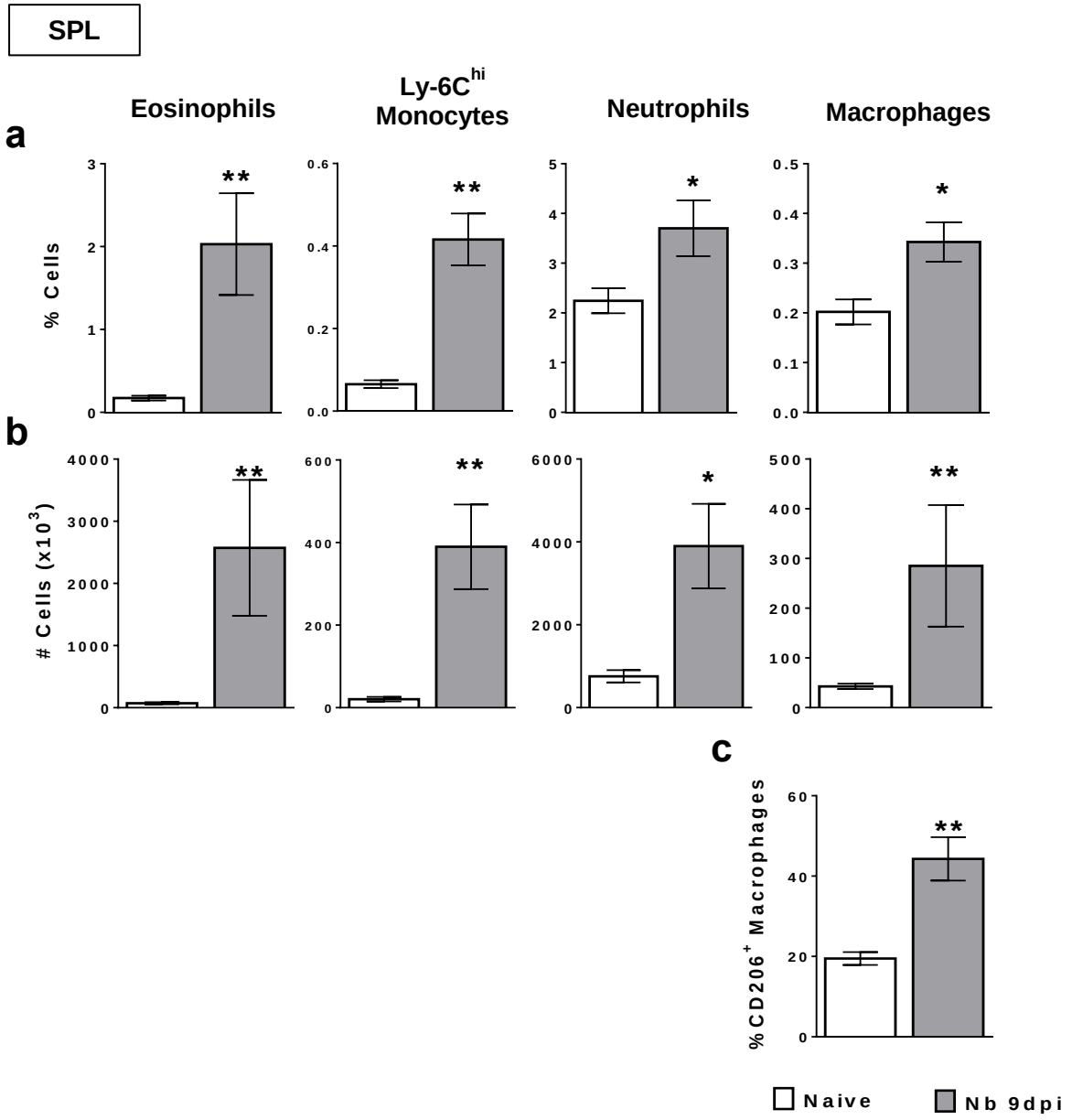
Supp Figure 3.3. iLN and Spleen T cell gating strategy: At day 3 post HSV-2 infection, iLNs and spleens were harvested and processed to a single cell suspension. Cells were stained with fluorochrome-conjugated antibodies and analysed by multi-colour flow cytometry. The following gating strategy was used to identify **(a)** $CD3^{+}CD4^{+}$ and **(b)** $CD3^{+}CD8^{+}$ T cells. Intracellular staining was used to identify **(c-d)** IFN-γ^{+} and **(e)** granzyme b^{+} T cells. Positive gates for intracellular markers were determined using 'fluorescent minus one' (FMO) controls.



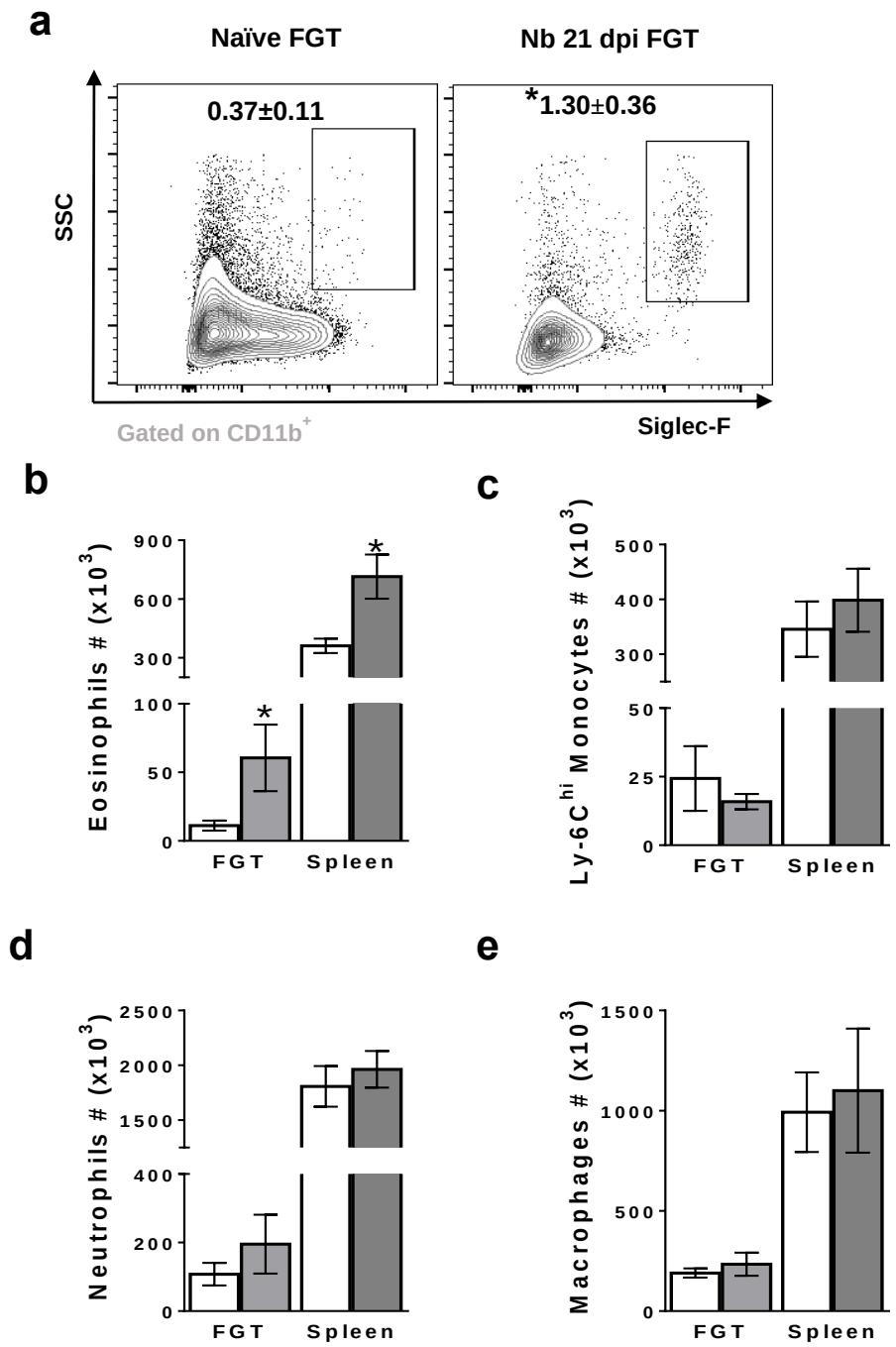
Supp Figure 3.4. ILC gating strategy for the FGT and iLN: At day 3 post HSV-2 infection, FGT and iLN were harvested and processed to a single cell suspension. Cells were stained with fluorochrome-conjugated antibodies and analysed by multi-colour flow cytometry. The following gating strategy was used to identify **(a)** $CD45^+$ cells, **(b)** Lineage (Lin; anti-mouse CD3, Ly-6G/Ly-6C, CD11b, CD45R/B220, TER-119/Erythroid cells)$^-$IL-7Rα$^+$ cells and **(c)** Lin$^-$ IL-7Rα$^+$icos$^+$ST2$^+$ ILC2s. Genital ILC2 gating is adapted from [225, 657].



