Influence of helminth infection on vaginal immunity

by

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DECLARATION

I, **Fidilia Millicent Achieng Omondi**, hereby declare that the work on which this dissertation/thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university.

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List of abbreviations

AAM	Alternatively activated macrophages
APC	Antigen presenting cells
B220	B cell isoform of 220 kDa
BCG	Bacille Calmette Guérin
CCL	Chemokine (C-C motif) ligand
CCR	Chemokine (C-C motif) receptor
CD	Cluster of differentiation
CVF	Cervicovaginal fluids
CXCR5	C-X-C chemokine receptor type 5
DC	Dendritic cells
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immunosorbent assay
FACS	Fluorescence activated cell sorter
FCS	Foetal calf serum
FI	Formalin inactivated
FOB	Follicular B cell
Foxp3	Forkhead box p3
FRT	Female Reproductive Tract
FSC	Forward scatter
GM-CSF	Granulocyte macrophage colony stimulating factor
HIV	Human Immunodeficiency virus
HPV	Human Papillomavirus
HREC	Human Research Ethics Committee

HSV-2	Herpes Simplex Virus Type 2
ICS	Intracellular staining
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IL-4Rα	Interleukin-4 receptor alpha
ILC	Innate lymphoid cells
ILN	Iliac lymph nodes
min	Minutes
MIP	Macrophage inflammatory protein
ml	Millilitre
MLN	Mesenteric lymph nodes
MOI	Multiplicity of infection
MST	Mediastinal lymph nodes
***N. brasiliensis*/ Nb**	*Nippostrongylus brasiliensis*
NK	Natural killer
PAMP	Pathogen associated molecular patterns
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD-1	Programmed cell death protein-1
SSA	Sub-Saharan Africa
ST(V)I	Sexually Transmitted (Viral) infections
STAT	Signal transducer and activator of transcription
T-reg	regulatory T cell

TAM	Tumour Associated Macrophages
Tfh	T follicular helper cells
TGF-β	Transforming growth factor β
Th	T helper
TLR	Toll-like receptor
TME	Tumour microenvironment
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organisation
WT	Wild type
µl	Microliter

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Abstract

Helminth infections induce systemic changes to host immunity and can impact unrelated infections, even those occurring at anatomical sites not normally colonised by the helminths. A few studies have shown that helminths can increase the risk of infection and pathology resulting from sexually transmitted viral infections in the female reproductive tract, however the evidence is limited and the scope of helminth infection on immunity and infection in the female reproductive tract has not been fully elucidated. In this thesis the impact of hookworm infection on immunity in the female reproductive tract and risk of Human Papillomavirus infection in humans was investigated. The influence of helminth infection on B and T cell responses in the female reproductive tract and how this impacts vaccine mediated responses to another viral infection of the female reproductive tract, Herpes Simplex Virus, Type 2 was also assessed in a mouse model.

To determine the risk of Human Papillomavirus among hookworm infected participants, we compared the prevalence of Human Papillomavirus infection among hookworm infected and uninfected women. Hookworm infected women were two times more likely to be Human Papillomavirus positive than women with no hookworm infection. Furthermore, hookworm infection was positively associated with the intensity of Human Papillomavirus infection.

To determine whether hookworm infection induced changes in vaginal immunity we employed multiplex assays to measure chemokine, cytokine and antibody levels in the vaginal flushes of our study participants. Hookworm infected women displayed an elevated mixed Type 1 (TNF-α, IL-2 and IL-12) and Type 2 (IL-4, IL-5, IL-13, eotaxin and elevated IgG4/ IgE ratio) immune response in the female reproductive tract in

comparison to uninfected women. Type 2 immunity was pronounced in hookworm and Human Papillomavirus co-infected women who maintained an elevated Type 2 signature (IL-4, IL-5, IL-13, eotaxin and elevated IgG4/ IgE ratio) and an increased Th2/Th1 ratio in comparison to uninfected women.

We then investigated the impact of primary helminth infection on B and T cell immunity in the female reproductive tract using the mouse model of hookworm infection, *Nippostrongylus brasiliensis*. *Nippostrongylus brasiliensis* infection of wild type BALB/c mice resulted in increased B cells, IgG1+ B cells and IgG1+ follicular B cells as well as increased effector memory T cells and T follicular helper cells in iliac lymph nodes, which drain the female reproductive tract. We then infected wild type BALB/c mice with *Nippostrongylus brasiliensis* and immunised them with formalin inactivated Herpes Simplex Virus, Type 2 then challenged them intravaginally with lethal dose Herpes Simplex Virus, Type 2. *Nippostrongylus brasiliensis* infection did not significantly impact B cell responses to vaccination and subsequent challenge though there was a trend towards lower B cell responses in mice that received *Nippostrongylus brasiliensis* treatment prior to vaccination. Mice that had prior *Nippostrongylus brasiliensis* infection, however, had significantly lower effector memory CD4+ T cells than mice that did not have helminth infection before vaccination.

In summary, this thesis demonstrates that helminth infection induces Type 2 associated immune changes in the female reproductive tract in humans and alters B and T cell populations in lymph nodes draining the female reproductive tract of mice. Furthermore, in humans, an increased risk of Human Papillomavirus infection and increased intensity of Human Papillomavirus infection was associated with hookworm infection. In mice, a dampening of Herpes Simplex Virus, Type 2 vaccine mediated

effector CD4 T cells responses and increased pathology following viral challenge was observed in mice previously infected with *Nippostrongylus brasiliensis*. The findings in this thesis highlight helminth infection as a significant risk factor for sexually transmitted viral infections and have implications for control of these infections among women living in helminth endemic areas.

Chapter 1: Literature review

1.1 Helminth infection

1.1.1 Burden of disease

Approximately 1.5 billion people are infected with helminths globally with a majority of these infections occurring in low and middle income nations in Asia, Sub-Saharan Africa (SSA) and the Americas (**Figure 1**; Pullan *et al.*, 2014).

Helminth infections include soil transmitted helminthiases (*Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus* and *Ancylostoma duodenale)*, schistosomiasis (*Schistosoma haematobium, Schistosoma mansoni* and *Schistosoma Japonicum*), filariasis (*Wuchereria bancrofti*) and onchocerciasis (*Onchocerca volvulus*), which cause significant morbidity in affected individuals (Murray *et al.*, 2012), Soil transmitted helminth infections alone account for approximately 5.2 million disability adjusted life years (DALYs) globally (Hotez *et al.*, 2014). Chronic infection with soil transmitted helminths results in anaemia, malnutrition, adverse pregnancy outcomes and in children, stunted physical growth (Bethony *et al.*, 2006; Brooker, Hotez and Bundy, 2008; Campbell *et al.*, 2016).

Successful treatment programs for helminth infections have been widely implemented in affected regions, however, high reinfection rates can dull the impact of these interventions (Jia *et al.*, 2012; Zerdo, Yohanes and Tariku, 2016). Furthermore, the burden of helminth infections overlaps with that of other infectious diseases in regions such as SSA (Mkhize-Kwitshana *et al.*, 2011;

Cadmusid *et al.*, 2020; Tadesse Boltena *et al.*, 2022) placing a huge constraint on public health systems.

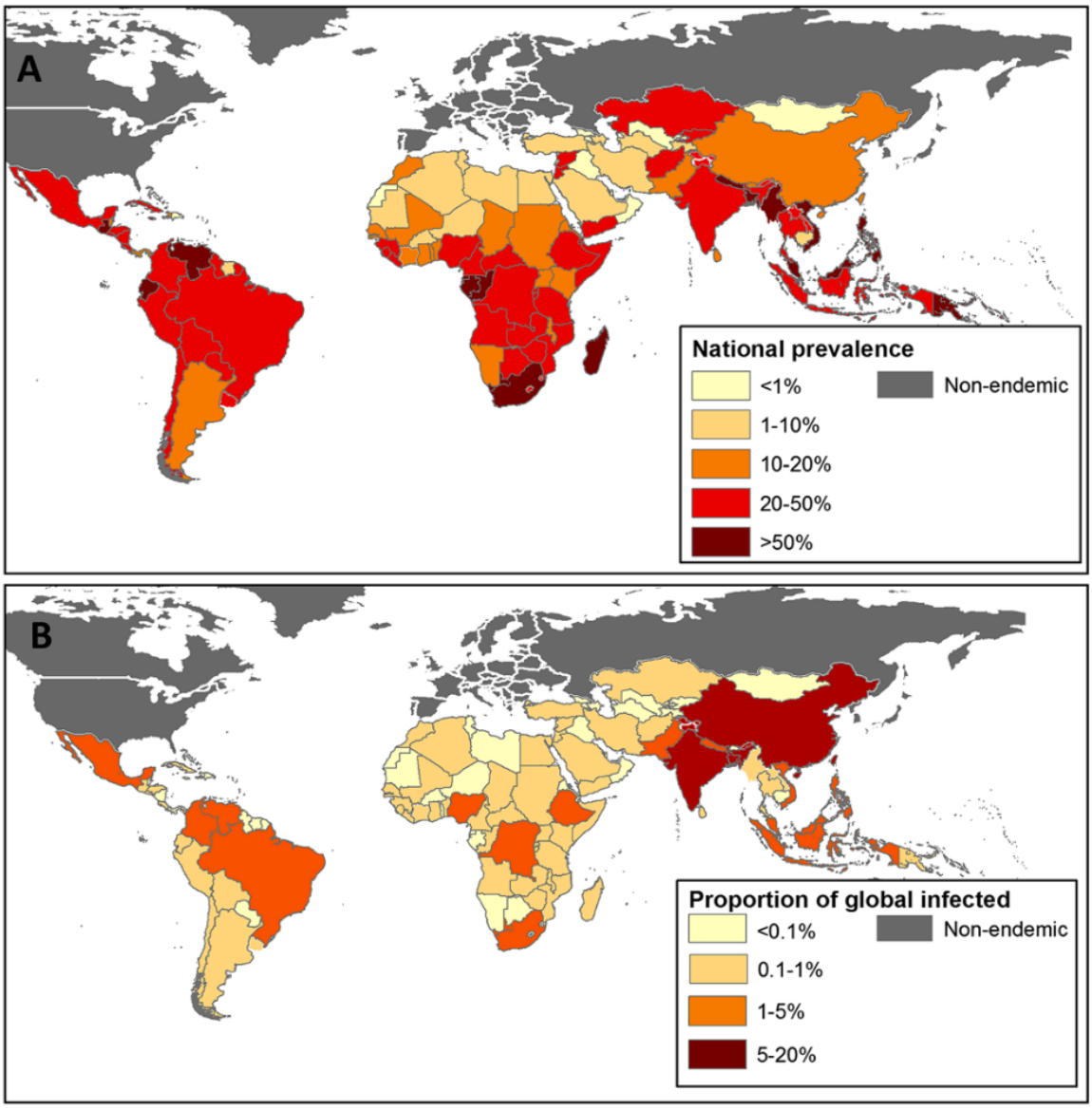


Figure 1: Global prevalence of soil-transmitted helminth infection in 2012. Taken from (Pullan *et al.*, 2014)

Helminths have the capacity to modulate immunity in the host with potentially harmful or beneficial outcomes for unrelated infections (McSorley and Maizels, 2012). Understanding how helminths induce immune responses in the host and how this impacts the response to other pathogens is critical for developing effective control strategies for both helminths and infectious diseases.

1.1.2 Innate immunity

Helminths are transmitted by insect bites, larval penetration of the skin or ingestion of food or water contaminated with eggs and larvae (Jourdan *et al.*, 2018). Migration of helminths through organs such as skin, gut, lungs and liver and production of helminth excretory and secretory (ES) products results in tissue damage triggering release of the alarmins IL-25, thymic stromal lymphopoietin (TSLP) and IL-33 from epithelial cells (Mishra *et al.*, 2014; Weatherhead *et al.*, 2020). These signals set off a cascade of cellular and soluble immune mediator responses in tissues that are predominantly Type 2 in nature aimed at controlling the infecting helminths.

Tuft cells, goblet cells and smooth muscle cells

Specialised epithelial cells, referred to as tuft cells, constitutively express IL-25, which is elevated following helminth infection, activating group 2 innate lymphoid cells (ILC2) to produce IL-13 (Howitt *et al.*, 2016; Von Moltke *et al.*, 2016). These cells intercept parasite excretory/secretory (ES) products via chemosensory receptors on their surface, and blocking of chemosensory signalling on these cells impairs Type 2 associated responses such as expansion of tuft cells, eosinophils, goblet cells and ILC2 required in defence against helminth infections (Howitt *et al.*, 2016).

Type 2 signalling in non-haematopoietic cells such as smooth muscle cells is also required in protection against helminth infection. Knockout of IL-4Rα on smooth muscle cells (SMC) delayed goblet cell hyperplasia and impaired expulsion of *N. brasiliensis* (Horsnell *et al.*, 2007), however, IL4Rα signalling in

SMC also contributed to lung pathology during *N. brasiliensis* infection (Horsnell *et al.*, 2011).

Goblet cells hyperplasia is a key feature of gastrointestinal helminth infection (Grencis, 2015). Goblet cells under the influence of type 2 cytokines (IL-4, IL-5 and IL-13) and the mucosal epithelial repair cytokine, IL-22, produce mucins that are required in expulsion of helminths from the gut (Hasnain *et al.*, 2010, 2011; Turner, Stockinger and Helmby, 2013; Fricke *et al.*, 2015).

Group 2 innate lymphoid cells (ILC2)

ILC2 are an innate cell population producing IL-5, IL-9 and IL-13 (Nussbaum *et al.*, 2013; Klose and Artis, 2016) and under the control of the transcription factor GATA3 (Hoyler *et al.*, 2012), that are important in immunity against intestinal nematode infection. Following *N. brasiliensis* infection, alarmin signals (IL-25, IL-33 and TSLP) induce activation of IL-5 and IL-13 secreting ILC2 in the gut (Moro *et al.*, 2010; Neill *et al.*, 2010) which contribute to worm clearance by promoting goblet cell hyperplasia (Moro *et al.*, 2010). Furthermore, neuronal signalling pathways involving ILC2 play an essential role in worm killing in the lung and worm expulsion from the gut in *N. brasiliensis* infection (Cardoso *et al.*, 2017; Klose *et al.*, 2017; Chu *et al.*, 2021; Roberts *et al.*, 2021), discussed in detail in section 1.4.3. IL-5 produced by ILC-2 also induces recruitment of eosinophils to the lung following *N. brasiliensis* infection (Nussbaum *et al.*, 2013).

Basophils

Basophils are induced in an IL3/IL3R dependent manner following helminth infection (Lantz *et al.*, 2008; Kim *et al.*, 2010) and have been demonstrated to

produce IL-4 and IL-13 in both the gut and lungs in the context of *N. brasiliensis* infection (Voehringer, Shinkai and Locksley, 2004).

Basophils are required for Th2 polarisation and subsequent worm expulsion in *Trichinella spiralis* and *Trichuris muris* infections (Perrigoue *et al.*, 2009; Giacomin *et al.*, 2012). In *T. muris* infection, Th2 polarisation is mediated by antigen presentation to T cells via MHCII expressed on basophils (Perrigoue *et al.*, 2009). Basophils are however not required for development of Th2 responses to primary *N. brasiliensis* and *H. polygyrus* infections, as basophil depletion did not abrogate development of IL-4 producing Th2 cells following infection (Kim *et al.*, 2010; Sullivan *et al.*, 2011; Schwartz *et al.*, 2014). In spite of this, cytokines produced by basophils still played an important role in immunity against *N. brasiliensis*. Mice deficient in IL-4 and IL-13 expression in both basophils and T cells (but not in only basophils or T cells), displayed elevated *N. brasiliensis* worm burdens, suggesting that basophil derived cytokines are non-redundant in protection against primary *N. brasiliensis* infection (Sullivan *et al.*, 2011). Furthermore, basophils promoted worm clearance in secondary *N. brasiliensis* infection in an IgE/IL-4/IL-13 dependent manner (Schwartz *et al.*, 2014). IL-4 produced by basophils was also important in activation of ILC2 in the lungs and skin during a Type 2 inflammatory response (Kim *et al.*, 2014; Motomura *et al.*, 2014).

Mast cells

Mast cells are required for both innate and adaptive immunity to *Heligmosomoides polygyrus bakeri* and *T. muris* in mice (Hepworth *et al.*, 2012). Mast cell depletion resulted in impaired alarmin (IL-25, IL-33 and TSLP)

production, highlighting a role early in the immune response following tissue invasion by helminths. A role for mast cells in subsequent adaptive responses to helminths is suggested by impaired Th2 responses and higher worm burdens in mast cell depleted mice (Hepworth *et al.*, 2012). The mechanism of protection induced by mast cells was demonstrated to be production of proteases that disrupt epithelial cell tight junctions, promoting shedding of worms attached to the intestinal epithelium in the *T. spiralis* model (Knight *et al.*, 2000; McDermott *et al.*, 2003; Lawrence *et al.*, 2004).

In *N. brasiliensis* infection, IL-9 stimulated mast cell expansion in the absence of IL-4, IL-5 and IL-13, similarly, IL-4 induced mastocytosis in IL-5/IL-9/IL-13 knockout mice (Fallon *et al.*, 2002). Mast cells were however non-essential for expulsion of *N. brasiliensis* as demonstrated in IL-9 knockout mice, defective in induction of mastocytosis but displaying worm burdens similar to WT mice (Townsend *et al.*, 2000).

Eosinophils

Eosinophils produce IL-4 and IL-13 in both the gut and lungs following *N. brasiliensis* infection (Voehringer, Shinkai and Locksley, 2004). A role for eosinophils in immunity to *N. brasiliensis* was demonstrated in transgenic mice overexpressing IL-5 that developed pronounced eosinophilia and enhanced resistance to *N. brasiliensis* infection (Dent *et al.*, 1999). Eosinophils also played a role in early resistance to secondary *N. brasiliensis* infection, where mice defective in eosinophilopoiesis developed higher worm burdens in the lungs in comparison to WT mice (Knott *et al.*, 2007). Eosinophils were however not essential for worm clearance from the gut in primary or secondary *N. brasiliensis*

infection (Knott *et al.*, 2007). Eosinophils are also part of the protective response against primary infection with *H. polygyrus* and *Brugia malayi*, where eosinophil deficient mice displayed significantly elevated worm burdens (Cadman *et al.*, 2014; Hewitson *et al.*, 2015).

In addition to Type 2 cytokine production, eosinophils secrete products that activate dendritic cells (DC) to promote Th2 differentiation (Yang *et al.*, 2008; Chu *et al.*, 2014) Eosinophil derived neurotoxin (EDN) activated DC via a Toll-like receptor (TLR) 2- myeloid differentiation factor 88 (MyD88) pathway (Yang *et al.*, 2008) and degranulation of eosinophil peroxidase promoted DC activation *in vitro* (Chu *et al.*, 2014).

Neutrophils

Neutrophils are an important part of the immune response against some helminth infections. In *Litomosoides sigmodontis* infected mice, IL-5 production resulted in neutrophil accumulation in the thoracic cavity and inflammatory neutrophils formed nodules around the worms eventually killing them (Al-Qaoud *et al.*, 2000). In addition, neutrophils, in co-operation with eosinophils in the *Strongyloides stercoralis* model, and macrophages in the *H. polygyrus* model, mediated worm destruction in infected mice (Anthony *et al.*, 2006).

Neutrophils recruited in an IL-17 dependent manner following *N. brasiliensis* infection promoted tissue damage in the lung, and neutrophil depletion prevented acute lung injury (Chen *et al.*, 2012). In this model, IL-4ra knockout mice displayed increased neutrophilia and lung tissue damage suggesting a role for IL-4ra signalling in controlling neutrophil inflammation in *N. brasiliensis* infection (Chen *et al.*, 2012). Though neutrophils promoted lung injury during *N.*

brasiliensis infection, they played an essential role in reduction of worm burdens in infected mice (Sutherland *et al.*, 2014). In addition IL-13 producing neutrophils were required for polarisation of M2 macrophages that displayed enhanced parasite killing *in vitro* and promoted *N. brasiliensis* expulsion (Chen *et al.*, 2014).

Macrophages

Macrophages play a role in direct killing of helminth larvae as well as in tissue repair following helminth infection (Lechner, Bohnacker and Esser-von Bieren, 2021). Type 2 cytokines (IL-4 and IL-13) from Th2 cells and innate cells activate macrophages during helminth infection skewing them towards an M2 phenotype (Anthony *et al.*, 2006; Chen *et al.*, 2014; Bouchery *et al.*, 2015). M2 macrophages, also referred to as alternatively activated macrophages (AAM), are characterized by expression of arginase 1, resistin like molecule (RELM) -α, Cluster of differentiation (CD) 206, YM1 and insulin-like growth factor (IGF) 1 (Inclan-Rico and Siracusa, 2018; Lechner, Bohnacker and Esser-von Bieren, 2021).

M2 macrophages mediate killing of *N. brasiliensis* larvae in the lungs of infected mice (Bouchery *et al.*, 2015) and persist long after infection has been cleared (Marsland *et al.*, 2008). In comparison to mice that received M2 macrophages from WT donors, mice that received macrophages deficient in Stat6 signalling had a higher number of viable worms in the lungs highlighting the role of M2 activation in worm killing in the *N. brasiliensis* model (Bouchery *et al.*, 2015). M2 macrophages also mediated protection against tissue damage in the lungs of *N. brasiliensis* infected mice, protection was dependent on RELM-α expression and Stat6 and IL4-Rα signalling (Chen *et al.*, 2012; Krljanac *et al.*, 2019).

Dendritic cells

Dendritic cells (DC) form the bridge between innate and adaptive immunity, and in helminth infection, they present helminth derived antigens to T cells, stimulating Th2 differentiation (Motran *et al.*, 2018). Treatment of DC with *N. brasiliensis* ES (NES) antigen *in vitro* and subsequent transfer of primed DC to naïve mice generated Th2 responses in recipient mice (Balic *et al.*, 2004). In this study, markers associated with promoting Th2 differentiation, CD86 and OX40L were upregulated on NES primed DC.

In contrast, in a chronic infection model with *H. polygyrus*, nonplasmacytoid CD11c lo CD103- DC induced an immunoregulatory environment in infected mice, that included diminished TLR responsiveness in DC, reduced antigen specific CD4 T cells and increased regulatory T cells and TGF-β production (Smith *et al.*, 2011). In addition, migratory CD103+ DC suppressed Type 2 associated immune responses such as Th2 differentiation, induction of AAM, eosinophilia and immunoglobulin (Ig) G1 class switching in *H. polygyrus* infected mice, these functions were restored upon deletion of the CD103+ DC transcription factor Batf3 in a knockout model (Everts *et al.*, 2016).

1.1.3 Adaptive immunity

Th2 cells

The main subset of CD4 T cells associated with helminth infection are Th2 cells. These cells are induced via a number of pathways following helminth infection. Innate immune cells such as DC and eosinophils present helminth ES to CD4 T cells, skewing their differentiation towards a Type 2 phenotype (Motran *et al.*,

2018). Type 2 cytokines IL-4, IL-5, IL-9 and IL-13 from innate immune cells such as eosinophils, basophils, mast cells and ILC-2 also promote development of Th2 cells (Henry, Inclan-Rico and Siracusa, 2017). In addition, Th2 cells can be directly activated by IL-25, IL-33 and TSLP bypassing T cell receptor engagement (Sorobetea, Svensson-Frej and Grencis, 2018).

Th2 cells secrete IL-4, IL-5, IL-9 and IL-13, cytokines which have been demonstrated in mouse models to play a role in resistance to helminth infections by promoting goblet cell hyperplasia, mucus production, smooth muscle contraction and worm expulsion (Henry, Inclan-Rico and Siracusa, 2017). In humans, Type 2 cytokines have been associated with worm clearance in *Ascaris lumbricoides* infected individuals (Turner *et al.*, 2003).

Th2 cells express IL-4Rα, the receptor that binds IL-13 and IL-4, on their surface. Knockout of IL-4Rα on CD4+ T cells did not impair worm expulsion in *N. brasiliensis* infected mice (Mearns *et al.*, 2008; Schmidt *et al.*, 2012), suggesting that expulsion mediated by Th2 cells was IL-4Rα independent. IL-4rα signalling in CD4 T cells rather contributed to tissue damage in the lung during primary *N. brasiliensis* infection, as mice deficient in IL-4Rα expression on CD4 T cells exhibited reduced lung pathology (Mearns *et al.*, 2008). In contrast to observations in primary infection, IL-4Rα signalling in lung resident CD4 T cells was required for protection against secondary *N. brasiliensis* infection (Thawer *et al.*, 2014).

Signal transducer and activator of transcription (STAT) 6 is a transcription factor expressed by Th2 cells and Type 2 associated myeloid cells (Walford and Doherty, 2013). STAT 6 knockout impaired Th2 responses to *N. brasiliensis*, *H.*

polygyrus, *T. muris* and *T. spiralis* infections (Urban *et al.*, 1998; Finkelman *et al.*, 1999; Goenka and Kaplan, 2011).

Regulatory CD4 T cells

Helminths induce regulatory responses in the host to dampen inflammation and enhance their survival. *In vitro* culture of splenocytes from naïve mice with *H. polygyrus* ES (HES) resulted in upregulation of expression of the T-regulatory (T reg) transcription factor Foxp3 on T cells (Grainger *et al.*, 2010). These T regs adoptively transferred into recipient mice were protective against allergic airway inflammation (AAI). Furthermore, a TGF-β mimic produced by *H. polygyrus* induced Foxp3+ Treg cells (Johnston *et al.*, 2017). TGF-β is a regulatory cytokine produced by innate immune cells such as macrophages, mast cells and granulocytes that exerts immunomodulatory functions on immune cells including T cells (Li *et al.*, 2006). In *S. mansoni* infected mice, T regs were located on the outer edges of granulomas and actively blocked IFN-γ and IL-10 production from CD25- CD4+ T cells (Layland *et al.*, 2010). In humans, experimental hookworm infection induced both systemic and mucosal regulatory (TGF-β and IL-10) responses (Gaze *et al.*, 2012).

T follicular helper (Tfh) cells

Tfh are located in the germinal centres where they interact with B cells providing B cell help (Reinhardt, Liang and Locksley, 2009). Tfh develop progressively during the course of helminth infection, undergoing distinct transcriptional and functional changes (Weinstein *et al.*, 2016). Tfh express the transcription factor Bcl6, surface CXCR5 and PD1 and produce IL-4 and IL-21 (Fairfax *et al.*, 2015;

Weinstein *et al.*, 2016). Tfh were demonstrated to be the main source of IL-4 in the mesenteric lymph nodes of *H. polygyrus* infected mice and were essential for induction of B cell antibody class switching to IgG1 and IgE following infection (King and Mohrs, 2009; Meli *et al.*, 2017). Tfh induced following *N. brasiliensis* infection produced IL-4 (Liang *et al.*, 2012; Weinstein *et al.*, 2016) and induced expansion of antibody producing B cells in draining lymph nodes and spleen of infected mice (Weinstein *et al.*, 2016).

B cells

B cells have varying roles in helminth infection, dependent on the infecting species. B cells were required for clearance of *H. polygyrus* but not *N. brasiliensis* infection, as B cell deficient *H. polygyrus* infected mice exhibited impaired worm clearance that was not observed in *N. brasiliensis* infected mice (Liu *et al.*, 2010). In addition, susceptibility to *L. sigmodontis* and filarial infection was increased in B1 B cell deficient mice (Ai-Qaoud, Fleischer and Hoerauf, 1998; Paciorkowski *et al.*, 2000).

B cells produce the antibodies IgE and IgG1 in mice and IgE and IgG4 in humans following helminth infection (Harris and Gause, 2011; McSorley and Maizels, 2012). Class switching in B cells to produce Type 2 associated antibodies is induced by the action of Th2 cytokines and interaction of B cells with Tfh in germinal centres (Haase and Voehringer, 2021). Antibodies can mediate protection against helminths by binding directly to parasites or by activating innate immune cells to promote worm expulsion (Zaini, Good-Jacobson and Zaph, 2021).

In addition to antibody production, B cells can also exert other effector functions in helminth infected mice. B cells in the draining lymph nodes and spleen of *N. brasiliensis* infected mice produced IL-13 in an IL-4Rα dependent manner, displayed increased cell surface expression of MHCII and CD86, and were required for protection against secondary infection (Horsnell *et al.*, 2013).

S. mansoni induced IL-10 producing regulatory B cells (B-regs) were reported to protect against AAI in mice in an IL-10 dependent manner (Smits *et al.*, 2007; Amu *et al.*, 2010). B-regs were also induced in H. polygyrus infection, that protected against AAI in recipient mice following adoptive transfer, though protection was IL-10 independent (Wilson *et al.*, 2010).

1.2 Sexually transmitted infections

1.2.1 Burden of disease

Sexually transmitted infections occur at high rates (**Figure 2**), with an estimated 373 million new cases of the four most common STI, chlamydia, gonorrhoea, syphilis and trichomoniasis and 604,000 cases of cervical cancer reported globally in 2020 (WHO, 2021). Estimates from 1990 to 2019 indicate a trend towards decreased age standardised STI incidence rates globally (Zheng *et al.*, 2022). Though a decreasing trend in the age standardised STI incidence rate was observed in SSA over this 30 year period, the region still had the highest age standardised STI incidence globally (Zheng *et al.*, 2022), suggesting that increased efforts are required to control STI.

Prevalence of chlamydia, gonorrhoea, Trichomonas and syphilis infections in SSA, estimated to be 4.7%, 1.4%, 6.6%, and 1.7%, respectively, in 2020 was

also higher than the global prevalence of these infections that was estimated at 3.2%, 0.7%, 2.7%, 0.6%, respectively (WHO, 2021).

STI are a major cause of morbidity, with 1.3 million DALYs are attributed to STI worldwide (Zheng *et al.*, 2022). Most STI are asymptomatic, meaning that infected individuals will fail to seek treatment or seek delayed treatment after infections have advanced to the point of causing noticeable complications (Mayaud and Mabey, 2004). STIs if left untreated can result in infertility, pelvic inflammatory disease and cancer (Walboomers *et al.*, 1999; Tsevat *et al.*, 2017; Fortner *et al.*, 2019). In addition, STIs increase the risk of acquiring HIV (Galvin and Cohen, 2004). STIs are also associated with pregnancy complications such as miscarriage, pre-term birth, stillbirth, low birth weight and congenital infections (Mullick *et al.*, 2005).

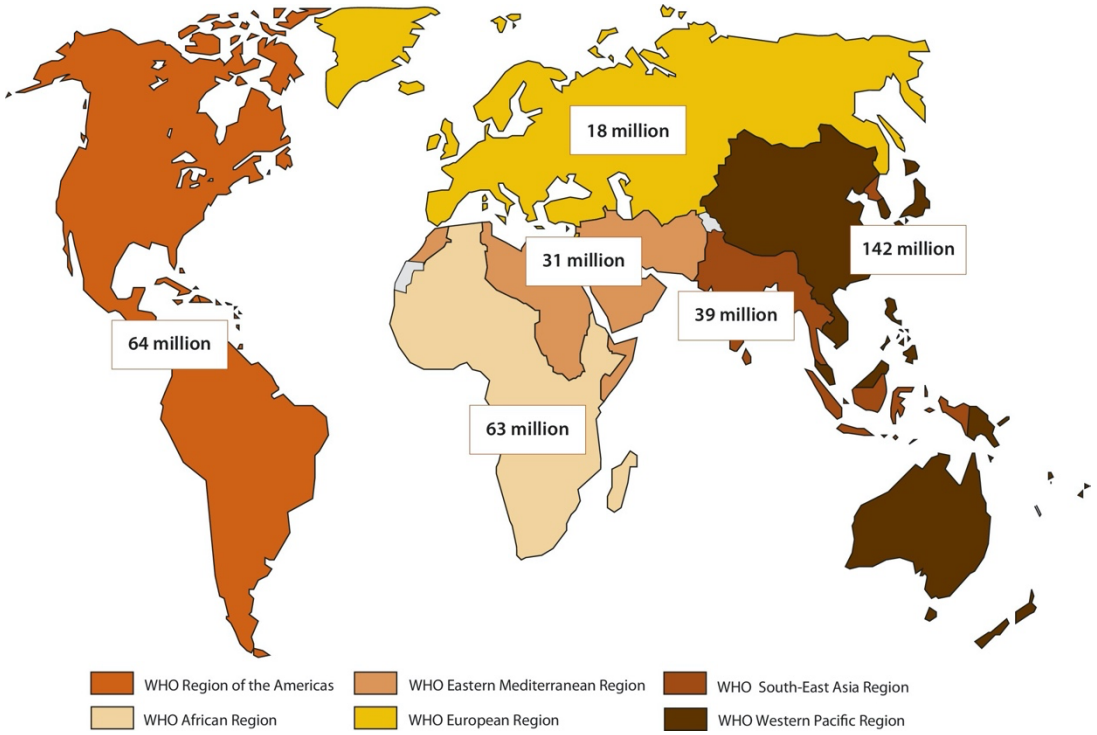


Figure 2: Global prevalence estimates of 4 curable STI infections (chlamydia, gonorrhoea, syphilis and trichomoniasis in 2012. Taken from (Gottlieb *et al.*, 2016).