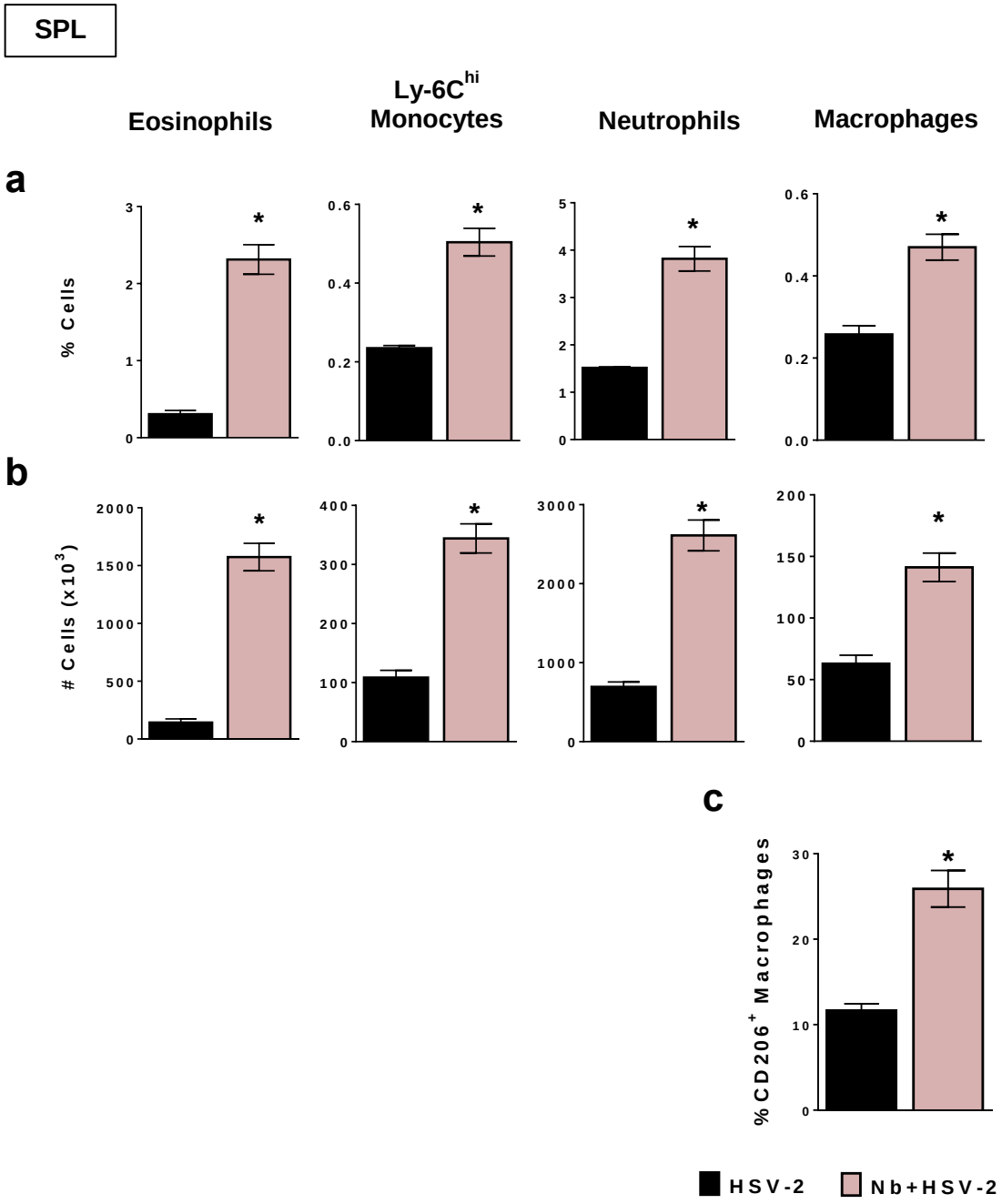
Supp Figure 3.5. FGT cell infiltration: At day 9 post Nb infection, FGT tissue was isolated and processed for flow cytometry analysis. Representative flow plots with proportions (mean±sem) and numbers of CD11b+ myeloid cells in the FGT of naïve and Nb infected mice. Data is representative of two independent experiments with 4 mice per group (mean±sem). Statistical significance was calculated by Mann Whitney t test. *p ≤ 0.05



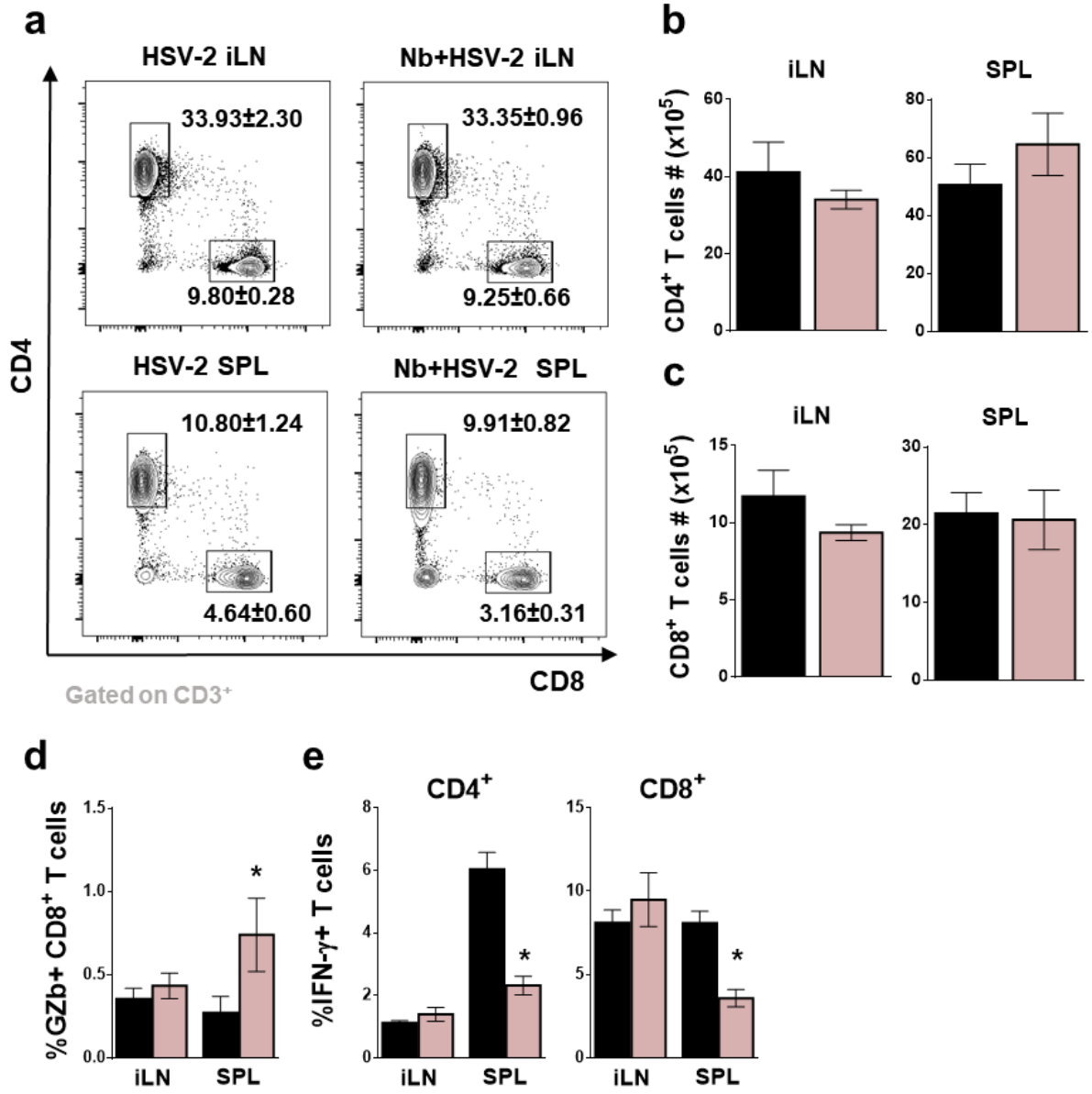
Supp Figure 3.6. Eosinophilia, elevated inflammatory monocytes, neutrophils and M2-like macrophages in the spleen, following Nb infection: At day 9 post Nb infection, spleen (SPL) myeloid cell populations were analysed by flow cytometry. **(a)** Cell proportions (mean±sem) and **(b)** numbers of splenic eosinophils ($CD11b^{+}Siglec\text{-}F^{+}SSC^{hi}$), $Ly\text{-}6C^{hi}$ monocytes ($CD11b^{+}Ly\text{-}6C^{hi}$), neutrophils ($CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{+}$), and macrophages ($CD11b^{+}F480^{+}$). **(c)** Percentage of $CD206^{+}$ (M2-like) splenic macrophages in naïve and Nb exposed mice. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$, **$p \leq 0.01$



Supp Figure 3.7. Nb-induced FGT Eosinophilia is present 21 days after infection: Hormone-synchronized female BALB/c mice were infected with Nb. At day 21 post Nb infection, FGT and spleen was isolated and analysed by flow cytometry. **(a)** Representative flow plots showing proportions (mean±sem) of FGT eosinophils ($CD11b^{+}Siglec\text{-}F^{+}SSC^{hi}$). Numbers of **(b)** eosinophils, **(c)** Ly-$6C^{hi}$ monocytes, **(d)** neutrophils and **(e)** macrophages at day 21 post infection. Data is representative of one experiment with 3 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$



Supp Figure 3.8. Nb-induced systemic inflammation present at day 3 post HSV-2 infection: At day 3 post HSV-2 infection, spleen (SPL) myeloid cell populations were analysed by flow cytometry. **(a)** Cell proportions (mean±sem) and **(b)** numbers of splenic eosinophils ($CD11b^{+}Siglec\text{-}F^{+}SSC^{hi}$), $Ly\text{-}6C^{hi}$ monocytes ($CD11b^{+}Ly\text{-}6C^{hi}$), neutrophils ($CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{+}$), and macrophages ($CD11b^{+}F480^{+}$). **(c)** Percentage of CD206+ (M2-like) splenic macrophages in HSV-2 only and Nb+HSV-2 coinfected mice. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$.