1.2.2 HSV-2 immune response

HSV-2 causes genital ulceration in infected individuals, these lesions can cause pain and are often recurrent (Gupta, Warren and Wald, 2007; Looker *et al.*, 2020). Recurrence is driven by virions that establish latency in the dorsal root ganglia early following HSV-2 infection (Cunningham *et al.*, 2006; Jaishankar and Shukla, 2016). In neonates, HSV-2 infection can cause severe neurological disease and/or death (Kimberlin and Baley, 2013). HSV-2 also increases the risk of acquiring HIV approximately 3- to 4- fold (Wald and Link, 2002). While there are antiviral treatments available for HSV-2, so far, there is no licensed HSV-2 vaccine (Ike *et al.*, 2020). To develop better control strategies for HSV-2 an intimate understanding of the immune responses to the virus is required, including the potential of other infections (such as helminth infections) to influence susceptibility to HSV-2, discussed in detail in section 1.4.

Innate immunity

The innate response to HSV-2 is initiated following recognition of pathogen associated molecular patterns (PAMP) by TLR9 and TLR2 on plasmacytoid dendritic cells pDC, which in turn produce type I interferons, IFN-α and IFN-β (Lund *et al.*, 2003, 2006). Upregulation of interferon related genes in the genital mucosa triggers antiviral mechanisms such as apoptosis of infected cells, immune cell recruitment, and inhibition of viral protein expression and viral replication (Taylor, Little and O'Brien, 1998; Mossman and Ashkar, 2005). Type I interferons also prevent tissue immunopathology resulting from matrix metalloproteinase production by macrophages during HSV-2 infection in mice (Lee *et al.*, 2022). In addition to Type I interferon production, DC prime cytotoxic

and IFN-γ producing CD8 T cells, required to control HSV-2 infection, in mice (Nelson *et al.*, 2011; Shin *et al.*, 2016). Depletion of pDC, resulted in impaired type I IFN and CD8+ T cell responses and decreased immune cell recruitment and NK cell activation following HSV-2 infection in mice (Swiecki *et al.*, 2013).

IFN-α activates natural killer (NK) cells which can kill HSV-2-infected cells or act as a source of IFN-γ early in infection (Milligan and Bernstein, 1997; Gill *et al.*, 2011). In mouse models, NK cells were demonstrated to be required in immune control of HSV-2, as their depletion resulted in increased susceptibility to infection and increased pathology following infection (Ashkar and Rosenthal, 2003; Nandakumar *et al.*, 2008). Cytotoxic CD8+ T cell responses were also impaired following NK cell depletion, suggesting a role for NK cells in augmenting CD8+ T cell responses to HSV-2, likely via IFN-γ production or other co-stimulatory mechanisms (Nandakumar *et al.*, 2008).

Requirement for NK cells and pDC in anti-HSV-2 immunity was also demonstrated in studies in humans that reported severe disease in HSV-2 infected individuals deficient in NK cells and pDC (Dalloul *et al.*, 2004).

Adaptive immunity

T cells play a key role in immunity against HSV-2. Vaccination with replication defective HSV-2 or a multivalent vaccine induced expansion of systemic and mucosal memory CD4+ T cells (Parr and Parr, 1998; Odegard *et al.*, 2016; Kim *et al.*, 2020), while T cell depletion resulted in impaired viral control in vaccinated mice following HSV-2 challenge (Parr and Parr, 1998; Kim *et al.*, 2020). In primary infection as well as vaccination models of HSV-2, protection conferred

by CD8 T cells was mediated by IFN-γ (Dobbs *et al.*, 2005; Shin *et al.*, 2016) production and cytotoxic activity (Nelson *et al.*, 2011). CD4+ T cells, on the other hand, were required for recruitment and priming of HSV-2 specific CD8+ T cells in the FRT in primary infection (Nakanishi *et al.*, 2009; Kumamoto *et al.*, 2011). In humans HSV-2 infection resulted in accumulation of effector memory CD4 and CD8 T cells in the FRT (Posavad *et al.*, 2017).

B cells also play a role in immunity to HSV-2. B cell-deficient (μMT and Igh-6tm 1Cgn), vaccinated mice displayed higher vaginal viral titres than control mice following HSV-2 challenge (Dudley, Bourne and Milligan, 2000; Parr and Parr, 2000). Viral titres were however reduced in immune B cell-deficient mice following passive administration of immune serum (Dudley, Bourne and Milligan, 2000; Morrison, Zhu and Thebeau, 2001). Despite a role in reducing viral titres following infection, B cell and antibody responses were not protective against mucosal infection (McDermott, Brais and Evelegh, 1990; Morrison, Zhu and Thebeau, 2001). The adaptive immune response to HSV-2 is discussed in further detail in Chapter 10.

There is currently limited information on how underlying helminth infection impacts primary and vaccine-mediated immunity to HSV-2. So far one study has demonstrated increased pathology in HSV-2 infected mice with prior helminth infection that was associated with a mucosal Type 2 immune response (Chetty *et al.*, 2021). Therefore, part of this thesis, will address B cell immunity and antibody responses elicited in a HSV-2 vaccine-challenge mouse model in the context of underlying helminth infection.

1.2.3 HPV immune response

Human papillomaviruses (HPVs) infect the skin and mucosa, while a large number of HPV infections can be cleared by the host immune system (Rodríguez *et al.*, 2008; Winer *et al.*, 2011), in a small number of individuals, persistent infection can progress to cancer (Zur Hausen, 2002). HPV can be classified into low-risk types, associated with anogenital warts and benign lesions (Egawa and Doorbar, 2017), and high-risk HPV types which are associated with oropharyngeal and anogenital cancers, including cervical cancer (zur Hausen, 2009). HPV employs several invasion strategies to escape immune destruction by the host (Steinbach and Riemer, 2018), and clearance of the virus depends on a balance of host immune responses (Wakabayashi *et al.*, 2019).

Innate immunity

Innate immunity to HPV is triggered by recognition of HPV PAMPS by pattern recognition receptors (PRR), including TLR3, TLR7, TLR8 and TLR9 on keratinocytes (Daud *et al.*, 2011; Scott *et al.*, 2015). This triggers cytokine and chemokine responses which recruit additional immune cells to the site of infection (Boccardo, Lepique and Villa, 2010; Karim *et al.*, 2013).

Dendritic cells play a role in HPV clearance as low proportions of pDC in peripheral blood in humans (accompanied by high T reg proportions) was positively associated with persistence of oncogenic HPV types (Strickler *et al.*, 2014). Dendritic cells present HPV antigens to CD8 T cells to promote cytolytic (Bellone *et al.*, 2009) and HPV-specific IFN-γ responses (Rudolf *et al.*, 2001). DC can also inhibit immunity to HPV by inhibiting CD8+ T cell responses. PD-L1, the

receptor for PD-1 is expressed on DC, and PD-L1/PD-1 interactions promote T cell anergy, exhaustion and apoptosis (Butte *et al.*, 2009; Francisco *et al.*, 2009). Furthermore, PD-L1 was upregulated in DC in women with squamous cell carcinoma of the head and neck (SCCHN; Ferris, 2015) and cervical cancer (Yang *et al.*, 2013) and this correlated with impaired cytotoxic CD8 T cell immunity, blocking PD-L1/ PD1 interactions was able to reverse this effect (Bashaw *et al.*, 2017).

M1 macrophages produce IFN-α and IL-12 which activate NK cells, Th1 cells and CD8 T cells to produce IFN-γ (Matikainen *et al.*, 2001), important in HPV control. Macrophages also kill virally infected cells by production of TNF-α and reactive nitrogen species (Routes *et al.*, 2005). M2 macrophages/ tumour associated macrophages (TAM), on the other hand, induced by IL-4, IL-13, IL-10 and TGF-β (Gordon and Martinez, 2010) promote tumour development following HPV infection through production of IL-10 and TGF-β (Giannini *et al.*, 1998; Lepique *et al.*, 2009), which creates an immunoregulatory environment characterised by increased FoxP3+ regulatory T cells (Van Esch *et al.*, 2015; Sun *et al.*, 2017) and suppressed IL-12 production, and subsequent suppression of NK, Th1 and cytotoxic CD8+ T cells (Lepique *et al.*, 2009; Gabrilovich, Ostrand-Rosenberg and Bronte, 2012) required in control of HPV. TAM numbers were positively associated with advanced disease stages in HPV infected individuals with cancerous lesions (Hammes *et al.*, 2007; Chen *et al.*, 2017). TAM also promote tissue regeneration, angiogenesis and metastasis, which are associated with tumour progression (Grivennikov, Greten and Karin, 2010).

Myeloid derived suppressor cells (MDSC) are immature mononuclear or polymorphonuclear myeloid cells with immunosuppressive roles in tumour microenvironments (TME; Umansky *et al.*, 2016). In a mouse model of HPV associated tumours, CD8 anti-tumour responses were inhibited by MDSC, this was mediated by altered antigen presentation via the MHCI pathway (Gabrilovich *et al.*, 2001). In humans, MDSC were significantly elevated in peripheral blood and tumour biopsies of SCCHN and cervical cancer patients in comparison to normal controls (Ma *et al.*, 2017; Wu *et al.*, 2018). *In vitro* experiments demonstrated suppression of CD4 and CD8 proliferative responses by MDSC derived from cervical cancer patients (Wu *et al.*, 2018). Furthermore, MDSC were associated with metastasis in cervical cancer (Wu *et al.*, 2018).

NK cells are important in immunity to HPV. NK cells perform cytotoxic functions to kill virally infected cells and produce cytokines such as IFN-γ, TNF-α, GM-CSF and chemokines such as MIP-1α which recruit additional immune cells to sites of infection (Waldhauer and Steinle, 2008). NK cells were fewer in early cervical lesions of women with HPV 16 infection in comparison to women with HPV 18 infection suggesting that HPV 16 inhibits NK responses in HPV associated cervical lesions (Zhang *et al.*, 2019). Furthermore, NK receptors, NKp30 and NKp46, were downregulated in circulating NK cells in women with cervical cancer and high grade cervical lesions (Garcia-Iglesias *et al.*, 2009), an indication that HPV 16 suppresses NK cell responses as a form of immune escape, to allow persistence and progression to cancer.

Adaptive immunity

CD4 T cells play an important role in immunity to HPV, evidenced by poor cervical cancer prognosis in HIV infected women, as HIV infection is characterised by CD4 T cell depletion (Harris *et al.*, 2005; Denny *et al.*, 2008; Singh *et al.*, 2009). Tumour infiltrating CD4+ T cells expressing PD-1 and ICOS with reactivity against E6 and E7 HPV proteins have been reported in SCCHN patients (Duhen *et al.*, 2022). Furthermore, cytokines produced by CD4+T cells, such as IFN-γ and TNF-α have previously been demonstrated to have a negative association with progression to cervical cancer (El-Sherif *et al.*, 2001; Bais *et al.*, 2007). Th2 immune responses are also induced by HPV infection and can function to enable immune evasion by HPV and subsequent persistence and progression to cervical cancer. Type 2 cytokines promote differentiation of TAM which contribute to development of HPV associated tumours (Giannini *et al.*, 1998; Lepique *et al.*, 2009). Type 2 cytokines levels in the blood and cervicovaginal fluids (CVF) were also positively associated with high grade cervical lesions (Al-Saleh *et al.*, 1998).

CD8+ T cells play antitumorigenic roles in HPV associated tumours. CD8+ T cell tumour infiltration was independently associated with prognosis of SCCHN and cervical cancer (Piersma *et al.*, 2007; Balermpas *et al.*, 2016). The oncogenic HPV protein E7 actively inhibited perforin expression in CD8+ T cells *in vitro* (Bhat *et al.*, 2018), suggesting that cytotoxic activity of CD8+ T cells inhibits tumour progression. Furthermore, HPV E2, E5 and E6 specific CD8+ T cells expressing PD-1 and low in granzyme and perforin gene expression were detected in SCCHN, and antigenic stimulation was able to restore proliferative

and effector functions of these CD8+ T cells (Eberhardt *et al.*, 2021). Consistent with this, previous clinical studies demonstrated that PD-1 blockade can boost effector CD8+ T cell function in TME (Heeren *et al.*, 2019; Balança *et al.*, 2020), highlighting role for PD-1 blockade in antitumour therapy.

T regs isolated from lymph node biopsies of cervical cancer patients were shown to suppress proliferative and cytokine (IFN-γ and IL-2) responses from CD25- responder T cells in co-culture (Van Der Burg *et al.*, 2007), suggesting that a immunoregulatory environment induced during HPV infection interferes with anti-tumour CD4 T cell responses. This is supported by an earlier study that demonstrated that T regs recruited to the TME in response to CCL22 produced by TAM, suppressed effector T cell antitumour responses in women with ovarian cancer (Curiel *et al.*, 2004). Furthermore, women with persistent HPV 16 infection had higher frequencies of circulating T regs than HPV negative women or women who had cleared the infection (Molling *et al.*, 2007).

Antibody responses are also important in immunity against HPV. Approximately 50-70% of patients with incident HPV 18, 16 and 6 infections seroconverted following infection, and most antibody responses to HPV 16 and HPV 18 persisted up to 4 years after infection (Carter *et al.*, 2000). Antibody responses induced by vaccination are more robust and persist longer than those induced by natural infection. Indeed, seroconversion occurred in approximately 90-100% of women who received the Cervavix and Gardasil HPV vaccines, and HPV16 and HPV18 antibodies persisted approximately to 9 - 12 years following vaccination (Artemchuk *et al.*, 2019; Hoes *et al.*, 2020). Furthermore, strong correlations between serum and CVF antibody levels (Petäjä *et al.*, 2011; Pattyn

et al., 2019) suggests that transudation of systemic antibodies into the FRT occurs following HPV vaccination. Antibodies binding to the surface protein L1 are hypothesised to protect against HPV invasion in the mucosa of the genital tract following HPV vaccination (Bissett, Godi and Beddows, 2016).

One study in Peru demonstrated that soil transmitted helminth infections were associated with increased risk of HPV (Gravitt *et al.*, 2016). Given the limited data on the impact of helminths on immunity and viral infection in the FRT, we wanted to determine how FRT immunity and risk of HPV is impacted by hookworm infection.

1.3 Helminth mediated immune modulation

In an attempt to escape immune destruction by the host, helminths have developed sophisticated strategies that involve direct modulation of host immunity to control inflammation (Maizels, 2020). This can have either beneficial or deleterious effects on the host's ability to respond to infections and disorders.

The "Hygiene Hypothesis" was developed based on observations of a family lineage over several decades (Strachan, 1989, 2000). Children with constant exposure to microorganisms earlier in life had lower incidence of asthma and hay fever later in life in comparison to children brought up in relatively more hygienic environments (Strachan, 1989, 2000), suggesting a link between microbial exposure and immune modulation to protect against allergy. The hygiene hypothesis has been expanded to include organisms such as parasites and other microbes (Maizels, 2020).

The scope of unrelated conditions impacted by helminth infections is wide, including, several autoimmune diseases and disorders such as multiple sclerosis, diabetes, inflammatory bowel disease, rheumatoid arthritis and infectious diseases (Maizels and McSorley, 2016).

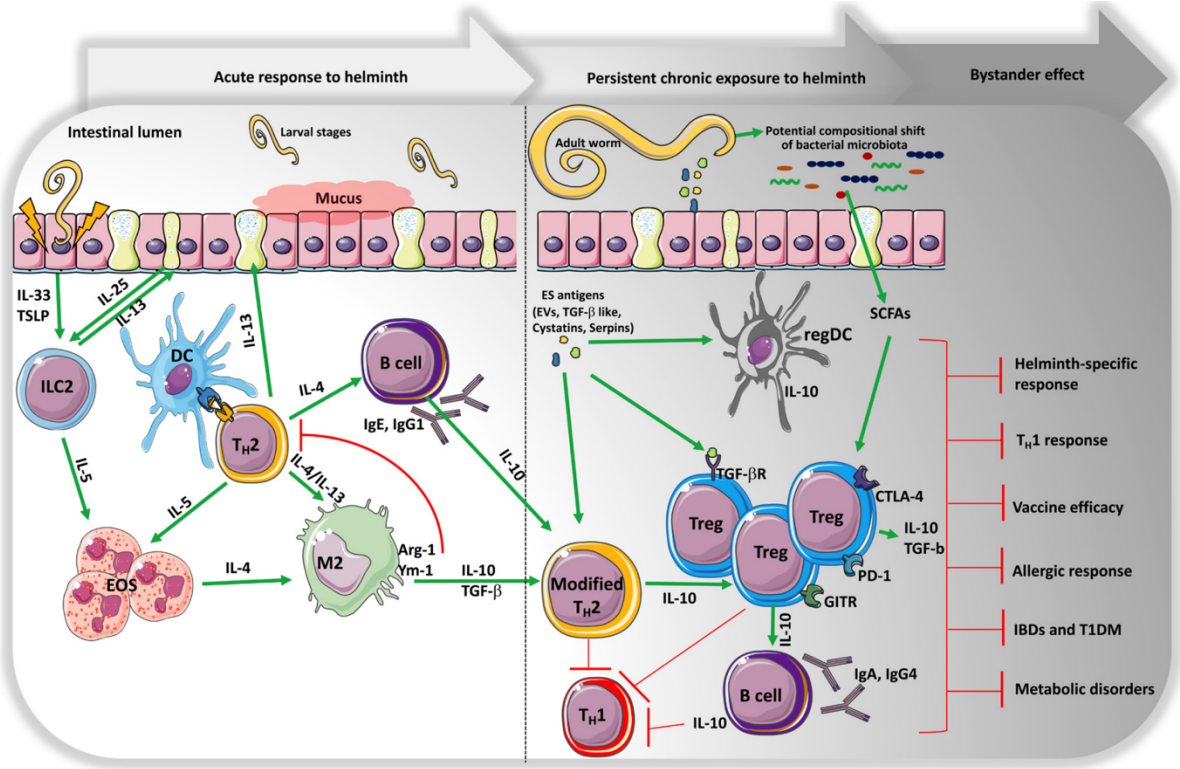


Figure 3: Helminth mediated immune modulation. Following infection, helminths and their products trigger alarmin (IL-25, IL-33 and TSLP) release by epithelial cells in the mucosa of colonised organs such as the lung or small intestine. Alarmins activate and promote differentiation of ILC2 and Th2 cells which produce Type 2 cytokines IL4, IL-5 and IL-13. Type 2 cytokines promote worm expulsion by inducing goblet cell hyperplasia, smooth muscle contraction, mucus secretion and eosinophil recruitment. Type 2 cytokines also promote differentiation of Type 2 macrophages and production of Type 2 associated antibodies IgG1, IgG4 and IgE. Persistent helminth infections result in a modified Type 2 immune response which modulates the Type 1 immune response, this response is characterised by upregulation of regulatory cytokines such as IL-10 produced by T regs, B regs and macrophages and regulatory DC and TGFβ produced by macrophages and T regs. This immunoregulatory environment can alter immunity to unrelated pathogens, vaccines, autoimmune diseases and disorders and allergies. Taken from (Gazzinelli-Guimaraes and Nutman, 2018).

The impact of helminth infections on bacterial and viral infections is discussed below.

1.3.1 Bacterial infections

Helminth infections can modulate immunity to unrelated bacterial infections as well as dampen immune responses to vaccines. In a mouse model, *N. brasiliensis* infection attenuated both vaccine-induced and natural immune responses to *Salmonella typhimurium* (Bobat *et al.*, 2014). In this study, bacterial burdens were elevated in mice with prior *N. brasiliensis* infection, accompanied by reduced IgG antibody titres in *S. typhimurium* porin vaccinated and *S. typhimurium* infected mice. Furthermore, *H. polygyrus*-induced metabolite alteration in the gut resulted in increased bacterial burdens in *S. typhimurium* co-infected mice (Reynolds *et al.*, 2017). The attenuated response to *S. typhimurium* was independent of Th2 or T reg induction, as gut bacterial burdens in *il4*, *stat6* and *rag1* deficient mice did not differ from those in WT co-infected mice (Reynolds *et al.*, 2017). Treatment of mice with anti-helminthic drugs to clear *H. polygyrus* before *S. typhimurium* infection rescued mice from susceptibility to *S. typhimurium* (Brosschot *et al.*, 2021). Drug treatment to clear *H. polygyrus* after establishment of *S. typhimurium* infection did not however reduce bacterial replication in the gut, suggesting that helminth infection interfered with establishment rather than persistence of infection once initial bacterial colonization occurred.

Susceptibility to *Mycobacterium tuberculosis* has also been linked to helminth infection. Having active TB was positively associated with being helminth infected in humans, with an even stronger association observed for individuals

with multiple helminth infections (Elias *et al.*, 2006). Furthermore, prevalence of intestinal nematodes, *A. lumbricoides, S. stercoralis. T. trichiura, A. duodenale and N americanus* was higher among individuals with pulmonary TB in comparison to TB uninfected individuals (Tristão-Sá *et al.*, 2002). Helminth mediated modulation of *M. tuberculosis* could be a result of suppression of Type 1 immunity, essential in protection against *M. tuberculosis* (O'Garra *et al.*, 2013). Indeed, stimulation of whole blood from helminth infected individuals with *M. tuberculosis* antigen yielded elevated IL-10 and reduced IFN-γ responses (Resende Co *et al.*, 2007).

Conflicting roles of helminths in latent TB infection (LTBI) have been reported. Hookworm infected individuals had a lower incidence of LTBI, and *in vitro* studies demonstrated enhanced killing of mycobacteria incubated in blood from helminth infected individuals. (O'Shea *et al.*, 2018). Furthermore, an inverse correlation between mycobacterial loads and eosinophil counts was reported in this study. Conversely, LTBI individuals infected with hookworms exhibited diminished *M. tuberculosis* specific Th1 and Th17 responses and enhanced Treg responses (George *et al.*, 2013). Th1 and Th17 cells are important in protective immunity to *M. tuberculosis* (Scriba *et al.*, 2008; O'Garra *et al.*, 2013). Th1 and Th17 cytokine responses and proliferative T cell responses to *M. tuberculosis* antigens were also lower in individuals with filarial infections (Stewart *et al.*, 1999; George *et al.*, 2014).

Studies in mouse models also reported conflicting evidence of helminth mediated modulation of immunity to mycobacteria. Underlying *N. brasiliensis* infection impaired innate immune responses to *M. tuberculosis* that was dependent on

induction of alternatively activated macrophages and IL-4Ra signalling (Potian *et al.*, 2011). In contrast, clearance of *Mycobacterium bovis* BCG from the lung was not altered by *N. brasiliensis* infection (Erb *et al.*, 2002). In another study *N. brasiliensis* infection induced reduction of *M. bovis* BCG bacterial burdens early in infection, with enhanced IFN-γ and IL-13 production, increased CD4+ T cells and transient elevation of neutrophils and macrophages in the lungs (Du Plessis *et al.*, 2013).

There is also conflicting evidence on the influence of helminth infections on responses to *M. tuberculosis* vaccines. Deworming prior to BCG vaccination, for example, was shown to result in enhanced IFN-γ and decreased TGF-β responses in PBMC (Elias *et al.*, 2008), suggesting TGF-β mediated suppression of the Th1 response to the BCG vaccine in individuals with helminth infection. In addition children born to women with filarial or schistosome infections displayed reduced IFNγ responses to BCG vaccination in comparison to children born to helminth uninfected mothers (Malhotra *et al.*, 1999). Furthermore, in a mouse model, *S. mansoni* infection prior to BCG vaccination resulted in elevated bacterial burdens following *M. tuberculosis* challenge (Elias *et al.*, 2005). In contrast other studies reported no impact of filarial (Lipner *et al.*, 2006) or schistosome (Wajja *et al.*, 2017) infection on *M. tuberculosis* antigen-specific responses in BCG vaccinated individuals.

Beneficial outcomes for bacterial infection in mice with underlying helminth infection have also been reported. *T. spiralis* infection protected mice against severe *Pseudomonas aeruginosa* induced pneumonia (Long *et al.*, 2022). *T. spiralis* infected mice displayed enhanced Th2 responses (IL-4, IL-15, IL-13 and

GATA3), accompanied by decreased levels of pro inflammatory cytokines (IL-1β and IL-6) and chemokines (CXCL1 and CXCL2) and reduced neutrophil recruitment to the lung following *P. aeruginosa* challenge (Long *et al.*, 2022).

1.3.2 Viral infections

Helminth infection can also alter immunity to viruses. *T. spiralis* infected mice were protected against inflammation induced damage in the lungs following influenza virus infection (Furze, Hussell and Selkirk, 2006). Here, *T. spiralis* infection resulted in diminished TNF-α responses in the host, and reduced infiltration of CD4 and CD8 T cells into the lungs. Suppression of inflammation was however independent of IL-10. In addition, mice in the chronic stage of infection with *S. mansoni* displayed low-grade lung inflammation, characterised by elevated IFN-γ, IL-12, TNF-α, IL-4, IL-5, IL-13, IL-6 and MIP-1α levels, along with increased goblet cell hyperplasia and mucus secretion (Scheer *et al.*, 2014). In this model, enhanced protection to respiratory viral infection was observed upon secondary infection with Influenza A virus or pneumonia virus that was dependent on TNF-α mediated induction of goblet cell hyperplasia and mucus production in the lung (Scheer *et al.*, 2014). In another study, *H. polygyrus* infection induced Type I interferon production in the gut and lungs, which protected mice in a microbiota dependent manner from subsequent respiratory syncytial virus (RSV) challenge (McFarlane *et al.*, 2017). *Rag1*$^{-/-}$ and *IL4ra-/-* mice were protected against infection, suggesting that Th2 immunity did not mediate protection. Instead, protection was dependent on Type I interferon signalling, and microbiota dependent mechanisms, as Ifnar*1*$^{-/-}$ and germ-free mice were more susceptible to RSV.

In contrast to the beneficial effect of helminths on the outcome of respiratory viral infections, the response to enteric viral infections was impaired by helminth co-infection. Murine norovirus (MNV) and *T. spiralis* co-infected mice displayed elevated MNV viral loads, reduced virus-specific CD8+ and CD4+ T cells and reduced polyfunctional CD8+ T cells (Osborne *et al.*, 2014). Defective immunity to MNV was independent of the microbiota, despite changes in gut microbiota composition induced by *T. spiralis* infection. Instead, impairment of MNV immunity was mediated by Th2 signalling and alternatively activated macrophages (AAM), induced by *T. spiralis* infection (Osborne *et al.*, 2014). In addition, helminth mediated susceptibility to MNV is also potentially dependent on tuft cell signalling, as treatment with IL-4 and IL-25 augmented MNV infection by increasing tuft cell proliferation (Wilen *et al.*, 2018). In this study tuft cells were identified as the main target cells for MNV replication in the gut.

Immunity to the flavivirus, West Nile Virus (WNV) was also impaired by helminth infection. *H. polygyrus bakeri* infection resulted in increased susceptibility to WNV that was mediated by type 2 associated immune responses i.e., Stat6, IL-4, IL-4Rα and tuft cells (Desai *et al.*, 2021). Alteration in the structural integrity of the small intestine and disruption of enteric neuronal networks resulted in increased bacterial translocation from the intestine and subsequent collapse in virus specific CD8+ T cell responses in *H. polygyrus bakeri* and WNV co-infected mice. These structural and biological changes were dependent on Type 2 signalling, as Stat6 knockout mice did not display changes in gut morphology, bacterial translocation to the spleen or impaired CD8+ T cell responses, all of which were induced by treatment of mice with an IL4 complex in the absence of *H. polygyrus bakeri* infection. Furthermore, decreased susceptibility to WNV in

tuft cell deficient mice and mice lacking IL-4Rα expression on tuft cells indicates that type 2 signalling in intestinal tuft cells was a crucial mediator of pathology in co-infection. Moreover, treatment of mice with IL-25 or succinate (ligand for the succinate receptor expressed on tuft cells) resulted in enhanced pathology to WNV.

There were contrasting reports on the impact of helminth infection on responses to murine gamma herpesviruses (MHV68 and MuVH4). Reese et al demonstrated that *H. polygyrus* infection and *S. mansoni* egg challenge induced reactivation of MHV68 from latency (Reese *et al.*, 2014). This was Type 2 mediated, as IL-4 and Stat6 signalling in infected macrophages blocked IFN-γ mediated suppression of MHV68. In contrast, expansion of virtual memory (T_{VM}) CD8+ T cells that protected against MuVH4 was observed in mice treated with *S. mansoni* eggs (Rolot *et al.*, 2018). Expansion was mediated by IL-4 signalling, as treatment of mice with IL-4 complexes led to an increase in virus-specific activated virtual memory CD8+ T cells, while helminth infection of *Il4ra*$^{-/-}$ mice did not alter T_{VM} CD8+ T cell numbers.

1.4 Helminth mediated immune modulation in the female reproductive tract (FRT)

1.4.1 Influence of helminth infections on fecundity

The impact of underlying helminth infection on reproduction has been explored in both mice and humans. *S. mansoni* infected mice mated in the Th2 phase of the response to the parasite bore foetuses with significantly lower weights in comparison to uninfected mice or mice mated in the Th1 or regulatory phase of

the response (Straubinger *et al.*, 2014). In addition, *S. mansoni* infected mice had significantly lower numbers of viable offspring in comparison to uninfected mice (Amano, Freeman and Colley, 1990). Trematode infections in invertebrates were also associated with reduced fecundity in the host (Hurd, 2001).

In humans, *S. japonicum* infection was associated with increased levels of inflammatory cytokines in maternal PBMC, placental blood and cord blood, with IL-1β and TNF-α from placental syncitiotrophoblasts being associated with lower birth weights (Kurtis *et al.*, 2011). In contrast, *S. mansoni* infection had no impact on birth outcomes in cohorts in Uganda and the Philippines, as no significant alterations in infant birth weight, congenital mortality or congenital anomalies were observed following albendazole or praziquantel treatment of mothers to clear schistosome infection (Ndibazza *et al.*, 2010; Olveda *et al.*, 2016).

A longitudinal follow up study in Bolivia demonstrated that nematode infections had contrasting impact on fecundity among women of reproductive age depending on the infecting species. Hookworm infection was associated with older age at first pregnancy and lower odds of subsequent pregnancy after the first (Blackwell *et al.*, 2015). In contrast, Ascaris infection, was positively associated with an earlier age at first pregnancy and increased likelihood of multiple pregnancies after the first pregnancy among women below the age of 32.