Supp Figure 3.9. Early genital T cell responses are not altered by prior Nb exposure: At day 3 post HSV-2 infection, iLN and spleen (SPL) T cell populations were analysed by flow cytometry. **(a)** Representative flow plots and proportions (mean±sem) of iLN and spleen CD4+ and CD8+ T cells. Numbers of **(b)** CD4+ and **(c)** CD8+ T cells in the iLN and spleen of virus only and coinfected mice. **(d)** GZb^+ CD8+ T cells and **(e)** IFN-γ-producing T cells in the iLN and spleen. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Cell proportions were calculated out of single cells acquired. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$

Chapter 4: Lack of IL-4Rα signalling enhances antiviral immunity and protects against vaginal HSV-2

4.1. Introduction

In Chapter 3 we observed Nb-induced genital eosinophilia and exacerbated HSV-2 pathology was independent of IL-4Rα signalling during coinfection. However, HSV-2 pathology was significantly reduced in IL-4Rα$^{-/-}$ mice infected with virus only, compared to wildtype counterparts. In this chapter we further investigated the effect of IL-4Rα signalling on HSV-2 immune control.

Cytokine signals are important mediators of cellular immune responses, driving cell activation and differentiation. Cytokines can be classified into type 1 or type 2 cytokines, based on the immune response they induce. Type 1 cytokines such as IFNs, typically induce pro-inflammatory responses important for anti-viral immunity [224, 276, 342, 593]. Alternatively, type 2 cytokines, including IL-4 and IL-13, are associated with parasitic helminth immunity and tissue repair [359, 403, 464, 488]. A counterbalance between Th1 and Th2 immunity has been described: IL-4/STAT6 signalling negatively regulates Th1 immunity, by inhibiting differentiation of naïve CD4$^+$ T cells into Th1 effector cells [623, 718] and IFN-γ expression by epigenetic modifications [568, 624, 625]; IL-4 also offsets pro-inflammatory macrophage responses by supressing TNF-α production [719] and inducing alternatively activated macrophages [25, 410, 487].

Studies have demonstrated the essential role of type 1 cytokine IFN-γ during HSV-2 immunity in the vaginal mucosa [224, 226, 250, 258]. Disruption of IFN-γ activity would significantly impair immune control during viral infections [568, 720-723]. Likewise, inhibition of IFN-γ antagonists would promote anti-viral immunity [724, 725]. Paludan *et al*., 1997 demonstrated a reduction in the IFN-γ-induced production of NO in HSV-2-infected macrophage cells *in vitro*, by IL-4 and IL-13 treatment. This study suggests that IL-4 and IL-13 antagonise IFN-γ signalling of iNOS transcription [720]. Additionally, Ghiasi *et al*., 1999 reported increased survival and reduced viral loads in ocular HSV-1-infected IL-4$^{-/-}$ mice, compared to WT[726]. In this chapter, we studied the role of IL-4Rα signalling during vaginal HSV-2 infection. IL-4Rα$^{-/-}$ mice, which have complete IL-4Rα deficiency and loss of IL-4-mediated signal transduction [402], have been used to study the role of IL-4/IL-4Rα signalling during infection and disease *in vivo* [489, 727-731]. To the best of our knowledge, the role of IL-4Rα during vaginal HSV-2 is not known. Here, we studied the influence of IL-4Rα signalling on vaginal HSV-2 pathology and protective immune responses.

4.2. Methods

All methods and analyses were performed as previously detailed in Chapter 3.

4.3. Results

4.3.1. Lack of IL-4Rα signalling, enhances IFN-γ production in uninfected mice

To investigate the influence of IL-4Rα signalling on female genital immunity, we treated female BALB/c wildtype (WT) and IL-4Rα$^{-/-}$ with 2 mg Depo Provera® subcutaneously, to synchronise their hormone cycles to the progesterone-dominant diestrus stage. Using multicolour flow cytometry, we assessed myeloid cells in the FGT, as well as NK cells and T cells in the iLN of WT and IL-4Rα$^{-/-}$ uninfected mice, to determine the influence of IL-4Rα signalling on genital immune homeostasis. During diestrus steady state, a subtle myeloid cell presence is evident in the genital tissue [230, 656, 732, 733]. Here, we observed no significant differences in the proportion and numbers of CD11b^{+}Ly-6G^{+} neutrophils, CD11b^{+}Ly-6C^{hi} inflammatory monocytes and CD11b^{+}F4/80^{+} macrophages, in the FGT of WT and IL-4Rα$^{-/-}$ naïve mice (Supp Figure 4.1). This suggests that lack of IL-4Rα signalling does not alter the genital myeloid compartment at homeostasis.

Next, we determined the effect of lack of IL-4Rα signalling on genital type 1 immune potential, at steady state. Equivalent proportions and numbers of iLN NK, CD4^{+} and CD8^{+} T cells were observed between experimental groups (Figure 4.1.1a and 4.1.2a, c). However, we observed significantly increased proportions and numbers of IFN-γ^{+} NK cells (Figure 4.1.1b) and CD4+ T cells (Figure 4.1.2b) in the iLN of IL-4Rα$^{-/-}$ naïve mice, compared to WT counterparts. This suggests that at steady state, mice that lack IL-4Rα signalling have an enhanced IFN-γ potential by NK and T helper cells, compared to immunocompetent mice. No significant differences in cytotoxic GZb potential by NK and CD8^{+} T cells were observed with lack of IL-4Rα signalling (Figure 4.1.1c and 4.1.2d).

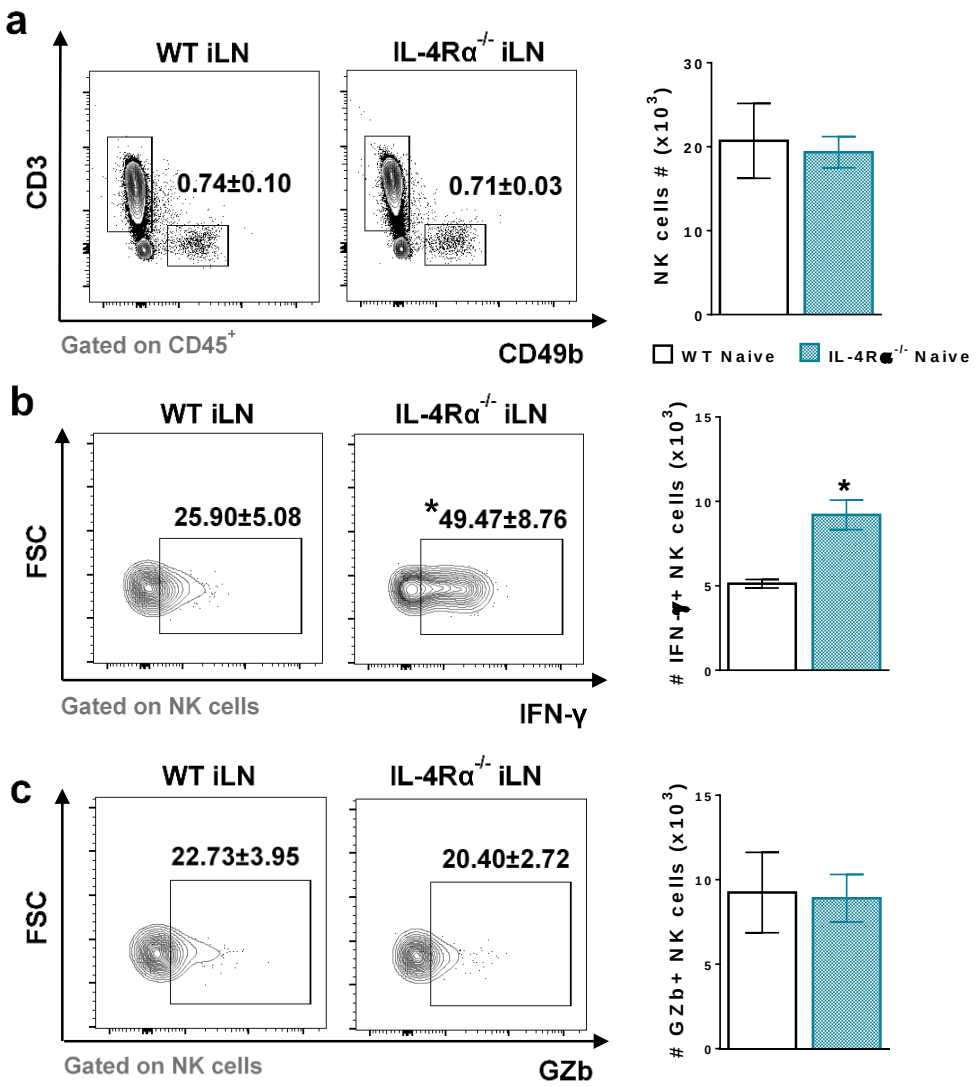


Figure 4.1.1. Lack of IL-4Rα signalling increases IFN-γ-producing NK cells in genital lymph nodes of uninfected mice: Female WT and IL-4Rα$^{-/-}$ mice were treated with 2 mg Depo Provera® prior to analysis. Mitogen-stimulated iLN cells were analysed by flow cytometry. **(a)** Representative flow plots showing the proportions (mean±sem) and numbers of NK cells in the iLN of uninfected WT and IL-4Rα$^{-/-}$ mice. Proportion and number of **(b)** IFN-γ^{+} and **(c)** Granzyme b^{+} NK cells in WT and IL-4Rα$^{-/-}$ uninfected mice. Data is representative of two independent experiments with 3-5 mice per group. Cell proportions were calculated out of (a) single cells acquired or (c, d) the parent population. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$.

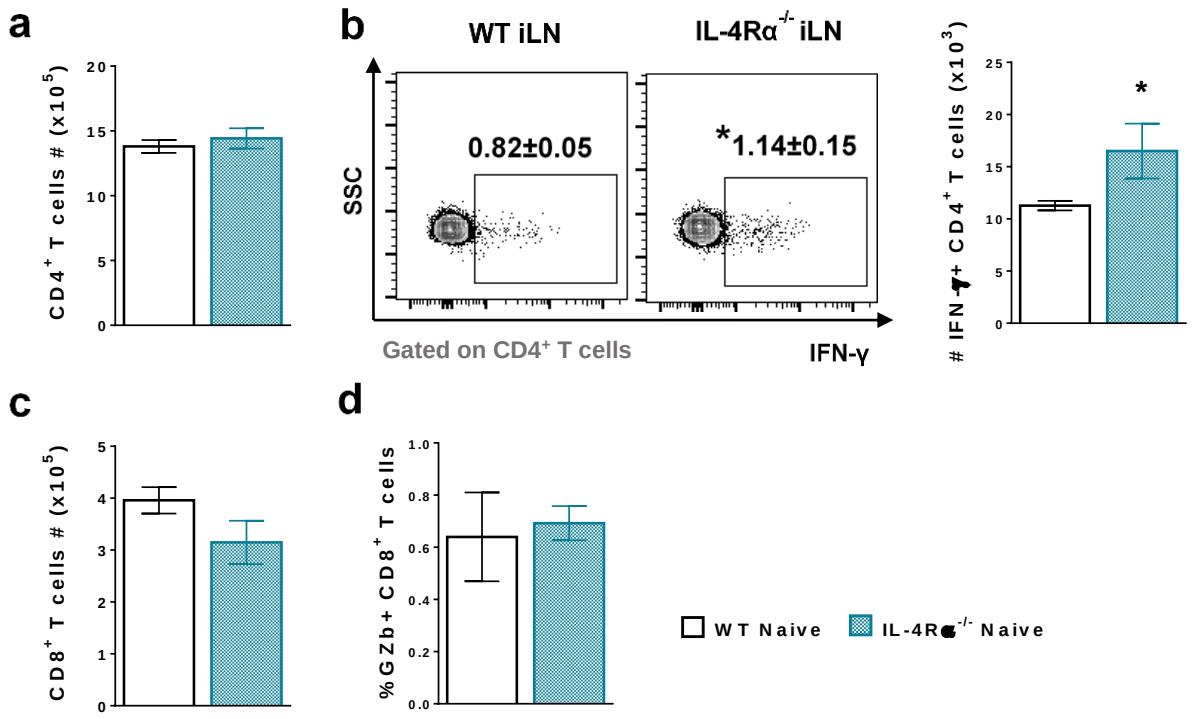


Figure 4.1.2. Increased IFN-γ-producing CD4$^+$ T cells in genital lymph nodes of IL-4Rα$^{-/-}$ uninfected mice: Naïve WT and IL-4Rα$^{-/-}$ iLN cells were mitogen-stimulated and analysed by flow cytometry. **(a)** Numbers of iLN CD4$^+$ T cells in WT and IL-4Rα$^{-/-}$ uninfected mice. **(b)** Representative flow plots showing proportions (mean±sem) and number of IFN-γ$^+$ CD4$^+$ T cells. **(c)** Number of CD8$^+$ T cells and **(d)** proportion of Granzyme b$^+$ CD8$^+$ T cells in the iLN of uninfected WT and IL-4Rα$^{-/-}$ mice. Data is representative of two independent experiments with 3-5 mice per group. Cell proportions were calculated out of the parent population. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$.

4.3.2. Reduced genital HSV-2 pathology with lack of IL-4Rα signalling

The increased steady state potential to produce IFN-γ with lack of IL-4Rα signalling, may influence susceptibility to vaginal HSV-2. To determine the effect of IL-4Rα signalling on HSV-2 pathology and immunity, we infected hormone-synchronised WT and IL-4Rα$^{-/-}$ mice with 5x10^5 PFU HSV-2, intravaginally under deep anaesthesia (Figure 4.2a). We assessed viral progression by daily illness scoring and early antiviral immunity at day 2-3 post HSV-2 infection. We observed significantly reduced genital pathology in IL-4Rα$^{-/-}$ mice from day 3 post HSV-2 infection, compared to WT mice (Figure 4.2b). As discussed previously, HSV-2 infects and ulcerates vaginal epithelium [655, 656]. Histological analysis of vaginal tissue at day 6 post HSV-2 infection suggests reduced epithelial ulceration in IL-4Rα$^{-/-}$ mice, compared to WT HSV-2 controls (Figure 4.2c). There is no difference in viral shedding at day 1-3 post HSV-2 infection (Figure 4.2d) however, it has been reported previously that early viral load does not correlate with altered HSV-2 pathology [225, 226]. These results suggest that lack of IL-4Rα signalling provides protection against vaginal HSV-2 infection.

With diminished HSV-2 pathology, we observed a significant increase in the proportions and numbers of pro-inflammatory Ly-6C^{hi} monocytes in IL-4Rα$^{-/-}$ FGT at day 3 post HSV-2 infection, compared to WT (Figure 4.3a). Ly-6C^{hi} inflammatory monocytes have been reported as key orchestrators of anti-HSV-2 immunity, via their response to type I IFN signals from infected epithelial cells [226, 230, 734]. Once rapidly recruited and activated in the vaginal mucosa, Ly-6C^{hi} monocytes release IL-18, which is important for NK cell activation and first wave IFN-γ production [226]. Further investigation is needed to determine whether Ly-6C^{hi} monocytes coordinate enhanced immune protection in mice that lack of IL-4Rα signalling. No significant differences were observed in genital neutrophil proportions and numbers, between experimental groups. Interestingly, we observed significantly increased proportions and numbers of FGT macrophage in HSV-2 infected IL-4Rα$^{-/-}$ mice, compared to WT counterparts (Figure 4.3b). Additionally, FGT macrophages from IL-4Rα$^{-/-}$ mice had significantly less CD206 expression, compared to WT macrophages. Vaginal levels of TNF-α, a classic type 1 cytokine produced by macrophages [735, 736] and DCs [248, 737], were significantly increased in IL-4Rα$^{-/-}$ mice at day 2 post HSV-2 infection, compared to WT counterparts (Figure 4.3c). This suggests that lack of IL-4Rα signalling increased levels of vaginal TNF-α and FGT macrophages, with reduced M2-like phenotype, during HSV-2 infection.