1.4.2 Modulation resulting from helminths that transit the FRT

Female Genital Schistosomiasis

Female Genital Schistosomiasis (FGS) is a complication in the urogenital tract arising from *S. haematobium* egg deposition in the urinary bladder and female reproductive tract (Odegaard and Hsieh, 2014). FGS causes significant morbidity in the host, characterised by symptoms such as ulceration, pain, vaginal itching, and haematuria (Hegertun *et al.*, 2013; Ismail *et al.*, 2014; Norseth *et al.*, 2014; Randrianasolo *et al.*, 2015). In addition, *S. haematobium* eggs lodged in the uterus or fallopian tubes can cause infertility (Downs *et al.*, 2011; Woodall and Kramer, 2018) and urogenital schistosomiasis has been associated with low birth weight in humans (Mombo-Ngoma *et al.*, 2017). *S. haematobium* infection has also been associated with bladder cancer, leading to its classification by the International Agency for Research on Cancer (IARC) as a group 1 carcinogen (Ishida and Hsieh, 2018; McManus *et al.*, 2018). Some studies have reported a link between FGS and cervical dysplasia (Kjetland *et al.*, 2010; Rafferty *et al.*, 2021), implying a potential impact on progression to cancer in HPV infected women, however more investigations are required to fully understand this.

Pathology in the urogenital tract in *S. haematobium* infections is a result of the immune response to the parasite eggs, which, through their excretory/secretory products, induce inflammation resulting in granuloma formation in resident tissue (McManus *et al.*, 2020). Granulomas recovered from experimental injection of *S. haematobium* eggs into the bladder wall in mice comprised eosinophils, macrophages and neutrophils (Fu *et al.*, 2012). Additionally, type 2 cytokines, IL-

4, IL-5 and IL-13 were elevated in the bladder and IL-4 was elevated in genital draining lymph nodes of egg treated mice (Fu *et al.*, 2012).

S. haematobium infection is associated with increased risk of HIV infection (Kjetland *et al.*, 2006; Downs *et al.*, 2011; Ndeffo Mbah *et al.*, 2013). One possible explanation for this is that *S. haematobium* induces genital lesions that likely provide easy entryway for HIV particles in the genital mucosa (Helling-Giese *et al.*, 1996; Swai *et al.*, 2006). Susceptibility to HIV could potentially also be due to inflammation induced by *S. haematobium* in the host (Helling-Giese *et al.*, 1996), resulting in recruitment of HIV target cells to genital tissue. This hypothesis is supported by the finding that *S. haematobium* infection resulted in increased CCR5 expression on monocytes in the genital tract (Kleppa *et al.*, 2014). CCR5 is an immune receptor that facilitates entry of HIV into target cells (Lederman *et al.*, 2006). Praziquantel treatment of women with FGS reduced the number of CCR5 expressing monocytes and CD4+ T cells in the FRT (Kleppa *et al.*, 2014).

Filariasis

Filariasis is a disease caused by parasites of the species *Wuchereria bancrofti, Brugia malayi* and *B. timori*. Filarial infection is positively associated with increased risk of HIV (Kroidl *et al.*, 2016). One possible mediator of this risk is immune activation driven by filarial parasites. Indeed, *W. bancrofti* infection resulted in increased frequencies of activated (HLA-DR+ and CD38+) and effector memory CD4 T (CD45RO+ CD27-) cells in peripheral blood of HIV negative individuals, these cells are potential targets for HIV infection (Kroidl *et al.*, 2019). This is consistent with findings from a previous study showing PBMC

from lymphatic filariasis patients were more susceptible to HIV infection in comparison to PBMC from the same individuals post treatment (Gopinath *et al.*, 2000).

Distinct immunological profiles are associated with the different clinical manifestations of filariasis. Individuals with filarial lymphoedema displayed increased systemic inflammation with elevated IL-17 and IFN-γ and suppressed FoxP3 in PBMC stimulated with *Brugia malayi* antigen (BmA; Babu, Bhat, Kumar, *et al.*, 2009). Patients with chronic filarial infections who are microfilaria positive (MF+), on the other hand, had a prominent Type 2 immune signature, with elevated IL-4, IL-5 and IL-13, detected in host PBMC stimulated with BmA (Babu, Kumaraswami and Nutman, 2005; Arndts *et al.*, 2012). In addition, the type 1 response (IFN-γ production) was dampened, in latently infected MF+ individuals (Babu, Kumaraswami and Nutman, 2005). This is a potential mechanism through which filariasis infections could impact immunity to HIV or other pathogens requiring Type 1-mediated immune control. Surprisingly, however, even though filarial parasites can infect the FRT, *Wuchereria bancrofti* infection was not associated with changes in fertility or pathology in the genital tract (Bernhard *et al.*, 2000).

1.4.3 Indirect modulation of immunity in the FRT

Earlier in this chapter the influence of helminth infection on immunity to unrelated pathogens is discussed, this involves modulation of immunity by parasites that infect co-colonised tissue. Systemic impact of helminth infections on immunity within anatomical compartments they do not occupy has also been explored using the *H. polygyrus* model, in which infection is restricted to the gut (Mohrs *et*

al., 2005). Infection with *H. polygyrus* enhanced protection against *Pseudomonas aeruginosa* in the lungs of co-infected mice, with a concomitant increase in CD4+ T cells, Th2 cytokines and goblet cell hyperplasia (Long *et al.*, 2019). Furthermore, *H. polygyrus* infection suppressed ovalbumin (OVA) induced allergic airway inflammation via induction of regulatory T cells (Wilson *et al.*, 2005). Excretory/secretory products of *H. polygyrus* also protected against harmful lung inflammation in the OVA-induced allergic airway inflammation model (McSorley *et al.*, 2012). This was characterised by suppression of Th1, Th2 and Th17 cytokines, as well as reduction in eosinophil recruitment to, and expression of arginase 1 and RELM-α in the lungs. Regulation of inflammation was hypothesised to be effected by both a TGF-β mimic found in *H. polygyrus* ES (Grainger *et al.*, 2010; Johnston *et al.*, 2017), as well as a heat stable immunomodulatory molecule that conferred protection following subsequent OVA challenge (McSorley *et al.*, 2012).

H. polygyrus infection was also protective against lung pathology induced by *N. brasiliensis* during the lung migrating phase of the parasite (Filbey *et al.*, 2019). Mucosal immunity induced in the gut by *H. polygyrus* was reflected in the lung, and protection against *N. brasiliensis* was via IL-33 mediated induction of IL-5 producing CD4 T cells and subsequent recruitment of eosinophils, furthermore, protection against *N. brasiliensis* persisted long after clearance of *H. polygyrus* from the gut (Filbey *et al.*, 2019).

Helminths can also exert immunomodulation in the host via a neuronal axis. Alarmin release, triggered by invasion of mucosal tissue by helminths results in activation of ILC2 which express receptors for neuroimmune mediators. Murine

ILC2 co-localised with cholinergic neuromedin U (NMU) expressing neurons in the gut (Klose *et al.*, 2017) and *in vitro* treatment of ILC2 with NMU rapidly enhanced IL-5, IL-13 (Cardoso *et al.*, 2017; Klose *et al.*, 2017) and IL-9 (Klose *et al.*, 2017) production, mediated by engagement of the NMU receptor (NMUR) on ILC-2. Administration of NMU *in vivo* also enhanced ILC2 and Type 2 responses resulting in enhanced *N. brasiliensis* worm expulsion from the gut and enhanced worm killing in the lung (Cardoso *et al.*, 2017; Klose *et al.*, 2017). Furthermore, *N. brasiliensis* infection or treatment of mice with "alarmins" (IL-33 and IL-25) resulted in increased acetylcholine (ACh) production (Roberts *et al.*, 2021) and upregulation of choline acetyltransferase (ChAT) expression (Chu *et al.*, 2021) on ILC2 in the lungs and gut. ChAT is the enzyme that regulates synthesis of the Ach (Oda, 1999). Transgenic mice with defective Ach and ChAT production in ILC-2 (Chu *et al.*, 2021; Roberts *et al.*, 2021) and mice deficient in the muscarinic receptor M3 which binds acetylcholine (Darby *et al.*, 2015; McLean *et al.*, 2016) exhibited impaired *N. brasiliensis* worm expulsion.

Soil transmitted helminths and Type 2 immunity in the FRT

Helminths induce Type 2 and regulatory immune responses in the host and have the capacity to modulate immunity to unrelated pathogens and in tissues they do or do not occupy at any stage during their life cycle (Maizels and McSorley, 2016). Few studies have investigated the influence helminth infections may have on immunity in the FRT and sexually transmitted viral infections (STVI).

In mice, *N. brasiliensis* induced a Type 2 response, with elevated IL-33, IL-4 and IL-5 and increased eosinophil infiltration into the FRT of infected in comparison to uninfected mice (Chetty *et al.*, 2021). Of note, this influence was apparent nine

days post infection, when *N. brasiliensis* had been completely cleared from the gut of infected mice, suggesting that the immunological signature helminths imprint in the FRT of the host persists after worm expulsion. This model further demonstrated increased pathology in previously *N. brasiliensis* infected mice challenged with HSV-2 (Chetty *et al.*, 2021). In these mice, pathology was associated with increased IL-33, IL-5, ILC2 and eosinophils. Depletion of IL-5 and eosinophils rescued mice with prior *N. brasiliensis* infection from enhanced pathology following HSV-2 infection. Pathology was however independent of IL-4ra signalling, as treatment of IL-4Ra knockout mice with *N. brasiliensis* and subsequent challenge with HSV-2 did not result in reduced pathology in comparison to WT mice. In addition, IFN-γresponses were suppressed in *N. brasiliensis* infected mice two days post infection with HSV-2.

The role of Type 2 immunity in enhanced susceptibility to HSV-2 infection has previously been demonstrated (Oh *et al.*, 2016). Vaginal dysbiosis induced by antibiotic treatment of mice resulted in tissue damage in the FRT that triggered IL-33 production in and increased ILC2 and eosinophil recruitment to the FRT (Oh *et al.*, 2016). Furthermore, IFN-γ responses were impaired in antibiotic treated mice and anti-viral effector CD4+ CD8+ T cells failed to traffic to the FRT of these mice (Oh *et al.*, 2016). The authors hypothesised that the Type 2 environment created by dysbiosis in the FRT suppressed IFN-γ production from local T cells and that defective IFN-γ production inhibited IFN-γ inducible chemokines CXCL9 and CXCL10, resulting in impaired recruitment of effector T cells to the FRT following HSV-2 challenge.

The mechanisms of induction of Type 2 immunity in the FRT were explored further through vaginal challenge with the serine peptidase, papain (Oh *et al.*, 2017). In this model, papain treatment resulted in elevated IL-33 production in vaginal epithelial cells, and activation of IL-5 producing ILC2. Furthermore, expansion of IL-4, IL-5 and IL-13 producing CD4+ T cells in the iliac lymph nodes and elevated IgG1 and IgE in vaginal washes was detected in papain treated mice. Induction of Type 2 immunity in the FRT was dependent on MyD88 signalling, as MyD88 deficient mice displayed diminished IL-4 production from CD4+ T cells and impaired antigen specific IgE production. Type 2 immunity was also dependent on dendritic cells expressing interferon regulatory factor 4 (IRF4), as mice deficient in IRF4 in $CD11c^{+}$ DC failed to launch Th2, IgG1 and IgE responses following papain challenge. TLR4 or IL-R1 signalling did not however play a role in induction of Type 2 immunity in the papain challenge model.

While Type 2 immunity was detrimental in HSV infection, it was demonstrated to protect against tissue damage in the upper genital tract (UGT) in mice following infection with *C. trachomatis* (Vicetti Miguel *et al.*, 2017). The mechanism of protection was induction of endometrial stromal cell (ESC) proliferation by IL-4 producing eosinophils in the UGT. In this model, IL-4 and IL-4ra knockout mice displayed enhanced UGT tissue damage in comparison to WT mice and further analysis revealed that majority (95%) of the IL-4 producing cells in the UGT were eosinophils. In addition, eosinophil depletion resulted in impaired proliferation of ESC.

In humans, a two-fold higher prevalence of HPV infection was reported among women infected with one or more of the soil-transmitted helminths (STH), *Trichuris trichiura, Ascaris lumbricoides, Ancylostoma duodenale* and *Strongyloides stercoralis* (Gravitt *et al.*, 2016). In this study, IL-4 was detected in cervicovaginal fluids of helminth infected women but not in that of uninfected women. Furthermore, there were positive correlations between vaginal IL-4 levels and levels of cytokines and chemokines involved in immunity against helminths, IL-25, IL-21, IL-5, IL-10, IL-8 and IL-31 (Gravitt *et al.*, 2016). The study by Gravitt et al. demonstrates a skewing of the immune response in the FRT of women with STH infection towards a Type 2 phenotype, that has previously been demonstrated to impair immunity to viral infection (requiring Type 1 immunity for control) in the FRT (Oh *et al.*, 2016; Chetty *et al.*, 2021). The potential impact of helminths on immunity in the FRT is summarised in **Figure 4**.

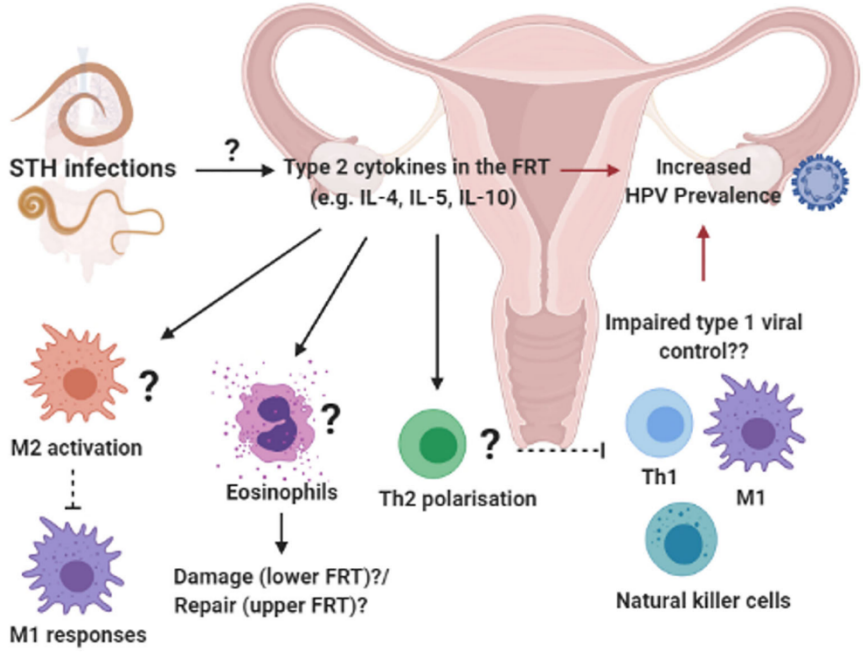


Figure 4: Systemic impact of soil transmitted helminth (STH) infection on immunity in the FRT. STH was associated with increased risk of HPV in women. Furthermore, Type 2 immunity was detected in the CVF of these women, and this was hypothesized to increase susceptibility to HPV by potentially dampening Type 1 immune responses. Taken from (Chetty *et al.*, 2020).

In contrast, Jacobs et al demonstrated that treatment of human cervical cell lines with *N. brasiliensis* somatic antigen resulted in impaired internalization of HPV-16 pseudovirus particles (Jacobs *et al.*, 2018). This was hypothesised to be due to a reduction in expression of cell surface vimentin on cervical cells induced by treatment with *N. brasiliensis* antigen. Infection of mice with *N. brasiliensis* also resulted in reduced vimentin expression in the FRT. Cell surface vimentin is a restriction factor previously demonstrated to facilitate uptake of HPV pseudovirions into cells (Schäfer *et al.*, 2017). In addition, cervical cancer cell migration was inhibited in cell lines treated with *N. brasiliensis* somatic antigen (Jacobs *et al.*, 2018). This suggests that helminth-HPV co-infection immunobiology is complex and more studies are required to understand this.

In summary limited data exists on the impact of helminth infections on immunity in the FRT and how this may alter the response to STVI. In this thesis, the impact of helminth infection on FRT immunity and STI is explored in a mouse model and in humans. This has important implications for female reproductive health, particularly in the SSA region where helminth infections and STVI occur at high rates.

Aims and Objectives

The overall aim of this thesis was to explore the systemic impact of helminth infection on immunity in the female reproductive tract (FRT) and susceptibility to sexually transmitted infections. First, the impact of hookworm infection on immunity in the FRT of humans was assessed and the association between hookworm infection and risk of Human Papillomavirus (HPV) infection was investigated. Next the impact of the gastrointestinal nematode *Nippostrongylus brasiliensis* on cellular immunity in the FRT was investigated in a mouse model. Further, the impact of *N. brasiliensis* infection on vaccine mediated immunity to another sexually transmitted viral infection Herpes Simplex Virus type 2 (HSV-2) was explored.

***Aim 1*:** To investigate the impact of hookworm infection on immunity in the human female reproductive tract (FRT) and risk of HPV infection

Specific objectives

1. To compare cytokine chemokine and antibody profiles in cervico-vaginal fluids (CVF) of hookworm infected and uninfected women. Immune profiles in the CVF of HPV infected, hookworm or HPV only infected and hookworm and HPV co-infected women were also assessed.
2. To compare the risk of HPV among hookworm infected and uninfected women

Rationale: Helminths can alter immunity to unrelated pathogens and at anatomical sites they do not normally colonise. Few studies in both mice and humans have demonstrated that helminths can impact immunity in the FRT by

inducing a Type 2 immune profile in the mucosa of the genital tract and that helminth infection associates with increased risk or susceptibility to sexually transmitted viral infections. We sought to determine whether infection with the hookworm, Ancylostoma duodenale, can exert systemic alterations on immunity in the FRT by using multiplex assays to measure cytokine, chemokine and antibody levels in the CVF of hookworm infected women and comparing to that in uninfected women. Immune profiles in CVF of HPV infected, hookworm or HPV mono infected and co-infected women were also established. Furthermore, risk of HPV infection among hookworm infected women was assessed in a regression analysis model.

***Aim 2*:** To investigate the impact of gastrointestinal nematode infection (*Nippostrongylus brasiliensis*) on immunity in the FRT of mice

Specific objectives

1. To determine whether *N. brasilienis* infection systemically alters B cell responses in the iliac lymph nodes (ILN), which drain the FRT in mice.
2. To determine whether *N. brasilienis* infection alters T cell responses in the ILN of infected mice.

Rationale: Helminth infections have been demonstrated to alter immunological profiles in the host even after infection has been cleared. This includes skewing the T-helper response towards a Th2 phenotype. In this model we investigated the impact of underlying N. brasilienis infection on B and T cell immunity in the iliac lymph nodes (ILN) draining the FRT of mice. Immune responses were investigated seven days post N. brasilienis infection when worm clearance from the gut had already commenced. Using flow cytometry, B and T cell subsets in

the ILN were phenotyped and compared between N. brasilienis infected and uninfected mice.

Aim 3: To investigate the impact of *N. brasiliensis* infection on B and T cell vaccine mediated responses to HSV-2, and pathology following HSV-2 challenge in mice

Specific objectives

1. To compare B cell responses in the draining lymph nodes (ILN) of *N. brasilienis*-infected and uninfected mice following vaccination with formalin-inactivated (FI) HSV-2 and subsequent HSV-2 challenge.
2. To compare T cell responses in the ILN of HSV-2 vaccinated *N. brasilienis*-infected and uninfected mice following HSV-2 viral challenge.
3. To compare pathology between HSV-2 vaccinated, *N. brasilienis*-infected and HSV-2 vaccinated, *N. brasilienis*-uninfected mice following HSV-2 challenge.

Rationale: Helminth infection-associated Th2 immunity would be expected to and, in certain contexts, has been shown to suppress antiviral immunity. An important and underworked area in such co-infection scenarios is how helminth infections alter B cell phenotypes and vaccine-associated genital immunity. We hypothesize that gastrointestinal helminth infections could alter B and T cell phenotypes in the female genital tract and influence pathology resulting from HSV-2 infection. To test this, a preclinical model was set up in which mice were infected with the murine hookworm N. brasiliensis then vaccinated with formalin inactivated (FI) HSV-2. B and T cells within lymph nodes draining the genital tract

were then phenotyped using flow cytometry to determine the effect of underlying helminth infection on the B cell response to HSV-2 vaccination and subsequent viral challenge.

Chapter 2: Materials and methods I (human)

2.1 Ethics statement

All participants taking part in this German Research Council (DFG)-funded study named GSAT were over 18 years of age at the time of recruitment, and all gave written informed consent prior sample collection. This study (HREC686/2021) is a sub-study of the parent protocols HREC696/2017 and HREC561/2016, that have received ethical approval from the UCT Human Research Ethics Committee. Ethical approval was also received from the Comité de Bioéthique pour la Recherche en Santé (CBRS) of the Ministry of Heath of Togo (N°26/2017/CBRS), and the Ethics Committee at the University Hospital of Bonn, Germany (Lfd. Nr. 273/16).

2.2 Study population

A total of 367, sexually active and premenopausal women, aged between 18-56 years were recruited from 6 villages (Sakalaoudè, Tseve, Fazao, Sagbadai, Alheridè and Kikimini) in the Central region of Togo in October 2019. This region was chosen as it is endemic for helminth infections, and would enable us to study the impact that helminth infections have on STI and immunity in the FRT. Of these women, 358 provided stool and urine samples, and skin snips, 240 provided blood samples, 205 provided vaginal swabs, cervical swabs and cytobrush samples. In order to obtain a more comprehensive overview of the patient cohort, an epidemiological-based survey was conducted at the beginning of the study to assess sociodemographic variables, sexual behaviour and vaginal hygiene practices. Pregnant and/or HIV positive women were excluded from the study. HIV positive women were directed to a local clinic for treatment.

2.3 *Parasitological examination for helminth diagnosis*

Microscopic examination of stool for parasitic eggs was performed using the Kato-Katz technique as previously described (Katz, N. Chaves, A. Pellegrino, 1972). Presence of *Schistosoma haematobium* eggs in urine was established by urine centrifugation (10 ml at 1500 rpm for 5 min) and examination of the pellet microscopically.

To determine the presence of *Onchocerca volvulus* microfilariae two skin samples (1-2mm diameter) obtained from left and right iliac crests were incubated in 100 µl of NaCl (0.9%) for 18-24 hrs at room temperature. The physiological water in which the skin snips were incubated was examined microscopically for microfilariae at 40x magnification.

2.4 *HIV screening and pregnancy tests*

HIV tests were performed using Alere Determine kits (Abbott, Rungis, France). Pregnancy tests were performed using Testsealabs® HCG pregnancy test strips (Hangzhou Testsea Biotechnology Co., Ltd, Hangzhou, China).

2.5 *Blood collection and plasma separation*

Blood was collected in 5 ml Ethylenediamine tetra-acetic acid (EDTA) tubes and centrifuged at 500xg, plasma was collected and stored at -20°C for later analyses of soluble cytokines, chemokines and antibodies.

A blood smear was also prepared on a slide, incubated in water for 15 minutes, fixed with methanol and stained with 10% Giemsa for 45 minutes to screen for microfilariae.

2.6 STI, GU and HPV diagnosis

One vaginal swab and one cervical swab were collected from each participant and stored in 1 ml of eNAT® medium (COPAN Italia S.P.A., Brescia, Italy) for PCR diagnosis of STI and Genito-urinary (GU) infections, and HPV, respectively. DNA was extracted using the Seegene Microlab Nimbus IVD automaton kit (Seegene Inc., Seoul, South Korea) according to manufacturer's instructions, using the DNA-extraction-program. Samples were screened for STI, GU and HPV using CFX96 Real-time PCR System (Bio-Rad Laboratories Inc.). Seegene Allplex™ STI Essential Assay was used for simultaneous detection and identification of 7 sexually transmitted pathogens using real-time PCR: *Chlamydia trachomatis* (CT), *Mycoplasma genitalium*, (MG) *Mycoplasma hominis* (MH), *Neisseria gonorrhoeae* (NG), *Trichomonas vaginalis* (TV), *Ureaplasma parvum* (UP) and *Ureaplasma urealyticum* (UU). The Allplex™ Genital Ulcer Assay was used to detect Cytomegalovirus (CMV), *Haemophilus ducreyi* (HD), Herpes simplex virus type 1 (HSV1), Herpes simplex virus type 2 (HSV2), *Lymphogranuloma venereum* (LGV), *Treponema pallidum* (TP) and Varicella-zoster virus (VZV). The Anyplex 2 HPV HR Detection kit was used to detect 14 high-risk HPV types: HPV 16, HPV 18, HPV 31, HPV 33, HPV 35, HPV 39, HPV 45, HPV 51, HPV 52, HPV 56, HPV 58, HPV 59, HPV 66, HPV 68. All reagents used for DNA extraction, and STI, GU and HPV diagnosis were manufactured by Seegene Inc. (Seoul, Republic of Korea).

2.7 *Measurement of plasma and cervico-vaginal fluid (CVF) chemokine and cytokine and antibody levels*

CVF was collected by flushing the vagina with 1 ml sterile PBS. To remove the cells, CVF was centrifuged at 1200 rpm for 5 min and supernatants were collected for assessment of cytokine, chemokine and immunoglobulin levels. In detail, levels of Type 1 cytokines (IFN-γ and TNF-α, IL-12p70 and IL-18, proinflammatory cytokines (IL-6 and IL-1β, type 2 cytokines (IL-5, IL-4 and IL-13), cell survival and growth factors (IL-2 and GMCSF), as well as chemokines (MIP-1α, MIP-1β, MCP-1, SDF-1α, GRO-α, Eotaxin, IP-10, RANTES and IL-8) were measured using the ProcartaPlex Human Th1/Th2 & Chemokine Panel 1 Luminex assay (Thermo Fisher Scientific, Vienna, Austria). In addition, IL-17A, IL-21, IL-22, eotaxin-2 and eotaxin-3 were analysed using a Human ProcartaPlex Mix&Match kit (Thermo Fisher Scientific). Moreover, levels of IgA, IgE, IgM, IgG1, IgG2, IgG3 and IgG4 were measured using the human antibody isotyping 7-Plex ProcartaPlex Panel (Thermo Fisher Scientific). All Luminex assays were analysed on the Luminex™ MAGPIX system (Luminex, Tokyo, Japan). In addition, IL-10 was measured by high sensitivity enzyme-linked immunosorbent assay (ELISA) using the High Sensitivity IL-10 Human ELISA Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. To normalise cytokine, chemokine and immunoglobulins levels relative to the protein concentration in the CVF, protein levels were measured using the Bio-Rad Protein Assay Dye reagent concentrate (Bio-Rad Laboratories, Hercules, USA). Individual analyte concentration was then expressed as ratio of the measured parameter and the total protein concentration of the sample.

2.8 *Analysis of HPV intensity*

To assess if hookworm infection influences HPV viral load, we applied a semi quantitative analysis (intensity grade 1-3) based on the cycle values from the HPV PCR assay. In detail, PCR readout assigned an intensity of 3 for samples that had viral copies detectable before 31 PCR cycles (high viral load), 2 for samples in which viral copies were detected between 31-39 PCR cycles (medium viral load), and 1 for samples in which virus was detected after 40 cycles (low viral load).

2.9 *Measurement of CVF protein levels*

Protein levels in the CVF were measured using the Bio-Rad Protein Assay Dye reagent concentrate (Bio-Rad Laboratories, Life Science Group, USA). Cytokine, chemokine and antibody levels measured in the Luminex assay were then normalised relative to the protein concentration in the sample. This was done by expressing the analyte concentration as a percentage of the total protein concentration of the sample.

2.10 *Statistical analyses*

Statistical tests to compare cytokine and chemokine levels between groups were performed on GraphPad Prism v8 software (GraphPad Software, Inc., La Jolla, USA). Mann Whitney U test was used to compare hookworm positive and hookworm negative groups; and Kruskal Wallis followed by Dunn's multiple comparisons test was used to compare HPV and hookworm single infected and co-infected groups. Univariate analysis to investigate the relationship between

hookworm positivity and intensity of HPV infection and logistic regression analyses to investigate the relationship between HPV and hookworm infection and immune parameters measured in the CVF, were performed on SPSS software (Statistical Package for Social Science) Version 21.0 (IBM SPSS statistics). Logistic regression analyses were performed as a stepwise logistic regression using thresholds of $p < 0.1$ for entry of variables into the model. The odds ratio (OR), 95% confidence intervals (CI) and p values were used as estimates of the effect of each variable.

Materials and methods II (mouse)

2.11 Animals

Wild Type (WT) female Balb/c mice were used for all *N. brasiliensis* and HSV-2 experiments. Mice were used at six weeks of age. Mice were maintained at the University of Cape Town (UCT) Research Animal Facility (RAF) in South Africa, under pathogen free conditions. This project was approved by the UCT Animal Ethics Committee (AEC), protocol number 018/002. Wistar rats were used for the maintenance of the *N. brasiliensis* life cycle. This project was approved by the UCT AEC, protocol number 018/037.

2.12 N. brasiliensis infection

The *N. brasiliensis* life cycle was maintained in male Wistar rats. Rats were infected with 5000 *N. brasiliensis* L3 larvae in 500μl of Phosphate Buffered Saline (PBS). At days six, seven and eight, rat faeces were collected and softened in water containing 5μg/ml of fungizone (fungizone controls fungal contamination). Faeces were then placed on sterile, moist filter paper in petri dishes and incubated at room temperature for one week. In one week, eggs are hatched, and larvae have migrated to the edges of the filter paper, from which they can be collected, washed, enumerated, and used for mouse infections.

Before *N. brasiliensis* infection, mouse oestrus cycles were synchronized by treatment with 2mg Depo-Provera® (Pfizer) (administered sub-cutaneously) on day 0. This minimized the variations between our mice which would be analysed for systemic impact of *N. brasiliensis* infection on immune responses in lymph

nodes draining the FRT. Seven days after Depo-Provera treatment, mice were infected with 500 *N. brasiliensis* L3 larvae. For analysis of B and T cell responses elicited in draining lymph nodes by *N. brasiliensis*, mice were killed on day 7 post infection and mediastinal (MST), mesenteric (MLN) and iliac (ILN) harvested for immunological analyses.

2.13 HSV-2

HSV-2 virus was cultured in African green monkey Kidney (Vero) cells (ATCC®CCL-81™, Manassas, VA, USA) as previously indicated (Blaho, Morton and Yedowitz, 2006). Briefly, HSV-2 at a multiplicity of infection (MOI) of 1 was used to infect a layer of 95% confluent Vero cells cultured in Dulbeco's Modified Eagle Medium (DMEM, Sigma-Aldrich®) supplemented with 10% Foetal calf serum (FCS), 100 U/ml penicillin, 100mg/ml streptomycin and 2mM glutamine. The cells were incubated at 37°C with 5% CO2 for two hours and excess virus washed off with serum free medium, the cells were then incubated in complete medium for two to three days until all cells displayed cytopathic effect (CPE). The cells were snap- frozen for 15 minutes and then warmed up at room temperature and resuspended in cold complete media. The stocks were stored at -80°C and titrated in plaque assays to determine the viral concentration.

2.14 Formalin inactivation of HSV-2

Live HSV-2 was inactivated by incubating in 0.1% Formalin at 37°C for 3 days with gentle shaking. Formalin was quenched by adding an equal volume of 0.5M glycine to the virus FI HSV-2 mixture. $1x10^6$ PFU of formalin inactivated (FI) HSV-2 in 200μl volume was then used to vaccinate mice subcutaneously.

2.15 Vaccination schedule

Two groups of six-week-old WT Balb/c mice received the FI HSV-2 vaccine. *N. brasiliensis* infected mice at day seven post-infection and mice that had no underlying *N. brasiliensis* infection. 21 days after the prime, mice were vaccinated subcutaneously with a booster dose ($1x10^6$ PFU) of FI HSV-2.

2.16 Challenge

Vaccinated (with or without underlying *N. brasiliensis* infection) were anaesthetized and infected intravaginally with $5x10^5$ PFU of HSV-2 strain G, WT unvaccinated mice were also included as controls and mice were killed six days post HSV-2 challenge and the ILN was harvested for cellular analyses.

2.17 Pathology scoring of HSV-2 infected mice

The pathology resulting from HSV-2 infection was scored on a scale of 0-5 based on one used by Gillgrass et al (Gillgrass *et al.*, 2005), where 0 = no infection, 1= mild redness of external vagina, 2= mild swelling and redness of external vagina, 3= severe swelling and redness of external vagina and surrounding tissue and hair loss in genital/perianal region, 4= genital ulceration accompanied by severe redness, swelling, and hair loss in the genital/perianal region and 5, severe genital ulceration spreading to surrounding tissue. Animals were sacrificed when they reached stage 4.

2.18 Flow cytometry

Single cell suspensions of draining lymph node samples were prepared by passing through a 40μm strainer. Cells were pelleted by centrifuging at 400 x *g* for 5 minutes and one million cells were added to v-bottomed wells for staining.

Cells were surface stained for B cell markers, CD19 (Alexa Fluor 488), CD45R/B220 (Brilliant Violet 421), CD23 (PerCPCy5.5), CD21 (PECy7), IgD (Brilliant Violet 650), IgM (Brilliant Violet 605) and CD138 (APCCy7), T cell markers, CD3 (Alexa Fluor 700), CD4 (PerCPCy5.5), CD44 (APC), CD62L (PE), CXCR5 (BV650) and PD1 (BV605). Antibody staining cocktails were made in staining buffer, PBS with 0.5% BSA, 2mM Ethylenediaminetetraacetic acid (EDTA) and 2% heat-inactivated rat serum. Cells were stained in the dark at 4°C in a staining volume of 50μl.

After surface staining for B cell markers, intracellular staining (ICS) was performed to detect IgG1 and IgG2a antibodies. Cells in v-bottomed plates were incubated in 200μl of Permwash buffer in the dark for 30 min. Cells were then washed and stained with anti IgG1 (PE) and IgG2a (APC) antibodies in a 50μl volume of Perm/Wash™ (BD Biosciences). Cells were washed twice in 150μl of Perm/Wash™ (BD Biosciences) and resuspended in 2% Paraformaldehyde (PFA) for acquisition. A summary of the antibodies used for staining is shown in **Table 19**.

Gating strategies for B and T cell phenotyping are presented in **Figure 5.** Cells were first gated on time against each of the markers, to monitor and exclude potential shifts in fluorescence that may have occurred during acquisition. Cells

were gated on single cells to exclude doublets. Lymphocytes were selected by gating on side scatter area (SSC-A) against forward scatter area (FSC-A). Plasma cells were gated directly from singlet and defined as B220-CD138+, plasmablasts were defined as B220 intermediate CD138+. CD3 negative cells were selected and B cells defined as CD3-B220+CD19+, Follicular B cells were defined as IgD+IgM+CD21+CD23+. From the lymphocyte gate, CD3+ CD4+ T cells were gated on. Effector memory CD4 T cells were defined as CD4+CD44+CD62L-, naïve CD4 T cells were defined as CD4+CD44-CD62L+. T follicular helper cells were defined as CD4+CXCR5+PD-1+.

Table 1: Antibodies used for flow cytometric analysis of B and T cell populations**.**

Marker	Fluorochrome	Clone	Manufacturer	Catalogue No
CD19	A488	6D5	Biolegend	115524
CD45R/B220	BV421	RA3-6B2	Biolegend	103239
CD21	PE-Cy7	7E9	Biolegend	123420
CD23	PerCPCy5.5	B3B4	Biolegend	101618
IgD	BV650	11-26c,2a	Biolegend	405721
IgM	BV605	RMM-1	Biolegend	406523
IgG1	PE	RMG1-1	Biolegend	406608
IgG2a	APC	RMG2a-62	Biolegend	407110
CD138	APC/Cy7	281-2	Biolegend	142530
CD3	A700	17A2	Biolegend	100216
CD4	PerCPCy5.5	GK1.5	Biolegend	100433
CD44	APCCy7	IM7	Biolegend	103027
CD62L	PE	MEL-14	Biolegend	104407
PD1	BV605	29F.1A12	Biolegend	135219
CXCR5	BV650	L138D7	Biolegend	145517

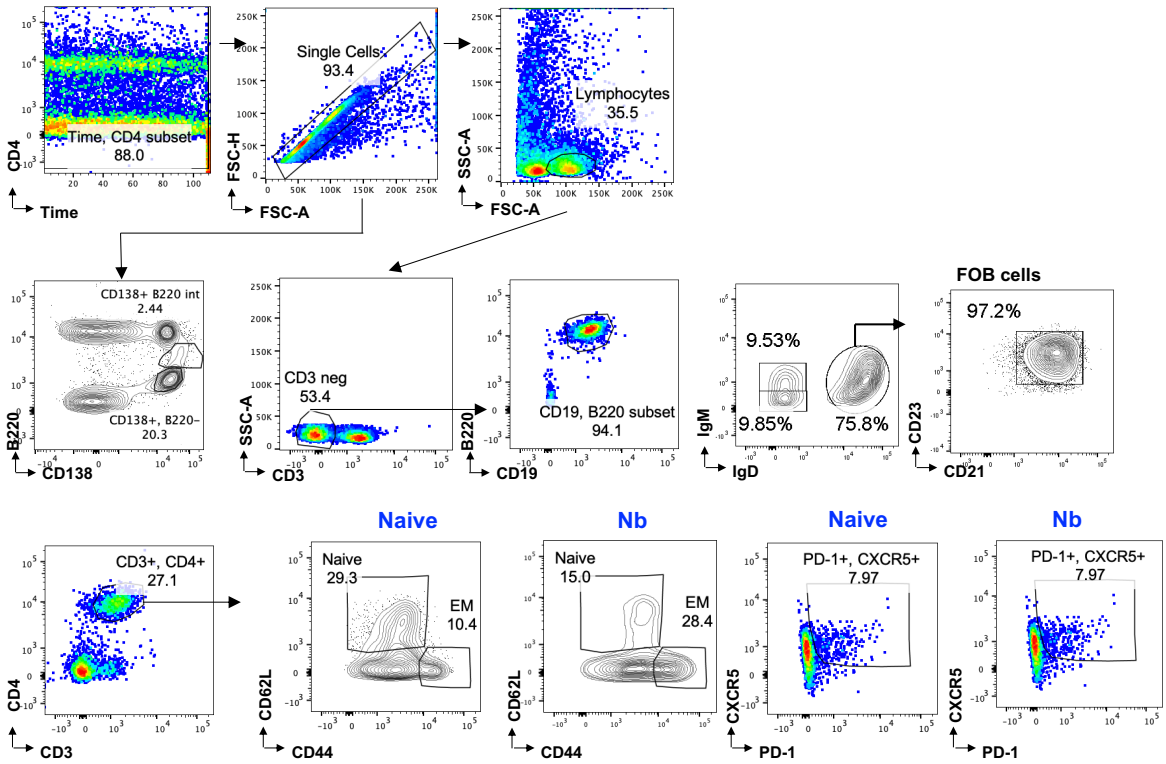


Figure 5: Gating Strategy

2.19 Statistical analyses

Flow cytometry data was acquired on a BD LSR Fortessa (BD Biosciences) using FACS diva software. Flow cytometry data was analysed on FlowJo™ v10.8.1 (BD Life Sciences). Statistical analysis was performed using PRISM software v8 (GraphPad Software, Inc., La Jolla, USA). Mann-Whitney U test was performed to compare two groups. P value <0.05 was considered statistically significant.

Chapter 3: Study participants, sociodemographic and female health characteristics of the cohort

3.1 Introduction

In sub-Saharan Africa (SSA) rates of female reproductive tract disease are amongst the highest in the world (WHO, 2018). Underlying this disease burden are high rates of sexually transmitted infections (STI), causing significant morbidity in affected populations (WHO, 2018). Cohorts assessing STI prevalence in Africa are often recruited in urban/peri urban settings in clinics where women seek sexual health services and there is limited data available for rural settings (Torrone *et al.*, 2018).

The WHO has outlined approaches to be taken towards achieving global control of STI in the WHO Global Health Sector Strategy on Sexually Transmitted Infections 2016–2021 (WHO, 2016). Collecting data on incidence and prevalence of STI is has been listed as the first step of this strategic plan. In order to achieve this, more incidence and prevalence surveys are required in SSA, especially in rural areas where there is limited access to healthcare and cases of STI may go unreported and untreated.

To date, several factors have been associated with increased risk of STI in SSA, including: Age <30 (Kharsany *et al.*, 2020; Semwogerere *et al.*, 2021), an early age of sexual debut, (Stöckl *et al.*, 2013; Wand *et al.*, 2020), being unmarried (Ginindza *et al.*, 2017; Semwogerere *et al.*, 2021), alcohol and drug use (WHO/UNAIDS, 2005), low level of education (Semwogerere *et al.*, 2021) and having multiple sexual partners (Mah and Halperin, 2010).

Understanding the prevalence of and risk factors that drive STI is critical in developing a targeted approach to controlling these infections in SSA. In this chapter the sociodemographic characteristics of a cohort of women recruited from 6 villages in central Togo are presented.

Results

3.2 Profile of cohort: sociodemographic indicators

For this study, 367 women were recruited, 205 consented to providing paired stool and vaginal samples (vaginal flush, cervical and vaginal swabs and cytobrushes; **Figure 6**). Two HIV positive participants were excluded leaving a total of 203 women. HPV results were available for 195 out of the 203 women.

Our cohort was recruited from a rural village in Togo. The median age of the population was 32 years. Majority of the participants were married (94.1%), non-smokers (99.5%) and not alcohol consumers (88.2%; **Table 2**).

Just over half of participants reported use of hormonal contraceptives (57.1%). Most participants had primary school or lower education (80.3%) and earned less than 27 dollars a month (82.3%).

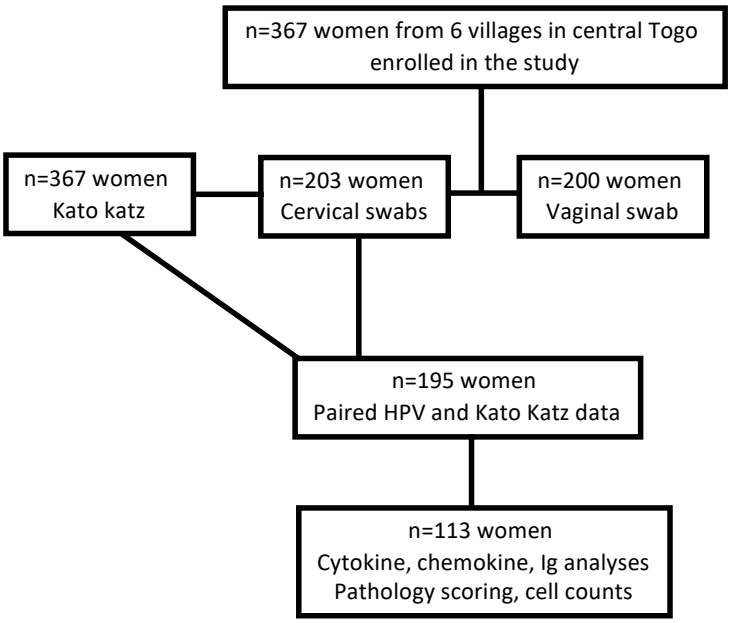


Figure 6: Study cohort Study participants recruited from 6 villages in the Central region of Togo

3.3 Sexual practices and vaginal hygiene

With respect to sexual reproductive health, 69.5% of women reported having had only one partner in their lifetime, 14.8% reported condom use and 52.2% reported sexual debut age between 10 and 20 years. In addition, 41% of women reported having a partner who had multiple sexual partners, with just over half the women (51%) reporting that their partner did not have multiple partners. Majority of the women (97%) did not engage in paid intercourse, those who did accounted for only 2.5% of our cohort.

Harsh vaginal hygiene practices including washing with soap, detergent and/or antiseptic were reported among 42.9% of participants in this cohort (**Table 3**). Furthermore, more than half of the women in this cohort (58.6%) used fingers or

a sponge to clean out the vagina, and 8.4% reported insertion of products into the vaginal canal.

Table 2: Cohort characteristics

Demographics		
Age	**Number (n=203)**	**%**
18-25	54	26.6
26-32	49	24.1
33-39	52	25.6
>40	48	23.6
Marital status		
Married	191	94.1
Single	3	1.5
In a relationship	4	2
Divorced	2	1
Widowed	3	1.5
Smoker		
Yes	1	0.5
No	202	99.5
Alcohol		
Yes	24	11.8
No	179	88.2
Hormonal contraceptive use		
Yes	116	57.1
No	84	41.4
N/A	3	1.5
Socioeconomic		
Level of education		
Primary School	76	37.4
Secondary school	24	11.8
Other	14	6.9
No	87	42.9
Not reported	2	1
Monthly income (USD)		
<27	134	66

27-62	10	4.9
62-124	2	1
No income	33	16.3
Don’t know	24	11.8
Vaginal hygiene practices		
Cleaning practices		
Water	50	24.6
Soap/detergent	9	4.4
Water and soap	69	34
Water, soap and antiseptics	9	4.4
Don't wash	63	31
Not reported	3	1.5
Objects used for intimate washing		
Finger	112	55.2
Sponge or other	7	3.4
I don’t do this	83	40.9
Not reported	1	0.5
Products introduced vaginally		
Yes	17	8.4
No	174	85.7
Not reported	12	5.9

Table 3: Sexual practices of women in the cohort

Sexual practices		
Number of partners	**Number (n=203)**	**%**
0	1	0.5
1	141	69.5
2	52	25.6
3	5	2.5
4	1	0.5
Not known	3	1.5
Condom use		
Every time	30	14.8
Most of the time	5	2.5
Never	137	67.5
Sometimes	28	13.8
Not reported	3	1.5
Partner has multiple partners		
Yes	85	41.9
No	104	51.2
Don't Know	8	3.9
Not reported	6	3.0
Paid intercourse		
Yes	5	2.5
No	196	96.6
Not reported	2	1.0
Age of first intercourse		
10-15	22	10.8
16-20	84	41.4
21-25	14	6.9
>25	1	0.5
Don't know	62	30.5
Not reported	20	9.9

3.4 Discussion

We identified sociodemographic characteristics of the participants in our cohort that match characteristics that have previously been reported to be risk factors for STI, for example, our participants had a low level of education and an early age of sexual debut. However, these factors were not associated with increased risk of STI in our cohort.

Independent factors associated with increased risk of STI in our cohort were identified as use of contraceptives, alcohol consumption, and having had more than one sexual partner at any point in the woman's life (Holali Ameyapoh *et al.*, 2021). After controlling for confounders in a multivariate logistic regression analysis, use of contraceptives was identified as an independent risk factor for STI (Holali Ameyapoh *et al.*, 2021). Hormonal contraceptives are hypothesised to increase the risk of STI by changing the structural biology of the FRT, they create a hypoestrogenic state that results in thinning of the vaginal epithelium and this has been associated with increased susceptibility to HSV-2 infection (Miller *et al.*, 2000; Quispe Calla *et al.*, 2016). Furthermore, women using progestin-based contraceptives displayed alterations in vaginal microbiota composition and increased inflammation in the FRT that was hypothesised to be a result of hypoestrogenism (Jespers *et al.*, 2017). Excessive inflammation in the FRT could induce barrier breaches and increase the risk of acquiring STI (Ghanem *et al.*, 2005; Straub, 2007). There is however conflicting evidence on the influence of contraceptive use on risk of STI. While a link to HSV-2 risk has been established (Grabowski *et al.*, 2015), no significant differences in HPV risk among women using different types of contraceptives was reported (Kiweewa *et*

al., 2019). Furthermore, there is no consensus on the influence of injectable progesterone, depot-medroxyprogesterone acetate (DMPA), on risk of HIV (Polis *et al.*, 2014; Baeten and Heffron, 2015). Other risk factors for STI such as being single, having multiple sexual partners were of low frequency among our participants.

There were similarities and differences between our cohort and cohorts recruited in sub-Saharan Africa, summarised in **Table 4**. Similarities include, alcohol use and smoking, which was low, <25% among women in our cohort and cohorts in West, Eastern and Southern Africa (Norris *et al.*, 2017; Dela *et al.*, 2019; Kharsany *et al.*, 2020). The age of sexual debut was also comparable between women in our cohort and those in West, South and Eastern Africa. Approximately 11% of the women in our cohort reported having had a sexual debut aged below 15 years, in South Africa 6% and in eastern Africa, 7-11% of adolescent females reported engagement in sexual activity aged <15 years old (Seff, Steiner and Stark, 2021). In Nigeria the proportion of adolescent girls with sexual debut <15 years (24%), was approximately double that observed in our Togolese cohort (Alawode *et al.*, 2021).

Table 4: Comparison of sociodemographic variables considered to be risk factors for STI in West, Eastern and Southern Africa

	Togo	West Africa	Eastern Africa	Southern Africa	References
Alcohol use	11.8%	21%	22-25%	9.1%	(Norris *et al.*, 2017; Dela *et al.*, 2019; Kharsany *et al.*, 2020)
No education	43%	4%	10-21%	54%	(Norris *et al.*, 2017; Torrone *et al.*, 2018; Dela *et al.*, 2019; Wand *et al.*, 2020)
Unmarried	6%	56%	76-86%	77%	(Torrone *et al.*, 2018; Dela *et al.*, 2019; Kharsany *et al.*, 2020; Wand *et al.*, 2020)
Sexual debut <20 years	11%	24%	6- 10%	84%	(Wand *et al.*, 2020; Alawode *et al.*, 2021; Seff, Steiner and Stark, 2021)
No condom use	68%	45%	37.2%	17%	(Barnabas *et al.*, 2018; Dela *et al.*, 2019; Wand *et al.*, 2020; Yuh *et al.*, 2020)
Hormonal contraceptive use	57%		20-40%	53%	(Torrone *et al.*, 2018; Wand *et al.*, 2020)
Harsh vaginal cleaning (antiseptics, soap, disinfectant, vinegar)	43%	43%	48.9%	32%	(Allen *et al.*, 2010; Lazarus *et al.*, 2019; Ziba *et al.*, 2019)
Objects and fingers used in vaginal cleaning	59%		99%	76%	(Allen *et al.*, 2010; Lazarus *et al.*, 2019)

In addition, the level of education we report among our study participants was similar to that observed in one South African cohort, with 43% the women in out

cohort having no education and 54% in a cohort of women in Kwa-Zulu Natal, South Africa reporting having no formal education (Wand *et al.*, 2020). Pooled data from studies in South Africa, in contrast, show that only 0.8% of women aged 15-24 and 6.9% of women aged 25-49 had no formal education (Torrone *et al.*, 2018), while in Eastern Africa <21% of women reported having no formal education(Norris *et al.*, 2017; Torrone *et al.*, 2018). In a Ghanaian cohort only 4% of women had no formal education (Dela *et al.*, 2019).

In contrast to our cohort where only 6% of women were unmarried, a cohort in Ghana comprised 56% unmarried women (Dela *et al.*, 2019) while cohorts in South Africa comprise a larger proportion of unmarried women, 92.1% of women aged 15-24 and 52% of women aged 25-49 were unmarried according to pooled data from studies investigating STI prevalence in South Africa (Torrone *et al.*, 2018) and in more recent estimates 86% and 77% of women reported to be unmarried in STI prevalence cohorts in Kwa-Zulu Natal province in South Africa (Kharsany *et al.*, 2020; Wand *et al.*, 2020). Marital status in our Togolese cohort also differed from what was observed in Eastern Africa where 86% of women aged 15-24 and 76% of women aged 25-49 were unmarried (Torrone *et al.*, 2018).

Condom use in our cohort was the inverse in comparison to cohorts in Southern and East Africa, with 68% of women in our cohort reporting never having used condoms, while in South Africa, more than 60% of women (Barnabas *et al.*, 2018; Wand *et al.*, 2020) and in Eastern Africa 62% of women (Yuh *et al.*, 2020) reported to have used condoms at least once. Condom use in our cohort was

also lower than that observed in Ghana where 45% of women reported never having used condoms (Dela *et al.*, 2019).

One limitation of our study was that while women reported use of hormonal contraceptives (57%), the type of contraceptive used was not specified by the study participants. Contraceptive use in our cohort was comparable to that of women in Southern and Eastern Africa, with just over half the women (53%) reporting use of injectable contraceptives in South Africa and between 20-40% of women using contraceptives in Eastern Africa (Torrone *et al.*, 2018; Wand *et al.*, 2020).

Another limitation of our study is that our cohort did not include adolescent females, however, young women aged 18-25 were included in the study. STI prevalence studies have previously shown adolescent girls and young women <25 to be at the highest risk of infection (Bruni *et al.*, 2010; Torrone *et al.*, 2018).

In our study 43% of women employed harsh vaginal cleaning practices such as use of soap and/or antiseptic with water, while 59% used fingers and objects for vaginal cleaning. Similar to what we reported in our cohort, a study in Ghana, showed that 43% of women use of harsh vaginal cleaning practices such as use of lemon juice, antiseptics and vinegar (Ziba *et al.*, 2019). In comparison to the Togolese data, a slightly lower proportion of women in a cohort in South Africa (32%) reported use of substances other than water for vaginal cleaning and a higher proportion (76%) used fingers and other objects to clean out the vagina (Lazarus *et al.*, 2019). Similar to our cohort, in Tanzania (East Africa), approximately half (48.9%) of women used soap and/or disinfectant and water

and more 99% used fingers and other objects for vaginal cleaning (Allen *et al.*, 2010).

In summary, with respect to income and recreational use of alcohol and cigarettes, education (in some regions), and vaginal cleaning practices, our cohort is similar to others in Sub-Saharan Africa. Sexual practices, on the other hand, differ between our rural cohort, and others in Sub-Saharan Africa which are recruited mostly in urban areas. Thus the influence of sociodemographic factors on STI as determined in this cohort (Holali Ameyapoh *et al.*, 2021) may not be reflected in cohorts in the greater Sub-Saharan Africa region but it provides a useful picture of the sexual health of women in rural Africa.

Chapter 4: Hookworm infection is associated with increased risk and intensity of Human Papillomavirus (HPV) infection

4.1 Introduction

Globally, an estimated 1.5 billion people are infected with helminths (Pullan *et al.*, 2014) with the largest burden of infection borne by LMIC (WHO, 2018). These infections are associated with significant morbidity in infected hosts with outcomes such as stunted physical development in children, malnutrition, chronic anaemia and adverse pregnancy outcomes in infected women (Bethony *et al.*, 2006; Brooker, Hotez and Bundy, 2008; Campbell *et al.*, 2016).

Distribution of STH varies according to the geographical region. According to a 2010 estimate (Pullan *et al.*, 2014), Sub-Saharan Africa (SSA) had the highest prevalence of hookworm infections (13.6%,) followed by Asia (7.5%) and Latin America and the Caribbean (LAC; 5.2%). Ascaris infections were highest in Asia (15.8%), with 14.7% prevalence reported for LAC and 13.6% for SSA. Trichuris infections were at 12.3%, 11.6% and 7.6% prevalence in LAC, SSA and Asia, respectively.

There is an overlap between the burden of helminths and STI in SSA with high prevalence of these infections reported in the region (Pullan *et al.*, 2014; Gottlieb *et al.*, 2016). According to a 2020 estimate, the prevalence of chlamydia, gonorrhoea, Trichomonas and syphilis infections in SSA was 4.7%, 1.4%, 6.6%, and 1.7%, respectively**.** Furthermore, the prevalence of treatable STI: gonorrhoea, chlamydia, trichomonas, and *Mycoplasma genitalium* over a 20-year-period (1999-2019) was estimated at 3.5%, 4.0%, 15.6%, and 10.2%,

respectively (Jarolimova *et al.*, 2022), while HPV prevalence among women with normal cytology in the region from the period of 1995-2009 stood at 24% (Bruni *et al.*, 2010).

While most STI are asymptomatic, they are a major cause of morbidity and can result in cancer, pelvic inflammatory disease, infertility and complications in pregnancy, furthermore they can cause congenital and neonatal infections (Walboomers *et al.*, 1999; Mullick *et al.*, 2005; Tsevat *et al.*, 2017; Fortner *et al.*, 2019).

Cervical cancer, which is associated with HPV infection (Walboomers *et al.*, 1999), is the fourth leading cause of death attributed to cancer globally, resulting in 342000 deaths among the 604000 cases reported in 2020 (Sung *et al.*, 2021). Majority of deaths from cervical cancer occur in LMIC (Randall and Ghebre, 2016).

The HPV types most commonly found in cancer lesions and responsible for oropharyngeal and anogenital cancers are classified as high-risk HPV types. They include HPV 16 and HPV-18, which have been implicated in approximately 70% of cervical cancer cases (de Sanjose *et al.*, 2010; Formana *et al.*, 2012) and HPV types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66 and 68 (Joura *et al.*, 2014; de Martel *et al.*, 2017).

The current HPV vaccines available include the bivalent Cervavix vaccine, designed to protect against HPV 16 and HPV18, the quadrivalent Gardasil, protective against HPV16, 18, 6 and 11 and the non-valent Gardasil®9 vaccine, which is targeted against HPV16, 18, 6, 11, 31, 33, 45, 52, 58 (Yusupov *et al.*, 2019). HPV 35, which is associated with high-grade Cervical Intraepithelial

Neoplasia (CIN3) and Invasive Cervical Cancer (ICC) in Sub-Saharan Africa (Carlander *et al.*, 2020; Pinheiro *et al.*, 2020) is not currently included in any of the available vaccine formulations. Furthermore, the distribution of high-risk HPV has been demonstrated to vary by region, disease state i.e. CIN or normal cytology, age and HIV infection status (McDonald *et al.*, 2014; Zhai and Tumban, 2016; Mcharo *et al.*, 2021).

It is important to investigate the prevalence of helminth infections and STI in affected populations, in order to provide information that can be used to develop effective control strategies for these infections. This thesis focuses specifically on how hookworm infection influences HPV infection.

Results

4.2 Prevalence of soil transmitted helminths

First, we examined the prevalence of helminth infection within the cohort. The hookworm *Ancylostoma duodenale* was the most prevalent helminth infection (20.2%; **Figure 7A**). The prevalence of *Schistosoma mansoni was* 0.5%, while *Ascaris lumbricoides, Trichuris trichiura* and *Onchocerca volvulus* were not detected in the women who were screened.

Majority of the hookworm infected women had egg counts between 1-10 (**75%; Figure 7B**), 13% had egg counts between 11-20, 3% between 21-30, 8% between 31-40, and 3% between 41-50 (**Figure 7B**).

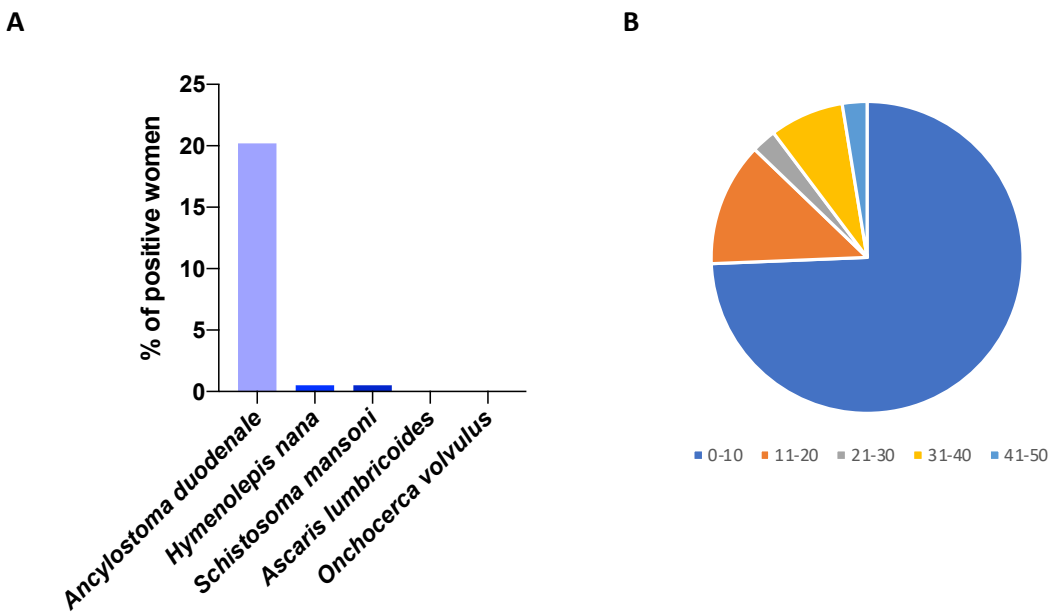
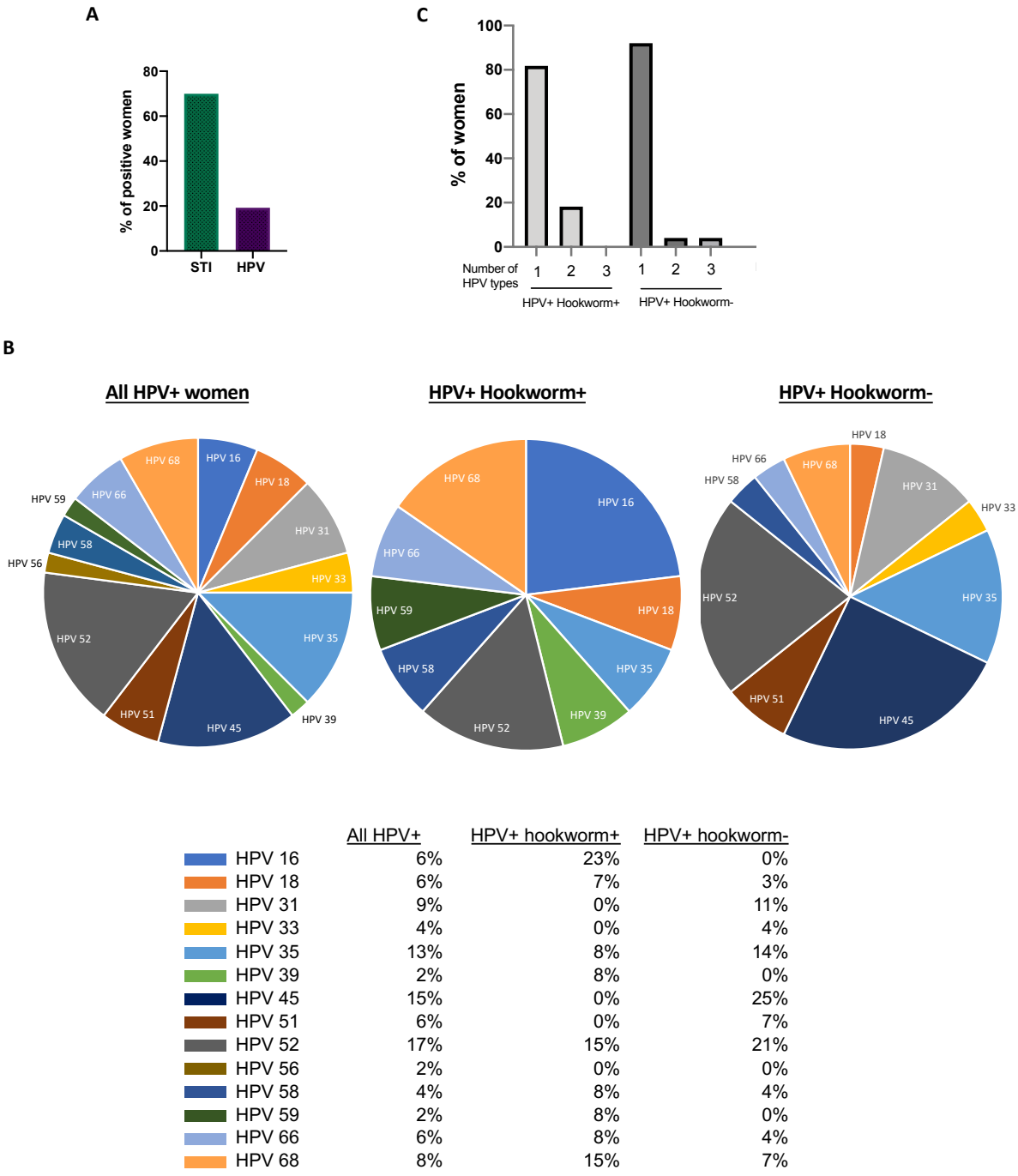


Figure 7: Overview of helminth infection. Graph demonstrating the helminth infection status in the cohort (A). Pie demonstrating the hookworm load among infected participants. Each bar represents the total percentage of women in each category of infection (n=203), each slice of the pie represents the percentage of women with egg counts within the indicated range (n=39).