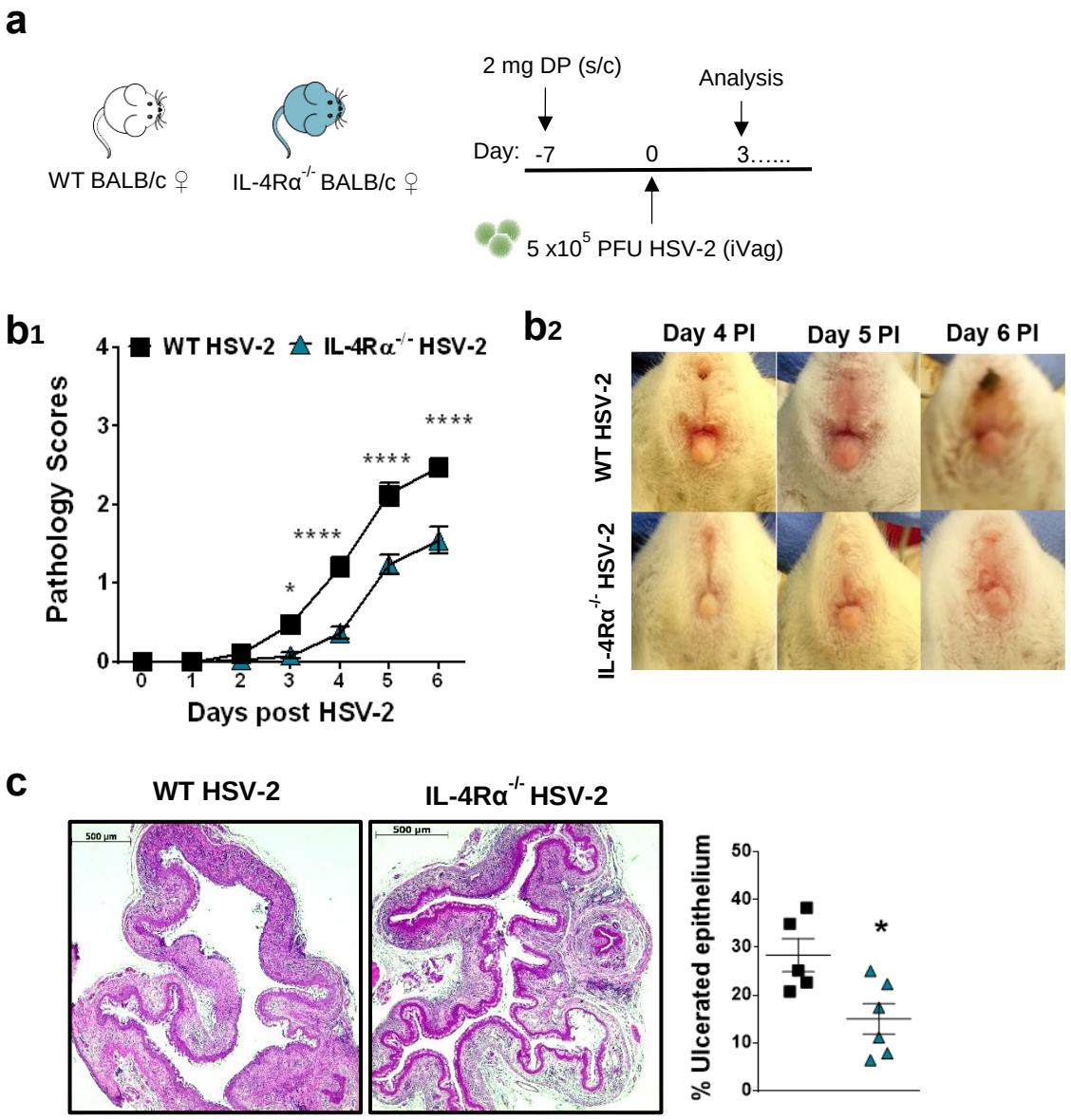


Figure 4.2. Lack of IL-4Rα signalling rescues HSV-2-associated pathology: (a) Female Wildtype (WT) BALB/c and IL-4Rα$^{-/-}$ mice were treated with 2 mg Depo Provera® (DP), 7 days prior to intravaginal HSV-2 infection (5x10^5 PFU). **(b)** HSV-2 progression was determined by daily pathology scoring following viral infection. **(c)** Representative histology images of ulcerated vaginal tissue (VT) in WT and IL-4Rα$^{-/-}$ mice, at day 6 post HSV-2 infection. Images were taken at 50x magnification. **(d)** Viral shedding (PFU/ml) was determined by plaque assay of vaginal lavages at day 1-3 post infection. Data is representative of two independent experiments with 5-6 mice per group (mean±sem). Statistical significance was calculated by two-way analysis of variance (ANOVA) with Bonferroni correction for multiple comparisons. *$p \leq 0.05$, ****$p \leq 0.0001$

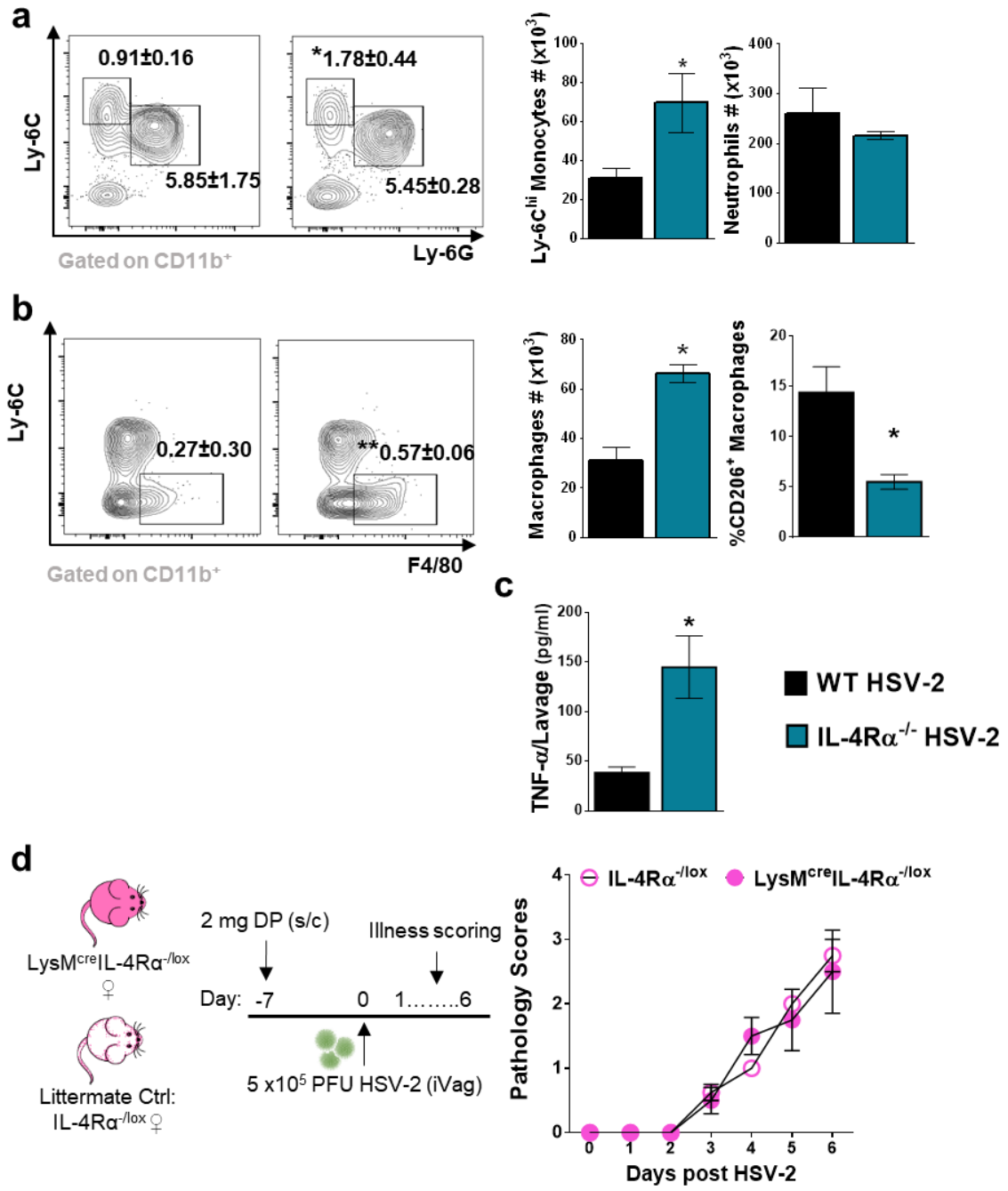


Figure 4.3. Increase in FGT inflammatory monocytes, macrophages and TNF-α in protected IL-4Rα$^{-/-}$ mice, following vaginal HSV-2 infection: At day 3 post HSV-2 infection, FGT myeloid cells were analysed by flow cytometry. Representative flow plots showing proportions (mean±sem) and numbers of **(a)** Ly-6C^{hi} monocytes, neutrophils, and **(b)** total and CD206^{+} macrophages, in the FGT of WT and IL-4Rα$^{-/-}$ mice. **(c)** Levels of TNF-α were measured in vaginal washes at day 2 post HSV2 infection, by ELISA. Data is representative of two independent experiments with 5-6 mice per group (mean±sem). Cell proportions are calculated out of single cells acquired. Statistical significance was calculated Mann Whitney t test. *$p \leq 0.05$. **$p \leq 0.01$. **(c)** Hormone (DP)-synchronised macrophage/neutrophil cell-specific IL-4Rα deficient mice (LysM^{cre}IL-4Rα$^{-/lox}$) and littermate controls (IL-4Rα$^{-/lox}$) were intravaginally infected with HSV-2. Viral progression was determined by daily pathology scoring. Data is representative of one experiment with 4 mice per group (mean±sem).

However, lack of IL-4Rα signalling on macrophages and neutrophils only (LysM^{cre}IL-4Rα$^{-/lox}$) was insufficient to protect against vaginal HSV-2 pathology, compared to littermate controls (Figure 4.3d). This suggests that lack of IL-4Rα signalling on macrophages is alone not responsible for protection against vaginal HSV-2 infection. Macrophage/neutrophil-specific IL-4Rα-deficient mice (LysM^{cre}IL-4Rα$^{-/lox}$) and littermate controls (IL-4Rα$^{-/lox}$) were kindly provided by the Frank Brombacher group, Division of Immunology, University of Cape Town.

4.3.3. Complete lack of IL-4Rα signalling enhances early antiviral immunity during vaginal HSV-2 infection

With diminished genital pathology, IL-4Rα$^{-/-}$ mice had significantly elevated levels of IFN-γ in vaginal washes at day 2 post HSV-2 (Figure 4.3a). During vaginal HSV-2 *in vivo* infection, first wave antiviral IFN-γ is produced by innate NK cells, and are essential for early viral control [224, 226, 238, 738]. Increased IFN-γ levels in IL-4Rα$^{-/-}$ mice was accompanied by significantly increased proportions and numbers of NK cells in the FGT and iLN at day 3 post HSV-2 infection, compared to WT mice (Figure 4.4b). Additionally, we observed a trend for increased intracellular IFN-γ and significantly elevated granzyme b potential in iLN NK cells from HSV-2 infected IL-4Rα$^{-/-}$ mice, compared to WT counterparts (Figure 4.3c). These results suggest that lack of IL-4Rα signalling enhances early anti-viral immunity in the FGT and associated lymph nodes, which may mediate increased viral control and protection. We did not observe any significant differences in iLN CD4^{+} and CD8^{+} T cell proportions and numbers in WT and IL-4Rα$^{-/-}$ mice, at day 3 post HSV-2 infection (Supp Figure 4.2a-c). As discussed previously, this was expected as adaptive responses against vaginal HSV-2 *in vivo* infection are typically prominent from day 5-6 post infection [224, 225, 258]. Interestingly, we observed increased intracellular IFN-γ in iLN CD4^{+} T cells from IL-4Rα$^{-/-}$ mice, compared to WT at day 3 post HSV-2 infection (Supp Figure 4.2d). Together, these data suggests that complete lack of IL-4Rα signalling enhances early anti-viral immunity and protection against vaginal HSV-2 infection. Further investigation is required to determine whether IL-4Rα signalling on NK cells alters IFN-γ production, as well as innate cytotoxicity.

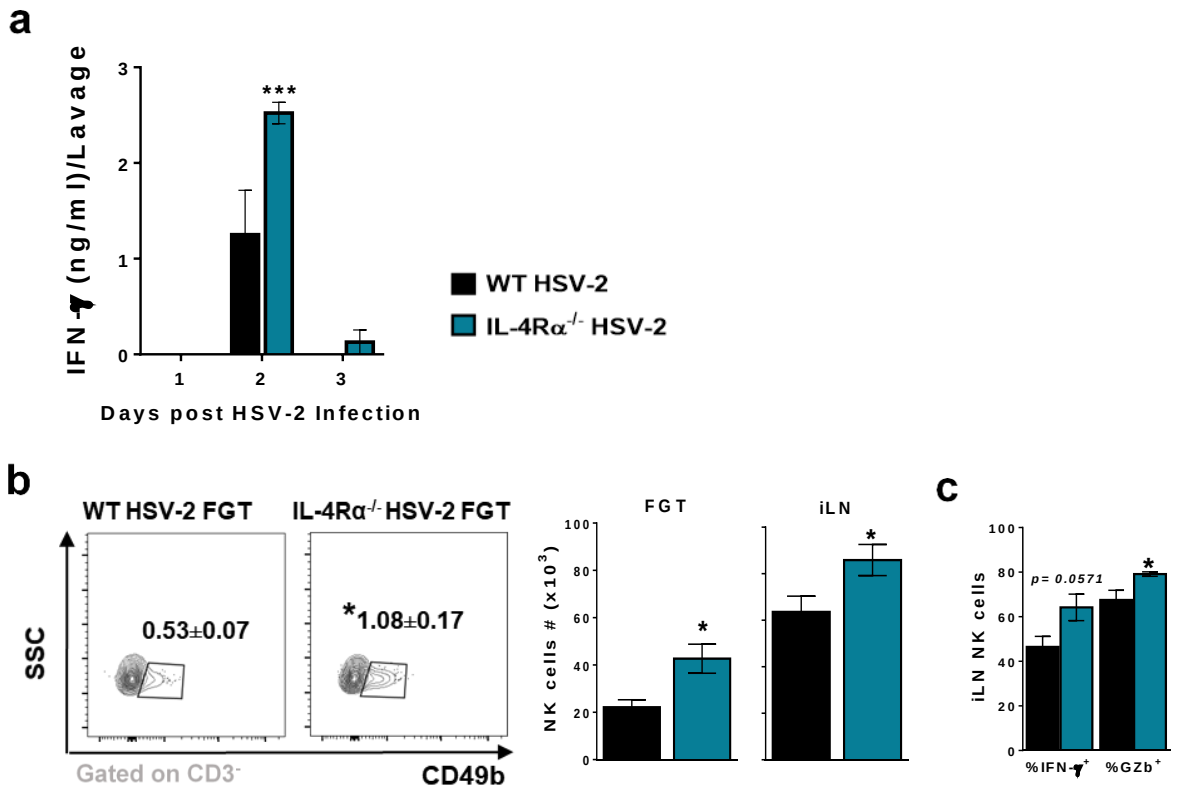


Figure 4.4. Reduced HSV-2 pathology is accompanied by heightened anti-viral immunity in IL-4Rα$^{-/-}$ mice: (a) At day 1-3 post HSV-2 infection, IFN-γ levels in vaginal lavages were determined by ELISA. At day 3 post HSV-2 infection, FGT and iLN cells were mitogen-stimulated and analysed by flow cytometry. **(b)** Representative flow plots showing proportions (mean±sem) and numbers of CD3^{-}CD49b^{+} NK cells in the FGT and iLN of WT and IL-4Rα$^{-/-}$ HSV-2 infected mice. **(c)** Percentage of IFN-γ^{+} and GZb^{+} NK cells in the iLN. Data is representative of two independent experiments with 5-6 mice per group (mean±sem). Cell proportions are calculated out of (b) single cells acquired or (c) the parent population. Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$. **$p \leq 0.01$. ***$p \leq 0.001$.

4.4. Discussion

IL-4 is a canonical type 2 cytokine, shown to supress type 1 responses [623-625, 718, 719]. As detailed previously, IL-4Ra chain is an essential molecule in both membrane-bound type I and II IL-4Rs and genetic deficiency in IL-4Rα abolishes IL-4 signalling, impairing Th2 immunity [396, 402-404, 727]. In this chapter, we showed that at steady state, complete lack of IL-4Rα signalling elevated intracellular IFN-γ levels of NK and $CD4^+$ T cells in genital associated lymph nodes. We also demonstrated that lack of IL-4R signalling protected against vaginal HSV-2 infection, likely via enhanced early antiviral IFN-γ responses. Several studies have reported the opposing effects of IL-4/IL-4R signalling on Th1 responses and disease susceptibility: Kopf *et al*., 1999 and Noben-Trauth *et al*., 1999 showed that mice genetically deficient for IL-4 or IL-4Rα, were resistant to *Leishmania major* infection, where resistance is mediated by Th1 immunity [727, 739, 740]; Saeftel *et al*., 2004 presented data suggesting the absence of IL-4 or IL-4Rα signalling increased innate type 1 immunity against *Plasmodium berghei* and survival in transgenic mice [741]; Redente *et al*., 2009 reported reduced lung tumour growth in IL-4Rα$^{-/-}$, compared to WT mice [742]; and Ripple *et al*., 2010 demonstrated that treatment with an IL-4R antagonist prevented RSV lung disease *in vivo*, associated with increased Th1 and reduced Th2 responses [743]. Moreover, children with IL-4 and IL-4R 'gain-of-function' polymorphisms, resulting in enhanced IL-4 activity, had increased severity of RSV disease [744]. During *in vivo* herpesvirus infection, Reese *et al*., 2014 established that helminth infection reactivates latent murine γ-HVs, via IL-4/STAT-6 competitively blocking the antiviral effect of IFN-γ at the viral genome, promoting viral replication [568]. Here, we show that mice deficient for IL-4Rα, have enhanced early IFN-γ responses, as well as an increased Ly-$6C^{hi}$ monocyte and NK cell presence in the FGT, following vaginal HSV-2 infection. To the best of our knowledge, the role of IL-4Rα signalling during vaginal HSV-2 infection, has not been reported previously.

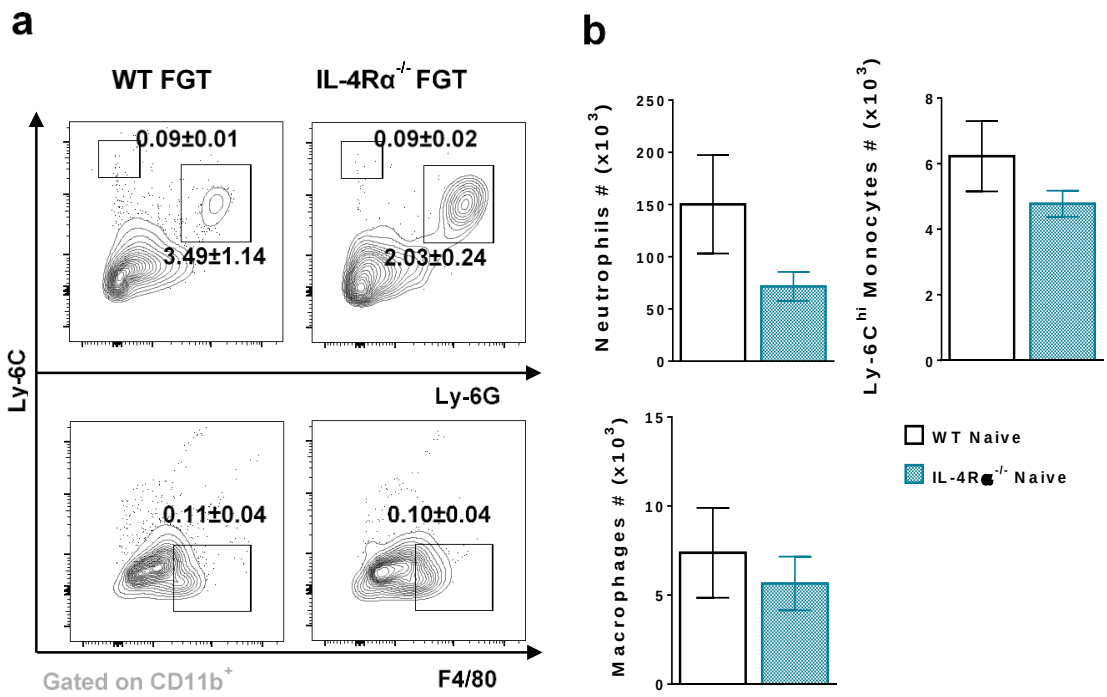
Paludan *et al*., 1997 studied the effect of IL-4 and IL-13 on IFN-γ-induced NO production in a murine macrophage cell line, infected with HSV-2. This study found reduced NO production in IFN-γ-treated, HSV-2-infected macrophages that were co-stimulated with IL-4/-13 [720]. Conflicting studies have reported the effects of IL-4 during ocular HSV-1 infection *in vivo*. Ghiasi *et al*., 1999 reported that IL-4$^{-/-}$ mice had lower HSV-1 viral PFUs in infected eyes, compared to WT BALB/c mice. Additionally, treatment with recombinant IL-4 (rIL-4) increased ocular viral replication in IL-4$^{-/-}$ mice [726]. This suggests that IL-4 action is detrimental during ocular HSV-1 infection. However, follow-up studies by the same research group demonstrated that replication-competent mutant HSV-IL-4, which expressed high levels of IL-4 in *in vitro* cell culture, was less virulent during ocular infection than WT HSV-1 [745], and was associated with the IL-4-induction of M2 macrophages [609].