4.3 HPV prevalence and type distribution

Next, we measured the prevalence of HPV among our study participants. Similar to the % hookworm positivity, approximately 1/5 of participants sampled were HPV positive (19.2%; **Figure 8A**). The qualitative/quantitative assay used to detect HPV types in this cohort is designed to screen for high risk HPV types. Among the women tested, the three most prevalent high risk HPV types were HPV 52, HPV 45 and HPV 35, at 16.6% and 14.6%, 12.5%, prevalence, respectively (**Figure 8B**). The other high risk HPV types screened for in the cohort (HPV 16, HPV 18, HPV 31, HPV 33, HPV 39, HPV 51, HPV 56, HPV 58, HPV 59, HPV 66, HPV 68) were also detected albeit at prevalence below 10% (**Figure 8B**).

Of note, a high prevalence of STI (70%) was observed in this cohort (**Figure 8A**). Individual STI prevalence in the study, recently published (Holali Ameyapoh *et al.*, 2021), was as follows: *Chlamydia trachomatis* (2.5%), *Mycoplasma genitalium* (3%), *Mycoplasma hominis* (17.5%), *Neisseria gonorrhoeae* (1.5%), *Trichomonas vaginalis* (2.5%), *Ureaplasma parvum* (50%), *Ureaplasma urealyticum* (26.5%), Cytomegalovirus (5%), *Haemophilus ducreyi* (0%), Herpes simplex virus (HSV) type 1 (0.5%), HSV-2 (0.5%), *Treponema pallidum* (0%) and Varicella-zoster virus (0%). The other STI will however not be covered in this thesis. As mentioned in the introduction, the main focus of the thesis is to investigate the impact of hookworm infection on immunity in the FRT and risk of HPV.



	All HPV+	HPV+ hookworm+	HPV+ hookworm-
HPV 16	6%	23%	0%
HPV 18	6%	7%	3%
HPV 31	9%	0%	11%
HPV 33	4%	0%	4%
HPV 35	13%	8%	14%
HPV 39	2%	8%	0%
HPV 45	15%	0%	25%
HPV 51	6%	0%	7%
HPV 52	17%	15%	21%
HPV 56	2%	0%	0%
HPV 58	4%	8%	4%
HPV 59	2%	8%	0%
HPV 66	6%	8%	4%
HPV 68	8%	15%	7%

Figure 8: Prevalence of STI and HPV. Overview of women with sexually transmitted infections (n=203) and HPV (n=195) in the cohort (A). Pie chart showing the proportion of women positive for the different HPV types tested (B). Overview of women infected with one or multiple HPV subtypes in the cohort (C). Each bar and each slice of the pie represents the total percentage of women in each category of infection (in the presence or absence of hookworm infection).

Interestingly, a higher proportion of HPV+ hookworm+ women were infected with HPV 16 (23%) and HPV18 (7%) in comparison to HPV+ hookworm– women among whom there were no HPV16 infections and only 3% HPV 18 infections (**Figure 8B**). In addition, majority of the women were found to be infected with only one HPV type, (82% of HPV+ hookworm+ women and 92% of HPV+ hookworm- women; **Figure 8C**). 18% of HPV+ hookworm+ and 4% of HPV+ hookworm- women were infected with two HPV types while 4% of HPV+ hookworm- women were infected with three HPV types.

Taken together, the HPV data from this cohort show that HPV type distribution varied between the overall cohort and women that were co infected with hookworm or not. Notably women with hookworm and HPV co-infection have a higher prevalence of the HPV types that result in more than 2/3 of cervical cancer cases (HPV 16 and HPV 18), in comparison to women with no underlying hookworm infection. In addition, the infection profile of participants in this cohort at the time of sampling show that infections are predominantly by a single HPV type.

4.4 Hookworm positive women are twice as likely to be HPV positive in comparison to hookworm uninfected women

Next, association between hookworm positivity and the risk for HPV infection was investigated. Women were categorised as either hookworm positive or hookworm negative and the percentage HPV prevalence among these two groups was determined. Women who were hookworm positive were found to be two times more likely to be HPV positive in comparison to hookworm uninfected women (**Figure 9**)**,** this was also confirmed in a univariate logistic regression

analysis (OR=2.22; 95% CI (0.999 - 4.945) p=0.05; **Table 5**). Furthermore, hookworm infection was found to be positively associated with the intensity of HPV infection (**Table 5**). HPV intensity is a semiquantitative measure of the viral load in the sample that was measured in the HPV PCR assay. The PCR readout assigns an intensity of 3 for samples that has viral copies detectable before 31 PCR cycles, 2 for samples in which viral copies are detected between 31-39 PCR cycles, and 1 for samples in which virus is detected after 40 cycles.

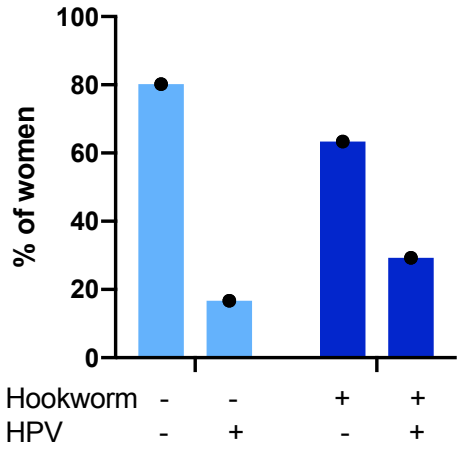


Figure 9: Women with hookworm infection have a 2x risk of acquiring HPV. Graph showing the percentage of HPV+ and HPV- women among hookworm- women (left, light blue n=157) and hookworm + women (right, dark blue n=38). Each bar represents the total percentage of women in each category of infection.

Table 5: Univariate analysis of the factors associated with hookworm infection in the cohort (n=195)

	Univariate	
	OR (95% CI)	**p value**
HPV positivity	2.22 (0.99 - 4.95)	**0.05**
HPV intensity	1.41 (1.02 - 1.95)	**0.04**
Age		
18-25	1	
26-32	0.57 (0.20 – 1.59)	0.28
33-39	0.56 (0.20– 1.55)	0.26
>40	1.18 (0.47 – 2.96)	0.73
Level of education		
No education	1	
Other	1.70 (0.45 - 6.40)	0.27
Primary	3.62 (0.64 -20.41)	0.40
Secondary	1.21 (0.31 – 4.73)	0.43

4.5 Number of partners is associated with increased risk while condom use is associated with reduced risk of HPV infection

We also investigated if the sociodemographic factors measured in our cohort were associated with HPV infection (**Table 6**). In a univariate analysis with a p threshold set to <0.2 and 95% CI we found that age 26-32 (OR= 0.48; 95% CI 0.16-1.39; p=0.18), live birth (OR= 0.11; 95% CI 0.01-1.30; p=0.08), knowledge that a partner has multiple partners (OR= 0.54; 95% CI 0.25-1.19; p=0.13), and condom use (OR= 0.16; 95% CI 0.05-3.51; p=0.003) were associated with low risk of HPV infection.

Factors that were associated with a high risk of HPV infection in the univariate analysis were number of partners (OR= 1.97; 95% CI 1.11-3.51; p=0.02) and use of soap/detergent for vaginal hygiene (OR= 4.69; 95% CI 1.01-21.85; p=0.049).

After adjusting for confounders in a multivariate logistic regression analysis with forward selection, condom use (OR= 0.098; 95% CI 0.02-0.44; p=0.002) was associated with low risk of HPV infection while number of partners in one's lifetime (OR= 2.51; 95% CI 1.28-4.91; p=0.007) was associated with increased risk of HPV infection.

Table 6: Univariate and multivariate analysis of factors associated with HPV infection in the cohort (n=195)

	Univariate		Multivariate	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
Age				
18-25	1			
26-32	0.48 (0.16 – 1.39)	**0.18**		
33-39	0.56 (0.20 – 1.55)	0.26		
>40	1.46 (0.59 – 3.59)	0.41		
Level of education				
No education	1			
Other	0.79 (0.25 – 2.46)	0.68		
Primary	0.34 (0.04 - 3.34)	0.36		
Secondary	0.92 (0.30 – 2.88)	0.89		
Alcohol				
No	1			
Yes	1.91 (0.73 – 5.04)	**0.19**		
Last pregnancy outcome				
Never gave birth	1			
Live birth	0.11 (0.01 - 1.30)	**0.08**		
Miscarriage	0.50 (0.01 – 19.56)	0.71		
Stillborn	0.50 (0.013-19.56)	0.71		
Contraception				
No	1			
Yes	1.21 (0.59 -2.49)	0.60		
Non-monogamous partner				
No	1			
Yes	0.54 (0.25 - 1.19)	**0.13**		
I don't know	0.57 (0.07 – 5.00)	0.61		
Condom use				
No	1			
Yes	0.16 (0.05 – 3.51)	**0.003**	0.098 (0.022-0.44)	**0.002**
Number of partners	1.97 (1.11 – 3.51)	**0.02**	2.51 (1.28-4.91)	**0.007**
Vaginal hygiene				
Water	1			
Water and soap	1.80 (0.68 - 4.79)	0.24		
Water, soap and antiseptics	0.00 (0.00-)	0.99		
Soap/detergent	4.69 (1.01 - 21.85)	**0.049**		
Don't wash	1.50 (0.54 - 4.16)	0.44		

In summary, HPV and hookworm infections were each prevalent in approximately 1/5 of women screened, and we demonstrate that women with hookworm infection have an increased risk (2x) of HPV infection as well as having a higher intensity of HPV infection. These findings highlight a need for helminth infection to be addressed as a significant risk factor for HPV infection in populations where helminth infections are endemic.

4.6 Discussion

The hookworm *Ancylostoma duodenale* was the most prevalent helminth infection in our cohort (20.2%). The hookworm prevalence in our study is higher than the cumulative prevalence (13.6%) reported in 2010 for SSA (Pullan *et al.*, 2014). Among children aged 5-14, there is evidence of a trend in reduction of hookworm infections globally, from 30% to 5%, in the past two decades (Sartorius *et al.*, 2021), however this trend towards lower hookworm prevalence is not is reflected in our adult cohort when compared to prevalence in 2010 (Pullan *et al.*, 2014).

The hookworm prevalence among our study participants is also higher in than that reported in some cohorts in West Africa. For example, Adu-Gyasi et al, reported a prevalence of 12.1% in Ghana (Adu-Gyasi *et al.*, 2018), while in Nigeria, estimates over the period 2005-2018 place hookworm infection prevalence at 16.8% (Yaro, Kogi and Luka, 2018).

Because our cohort comprises only women, the high hookworm prevalence we observe in comparison to other studies raises the possibility that the women in our cohort were predisposed to hookworm infection. This has however not been

reported for studies in Africa (Behnke *et al.*, 2000; Raso *et al.*, 2006), where being male was reported as a risk factor for hookworm infection. Furthermore, gender as a risk factor for hookworm infection could be explained by the fact that occupations involving high exposure to hookworms, such as building houses with mud, are assigned to men in the population (Behnke *et al.*, 2000; Hossain and Bhuiyan, 2016). In our cohort, we do not have information concerning the different occupational assignments based on gender, thus we cannot determine whether the high prevalence of hookworm infection observed in our female cohort is as a result of their occupational engagements putting them at higher risk of hookworm exposure.

The high prevalence of hookworm infection among our study participants could also be because the cohort was recruited in a rural area. High temperatures in tropical regions, combined with a rural setting with potentially limited access to proper sanitation elevates the risk of infection (Loukas *et al.*, 2016).

Interestingly, we did not detect *Onchocerca volvulus* among our study participants, this is likely reflective of Togo moving closer to complete elimination of onchocerciasis, following a targeted mass drug administration program in place for over two decades (Komlan *et al.*, 2018), though few infections have still been reported in some regions.

There was a high prevalence of STI in our cohort (70%). However, the prevalence of chlamydia, gonorrhoea, trichomoniasis and syphilis we report in our cohort (2.5%, 1.5%, 2.5%, 0%) was lower (with the exception of gonorrhoea prevalence, which was comparable to the global prevalence) than the global estimates for 2020 for these infections, at 4.7%, 1.4%, 6.6%, and 1.7%,

respectively. Furthermore, the individual prevalence of the four most common treatable STI (gonorrhoea, chlamydia, trichomoniasis and *Mycoplasma genitalium*) we reported in our study 1.5%, 2.5%, 2.5%, 3%, respectively were lower than prevalence estimates reported for these infections in SSA for the period of 1999-2019, 3.5%, 4.0%, 15.6% and 10.2%, respectively (Jarolimova *et al.*, 2022). The lower prevalence we observe in our cohort in comparison to the WHO estimates or data from the pooled studies reported by Jarolimova *et* al (Jarolimova *et al.*, 2022) for SSA could be due to variation in sampling sites, with our study being focused on a single rural site and therefore not representative of the prevalence in all of SSA. The difference could also potentially be attributed to the fact that we did not include HIV positive participants in our study. HIV positive participants have been shown to have higher risk of STI (Passmore, Jaspan and Masson, 2016; Kharsany *et al.*, 2020). The prevalence of STI in our study was also lower than that reported in a cohort of female sex workers in Togo where NG*,* CT*,* MG *and* TV prevalence was at 4.2%, 6.1%, 5.5% and 6.5%, respectively (Ferré *et al.*, 2019), the higher STI prevalence in the latter cohort could be attributed to the participants being engaged in high-risk sexual behaviour.

HPV prevalence in our cohort (19.2%) is lower than that observed in some cohorts in Togo (Ferré *et al.*, 2019; Kuassi-Kpede *et al.*, 2021) that reported 32.9% and 53.3% prevalence, respectively. The study by Ferré et al was performed in FSW, HPV risk has previously been shown to be higher in women with multiple sexual partners (Roset Bahmanyar *et al.*, 2012). The study by Kuassi-Kpede *et al* was carried out in an urban setting, the capital Lomé, in comparison to our study that was carried out in rural Togo, and our results

indicate that there is a potentially higher risk of HPV infection among women living in urban areas in Togo, however, this requires further investigation.

The HPV prevalence in our study is also lower than that reported in other studies that estimate HPV prevalence at 24% in women with normal cytology in sub-Saharan Africa over the period of 1995-2009 (Bruni *et al.*, 2010) and 25-33% in more recent studies in West and South Africa (Mbatha *et al.*, 2017; Krings *et al.*, 2019).

The HPV prevalence we observe in our cohort (19.2%) is however comparable to that in cohorts in Kenya and South Africa, that reported HPV prevalence of 18.2% and 21.4%, respectively, among HIV negative women (Sweet *et al.*, 2020; Taku *et al.*, 2020). Moreover, in these studies, a higher HPV prevalence was reported in HIV positive women in comparison to HIV uninfected women. The link between HIV and increased HPV risk has previously been established (Veldhuijzen *et al.*, 2011; McDonald *et al.*, 2014). This could explain the relatively lower prevalence of HPV in our study cohort as it did not include HIV positive women, though other factors may also play a role.

Furthermore, the HPV prevalence observed in our in our cohort, which comprised women aged 18-49, was lower in comparison to studies carried out in younger cohorts in eastern, western and southern Africa (Watson-Jones *et al.*, 2013; Mbulawa *et al.*, 2021). HPV prevalence has previously been demonstrated to vary with age, with a higher prevalence observed among adolescent girls and women under 25 (Bruni *et al.*, 2010).

The most prevalent HPV types in our cohort were HPV 52, 45 and 35. In Togo, Ferré et al reported HPV types 58, 35 and 16 (Ferré *et al.*, 2019) and Kuasi-

Kpede et al reported HPV types 56, 51, 31, 52 and 35 (Kuassi-Kpede *et al.*, 2021) as most prevalent. In Ghana, Kenya and South Africa HPV types 16, 52 and 35 (Krings *et al.*, 2019), HPV types 52, 35 and 51 (Sweet *et al.*, 2020) and HPV types 16, 51, 18 and 35 (Mbatha *et al.*, 2017), respectively, were most prevalent. These data indicate that the high-risk HPV types vary by cohort and geographical location, with the most frequently reported HPV types in SSA being HPV 35 and HPV 52. Interestingly, the increased prevalence of HPV 16 and HPV 18 that we observe in hookworm infected in comparison to hookworm uninfected women could imply that these women are at a higher risk of cancer, due to the majority of cervical cancer cases being attributed to these two HPV types (de Sanjose *et al.*, 2010), though this reqiures further investigation.

We found that women in our cohort were up to two times more likely to be HPV positive. This is consistent with the study by Gravitt *et al.*, where they reported an increased risk of HPV among women infected with soil-transmitted helminths (Gravitt *et al.*, 2016). Helminth infection has previously been demonstrated to promote reactivation of latent virus (Reese *et al.*, 2014) and our data provides evidence that this extends to promoting an increased intensity of viral infection as demonstrated in hookworm and HPV co-infected women. Further understanding of how helminth infections may alter host and viral biology is currently lacking and requires further investigation. Type 2 signalling induced by helminths may suppress antiviral immune responses. For example, IL-4 mediated induction of alternatively activated macrophages is associated with impairment of CD8+ T cells specific for murine norovirus (Osborne *et al.*, 2014). In addition, alteration to the structural integrity of the small intestine induced by

type 2 signalling resulted in increased microbial translocation and collapse of West Nile virus specific CD8+ T cells (Desai *et al.*, 2021).

Risk factors for HPV infection include other infections that cause disruption of the vaginal epithelium such as *Chlamydia trachomatis* and HIV (Samoff *et al.*, 2005). In our study, HIV positive women were excluded, and none of the 195 women screened for HPV were *C. trachomatis* positive, thus *C. trachomatis* was not associated with HPV infection in this cohort. Vaginal trauma and injury as a result of sexual activity, and insertion of objects (including cleaning products) into the vagina are also potential risk factors for HPV as they can cause epithelial microabrasions (Cottrell, 2010). In our study, while harsh vaginal cleaning practices were common, no association between them and HPV positivity were found after controlling for confounding factors.

We however found that HPV risk was positively associated with the number of sexual partners and inversely associated with use of condoms after controlling for confounders in a multivariate regression analysis. This is consistent with other studies that list unprotected sex and multiple sexual partners as risk factors for HPV infection (Roset Bahmanyar *et al.*, 2012; Tran *et al.*, 2015).

In summary, we observed a high prevalence of hookworm infection among our study participants in rural Togo. Though STI and HPV prevalence was slightly lower than that reported for other countries in SSA, a significant proportion of women are still infected and information concerning prevalence of these infections is important in informing public health strategies to reduce the burden of disease and morbidity associated with these infections in the region, particularly in rural areas where access to healthcare remains a challenge.

Furthermore, we demonstrated that infection with hookworms was a significant risk factor for HPV infection and this highlights the need to take into account hookworm infection when designing control strategies for HPV infection. In addition, we detected a distribution of HPV types that has both similarities and differences to that reported in some cohorts in SSA. Importantly, HPV 35 that is implicated in cervical cancer progression in SSA, and not included in any current HPV vaccine, is one of the predominant HPV types we detected in our cohort. This highlights a need to develop better HPV vaccines specific for each population according to the type distribution.

Chapter 5: Hookworm infection induces a Type 2/ Type 1 signature in the female reproductive tract of infected women

5.1 Introduction

Human hookworm infections are initiated by L3 larval penetration of the skin (as well as oral ingestion for *Ancylostoma duodenale*), they travel via the circulation to the lungs, are coughed up and swallowed into the digestive system. In the lumen of the small intestine they moult into adults and attach to the intestinal wall, reproduce and lay eggs that are passed out in the faeces (Gaze, Bethony and Periago, 2014; Loukas *et al.*, 2016).

Experimental animal models have provided an in-depth understanding of the immune response to hookworms that arises from larval penetration of the skin, migration through lungs and tissue damage in small intestine (Bouchery *et al.*, 2017). In the mouse model of hookworm, *Nippostrongylus brasiliensis*, the parasite and the excretory/secretory enzymes they release to facilitate breaching of epithelial barriers trigger release of Interleukin(IL)-33, IL-25 and Thymic stromal lymphopoietin (TSLP; Allen and Sutherland, 2014). This induces accumulation of eosinophils, mast cells, neutrophils and basophils that produce IL-4 and IL-5 (Voehringer, Shinkai and Locksley, 2004; Obata-Ninomiya *et al.*, 2013; Chen *et al.*, 2014; Bouchery *et al.*, 2020). These cytokines recruit additional myeloid cells and IL-4, IL-5, IL-13 and IL-9 producing ILC2 and Th2 cells to the site of infection in addition to polarising tissue-resident macrophages towards an M2 phenotype (Obata-Ninomiya *et al.*, 2013; Bouchery *et al.*, 2015). Type 2 immune responses induced by hookworm infection in the lung contribute

to parasite killing and tissue damage (Mearns *et al.*, 2008; Endo *et al.*, 2015) and in the intestine, they promote goblet cell hyperplasia and worm expulsion in a STAT6/IL-4Rα dependent manner (Kaplan *et al.*, 1996; Urban *et al.*, 1998). The regulatory cytokine TGF-β has also been demonstrated to be required in limiting emphysematous inflammation in the lungs following *N .brasiliensis* infection (Heitmann *et al.*, 2012).

In humans, the cytokine response to hookworms consists of Type 1 (IFN-γ, TNF-α and IL-2, IL-12), Type 2 (IL-4, IL-5, IL-13) and regulatory (IL-10 and TGF-β) cytokines, detected in peripheral circulation (Pit *et al.*, 2000, 2001; Geiger *et al.*, 2004; Quinnell, Bethony and Pritchard, 2004; Gaze, McSorley, Daveson, Jones, Jeffrey M Bethony, *et al.*, 2012). A mixed type 1 and Type 2 immune response has also been detected in the duodenal mucosa in humans (Gaze, McSorley, Daveson, Jones, Jeffrey M Bethony, *et al.*, 2012).

In humans and in animal models, levels of the antibody, IgE, were inversely correlated to hookworm load, fecundity and weight (Pritchard, Quinell and Walsh, 1995; Bethony *et al.*, 2005) and IgE receptor deficient mice displayed impaired larval trapping in the skin during secondary *N. brasiliensis* infection (Obata-Ninomiya *et al.*, 2013). However, an elevated IgG4 response positively correlated with a high intensity of hookworm infection (Palmer, Bradley and Bundy, 1996), suggesting that hookworms skew the antibody response to favour an IgG4 over the strongly antiparasitic IgE response. Additionally, IgG1, IgM, IgD and IgA antibodies were also shown to bind both anthropophilic and zoonotic hookworm antigens (Pritchard, Quinell and Walsh, 1995).

These data collectively show that hookworms imprint a type 2 immune response in the host. Given that helminths can alter immunity to unrelated pathogens (Osborne *et al.*, 2014; Reese *et al.*, 2014), and at sites not normally colonized by the helminths (Gravitt *et al.*, 2016; Chetty *et al.*, 2021), we sought to investigate whether hookworms, the most prevalent helminth infection in our cohort, and that which increases the risk of HPV up to 2-fold (as reported in Chapter 4), could alter immunity in the unrelated female reproductive tract.

Results

5.2 Type 2 and Type 1 cytokines are elevated in the female reproductive tract (FRT) of women with hookworm infection

First, we measured levels of Type 1, Type 2 and proinflammatory cytokines in the FRT of our study participants. Elevated classic Type 2 cytokines: IL-5 (median 30.81 vs 0.00 pg/mg of total protein p=0.006), IL-13 (median 11.82 vs 3.16 p=0.004) and IL-4 (median 10.67 vs 0.00 pg/mg of total protein p=0.002) were detected in the cervico-vaginal fluids (CVF) of hookworm infected in comparison to hookworm uninfected women (**Figure 10A**).

Type 1 cytokines TNF-α (median 37.68 vs 15.86 pg/mg of total protein p=0.0002) and IL-12 (median 13.26 vs 3.36 pg/mg of total protein p <0.0001), GM-CSF (median 75.79 vs 0.00 pg/mg of total protein p=0.005), IL-2 (median 33.39 vs 14.79 pg/mg of total protein p=0.02) and the regulatory cytokine IL-10 (median 0.29 vs 0.00 pg/mg of total protein p=0.048), were also elevated in the FRT of hookworm positive women when compared to uninfected women (**Figure 10A**).

No differences in CVF levels of IFN-γ, IL-18, IL-6, IL-1β, IL-17, IL-21 and IL-22 were observed between hookworm positive and hookworm negative women (**Figure 10A**).

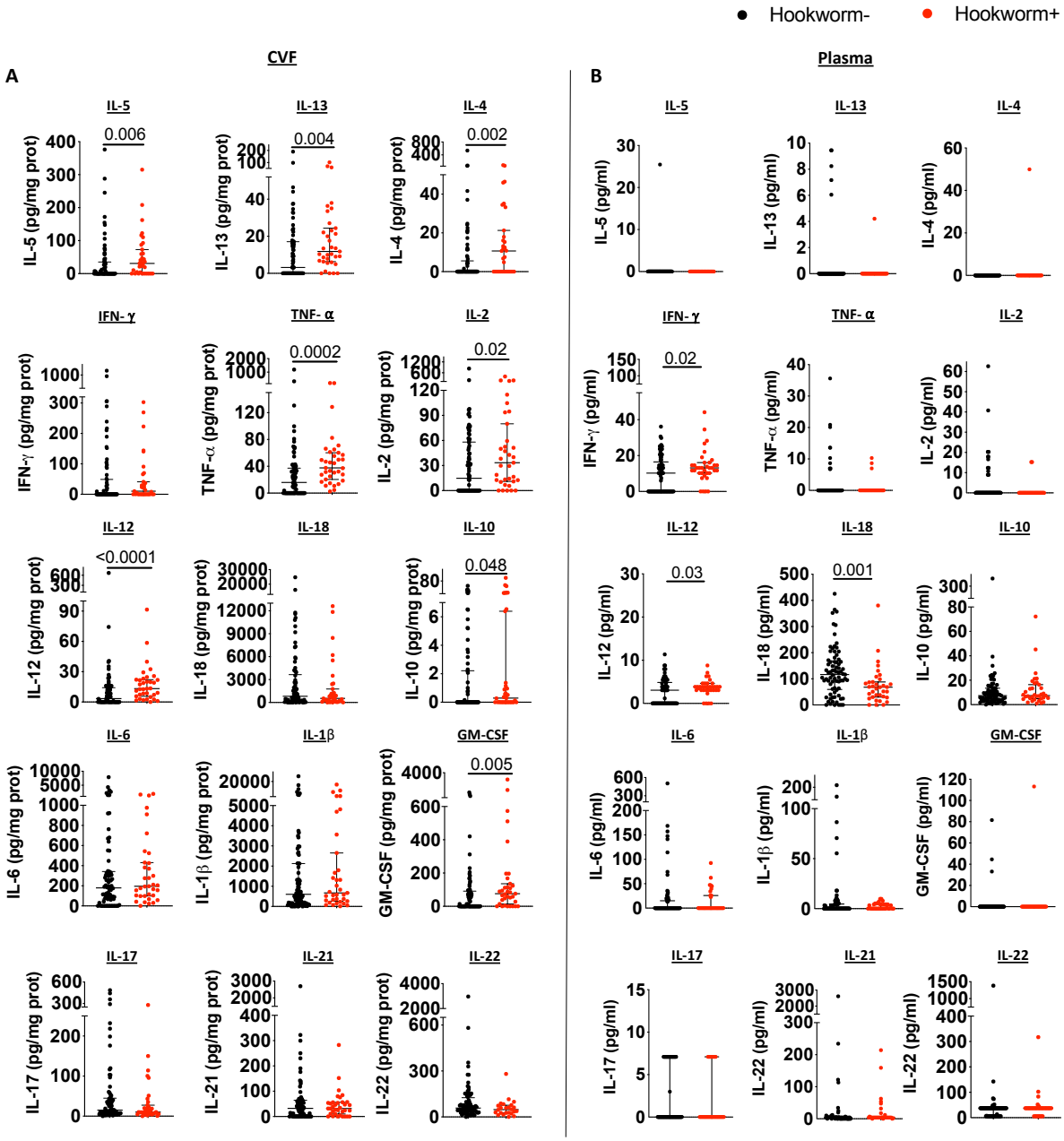


Figure 10: Hookworm infection induces type 2 immune responses in the FRT of humans. IL-5, IL-13, IL-4. IFN- γ ,TNF- α, IL-2, IL-12, Il-18, IL-10, IL-6, IL-1β, GM-CSF, IL-17, IL-21, and IL-22 levels in the CVF (A) and plasma (B) of hookworm - and hookworm + women. Each dot represents a single individual, horizontal bars indicate the median and IQR. Hookworm- (n=78) hookworm+ (n=35). Mann Whitney U test was used to compare hookworm- and hookworm+ groups.

5.3 Eotaxin and MIP-1α are elevated in the FRT of hookworm-infected women

Eotaxin, a chemokine that is involved in eosinophil recruitment to sites of infection, and MIP-1α were elevated in the FRT of hookworm infected women (Eotaxin: median 2.46 vs 0.00 pg/mg of total protein p=0.02; MIP-1α: median 29.54 vs 7.19 pg/mg of total protein p<0.0001; **Figure 11A**), while RANTES, IL-8, MIP-1β, MCP-1, GRO-α, SDF-1α, IP-10, Eotaxin-2 and Eotaxin-3 levels did not differ between hookworm infected and uninfected women (**Figure 11A**).

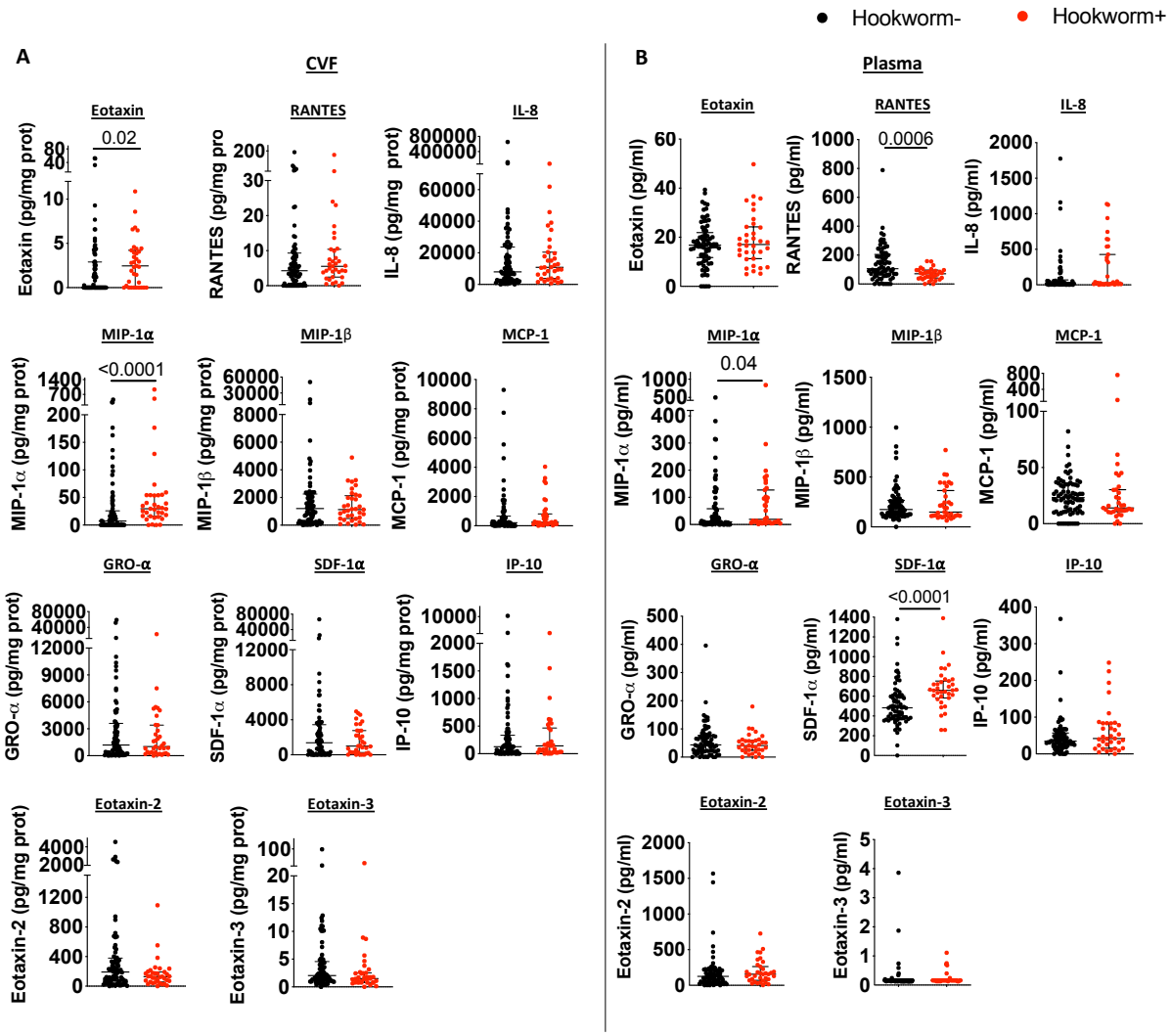


Figure 11: Eotaxin and MIP-1α are elevated in the FRT of hookworm egg-positive women. Eotaxin, RANTES, IL-8, MIP-1α, MIP-1β, MCP-1, GRO-α, SDF-1α, IP-10, Eotaxin-2 and Eotaxin-3 levels in CVF (A) and plasma (B) of hookworm - and hookworm + women. Each dot represents a single individual, horizontal bars indicate the median and IQR. Hookworm- (n=78) hookworm+ (n=35). Mann Whitney U test was used to compare hookworm- and hookworm+ groups.

5.4 *IgG4/ IgE ratio is significantly elevated in the FRT of hookworm positive women*

Having observed an elevated Type 2 cytokine response in the FRT of hookworm-infected women we next moved on to determine if antibody responses in the CVF

were also altered in hookworm infection. The helminth-associated antibodies, IgG4 and IgE, did not differ between hookworm infected and uninfected participants (**Figure 12A**), however the vaginal IgG4/IgE ratio was elevated in women with hookworm infection in comparison to hookworm uninfected women (median 2164 vs 957.5pg/mg of total protein p=0.04; **Figure 12A**). Elevated IgG4/IgE ratio is a common feature of helminth infection whereby helminths favour an IgG4 antibody response over the more strongly anti-parasitic IgE response, with higher worm burdens reported in patients with elevated IgG4 (Turner *et al.*, 2005; Figueiredo *et al.*, 2012).

In addition, IgG2 levels were higher in the FRT of hookworm positive versus hookworm negative women (median $1.4x10^9$ vs $1.7x10^8$ pg/mg of total protein p=0.01; **Figure 12A**). No differences in levels of IgG1, IgG3, IgM and IgA were observed between hookworm infected and uninfected women (**Figure 12A**).

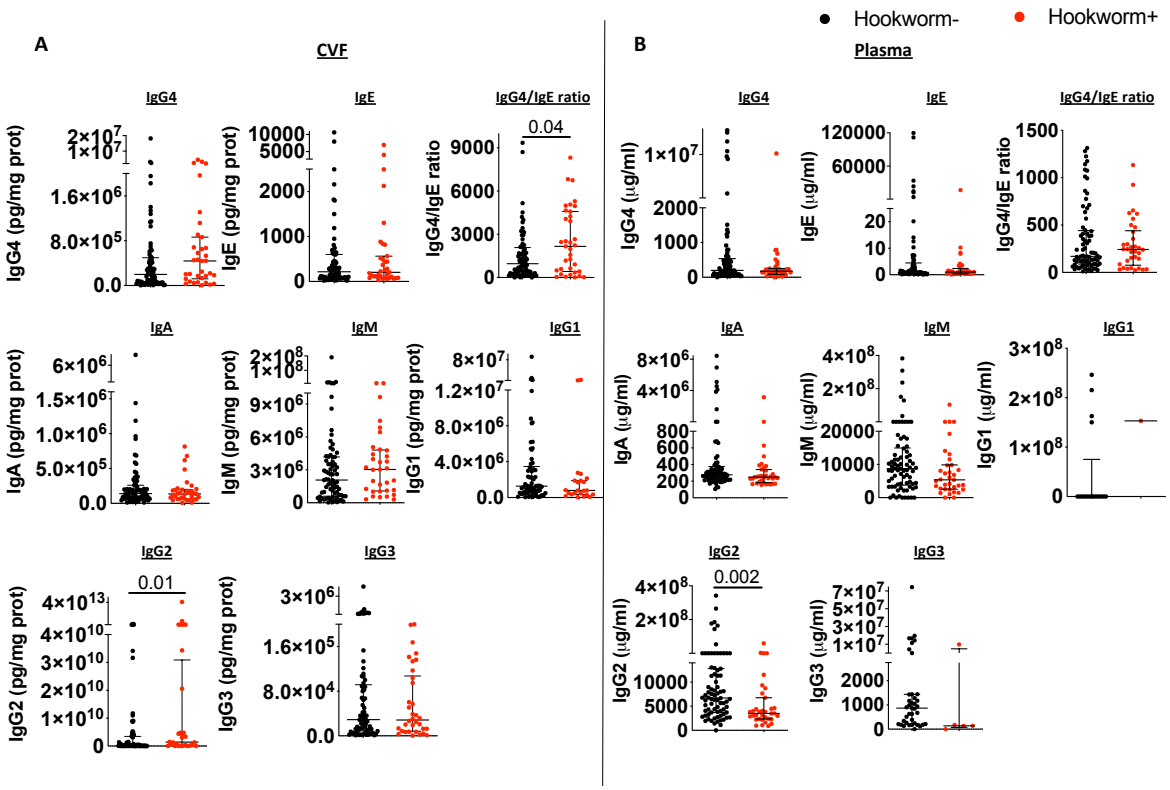


Figure 12: IgG4/IgE ratio is elevated in FRT of hookworm egg-positive women. IgG4, IgE, IgG4/IgE ratio, IgA, IgM, IgG1, IgG2 and IgG3 levels in CVF (A) and plasma (B) of hookworm - and hookworm + women. Each dot represents a single individual, horizontal bars indicate the median and IQR. Hookworm- (n=78) Hookworm+ (n=35). Mann Whitney U test was used to compare hookworm- and hookworm+ groups.

We also observe elevated IL-5/IFN-γ (median 0.97 vs 0.25; p=0.006) and IL-13/IFN-γ ratios (median 0.43 vs 0.16; p=0.04) in the CVF of hookworm infected in comparison to uninfected women (**Figure 13A**).

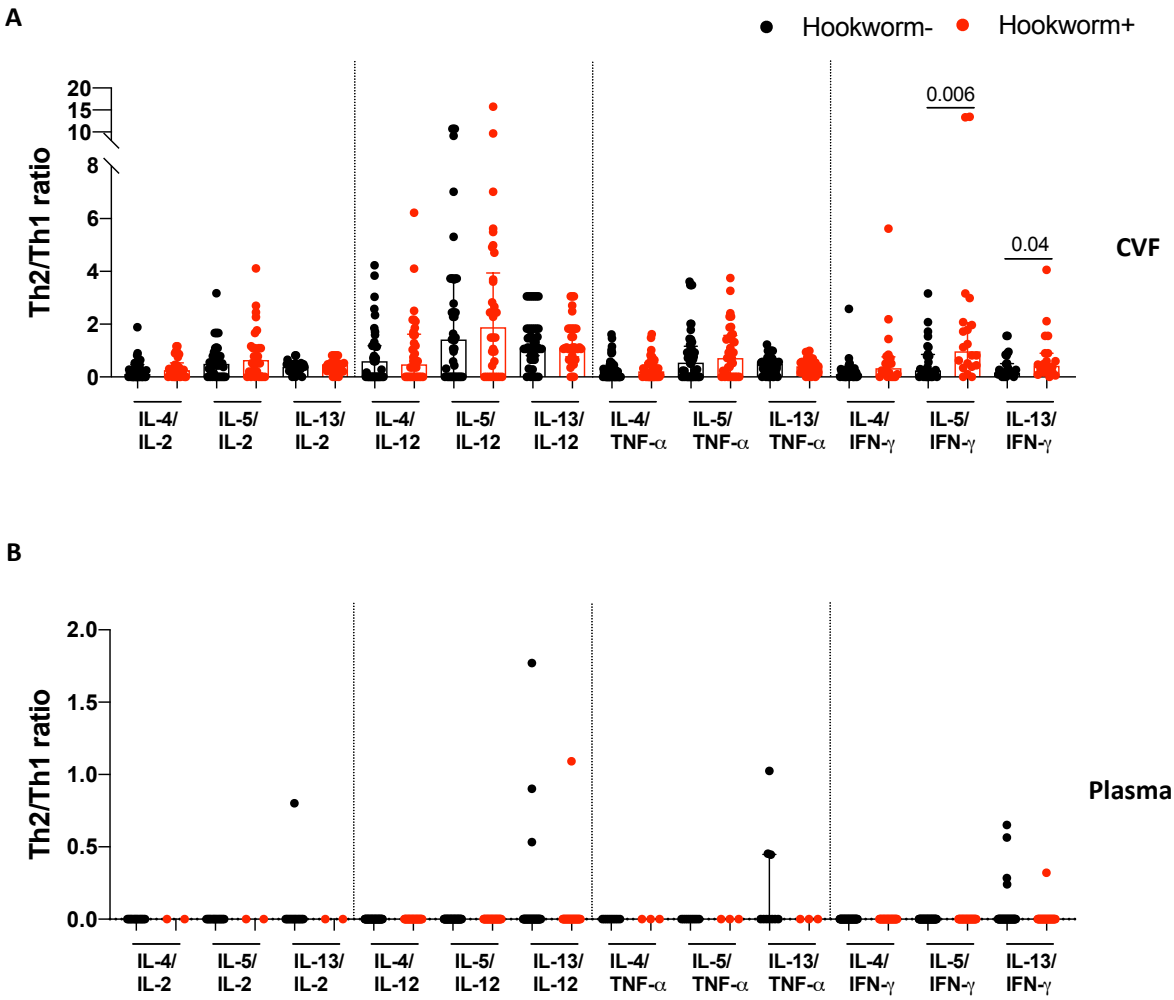


Figure 13: IL-5/IFN-γ and IL-13/IFN-γ ratios are elevated in the CVF of hookworm infected in comparison to hookworm uninfected women. Th1/Th2 ratio in CVF (A) and plasma (B) of hookworm - and hookworm + women. Each dot represents a single individual, horizontal bars indicate the median and IQR. HPV- (n=78) HPV+ (n=35). Mann Whitney U test was used to compare hookworm- and hookworm+ groups.

Collectively, this data shows that hookworm infection can systemically alter immunity in the FRT by inducing a type 2 immune signature consisting of an elevated Type 2 cytokine, chemokine and antibody signature.

5.5 Systemic immune response to hookworm infection

Cytokine, chemokine, and antibody responses in hookworm infected and uninfected women were also measured in plasma ex vivo to determine whether the Type 2 signature that we detect in the mucosa of the FRT is also apparent systemically.

Interestingly, systemic IL-5, IL-13 and IL-4 levels were undetectable in a majority of the samples tested in the Luminex assay (**Figure 10B**). However, elevated IFN-γ (median 13.14 vs 10.25 pg/ml p=0.02) and IL-12 (median 3.86 vs 3.07 pg/ml p=0.03), and reduced IL-18 (median 67.99 vs 116.2 pg/ml p=0.001) was observed in the plasma of hookworm infected in comparison to hookworm uninfected women (**Figure 10B**). There were no differences in the levels of TNF-α, IL-2, IL-10, IL-6, IL-1β, GM-CSF, IL-17, IL-21 and IL-22 levels between hookworm infected and uninfected women.

MIP-1α and SDF-1α were elevated (MIP-1α: median 9.72 vs 19.02 pg/ml p=0.04; SDF-1α: median 659.7 vs 482.1 pg/ml p<0.0001) and RANTES decreased (median 105.7 vs 71.18 pg/ml p=0.0006) in the plasma of hookworm infected compared to uninfected women (**Figure 11B**).

Similar to the results observed in the CVF, no change in levels of helminth-associated antibodies, IgG4 and IgE, was detected in the plasma of hookworm positive versus hookworm negative women (**Figure 12B**). We also did not detect

an elevated IgG4/IGE ratio in the plasma of hookworm infected women (**Figure 12B**). IgG2, on the other hand, was decreased in the plasma of hookworm infected compared to uninfected women (median 3502 vs 6719 pg/ml p=0.002) and there were no differences in the levels of systemic IgG1, IgG3, IgM and IgA between hookworm infected and uninfected women (**Figure 12B**). Th2/Th1 ratios were not altered in the plasma of hookworm infected in comparison to hookworm uninfected women (**Figure 13B**).

5.6 Hookworm infections are positively associated with IgG4 and IgG4/IgE ratio in the FRT

Regression analysis was performed to investigate the association between hookworm infection and the immune parameters measured in the FRT. In a univariate regression analysis with a p value threshold set at 0.2, hookworm infection was positively associated with MIP-1α (OR=1.003, 95% CI 0.999-1.007, p=0.136), IL-5 (OR=1.004, 95% CI 0.998-1.009, p=0.186), GM-CSF (OR=1.000, 95% CI 1.000-1.002, p=0.109), IL-10 (OR=1.024, 95% CI 0.993-1.056, p=0.129), IgG1 (OR=1.000, 95% CI 1.000-1.000, p=0.169) and IgG4/IgE ratio (OR=1.000, 95% CI 1.000-1.000, p=0.014) and negatively associated with eotaxin-2 (OR=0.998, 95% CI 0.996-1.000, p=0.098; **Table 7**).

Immune parameters that were significant in the univariate analysis were then entered into a multivariate logistic regression model with forward selection. After adjustment for multiple comparisons, only the IgG4/IgE ratio was found to be positively associated with hookworm infection (aOR=1.000, 95% CI 1.000-1.000, p=0.009; **Table 7**).

After adjusting for age, level of education, number of partners, non-monogamous partners, vaginal hygiene techniques, contraception, only the VF IgG4/IgE ratio (OR=1.000, 95% CI 1.000-1.001, p=0.006) was positively associated with hookworm infection (**Table 8**).

Table 7: Logistic regression analysis testing the association between hookworm infection and immune parameters measured in the FRT (n=113).

	Univariate		Multivariate	
	OR (95% CI)	**p value**	**aOR (95% CI)**	**p value**
MIP-1α	1.003 (0.999 - 1.007)	**0.136**		
SDF1α	1.000 (1.000 - 1.000)	0.257		
IL-1β	1.000 (1.000 - 1.000)	0.649		
IL-2	1.002 (0.998 - 1.006)	0.384		
IL-4	1.001 (0.994 - 1.008)	0.802		
IL-5	1.004 (0.998 - 1.009)	**0.186**		
IP-10	1.000 (0.999 - 1.000)	0.631		
IL-6	1.000 (0.999 - 1.000)	0.444		
IL-8	1.000 (1.000 - 1.000)	0.631		
Eotaxin	0.993 (0.938 - 1.050)	0.799		
IL-12	1.000 (0.994 - 1.006)	0.956		
IL-13	1.010 (0.994 - 1.027)	0.217		
RANTES	1.002 (0.987 - 1.017)	0.833		
IFN-γ	0.999 (0.996 - 1.002)	0.529		
GM-CSF	1.001 (1.000 - 1.002)	**0.109**		
TNF-α	1.000 (0.997 - 1.004)	0.784		
MIP-1β	1.000 (1.000 - 1.000)	0.423		
MCP-1	1.000 (1.000 - 1.000)	0.944		
GRO-α	1.000 (1.000 - 1.000)	0.426		
IL-18	1.000 (1.000 - 1.000)	0.335		
IL-10	1.024 (0.993 - 1.056)	**0.129**		
Eotaxin-2	0.998 (0.996 - 1.000)	**0.098**		
Eotaxin-3	0.999 (0.020 - 6.146)	0.476		
IL-17	1.000 (0.990 - 1.002)	0.227		
IL-21	1.000 (0.993 - 1.004)	0.557		
IL-22	1.000 (0.994 - 1.003)	0.477		
IgG1	1.000 (1.000 - 1.000)	**0.169**		
IgG2	1.000 (1.000 - 1.000)	0.383		
IgG3	1.000 (1.000 - 1.000)	0.229		
IgG4	1.000 (1.000 - 1.000)	0.792		
IgE	1.000 (1.000 - 1.000)	0.735		
IgA	1.000 (1.000 - 1.000)	0.411		
IgM	1.000 (1.000 - 1.000)	0.626		
IgG4/IgE ratio	1.000 (1.000 - 1.000)	**0.014**	1.000 (1.000-1.001)	**0.009**

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

Table 8: Multiple logistic regression analysis testing the association between hookworm infection and immune parameters measured in the FRT (n=113) after adjusting for sociodemographic factors.

	aOR (95% CI)	p value
IgG4/IgE ratio	1.000 (1.000 – 1.001)	**0.006**

p-value threshold is 0.05. Significant values are in bold.

5.7 Discussion

We found that hookworm infection induces a Type 2 immune profile in the FRT (IL-4, IL-5, IL-13, IL-10, Eotaxin, elevated IgG4/IgE ratio). Elevated IL-5/IFN-γ and IL-13/IFN-γ ratios were also observed in the CVF of hookworm infected women. This is consistent with findings by Gravitt et al., (Gravitt *et al.*, 2016) where they reported detection of Type 2 cytokines in the FRT in women infected with soil transmitted helminths in Peru. Elevated Type 2 immunity in the FRT has also been demonstrated in mice infected with *N. brasiliensis*, that displayed increased eosinophilia and elevated IL-5 in the FRT mucosa (Chetty *et al.*, 2021).

Interestingly, we found that the Type 2 immune signature mounted in the FRT was not reflected systemically, where Type 2 cytokines were undetectable. This is in contrast to studies that reported detection of Type 1 and Type 2 immune responses to hookworms both systemically and in the mucosa of the small intestine (Quinnell *et al.*, 2004; Gaze, McSorley, Daveson, Jones, Jeffrey M Bethony, *et al.*, 2012). A possible explanation for why we were unable to detect Type 2 cytokines systemically is because we measured cytokine responses in the plasma *ex vivo* without stimulation. The study by Gaze and colleagues does show that baseline immune responses to hookworm infection were low (IL-4 and IL-13) or absent (IL-5) and there were no apparent differences in Th1 and Th2 levels between hookworm infected and uninfected participants (Gaze, McSorley,

Daveson, Jones, Jeffrey M. Bethony, *et al.*, 2012). They, and others however report mixed systemic Type1 and Type 2 cytokine responses in hookworm infected individuals following antigen stimulation of PBMC (Quinnell *et al.*, 2004; Geiger *et al.*, 2008; Gaze, McSorley, Daveson, Jones, Jeffrey M. Bethony, *et al.*, 2012). Our study thus represents baseline immune responses to hookworms in the blood and mucosa in the absence of antigen stimulation.

Increased eotaxin, MIP-1α, MIP-1β and RANTES in PBMC from children co-infected with hookworms, *Entamoeba* and schistosomes has previously been reported (Hamm *et al.*, 2009). In line with this, we found increased MIP-1α in the plasma of hookworm infected women, but in contrast to the study by Hamm et al., we observed diminished systemic RANTES in hookworm infected in comparison to hookworm uninfected women, and no change in eotaxin and MIP-1β. The disparity in the chemokine responses observed in our study and those reported by *Hamm et al.*, could be because the latter study investigated responses to polyparasite infection, whereas in our cohort, few women were co-infected with hookworm and other parasites and the data that we report is in participants largely infected with only hookworms.

We detected elevated eotaxin and MIP-1α in the FRT of hookworm infected women. The hookworm *Necator americanus* was previously demonstrated to induce eotaxin and MIP-1α production that functions in concert with IL-5 to recruit eosinophils into the lung tissue of infected mice (Culley *et al.*, 2002). Our data therefore suggests that the mucosal immune response elicited by hookworms at sites of larval migration can also be detected at unrelated mucosal sites such as

the FRT, further reinforcing the evidence of the wide scope of helminth mediated immune modulation in the host.

We did not detect elevated IgE or IgG4 responses in the FRT hookworm infected in comparison to uninfected women, however we observed an elevated IgG4/IgE ratio, this is in line with previous studies that have reported an elevated worm burden to be positively associated with IgG4 (Palmer, Bradley and Bundy, 1996), and suggesting that hookworms may favour an IgG4 response as a form of immune escape. This phenomenon has also been reported in *Ascaris* and schistosome infections (Turner *et al.*, 2005; Figueiredo *et al.*, 2012).

Though we found elevated Type 2 immune mediators in the FRT of hookworm infected women when compared to hookworm uninfected women, we did not find any associations between hookworm infection and Type 2 cytokines in the CVF in a multivariate logistic regression analysis. Only IgG4 and the IgG4/IgE ratio (Type 2 associated antibody signatures) were positively associated with hookworm infection after controlling for sociodemographic and biological factors that could impact immune parameters in the FRT. This could imply that there are other confounding factors we did not measure in this study that could mask associations between hookworm infection status and Type 2 immunity in the FRT.

In summary we found that hookworm infection induced a Type 2 immune signature in the FRT that could potentially impact immunity to viral pathogens such as HPV that require Type 1 immunity for control. The mechanisms of induction of this Type 2 response in uncolonized tissue are not yet understood, but it is possible that the secretory products produced by helminths are

transported via blood, or lymphatics to distal sites where immune responses to these products are then mounted (Zarek and Reese, 2021).

Chapter 6: Human Papillomavirus (HPV) infection induces a Type 1 response systemically and a mixed Type 1 and Type 2 immune response in the female reproductive tract of infected women

6.1 Introduction

HPV infection in the female reproductive tract (FRT) requires microwounds in the vaginal epithelium that allow entry of virions into target basal keratinocytes where they replicate (Stanley, 2010). A small percentage (~10%) of infections with high risk HPV types can persist for decades and go on to develop into cervical cancer (Zur Hausen, 2002). On the other hand, majority of HPV infections are cleared by host immunity, but this happens approximately 1-2 years after infection (Rodríguez *et al.*, 2008; Winer *et al.*, 2011) suggesting that HPV employs immune evasion strategies that facilitate replication within the host without detection. Strategies for immune evasion include virion replication without inducing cell death, suppression of HPV gene expression, altered antigen presentation to evade cytotoxic T cell responses, interference with interferon signalling pathways in keratinocytes (mediated by early HPV proteins, E6 and E7) and induction of regulatory T cell responses (Steinbach and Riemer, 2018).

The innate immune response to HPV in the cervix is initiated by upregulation of pattern recognition receptors (PRRs) such as TLR3, TLR7, TLR8 and TLR9 on keratinocytes that recognize HPV pathogen associated molecular patterns (PAMPs) and are associated with viral clearance of HPV16 (Daud *et al.*, 2011; Scott *et al.*, 2015). PRR signalling induces proinflammatory cytokines and chemokines such as IFN-α, IFN-β, IFN-γ, TNF-α, IL-1β, MIP-3α and RANTES

from keratinocytes, macrophages and NK cells (Boccardo, Lepique and Villa, 2010; Karim *et al.*, 2013). Notably, HPV has been demonstrated to interfere with the transcription factors NF-kappa-B (NF-kB) and interferon regulatory factor 3 (IRF3) thus diminishing proinflammatory responses (Karim *et al.*, 2013). Overexpression of IL-1β on the other hand has been associated with metastasis and poor prognosis in cervical cancer (Lewis *et al.*, 2006). These reports indicate that a delicate balance in the inflammatory response to HPV is required for immune control.

The importance of CD4+T cells in immunity to HPV is evidenced by increased prevalence and persistence of HPV infection and increased precancerous lesions in HIV positive individuals (Harris *et al.*, 2005; Denny *et al.*, 2008; Singh *et al.*, 2009). Furthermore, women with higher grades of cervical neoplasia had fewer CD4+ T cells (Steele *et al.*, 2005).

The CD4+ T cell response to natural HPV infection consists of Th1, Th2 and regulatory T cell responses (Zhou, Tuong and Frazer, 2019). Vaccination with the bivalent Cervavix vaccine results in elevated levels of Type 1 (IFN-γ, TNF-α, IL-2, IL-17), Type 2 (IL-5, IL-13) and regulatory (IL-10) cytokines in the FRT (Pasmans *et al.*, 2019). Bias towards Type 1 cytokines such as IL-2, IL-12, TNF-α, IFN-γ was associated with decreased progression of cervical lesions (El-Sherif *et al.*, 2001; Bais *et al.*, 2007), while high grade intraepithelial lesions and progression to cancer in HPV-infected individuals was associated with increased regulatory cytokines, IL-10 and TGF-β, and Type 2 cytokines, IL-4, IL-5 and IL-13 (Clerici *et al.*, 1997; Al-Saleh *et al.*, 1998; Peghini *et al.*, 2012) both in circulation and in the cervix. Furthermore, women with high risk HPV types were

demonstrated to have elevated CVL levels of IL-5, IL-9 and IL-13 in comparison to HPV negative women (Marks *et al.*, 2011).

The humoral response to natural HPV infection comprises IgG and IgA antibodies (Veress *et al.*, 1994; Carter *et al.*, 2000), these antibodies appear in low titres systemically and in the cervix, and seroconversion takes more than 6 months to occur (Carter *et al.*, 2000; Onda *et al.*, 2003). HPV vaccination elicits IgG, and IgA in both serum and CVF but in higher titres than would natural infection, with the predominant IgG subclasses induced being IgG1 and IgG3 (Scherpenisse *et al.*, 2013). Furthermore, vaccination with the Cervavix and Gardasil vaccines induce robust systemic HPV-specific antibody responses in approximately 90-100% of patients and persist between 9-12 years after vaccination (Artemchuk *et al.*, 2019; Hoes *et al.*, 2020). Of note the systemic antibody response to vaccination correlated strongly to that in cervicovaginal fluids (Petäjä *et al.*, 2011; Pattyn *et al.*, 2019), suggesting that antibody in serum was able to cross the mucosal barrier following vaccination. Moreover, vaccination provides protection against HPV infection and cervical cancer. Collectively these data suggest a role for antibodies in protection against HPV associated cervical cancer.

Given that a delicate immune balance is required in control of HPV infection, with a Th1 biased response associated with clearance and a Th2 skewed phenotype associated with viral persistence and poor prognosis in patients with cervical intraepithelial neoplasia (CIN), investigating immune profiles in HPV infected women has possible value in predicting viral persistence and HPV infection outcomes. This can help in improving control strategies for HPV.

6.2 Type 2 (IL-5, IL-4 and IL-13) and Type 1 (IL-12, TNF-α, IL-2) cytokines elevated in FRT of HPV positive women

The immune response in the FRT of HPV positive women was compared to that in HPV negative women. Elevated Type 2 cytokines: IL-5 (median 19.94 vs 0.00 pg/mg of total protein p=0.0095), IL-13 (median 14.67 vs 3.35 pg/mg of total protein p=0.0007) and IL-4 (median 4.36 vs 0.00 pg/mg of total protein p=0.037; **Figure 14A**) were detected in the FRT of HPV positive women in comparison to uninfected women.

Furthermore, GM-CSF (median 82.19 vs 0.00 pg/mg of total protein p=0.002), TNF-α (median 34.37 vs 15.04 pg/mg of total protein p=0.0002), IL-2 (median 51.24 vs 8.03 pg/mg of total protein p<0.0001) and IL-12 (median 13.54 vs 3.55 pg/mg of total protein p=0.0005; **Figure 14A**) were increased in the cervico-vaginal fluids (CVF) of HPV positive versus HPV uninfected women**.** Regulatory IL-10 was however not increased in the FRT of HPV positive in comparison to HPV uninfected women (**Figure 14A**).

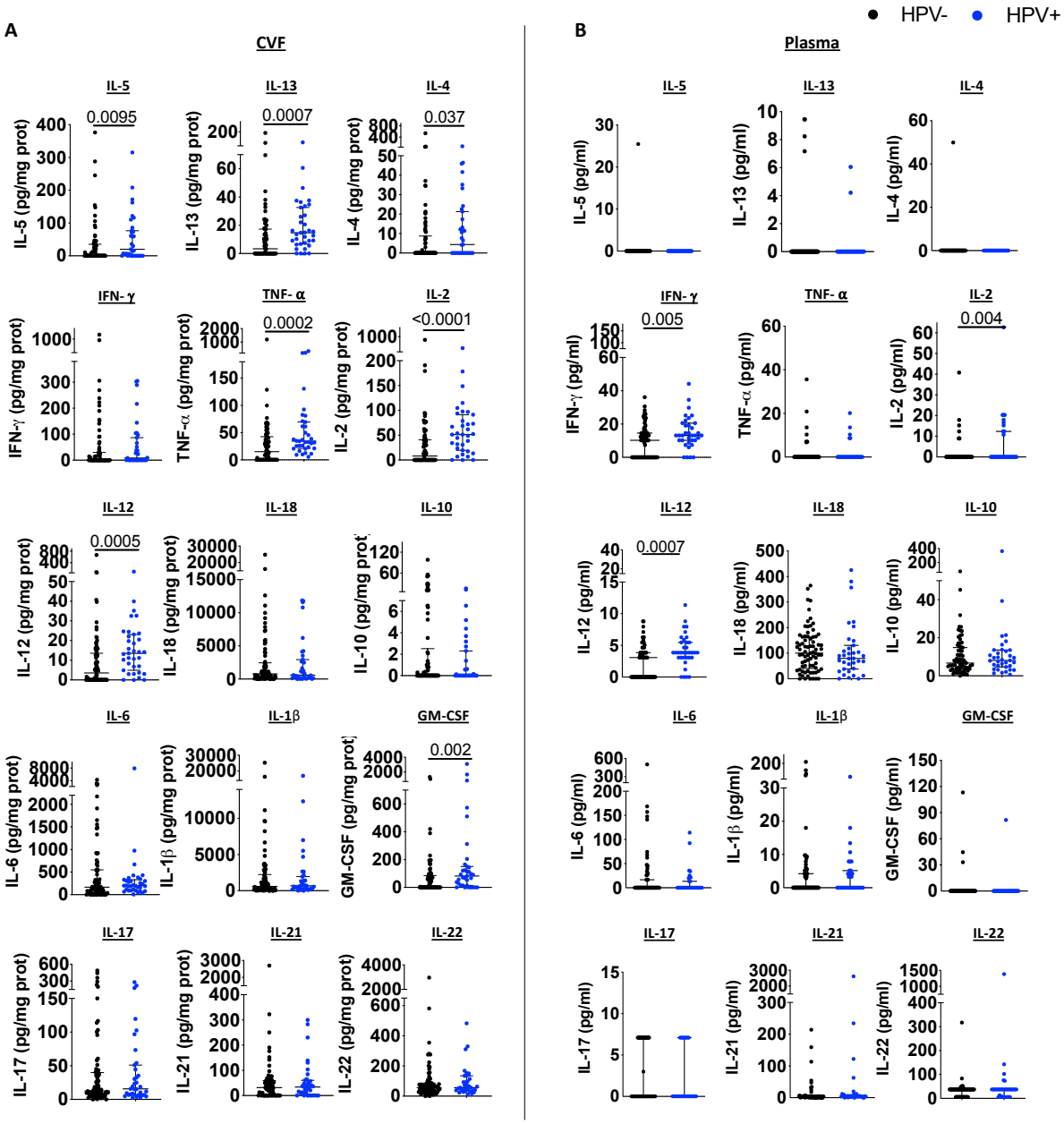


Figure 14: Type 1 and Type 2 immune responses are elevated in the FRT of HPV-infected women. IL-5, IL-13, IL-4. IFN- γ ,TNF- α, IL-2, IL-12, Il-18, IL-10, IL-6, IL-1β, GM-CSF, IL-17, IL-21 and IL-22 levels in the CVF (A) and plasma (B) of HPV - and HPV + women. Each dot represents a single individual, horizontal bars indicate the median and IQR. Hookworm- (n=78) hookworm+ (n=35). Mann Whitney U test was used to compare HPV- and HPV+ groups.