Conversely, we observed significantly increased genital macrophages with reduced levels of CD206 expression (M2-like), as well as increased vaginal IFN-γ and TNF-α levels, in protected IL-4Rα$^{-/-}$ HSV-2 infected mice. Lee *et al*., 2017 reported less HSV-1 replication and increased production of type 1 cytokines IFN-γ and TNF-α, by *in vitro* and *ex vivo* polarised M1 macrophages, compared to M2 polarised [746]. Lack of IL-4Rα signalling during vaginal HSV-2 infection may reduce M2 polarisation, in theory increasing the proportion of M1-polarised genital macrophages and thus type 1 cytokine production, compared to immunocompetent mice. However, we found that lack of IL-4Rα signalling on macrophages/neutrophils alone did not provide protection against vaginal HSV-2 infection, as seen in complete IL-4Rα deficient mice. Our data suggests that with lack of IL-4Rα signalling, increased genital macrophages with reduced M2 surface markers, may contribute to early antiviral immunity however, they are insufficient to control vaginal HSV-2.

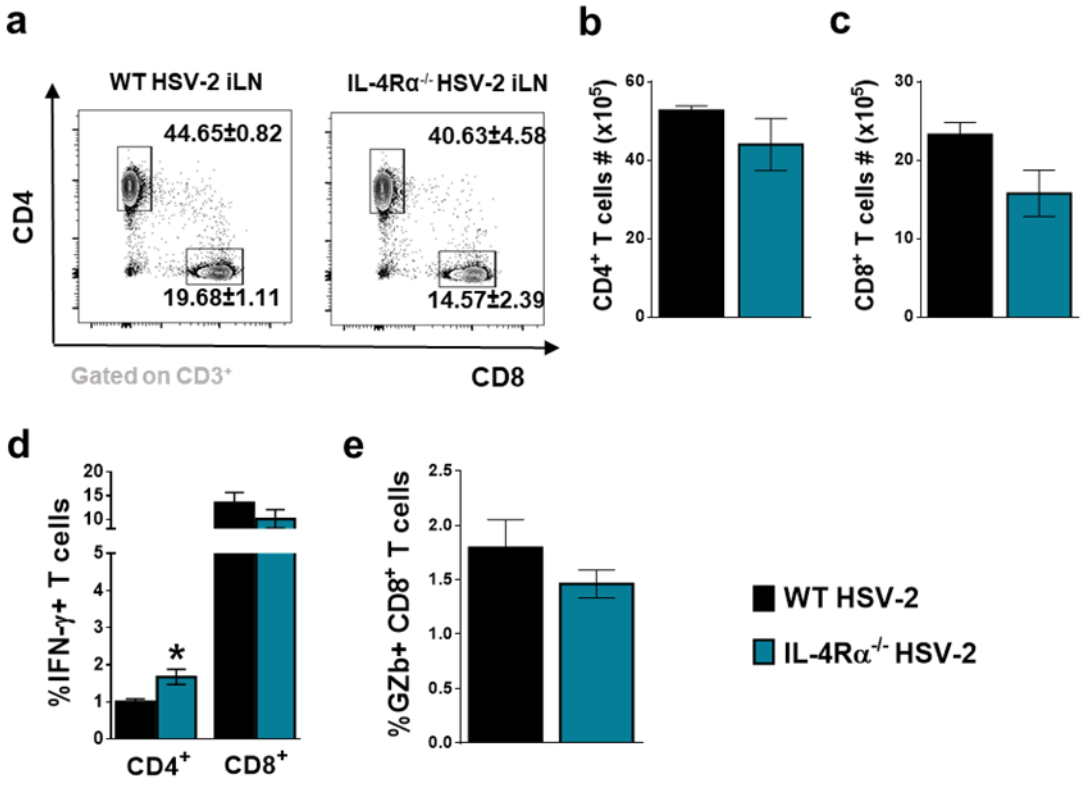
During primary vaginal HSV-2 infection, recruitment of Ly-6Chi monocytes are crucial for mounting antiviral Th1 immunity [734]. Here, we found that absence of IL-4Rα signalling during vaginal HSV-2 infection, was accompanied by elevated levels of Ly-6C^{hi} inflammatory monocytes in genital tissue. Lee *et al*., 2017 established that recruited Ly-6C^{hi} inflammatory monocytes respond to early type I IFN signals from infected vaginal epithelial cells and produce IL-18, which activates vaginal NK cells to produce first wave IFN-γ responses [226]. Further investigation is required to determine whether elevated genital Ly-6C^{hi} monocytes are mediating protection during vaginal HSV-2 infection, in the absence of IL-4Rα signalling. Studies have demonstrated that the absence or depletion of NK cells, significantly impairs control of vaginal HSV-2 infection [226, 238, 239]. Moreover, mice genetically deficient of IFN-γ production are more susceptible to HSV-2, with higher mortality rates compared to immunocompetent mice [238]. Interestingly, Kiniwa *et al*., 2016 demonstrated that IL-4 overexpression *in vivo*, induced NK cells with distinctive characteristics and enhanced IFN-γ production, compared to conventional NK cells [716]. Conversely, we observed an elevated genital NK cell presence in HSV-2-infected mice that lacked IL-4Rα signalling, which is likely the main supply of increased vaginal IFN-γ during early viral infection [224, 226, 232]. Additionally, TNF-α has been shown to activate NK cells and augment cytokine production [747, 748]. Rossol-Voth *et al*., 1991 demonstrated that TNF-α pre- and post-treatment increased survival of intraperitoneal HSV-1 infection, with resistance associated with NK cell activation [749]. Further investigation is needed to confirm whether enhanced genital NK cell and type 1 cytokine responses, with lack of IL-4Rα signalling, mediates early immune protection during vaginal HSV-2 infection.

In conclusion, in this chapter we demonstrated that impaired canonical Th2 immune signalling, enhances Th1 responses and protects against vaginal HSV-2 infection. It is not known whether *in vivo* findings translates to human HSV-2 infections. Further study is needed to determine whether manipulation of host immunity towards Th1 responses, could improve HSV-2 outcome, as well as the negative implications of such measures on host homeostasis.

4.5. Supplementary information



Supp figure 4.1. Lack of IL-4Rα signalling does not significantly alter the genital myeloid compartment in uninfected mice: Female WT and IL-4Rα$^{-/-}$ mice were hormone synchronised prior to analysis. FGT myeloid cell populations were determined by flow cytometry. **(a)** Representative flow plots showing the proportions (mean±sem) of Ly-6C^{hi} Monocytes (CD11b^{+}Ly-6C^{hi}), neutrophils (CD11b^{+}Ly-6G^{+}Ly-6C^{+}) and macrophages (CD11b^{+}F480^{+}). **(b)** Number of neutrophils, Ly-6C^{hi} monocytes and macrophages in the FGT. Data is representative of two independent experiments with 3-5 mice per group. Cell proportions are calculated out of single cells acquired. Statistical significance was calculated by Mann Whitney t test.



Supp figure 4.2. No significant differences in CD8$^+$ T cell responses with lack of IL-4Ra signalling during early HSV-2 infection: iLN lymphocyte populations were analysed by flow cytometry at day 3 post HSV-2. **(a)** Representative flow plots and proportions (mean±sem) of CD4+ and CD8+ T cells. Numbers of **(b)** CD4$^+$ and **(c)** CD8$^+$ T cells. **(d)** IFN-γ production by iLN T cells. **(e)** GZb$^+$ CD8$^+$ iLN T cells. Data is representative of two independent experiments with 4-5 mice per group (mean±sem). Cell proportions are calculated out of single cells acquired (a) or the parent population (d, e). Statistical significance was calculated by Mann Whitney t test. *$p \leq 0.05$

Chapter 5: Concluding Remarks

5.1. Summary of Results

Through our *in vivo* studies, we have demonstrated the bystander effects of nematode exposure on subsequent herpesvirus infections, in colonized and uncolonized biological compartments. We showed that nematode colonisation of the lung, enhanced virus-specific effector $CD8^+$ T cell responses against subsequent MuHV-4 respiratory infection, reducing virus-associated weight loss and viral replication in coinfected mice. Along with increased numbers, virus-specific effector CD8 T cells displayed elevated cytokine production in coinfected mice, when stimulated with viral antigens. This supports published works that demonstrate helminth infection induces $CD8^+$ T_{VMs} via IL-4, which confers broad protection against subsequent viral and bacterial infection [161, 162]. In contrast, we provided preliminary evidence of an IL-4-independent increase in MuHV-4 reactivation in the female genital region of mice previously exposed to the helminth, despite enhanced primary viral control in the lung. This presents the complexity of nematode-induced bystander immunity in colonized and uncolonized tissues.