6.3 Chemokines and antibodies are not significantly altered in the FRT of HPV positive women

To determine whether HPV infection induced inflammation in the FRT, we measured CVF levels of pro-inflammatory chemokines. There were no differences observed in vaginal chemokine levels between HPV infected and uninfected women (**Figure 15A**). In addition, antibody levels in the FRT did not differ significantly between HPV positive and HPV negative women (**Figure 16A**).

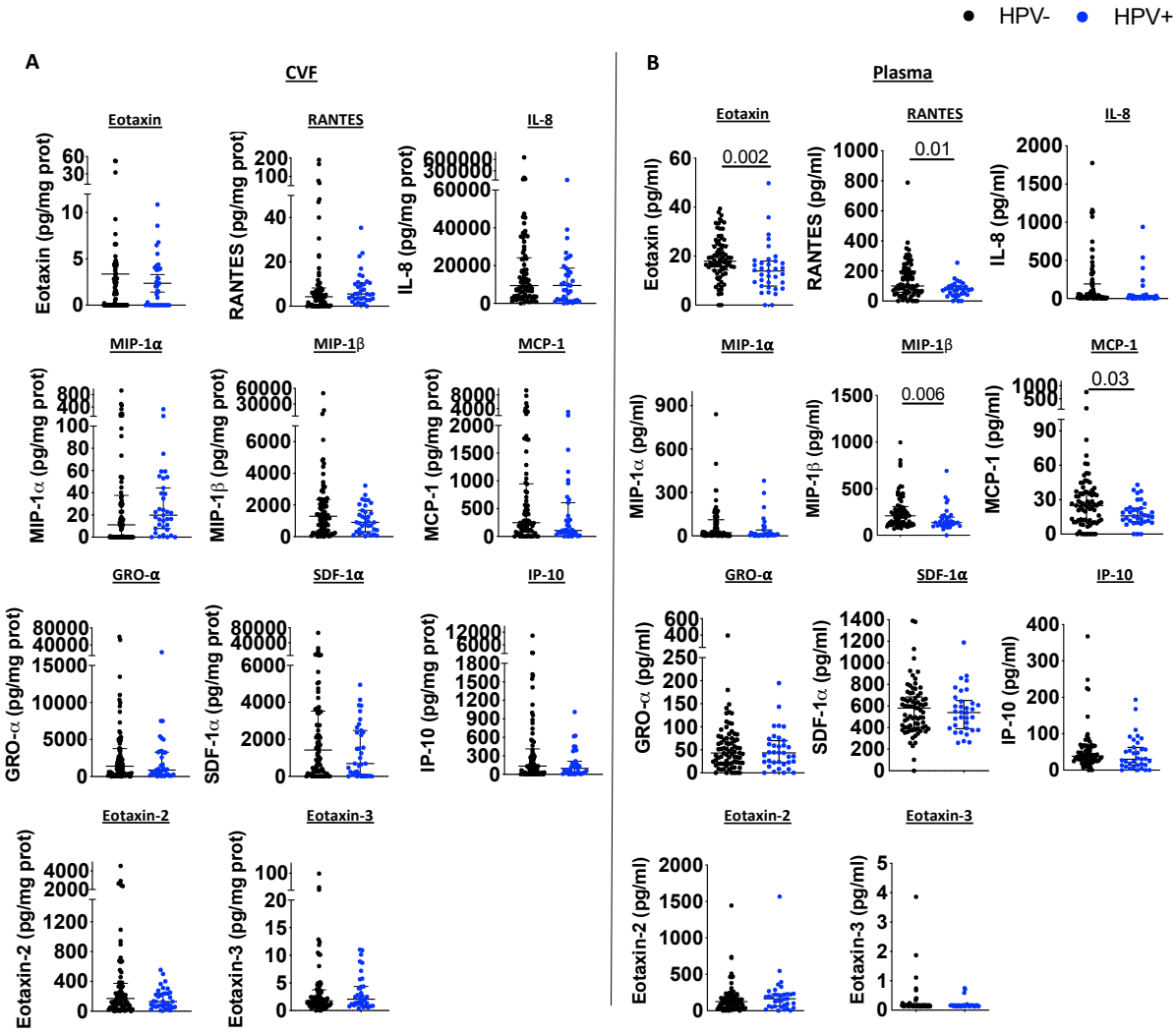


Figure 15: Systemic proinflammatory chemokines are decreased in HPV+ women. Eotaxin, RANTES, IL-8, MIP-1α, MIP-1β, MCP-1, GRO-α, SDF-1α, IP-10, Eotaxin-2 and Eotaxin-3 levels in CVF (A) and plasma (B) of HPV - and HPV+ women. Each dot represents a single individual, horizontal bars indicate the median and IQR. Hookworm- (n=78) hookworm+ (n=35). Mann Whitney U test was used to compare hookworm- and hookworm+ group.

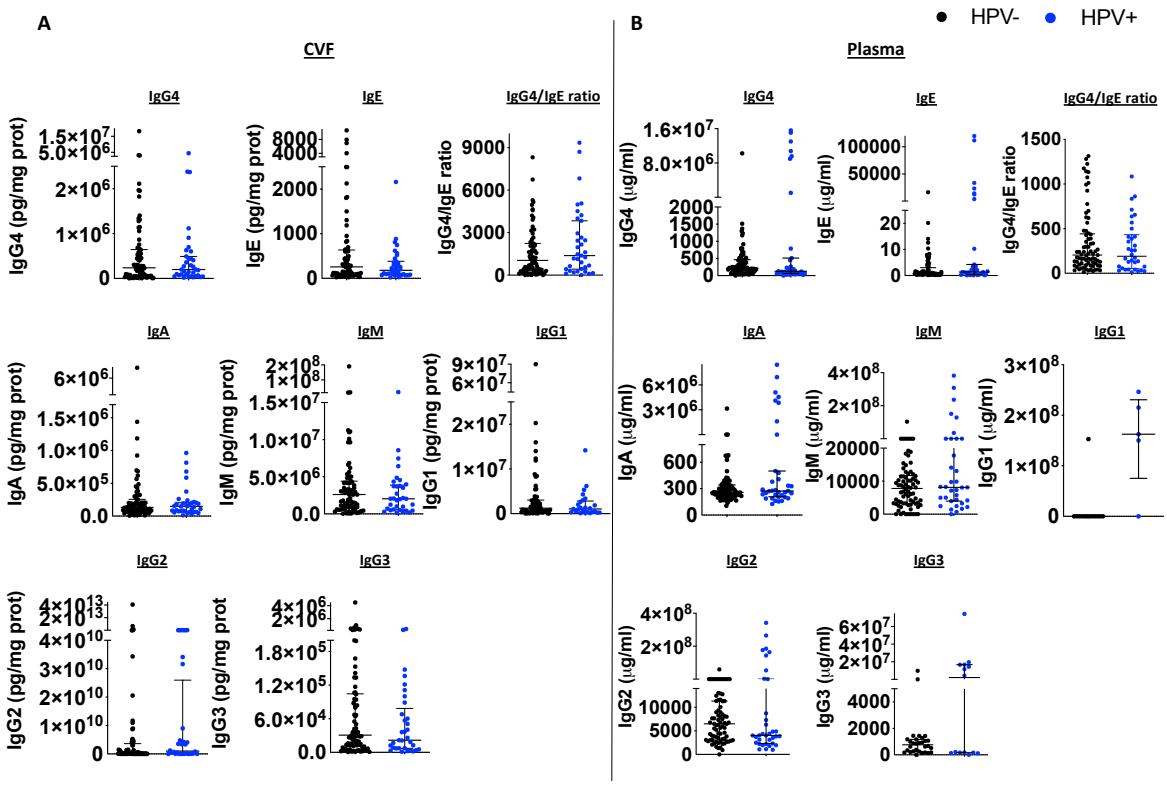


Figure 16: Antibodies are not altered in FRT of HPV-positive women. IgG4, IgE, IgG4/IgE ratio, IgA, IgM, IgG1, IgG2 and IgG3 levels in CVF (A) and plasma (B) of HPV - and HPV + women. Each dot represents a single individual, horizontal bars indicate the median and IQR. Hookworm- (n=78) Hookworm+ (n=35). Mann Whitney U test was used to compare hookworm- and hookworm+ groups.

6.4 *Type 1 but not Type 2 cytokines are elevated in the plasma of HPV positive women*

Type 2 cytokines were undetected in the plasma of HPV positive and HPV negative women (**Figure 14B**). However, IFN-γ (median 13.14 vs 10.25 pg/ml p=0.005), IL-2 (median 0.00 vs 0.00 pg/ml p=0.004) and IL-12 (median 3.86 vs 3.07 pg/ml p=0.0007) were elevated in the plasma of HPV positive in comparison to HPV negative women (**Figure 14B**).

Chemokines, Eotaxin (median 13.93 vs 17.92 pg/ml p=0.002), RANTES (median 77.26 vs 100.5 pg/ml p=0.01), MIP-1β (median 136.3 vs 208.6 pg/ml p=0.006) and MCP-1 (median 15.89 vs 24.95 pg/ml p=0.03) were decreased in the plasma of HPV infected in comparison to uninfected women (**Figure 15B**). There were no differences in systemic antibody levels between HPV positive and HPV negative women (**Figure 16B**).

6.5 Th2/Th1 ratio does not differ between HPV positive and HPV negative women

We next compared the ratio of Th1 cytokines to Th2 cytokines in the plasma and CVF of HPV infected in comparison to HPV uninfected women *i.e.,* Type 2: IL-4, IL-5 and IL-13 vs Type 1: IFN-γ, IL-2, IL-12 and TNF-α. The Th2/Th1 ratios in the CVF and plasma did not differ between HPV+ and HPV- women (**Figure 17A** and **Figure 17B**).

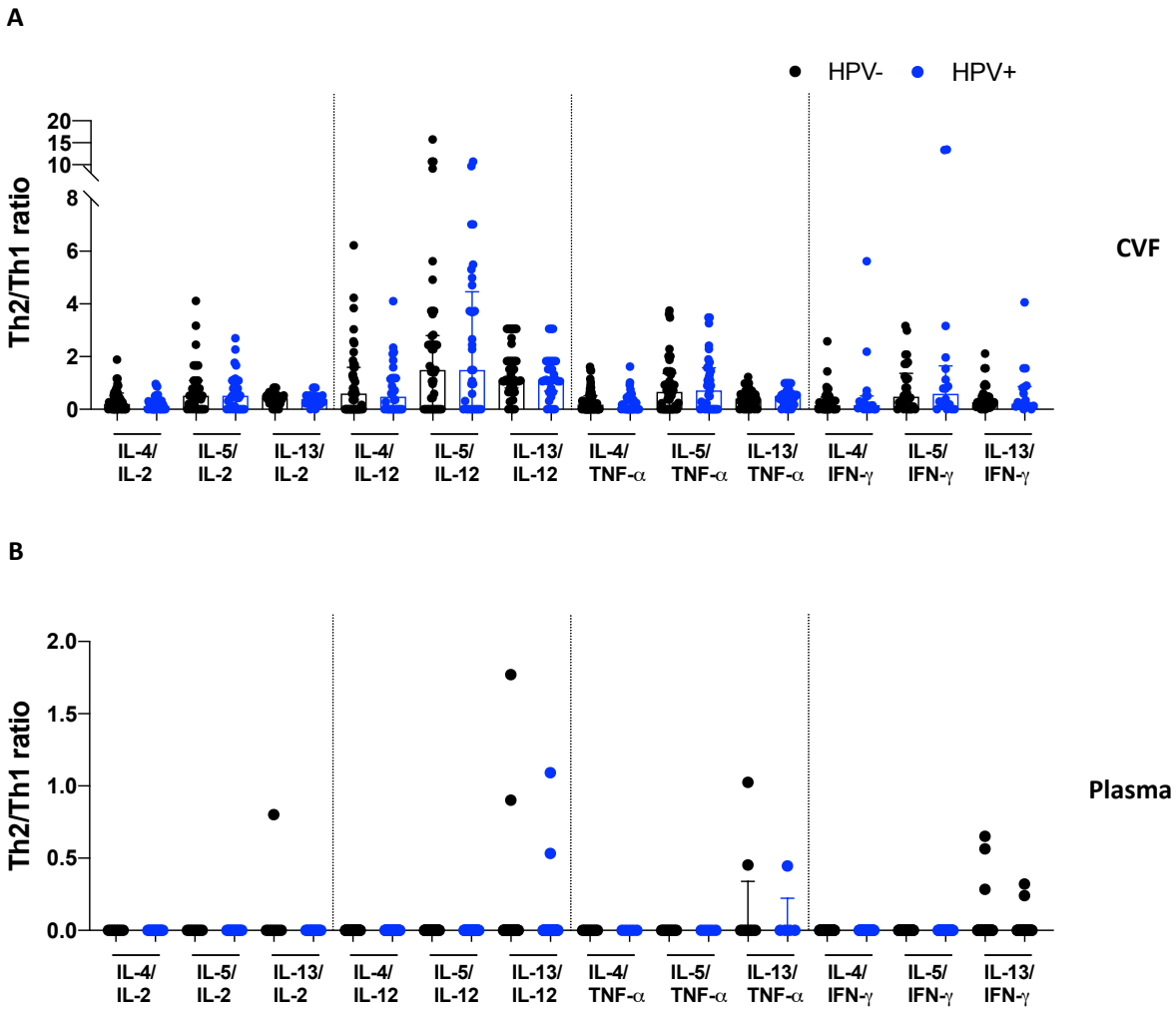


Figure 17: CVF Th2/Th1 ratios do not differ between HPV-positive and HPV- women. Th1/Th2 ratio in CVF (A) and plasma (B) of HPV - and HPV + women. Each dot represents a single individual, horizontal bars indicate the median and IQR. HPV- (n=78) HPV+ (n=35). Mann Whitney U test was used to compare HPV- and HPV+ groups.

6.6 GM-CSF is positively associated with HPV infection

We next tested the association between HPV and immune mediators measured in the FRT (**Table 9**). First a univariate analysis was performed, SDF-1α (OR 1.000, 95% CI 1.000-1.000, p=0.117), IL-2 (OR 1.004, 95% CI 0.998-1.009, p=0.185), IL-5 (OR 1.005, 95% CI 0.999-1.011, p=0.095), IL-13 (OR 1.012, 95% CI 0.995-1.029, p=0.164), GM-CSF (OR 1.001, 95% CI 0.999-1.011, p=0.078),

MIP-1β (OR 1.000, 95% CI 0.999 - 1.000, p=0.078), MCP-1 (OR 1.000, 95%CI 1.000 - 1.000, p=0.113) and IgG4/IgE ratio (OR 1.000, 95% CI 1.000 - 1.000, p=0.101) were positively associated with HPV positive status (**Table 9**).

On the other hand, IP-10 (OR 0.999, 95% CI 1.000-1.003, p= 0.115), IL-10 (OR 0.951, 95% CI 0.997 - 1.020, p=0.158), Eotaxin-2 (OR 0.998, 95% CI 0.922 - 1.000, p =0.107), IgE (OR 0.999, 95% CI 0.999 - 1.000, p=0.159) were negatively associated with HPV infection (**Table 9**).

After adjusting for multiple comparisons in a multivariate regression analysis with forward selection, GM-CSF (aOR=1.002, 95% CI 1.000-1.004, p=0.043) and MIP-1β (aOR=1.000, 95% CI 0.999-1.000, p=0.037) were found to be positively associated with HPV infection (**Table 9**).

After correcting for age, level of education, number of partners, non-monogamous partners, vaginal hygiene techniques and contraception, IL-5 was positively associated with HPV infection (OR 1.013, 95% CI 1.003-1.022, p value 0.007), while MCP-1 was negatively associated with HPV infection (OR 0,999, 95% CI 0.998-1.000, p value 0.043; **Table 10**).

Table 9: Logistic regression analysis assessing the association between HPV positivity and immune parameters measured in the FRT (n=113)

	Univariate		Multivariate	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
MIP-1α	0.998 (0.994 - 1.000)	0.446		
SDF1α	1.000 (1.000 - 1.000)	**0.117**		
IL-1β	1.000 (1.000 - 1.000)	0.945		
IL-2	1.004 (0.998 - 1.009)	**0.185**		
IL-4	1.000 (0.992 - 1.008)	0.936		
IL-5	1.005 (0.999 - 1.011)	**0.095**		
IP-10	0.999 (0.998 - 1.000)	**0.115**		
IL-6	1.000 (1.000 - 1.000)	0.826		
IL-8	1.000 (1.000 - 1.000)	0.236		
Eotaxin	0.979 (0.915 - 1.046)	0.527		
IL-12	1.000 (0.993 - 1.006)	0.955		
IL-13	1.012 (0.995 - 1.029)	**0.164**		
RANTES	0.990 (0.966 - 1.013)	0.386		
IFN-g	1.000 (0.997 - 1.002)	0.858		
GM-CSF	1.001 (1.000 - 1.003)	**0.07**	1.002(1.000-1.004)	**0.043**
TNF-α	1.001 (0.998 - 1.004)	0.475		
MIP-1β	1.000 (0.999 - 1.000)	**0.078**	1.000 (0.999-1.000)	**0.037**
MCP-1	1.000 (1.000 - 1.000)	**0.113**		
GRO-α	1.000 (1.000 - 1.000)	0.424		
IL-18	1.000 (0.887 - 1.000)	0.916		
IL-10	0.951 (0.997 - 1.020)	**0.158**		
Eotaxin-2	0.998 (0.922 - 1.000)	**0.107**		
Eotaxin-3	0.981 (0.994 - 1.043)	0.54		
IL-17	0.999 (0.997 - 1.004)	0.77		
IL-21	1.000 (0.998 - 1.002)	0.665		
IL-22	1.000 (1.000 - 1.001)	0.706		
IgG1	1.000 (1.000 - 1.000)	0.255		
IgG2	1.000 (1.000 - 1.000)	0.601		
IgG3	1.000 (1.000 - 1.000)	0.223		
IgG4	1.000 (1.000 - 1.000)	0.599		
IgE	0.999 (0.999 - 1.000)	**0.159**		
IgA	1.000 (1.000 - 1.000)	0.548		
IgM	1.000 (1.000 - 1.000)	0.456		
IgG4/IgE ratio	1.000 (1.000 - 1.000)	**0.101**		

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

Table 10: Multiple logistic regression analysis testing the association between HPV infection and immune parameters measured in the FRT (n=113) after adjusting for sociodemographic factors.

	aOR (95% CI)	p value
IL-5	1.013 (1.003 – 1.022)	**0.007**
MCP-1	0.999(0.998-1.000)	**0.043**

p-value threshold is 0.05. Significant values are in bold.

6.7 Discussion

Similar to what has been reported previously (Bais *et al.*, 2007) , we detect both Type 1 and Type 2 cytokines in the FRT of HPV infected women. Interestingly, HPV-infected women did not have elevated IL-10 in the CVL. Furthermore, we didn't find any differences in the Th2/Th1 ratio between HPV-positive and HPV-negative women. Elevated Th2/Th1 ratios (Bais *et al.*, 2007; Peghini *et al.*, 2012; Lin *et al.*, 2020) and upregulated IL-10 (Berti *et al.*, 2017) are associated with late stage cervical lesions. Our data therefore may suggest that at the time of sampling, the women in our study did not have advanced HPV-associated lesions. Staging of cervical lesions, however, requires cervical cytological examination (Nayar and Wilbur, 2015). Even so, the elevated Type 2 signature we observe in the FRT of HPV infected women could be a potential risk for development of severe HPV associated cervical lesions (Clerici *et al.*, 1997; Al-Saleh *et al.*, 1998; Peghini *et al.*, 2012).

We were able to detect elevated Type 2 responses in the CVF but not in the plasma of HPV-infected women. Type 2 cytokines may not be readily detected in the plasma, because they are produced at very low levels, requiring antigen stimulation to unmask. In contrast, the FRT mucosa is a site of constant exposure

to antigen, thus cytokine production can be detected at levels that do not require restimulation with antigen to detect.

We did not observe elevated chemokines or pro-inflammatory cytokines in the FRT of HPV infected in comparison to uninfected women, this is consistent with reports that HPV can limit inflammation in the cervix as a means to evade the immune system (Steinbach and Riemer, 2018).

HPV infection was positively associated with IL-5 CVF levels in a regression analysis testing the association between HPV positivity and levels of immune markers in the FRT. We were likely unable to detect more significant associations between HPV status and CVF immune markers (that were significantly elevated in the FRT of HPV positive women in pairwise comparison analysis using the Mann Whitney U test) because of factors not addressed in this analysis. Factors such as hormonal changes induced in the FRT during the menstrual cycle, variation in vaginal microbiota, could all impact immune mediators in the genital tract (Cummins and Doncel, 2009).

The Type 2 cytokine milieu we observe in the FRT in hookworm infection (as reported in Chapter 5) may be disrupting FRT epithelium allowing for higher rates of HPV infection. The ability of Type 2 cytokines to induce epithelial barrier disruption has previously been demonstrated in nasal and lung epithelia of mice (Ahdieh, Vandenbos and Youakim, 2001; Steelant *et al.*, 2018). Here blockade of Type 2 signalling resulted in restoration of the barrier integrity (Ahdieh, Vandenbos and Youakim, 2001; Steelant *et al.*, 2018). Mechanistic studies are however required to determine precisely how Type 2 cytokines impact FRT mucosal barrier integrity and subsequent risk of HPV infection.

Furthermore, it would be worthwhile to follow up HPV-infected women in longitudinal studies to better understand how the immune environment in the FRT changes over time. For the women in our cohort, for instance, a vaginal Type 2 immune signature was induced by hookworm infection, this could have long term implications for HPV infected women, as a sustained Type 2 immune profile in the FRT could have negative outcomes for the progression of HPV infection.

Chapter 7: Hookworm and HPV co-infected women have a pronounced Type 2 immune signature in the female reproductive tract

7.1 Introduction

Soil transmitted helminth (STH) infections in low- and middle-income countries (LMIC) occur at high rates (WHO, 2020). Disproportionately high incidence and mortality (>85%) of Human Papillomavirus (HPV)-associated cervical cancer (CC) also occur in these areas (Hull *et al.*, 2020). In Chapter 4, helminth infection was shown to be associated with a 2x greater risk of HPV infection, consistent with the study by Gravitt et al., where they identify STH infections as a risk factor for HPV (Gravitt *et al.*, 2016).

Helminth infections can modulate host immunity to a range of viral infections. This impact can be detrimental to the host, for example, *Trichinella spiralis* infection impaired CD8+ T cell responses to murine norovirus, via induction of alternatively activated macrophages in co-infected mice (Osborne *et al.*, 2014). Additionally, *H. polygyrus* infection resulted in reactivation of latent murine gamma herpesvirus (MHV68), where IL-4 induction and STAT6 mediated IFN-γ blockade were the mechanisms impairing antiviral immunity (Reese *et al.*, 2014). Furthermore, *H. polygyrus bakeri i*nfection exacerbated pathology following West Nile Virus infection that was dependent on IL-4Rα signalling in intestinal tuft cells (Desai *et al.*, 2021).

Beneficial effects of helminth infections to the host have also been reported, for example, treatment of mice with *Schistosoma mansoni* eggs prior to viral challenge resulted in virtual CD8+ memory T cell expansion and enhanced

control of murine gammaherpesvirus4 (Rolot *et al.*, 2018). In addition, *T. spiralis* infection ameliorated influenza A virus induced pathology in the lung by limiting TNF-α production and CD4 and CD8 T cell infiltration into the airway (Furze, Hussell and Selkirk, 2006), while *H. polygyrus* infection induced Type I interferons in the lung that limited Respiratory Syncytial Virus (RSV) replication and associated lung inflammation (McFarlane *et al.*, 2017). These studies demonstrate that the immune modulation helminths induce in the host is pathogen dependent, and that the immune response triggered by helminth infection could benefit or harm the host dependent on what constitutes protective immunity against the co-infecting virus.

The influence of helminths on unrelated female reproductive tract (FRT) infections in the host could be direct, such as in co-colonised tissue. For example, *Schistosoma haematobium* infected women have a three-fold higher risk of acquiring HIV (Kjetland *et al.*, 2006; Ndhlovu *et al.*, 2007; Downs *et al.*, 2011), likely a result of *S. haematobium* egg related genital pathology induced by eggs lodged the mucosa of the urogenital tract (Odegaard and Hsieh, 2014). Genital filariasis caused by *Wuchereria bancrofti* has also been associated with increased risk of HIV (Kroidl *et al.*, 2019). This may be driven by increased recruitment of $CD4^+$ T cells (target cells for HIV infection) to the vagina from filaria infected lymph nodes draining the FRT.

Indirect impact of helminth infections that neither colonise nor transit the urogenital tract or associated lymphoid organs on vaginal immunity and ability to control viral infection/pathology has been reported. In a mouse model, infection with the murine hookworm *Nippostrongylus brasiliensis* resulted in heightened pathology following subsequent HSV-2 infection which was driven by a helminth

promoted vaginal IL-5, IL-33 and eosinophil response (Chetty *et al.*, 2021). Related to this, the influence of Type 2 immunity on viral infection in the FRT was demonstrated in a study by Oh et al., where dysbiosis in the vaginal tract of mice resulted in IL-33 driven induction of IL-5 production and ILC-2 and eosinophil accumulation (Oh *et al.*, 2016). This, coupled with impaired recruitment of IFN-γ producing effector CD4+ and CD8+T cells to the FRT resulted in impaired control of HSV-2 (Oh *et al.*, 2016). How helminth infections influence vaginal immunity and whether this increases susceptibility to viral infection has not been widely reported.

7.2 Type 2 immune signature is pronounced in the FRT of hookworm and HPV co-infected women

We wanted to determine whether the Type 2 immune responses we observed in hookworm infected and HPV infected women was also a feature of co-infection. Indeed, VF levels of all three Type 2 cytokines (IL-4, IL-5 and IL-13) were significantly elevated in the VF of HPV and hookworm co-infected women in comparison to women with no hookworm or HPV infection (IL-5: median 72.89 vs 0.00 pg/mg of total protein p=0.0006; IL-13: median 22.09 vs 0.00 pg/mg of total protein p=0.006; IL-4: median 13.14 vs 0.00 pg/mg of total protein p=0.004 and **Figure 18A**).

IL-12 (median 22.09 vs 0.00 pg/mg of total protein p=0.0002), TNF-α (median 49.95 vs 0.00 pg/mg of total protein p=0.0004), GM-CSF (median 114.3 vs 0.00 pg/mg of total protein p=0.003) and IL-2 (median 93.67 vs 0.00 pg/mg of total protein p=0.0004; **Figure 18A**) were also elevated in the FRT of HPV and hookworm co-infected women in comparison to uninfected women.

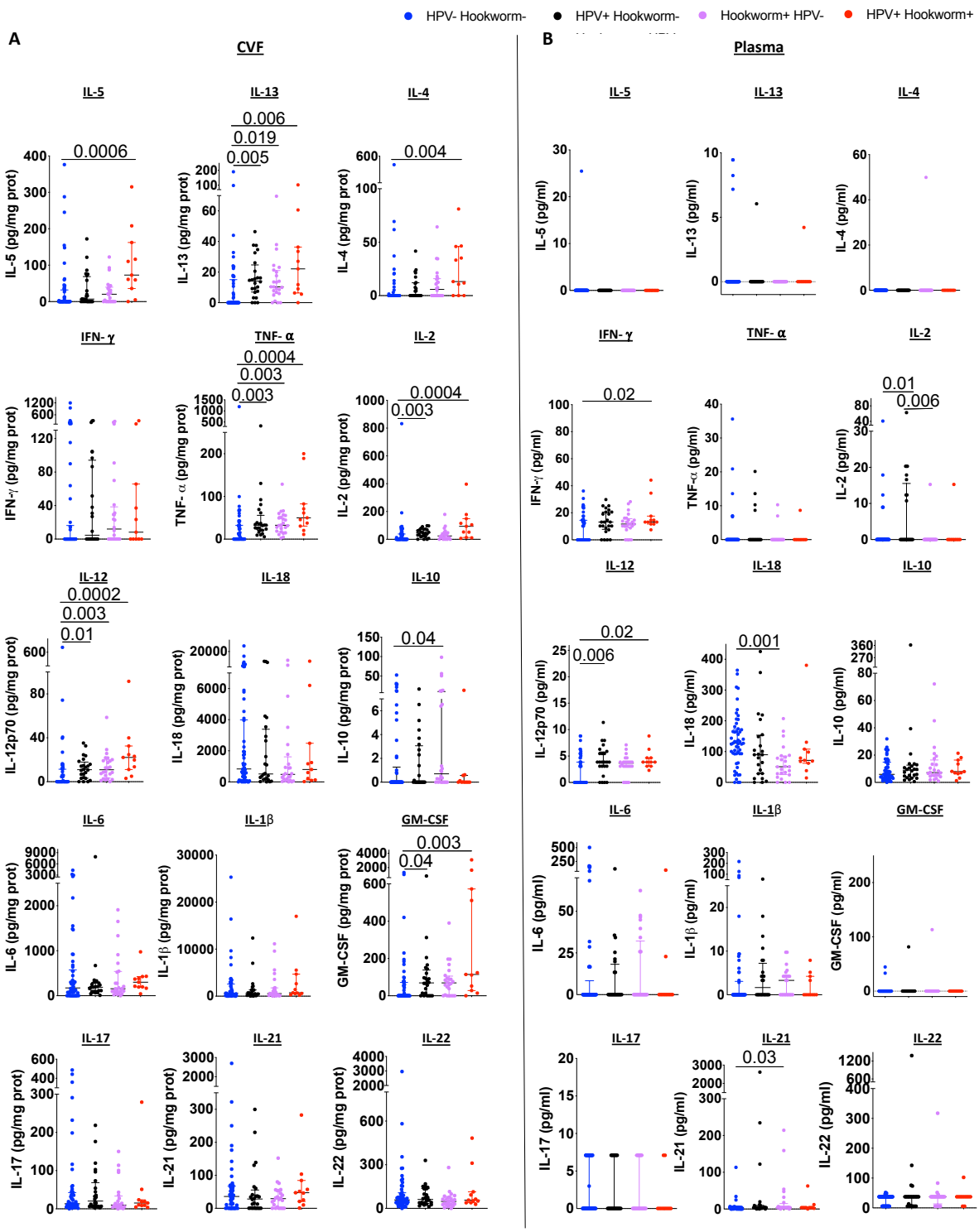


Figure 18: Type 2 immune responses are elevated in the FRT of HPV and Hookworm co-infected women. IL-5, IL-13, IL-4. IFN- γ ,TNF- α, IL-2, IL-12, IL-18, IL-10, IL-6, IL-1β, GM-CSF, IL-17, IL-21 and IL-22 levels in the vaginal flushes (A) and plasma (B) of HPV and hookworm negative, single-infected and co-infected women. Each dot represents a single individual, horizontal bars indicate the median and IQR. HPV- hookworm- (n=54), HPV+ hookworm- (n=24), Hookworm+ HPV- (n=24), HPV+ hookworm+ (n=11). Kruskal Wallis with

Dunn's multiple comparisons test were used to compare HPV and hookworm negative, single-infected and co-infected groups.

7.3 Chemokine responses in VF of HPV and hookworm and HPV co-infected women

Next, we measured chemokine levels in VF. Eotaxin and MIP-1α were elevated in the FRT of HPV and hookworm co-infected women in comparison to uninfected women (eotaxin: median 4.07 vs 0.00 pg/mg of total protein p=0.04; MIP-1α: median 25.27 vs 0.00 pg/mg of total protein p=0.03; **Figure 19A**). There were no differences in VF levels of RANTES, IL-8, MIP-1β, MCP-1, GRO-α, SDF-1α, IL-10, eotaxin-2 and eotaxin-3 between co-infected and women with no HPV or hookworm infection.

7.4 Systemic cytokine and chemokine responses in hookworm and HPV co-infected women

IFN-γ (median 13.14 vs 0.00 pg/ml p=0.02) and IL-12 (median 3.86 vs 0.00 pg/ml p=0.02) were the only cytokines elevated in the plasma of HPV and hookworm co-infected women in comparison to women with no hookworm and no HPV infection (**Figure 18B**). There were no differences in systemic chemokine levels between hookworm and HPV co-infected women and uninfected women (**Figure 19B**).