Further investigation into the systemic effects of nematode infection on female genital immunity revealed an imprint of helminth-associated type 2 immunity in uncolonized genital tissue. Specifically, elevated type 2 cytokine levels and long-lasting eosinophilia, associated with bystander genital tissue damage in nematode infected mice. We determined the implications of nematode-induced alterations to genital immunity, on a subsequent mucosal infection with HSV-2. Prior nematode infection exacerbated subsequent vaginal HSV-2 inflammation and pathology in an IL-4Rα-independent manner. Along with increased viral pathology, we observed reduced early antiviral IFN-γ and NK cell responses in coinfected mice. Antibody-depletion of eosinophils rescued nematode-exacerbated HSV-2 pathology, associated with reduced vaginal IL-33 and increased IFN-γ responses. Exacerbated viral pathology in coinfected mice was likely mediated by a cycle of genital eosinophilia inducing bystander tissue damage, elevating alarmin IL-33 responses, which likely signal genital ILC2s to produce IL-5, promoting further eosinophil activation. Similar observations were presented by Oh *et al*., 2016, where oral antibiotic-induced vaginal dysbiosis increased HSV-2 pathology and impaired antiviral immunity, mediated by IL-33 and associated with ILC2s, IL-5 and genital eosinophilia [225]. It remains unclear how nematode infection systemically induces Th2 immunity in non-colonized genital tissue. The high prevalence of genital herpes in STH endemic regions suggests that *in vivo* findings may have clinical implications, particularly for at risk women in Sub-Saharan Africa [200, 293, 352, 357]. This has been demonstrated by Gravitt *et al*., 2015, where women from STH-endemic regions had increased prevalence of HPV and a helminth-

associated cytokine signature in cervical fluids [567]. Importantly, the results of this thesis demonstrate local and systemic bystander changes to host immunity following STH infection, and the both positive and negative implications on subsequent herpesvirus infections in colonized and uncolonized biological compartments. Interestingly, we observed significantly diminished HSV-2 genital pathology in mice that lacked IL-4Rα signalling, with elevated protective Th1 responses compared to immunocompetent counterparts. These data demonstrate the counter-regulation between Th1 and Th2 immune responses and how manipulation of this balance could be beneficial for host defences.

5.2. Future work

The body of work presented in this thesis demonstrates nematode-induced bystander immunity in colonized and uncolonized host tissues and the effects on subsequent herpesvirus infections. However, there are unanswered questions and further investigation needed.

The induction of $CD8^+$ T cells by helminth infection has been further investigated and published by Rolot *et al*., 2018. Here, helminth-driven CD8+ T_{VMs} were characterised by the surface expression of $CD44^{hi}CD62L^{hi}CXCR3^{hi}CD49d^{lo}$ and further phenotyped by transcriptomic analysis, which revealed that helminth-induced $CD8^+$ T_{VMs} have altered gene expression associated with enhanced function [162]. We also observed heightened MuHV-4 reactivation in the genital region of mice previously exposed to Nb however, further study is needed to determine how prior nematode exposure heightens genital reactivation of MuHV-4, in an IL-4-independent manner. This preliminary finding contradicts published work by Reese *et al*., 2014, where increased reactivation of murine γ-HV was observed with prior helminth infection, mediated by IL-4/STAT-6 competitively inhibiting IFN-γ action [568]. Additional analysis is needed to confirm whether heightened genital reactivation with prior nematode exposure, is independent of helminth-induced IL-4 action. Preliminary analysis suggests an increase in latency reservoirs e.g. B cells and DCs, in the lung with prior parasite infection, which may facilitate increased latency and reactivation in coinfected mice. However, analysis of genital viral reservoirs is required to determine their role during heightened genital reactivation of MuHV-4 in nematode-exposed mice.

The results presented in Chapter 3, demonstrating nematode-induced type 2 immunity, namely increased IL-4, IL-5, ILC2s and eosinophilia in uncolonized FGT, are very interestingly and novel findings. However, further investigation is needed to determine how nematode infection alters immunity in uncolonized biological compartments. It is possible that the systemic effect we observed is a result of circulation of nematode antigen or ES products. Nb antigens and ES products are not fully characterised however, studies have reported the induction of type 2 immunity by Nb products [387, 500].

The lifecycle of Nb involves the transition of L3 larvae from the skin to the lungs, where they moult into L4 larvae and thereafter migrate to the intestine and development into adult worms [359]. During these events, somatic antigen or ES products could enter circulation, and influence uncolonized biological compartments. Further analysis for the presence of Nb antigen or ES products in genital tissue is needed to determine whether direct nematode antigen stimulation induces Th2 immunity in the FGT. Alternatively, systemic cytokine and chemokine responses during Nb infection, could induce bystander Th2 immunity in uncolonized genital tissue. In support of this hypothesis, studies have reported increased serum levels of type 2 cytokines IL-4 [750], IL-5 [751] and IL-13 [479] during Nb infection. Kinetic analysis of serum and genital type 2 cytokine levels will reveal whether serum cytokine levels correlate with genital levels during Nb infection. Additionally, further investigation is needed to support preliminary findings of increased non-haematopoietic cell 'death' in genital tissue following nematode infection. In depth flow cytometry and immunohistochemistry (IHC) analysis of the viability and integrity of vaginal non-haematopoietic cells (e.g. epithelial, endothelial and stromal cells) is needed to confirm whether nematode exposure alters vaginal tissue viability/integrity, and if nematode-induced genital eosinophilia mediates tissue disruption.

We provided evidence which suggests nematode-induced genital eosinophilia mediates exacerbated genital inflammation and pathology during subsequent HSV-2 infection. Further phenotypic characterisation of eosinophils by transcriptomic analysis would determine their gene expression profile and related effector functions. This could identify how genital eosinophils mediate bystander tissue damage and heightened HSV-2 pathology in coinfected mice. Oh *et al*., 2016 demonstrated how antibiotic-induced vaginal dysbiosis resulted in IL-33-mediated exacerbated HSV-2 pathology, associated with increased genital eosinophils and impaired antiviral immunity [225]. McFarlane *et al*., 2017 reported alterations in host gut microbiota by nematode infection, which conferred protection against lung RSV infection by systemic type I IFN induction [470]. To the best of our knowledge, whether nematode infection alters vaginal microbiota is not known. Analysis of vaginal microbiota following Nb infection will determine whether nematode infection induces vaginal dysbiosis and whether this contributes to Th2 immunity in the FGT and exacerbated HSV-2 pathology in coinfected mice.

Lastly, significantly diminished vaginal HSV-2 pathology in mice that lack complete IL-4Rα signalling is also an interestingly and novel result. Our findings suggest enhanced early antiviral responses mediate protection, namely increased genital IFN-γ and NK cell levels in IL-4Rα$^{-/-}$ mice, compared to immunocompetent counterparts. However, additional investigation is needed such as functional assays (e.g. *ex vivo* cytotoxicity assays) and transcriptomic analysis of NK cells isolated from HSV-2-infected WT and IL-4Rα$^{-/-}$ mice, would reveal whether lack of IL-4Rα signalling enhances NK cell function during vaginal HSV-2 infection.

Appendix I: List of media and buffers

10x Phosphate-buffered saline (PBS)

80 g NaCl

2g KCl

14.4g Na_2HPO_4 or 18g $Na_2HPO_4.2H_2O$

2.4g KH_2PO_4

Dissolve above in 1L distilled H_2O and adjust Ph to 7.2-7.4. To make 1x PBS, mix 100mL of 10X PBS with 900mL distilled H_2O. Filter sterile (0.22 µm filter) and store at room temperature.

Complete Media

440 mL DMEM (Gibco™)

5 mL Penicillin/streptomycin (100X)

5 mL Glutamine (200mM)

50 mL heat-inactivated Fetal Calf Serum (Gibco™)

Filter sterilise and store at 4º C.

Digestion media

Complete media

1% HEPES

20 µg/ml Liberase™ TL (Roche)

Filer sterile and store at 4º C.

RIPA lysis buffer

150 mM NaCl

1% Triton X-100

0.5% Sodium deoxycholate

0.1% Sodium dodecyl sulphate (SDS)

50 mM Tris, pH 8.0

MACS buffer

0.745g (2mM) EDTA

5g (0.5%) BSA

Add to 1L 1X PBS. Filter sterilise and store at 4°C.

Paraformaldehyde (PFA, 2%)

27 mL 37% Formaldehyde solution (Sigma-Aldrich®)

473 mL 1x PBS

Mix and adjust pH to 7.2. Store at room temperature

Permeabilization buffer

5 mL 10x Permeabilization buffer (eBioscience™)

45 mL distilled H_2O

Mix and store at 4°C.

ELISA coating buffer

1.6g Na_2CO_3

2.9g $NaHCO_3$

4.2g NaCl

Make up to 1L distilled H_2O and adjust the pH to 9.5. Filter sterilised and store at 4°C.

ELISA assay buffer

5g BSA (1%w/v)

Dissolve in 500mL 1x PBS and store at 4°C.

ELISA wash buffer (20x)

20g KCl

20g KH_2PO_4

144g $Na_2HPO_4.2H_2O$

800g NaCl

50mL Tween 20

Make solution up to 5L with distilled H2O. To make 1x solution, add 1L of the 20x solution to 19L distilled H_2O.

ELISA substrate (for horseradish peroxidase conjugates)

Peroxidase Substrate Solution B (Roche)

TMB Peroxidase Substrate Solution A (Roche)

Just before use, mix equal volumes of TMB Peroxidase Substrate Solution A with

Peroxidase Substrate Solution B.

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