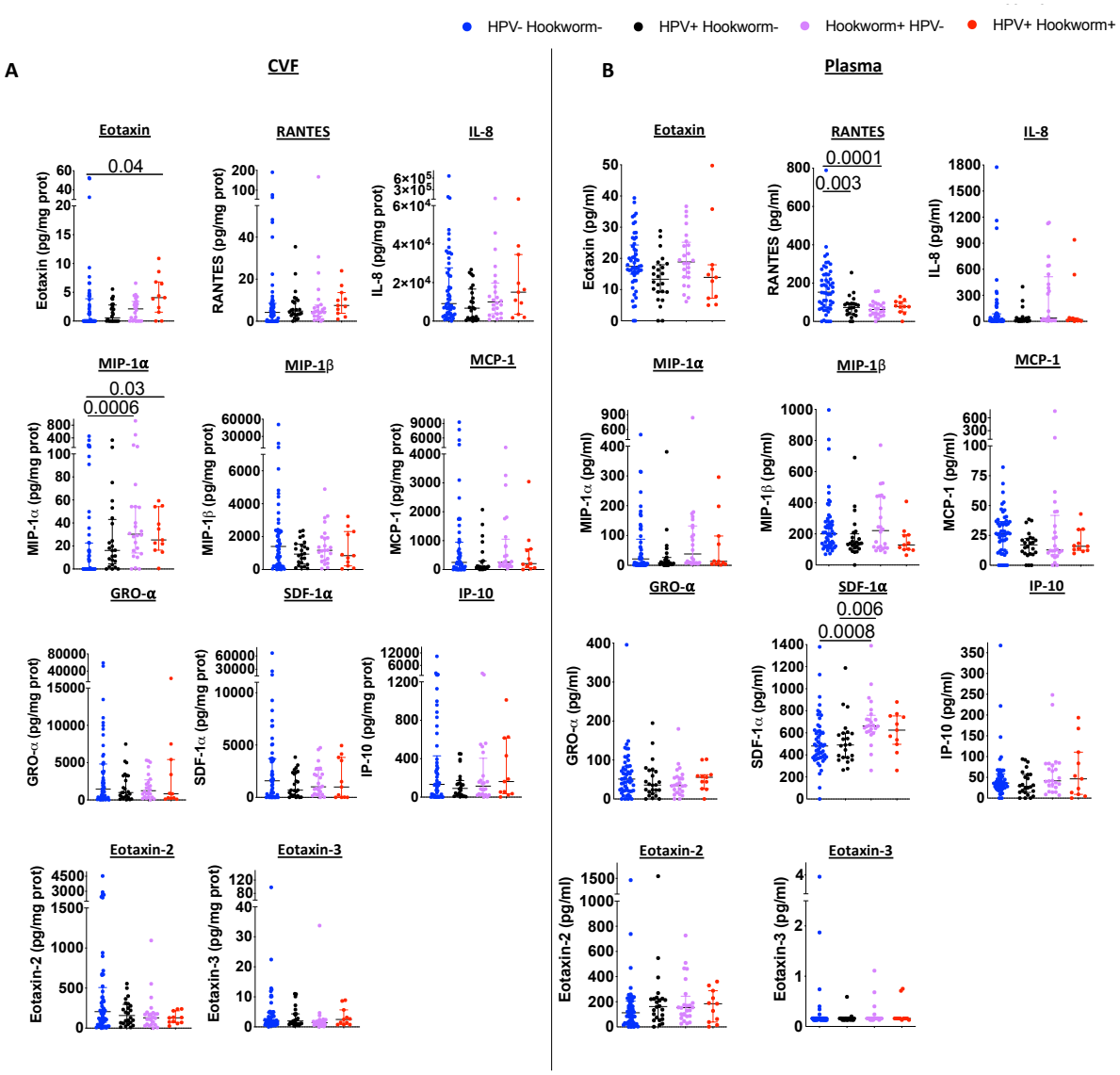


Figure 19: Eotaxin and MIP-1α are elevated in the FRT of HPV and hookworm co-infected women. Eotaxin, RANTES, IL-8, MIP-1α, MIP-1β, MCP-1, GRO-α, SDF-1α, IP-10, Eotaxin-2 and Eotaxin-3) levels in vaginal flushes (A) and plasma (B) levels in vaginal flushes of HPV and hookworm negative, single-infected and co-infected women. Each dot represents a single individual, horizontal bars indicate the median and IQR. HPV- hookworm- (n=54), HPV+ hookworm- (n=24), Hookworm+ HPV- (n=24), HPV+ hookworm+ (n=11). Kruskal Wallis with Dunn's multiple comparisons test were used to compare HPV and hookworm negative, single-infected and co-infected groups.

7.5 IL-13 and Type 1 cytokines elevated in VF of women with only hookworm or only HPV infection

Among women with only hookworm infection IL-13 was the only Type 2 cytokine that was elevated in the VF in comparison to hookworm uninfected women (median 10.37 vs 0.00 pg/mg of total protein p=0.019; **Figure 18A)**. Additionally, IL-12 (median 10.86 vs 0.00 pg/mg of total protein p=0.003), TNF-α (median 32.59 vs 0.00 pg/mg of total protein p=0.003), IL-10 (median 0.70 vs 0.00 pg/mg of total protein p=0.04; **Figure 18A**) and MIP-1α (median 30.22 vs 0.00 pg/mg of total protein p=0.0006; **Figure 19A**) were elevated in the VF of women with only hookworm infection in comparison to uninfected women.

Similar to women with only hookworm infection, women with only HPV infection also had elevated VF IL-13 levels in comparison to uninfected women (median 14.16 vs 0.00 pg/mg of total protein p=0.005; **Figure 18A**). Additionally, GM-CSF (median 69.09 vs 0.00 pg/mg of total protein p=0.04), TNF-α (median 32.28 vs 0.00 pg/mg of total protein p=0.003), IL-2 (median 46.66 vs 0.00 pg/mg of total protein p=0.003) and IL-12 (median 10.92 vs 0.00 pg/mg of total protein p=0.01; **Figure 18A**) were elevated in VF of women with only HPV in comparison to women with no hookworm or HPV infection.

7.6 Systemic cytokine and chemokine responses in women with only hookworm or only HPV infection

Women with only hookworm infection had elevated plasma levels of SDF-1α (median 662.7 vs 481 pg/ml p=0.0008; **Figure 19B**), and decreased IL-18 (median 50.90 vs 126.9 pg/ml p=0.001; **Figure 18B**) and RANTES (median 77.7

vs 152.6 pg/ml p=0.0001; **Figure 19B**) in comparison to those with no hookworm or HPV infection.

Women with only HPV infection, on the other hand, had elevated systemic IL-12 (median 3.86 vs 0.00 pg/ml p=0.006) and IL-2 (median 0.00 vs 0.00 pg/ml p=0.01) in comparison to women with no hookworm or HPV infection (**Figure 18B**).

7.7 Vaginal and systemic antibody levels are not altered in women with only HPV infection and HPV and hookworm co-infected women

We did not observe any differences in VF and systemic antibody levels between HPV and hookworm co-infected women and uninfected women or between HPV infected women and uninfected women (**Figure 20A** and **Figure 20B**), we however observed elevated IgG2 in the VF of hookworm infected and uninfected women (median $1.4x10^9$ vs $1.2x10^8$ pg/mg of total protein p=0.04; **Figure 20A**).

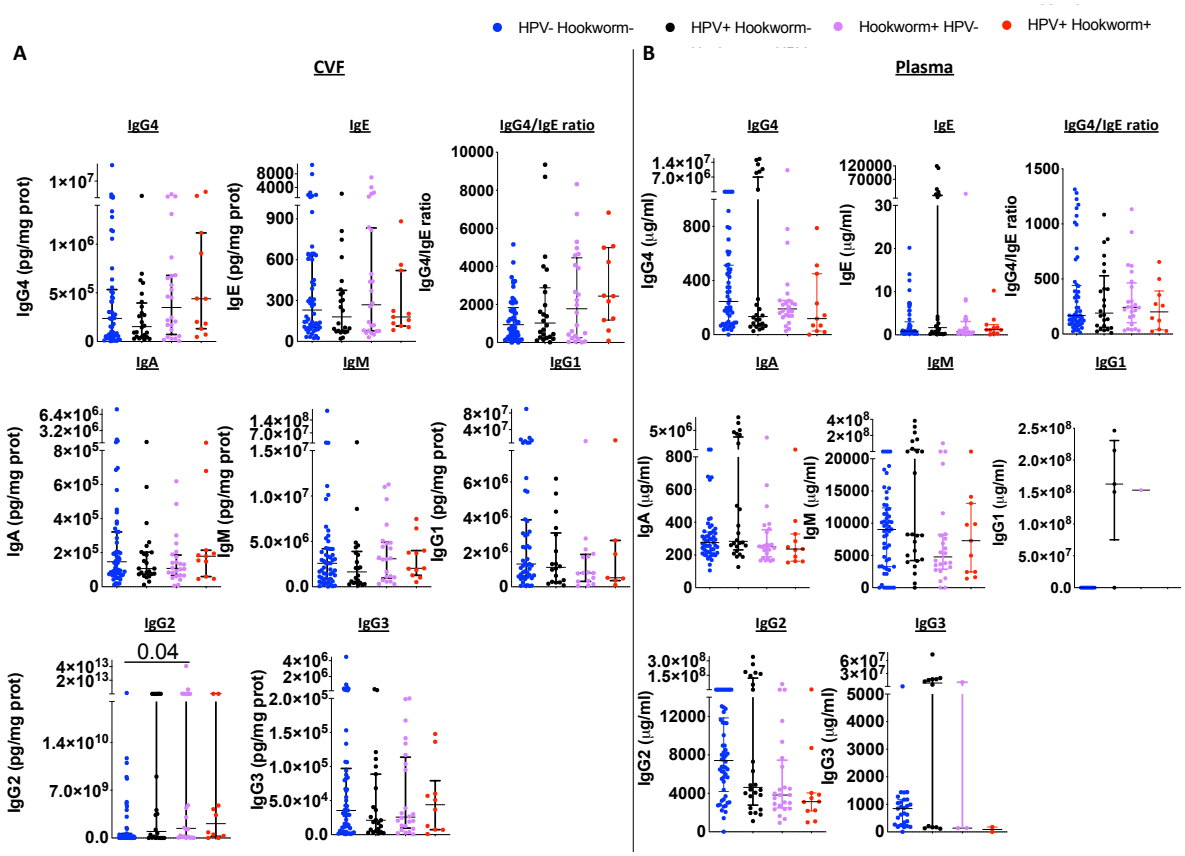


Figure 20: IgG2 is elevated in the FRT of hookworm single-infected women. IgG4, IgE, IgG4/IgE ratio, IgA, IgM, IgG1, IgG2 and IgG3 levels in vaginal flushes (A) and plasma (B) of HPV and hookworm negative, single-infected and co-infected women. Each dot represents a single individual, horizontal bars indicate the median and IQR. HPV- hookworm- (n=54), HPV+ hookworm- (n=24), Hookworm+ HPV- (n=24), HPV+ hookworm+ (n=11). Kruskal Wallis with Dunn's multiple comparisons test were used to compare HPV and hookworm negative, single-infected and co-infected groups.

7.8 Th2/Th1 ratio is elevated in the VF of hookworm and HPV co-infected women in comparison to women infected with only HPV

The ratio of Th2 cytokines to Th1 cytokines in the plasma and VF of women infected with only hookworms or HPV, hookworm and HPV co-infected women or uninfected women were compared (Type 2: IL-4, IL-5 and IL-13 and Type 1: IFN-γ, IL-2, IL-12 and TNF-α). The IL-5/IL-2, IL-5/IL-12, IL-5/ TNF-α and IL-5/IFN-

γ ratios were significantly elevated in the VF of HPV and hookworm co-infected women in comparison to women with only HPV infection (IL-5/IL-2 median 1.05 vs 0.31 p= 0.009, IL-5/IL-12 median 3.69 vs 0.59 p= 0.03, IL-5/ TNF-α median 1.57 vs 0.26 p= 0.01, IL-5/IFN-γ median 2.57 vs 0.22 p= 0.002; **Figure 21.1A**). Plasma Th2/Th1 ratios did not differ between hookworm and HPV co-infected and HPV only infected women (**Figure 21.1B**). The IL-5/IFN-γ ratio was elevated in the CVF of HPV+ Hookworm+ women in comparison to women with no hookworm and no HPV infection (IL-5/IFN-γ median 2.57 vs 0.26 p= 0.008; **Figure 21.1C**). There were no differences in Th2/Th1 ratios in the plasma of HPV+ Hookworm+ and HPV- Hookworm- women (**Figure 21.1D**). CVF and plasma Th2/Th1 ratios also did not differ between HPV+ Hookworm- and HPV- Hookworm- (**Figure 21.2E-F**) or HPV- Hookworm+ and HPV- Hookworm- (**Figure 21.2G-H**) women.

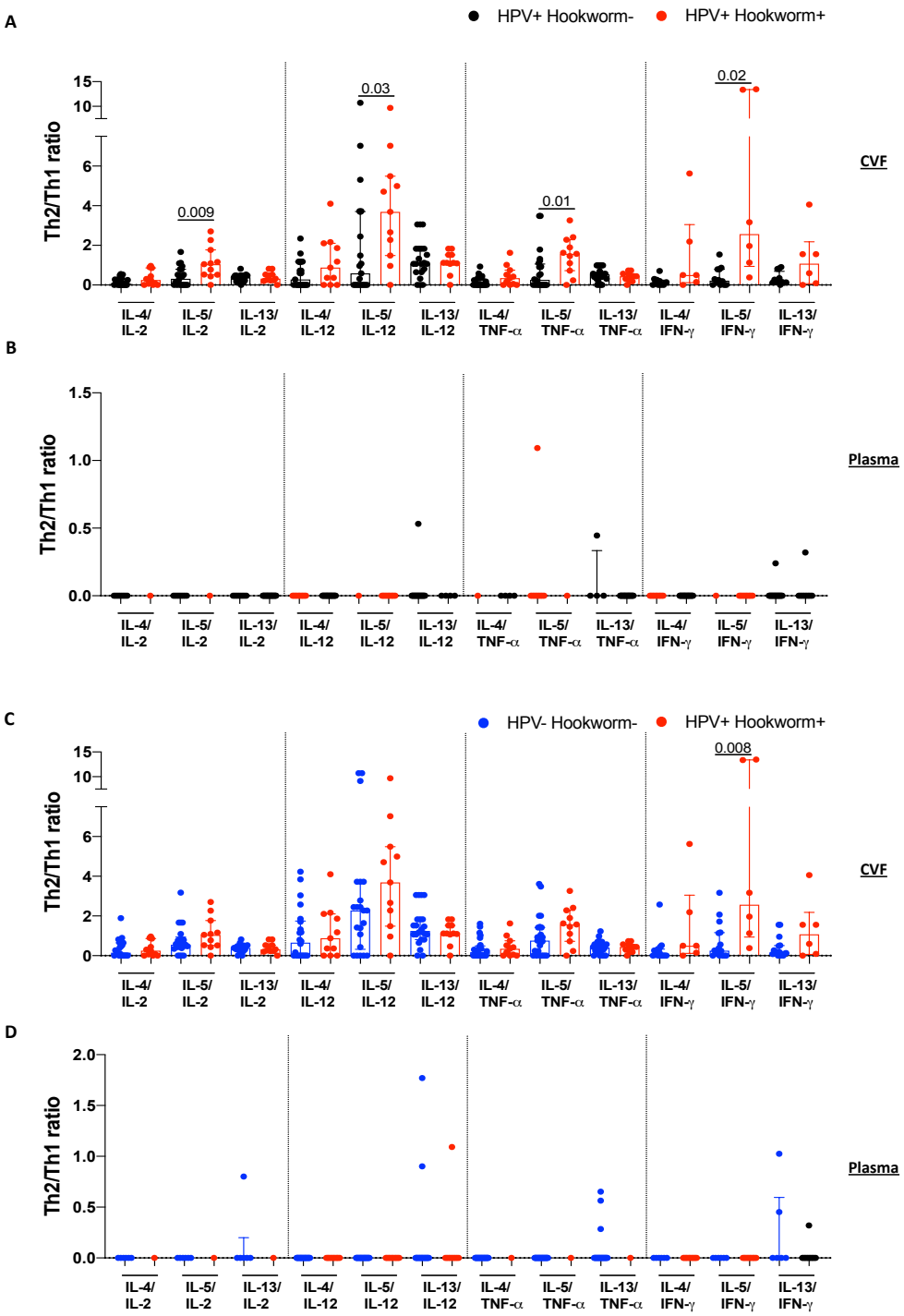


Figure 21.1: Th2/Th1 ratios are elevated in VF of hookworm and HPV co-infected women in comparison to HPV-infected women. Th2/Th1 ratios in vaginal flushes and plasma of HPV+ Hookworm+ and HPV+ Hookworm- (A and B), HPV+ Hookworm+ and HPV- Hookworm- (C and D). Each dot represents a single individual, horizontal bars indicate the median and IQR. HPV- hookworm- (n=54), HPV+ hookworm- (n=24), HPV+ hookworm+ (n=11). Kruskal Wallis with Dunn's multiple comparisons test were used to compare HPV and hookworm negative, single-infected and co-infected groups.

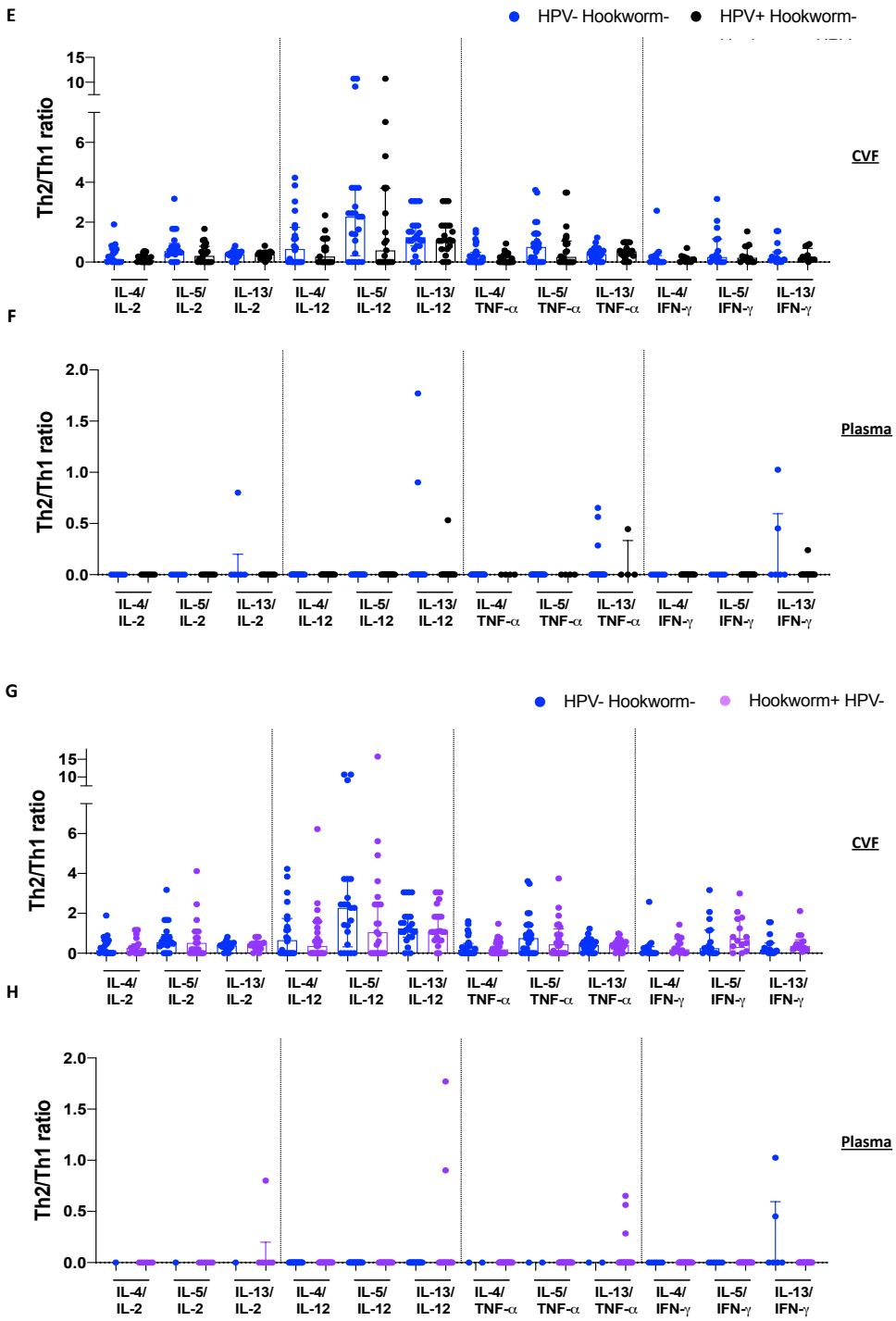


Figure 21.2: Th2/Th1 ratios are elevated in VF of hookworm and HPV co-infected women in comparison to HPV-infected women. Th2/Th1 ratios in vaginal flushes and plasma of HPV+ Hookworm- and HPV- Hookworm- (E and F), HPV- Hookworm+ and HPV- Hookworm- (G and H). Each dot represents a single individual, horizontal bars indicate the median and IQR. HPV- hookworm- (n=54), HPV+ hookworm- (n=24), Hookworm+ HPV- (n=24). Kruskal Wallis with Dunn's multiple comparisons test were used to compare HPV and hookworm negative, single-infected and co-infected groups.

7.9 VF GM-CSF and IgG4/IgE ratio are positively associated with being hookworm+ HPV+

Logistic regression analysis was used to test the associations between analytes measured in the VF and the infection status of the women (i.e., uninfected, infected with only hookworm or HPV, or hookworm and HPV co-infected).

First the association between immune marker levels and being either hookworm+ HPV+ or hookworm- HPV- was investigated in binary logistic regression models. Univariate analysis revealed a positive association between IL-2 (OR 1.004, 95% CI 0.999 - 1.009, p=0.148), IL-5 (OR 1.008, 95% CI 1.001 - 1.015, p=0.020), IL-13 (OR 1.014, 95% CI 0.996 - 1.033, p=0.132), GM-CSF (OR 1.002, 95% CI 1.000 - 1.004, p=0.034) and the IgG4/IgE ratio (OR 1.001, 95% CI 1.000 - 1.001, p=0.005) and being hookworm+ HPV+, and a negative association between eotaxin-2 (OR 0.996, 95% CI 0.992 - 1.001, p=0.148) and being hookworm+ HPV+ (**Table 11**) in comparison to having no hookworm and no HPV infection.

After adjustment for multiple comparisons in a multivariate analysis the IgG4/IgE ratio (aOR 1.000, 95% CI 1.000 - 1.001, p=0.019) was positively associated with being hookworm+ HPV+ in comparison to being hookworm- HPV- (**Table 11**). After correcting for potential confounders such as age, level of education, vaginal cleaning practices, contraception, number of partners and non-monogamous partners, GM-CSF (aOR 1.003, 95% CI 1.000 - 1.005, p=0.035 and the IgG4/IgE ratio (aOR 1.001, 95%CI 1.000 – 1.001, p=0.022) were positively associated with HPV and hookworm co-infection in comparison to being uninfected (**Table 12**).

Table 11: Logistic regression analysis assessing the association between immune parameters measured in the FRT and being either hookworm+ HPV + (n=11) or hookworm- HPV- (n=54).

	Univariate		Multivariate	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
MIP-1α	0.999 (0.990 - 1.009)	0.883		
SDF1α	1.000 (1.000 - 1.000)	0.404		
IL-1β	1.000 (1.000 - 1.000)	0.503		
IL-2	1.004 (0.999 - 1.009)	**0.148**		
IL-4	1.002 (0.994 - 1.010)	0.657		
IL-5	1.008 (1.001 - 1.015)	**0.020**		
IP-10	1.000 (0.998 - 1.001)	0.619		
IL-6	1.000 (0.998 - 1.001)	0.460		
IL-8	1.000 (1.000 - 1.000)	0.728		
Eotaxin	1.005 (0.944 - 1.070)	0.882		
IL-12	1.001 (0.994 - 1.008)	0.797		
IL-13	1.014 (0.996 - 1.033)	**0.132**		
RANTES	0.994 (0.963 - 1.027)	0.720		
IFN-g	0.999 (0.996 - 1.003)	0.793		
GM-CSF	1.002 (1.000 - 1.004)	**0.034**		
TNF-α	1.001 (0.998 - 1.004)	0.521		
MIP-1β	1.000 (0.999 - 1.000)	0.389		
MCP-1	1.000 (0.999 - 1.000)	0.521		
GRO-α	1.000 (1.000 - 1.000)	0.800		
IL-18	1.000 (1.000 - 1.000)	0.712		
IL-10	0.948 (0.829 - 1.083)	0.430		
Eotaxin-2	0.996 (0.992 - 1.001)	**0.148**		
Eotaxin-3	0.969 (0.848 - 1.107)	0.642		
IL-17	0.998 (0.990 - 1.006)	0.602		
IL-21	1.000 (0.997 - 1.002)	0.770		
IL-22	1.000 (0.998 - 1.002)	0.838		
IgG1	1.000 (1.000 - 1.000)	0.459		
IgG2	1.000 (1.000 - 1.000)	0.877		
IgG3	1.000 (1.000 - 1.000)	0.331		
IgG4	1.000 (1.000 - 1.000)	0.810		
IgE	0.999 (0.997 - 1.000)	0.435		
IgA	1.000 (1.000 - 1.000)	0.710		
IgM	1.000 (1.000 - 1.000)	0.744		
IgG4/IgE ratio	1.001 (1.000 - 1.001)	**0.005**	1.000 (1.000 - 1.001)	**0.019**

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

Table 12: Multiple logistic regression analysis testing the association between immune parameters measured in the FRT and being either hookworm+ HPV+ (n=11) or hookworm- HPV- (n=54) after adjusting for sociodemographic factors.

	aOR (95% CI)	p value
GM-CSF	1.003 (1.000 - 1.005)	**0.035**
IgG4/IgE ratio	1.001 (1.000 – 1.001)	**0.022**

p-value threshold is 0.05. Significant values are in bold.

We also tested the association between immune marker levels and being either hookworm+ HPV+ or hookworm- HPV+ and being either hookworm+ HPV+ or hookworm+ HPV-.

Univariate analysis testing the association between immune markers and being hookworm+ HPV+ or hookworm- HPV+ showed that IL-2 (OR 1.018, 95% CI 0.999 - 1.037, p=0.059), IL-4 (OR 1.062, 95% CI 1.009 - 1.119, p=0.022), IL-5 (OR1.017, 95% CI 1.003 - 1.031, p=0.020), IP-10 (OR 1.003, 95% CI 1.000 - 1.007, p=0.081), IL-8 (OR 1.000, 95% CI 1.000 - 1.000, p=0.061), eotaxin (OR 1.627, 95% CI 1.121 - 2.363, p=0.010), IL-12 (OR 1.069, 95% CI 0.997 - 1.147, p=0.062), IL-13 (OR 1.031, 95% CI 0.991 - 1.073, p=0.132), GM-CSF (OR 1.002, 95% CI 0.999 - 1.005, p=0.138) and IgG4 (OR 1.000, 95% CI 1.000 - 1.000, p=0.112) were positively associated with being hookworm+ HPV+ in comparison to being hookworm- HPV+ (**Table 13**).

After adjusting for multiple comparisons in a multivariate analysis, only eotaxin (aOR 1.748, 95% CI1.127 – 2.712, p=0.013) was positively associated with being hookworm+ HPV+ in comparison to being hookworm- HPV+ (**Table 13**). When the data was adjusted for potential confounding sociodemographic factors, no associations were observed between immune parameters and being hookworm+ HPV+ in comparison to being hookworm- HPV+.

Table 13: Logistic regression analysis assessing the association between immune parameters measured in the FRT and being either hookworm+ HPV+ (n=11) or hookworm- HPV+ (n=24).

	Univariate		**Multivariate**	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
MIP-1α	0.998 (0.984 - 1.012)	0.996		
SDF1α	1.000 (1.000 - 1.001)	0.353		
IL-1β	1.000 (1.000 - 1.000)	0.246		
IL-2	1.018 (0.999 - 1.037)	**0.059**		
IL-4	1.062 (1.009 - 1.119)	**0.022**		
IL-5	1.017 (1.003 - 1.031)	**0.020**		
IP-10	1.003 (1.000 - 1.007)	**0.081**		
IL-6	1.000 (0.999 - 1.001)	0.715		
IL-8	1.000 (1.000 - 1.000)	**0.061**		
Eotaxin	1.627 (1.121 - 2.363)	**0.010**	1.748 (1.127 – 2.712)	**0.013**
IL-12	1.069 (0.997 - 1.147)	**0.062**		
IL-13	1.031 (0.991 - 1.073)	**0.132**		
RANTES	1.035 (0.943 - 1.156)	0.456		
IFN-g	1.000 (0.991 - 1.008)	0.907		
GM-CSF	1.002 (0.999 - 1.005)	**0.138**		
TNF-α	1.005 (0.994 - 1.016)	0.354		
MIP-1β	1.000 (0.999 - 1.001)	0.488		
MCP-1	1.001 (0.999 - 1.002)	0.338		
GRO-α	1.000 (1.000 - 1.000)	0.250		
IL-18	1.000 (1.000 - 1.000)	0.892		
IL-10	0.949 (0.759 - 1.187)	0.649		
Eotaxin-2	0.996 (0.990 - 1.002)	0.225		
Eotaxin-3	1.001 (0.797 - 1.255)	0.996		
IL-17	0.999 (0.987 - 1.010)	0.809		
IL-21	1.003 (0.994 - 1.012)	0.563		
IL-22	1.004 (0.996 - 1.011)	0.327		
IgG1	1.000 (1.000 - 1.000)	0.618		
IgG2	1.000 (1.000 - 1.000)	0.732		
IgG3	1.000 (1.000 - 1.000)	0.705		
IgG4	1.000 (1.000 - 1.000)	**0.112**		
IgE	1.000 (1.000 - 1.000)	0.783		
IgA	1.000 (1.000 - 1.000)	0.436		
IgM	1.000 (1.000 - 1.000)	0.729		
IgG4/IgE ratio	1.001 (1.000 - 1.001)	0.336		

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

A univariate analysis to test association between immune markers and being hookworm+ HPV+ or hookworm+ HPV- revealed IL-2 (OR 1.029, 95% CI 1.006 - 1.053, p=0.014), IL-4 (OR 1.059, 95% CI 1.005 - 1.115, p=0.031), IL-5 (OR 1.028, 95% CI 1.006 - 1.050, p=0.012), eotaxin (OR 1.431, 95% CI 1.029 – 1.989, p=0.033), IL-12 (OR 1.046, 95% CI 0.992 - 1.104, p=0.098), IL-13 (OR 1.049, 95% CI 0.995 - 1.106, p=0.076), GM-CSF (OR 1.006, 95% CI 0.999 - 1.013, p=0.102), TNF-α (OR 1.031, 95% CI 0.997 - 1.066, p=0.077), IL-21 (OR 1.011, 95% CI 0.995 - 1.028, p=0.174), IL-22 (OR 1.007, 95% CI 0.998 - 1.016, p=0.139) and IgA (OR 1.000, 95% CI 1.000 - 1.000, p=0.112) to be positively associated with being hookworm+ HPV+ in comparison to being hookworm+ HPV- (**Table 14**).

After adjusting for multiple comparisons in a multivariate regression analysis model, IL-5 (aOR 1.028, 95% CI 1.006 - 1.050, p=0.012) was associated with being hookworm+ HPV+ in comparison to being hookworm+ HPV-. Correction for potential confounding sociodemographic factors resulted in no associations between VF immune mediators and being hookworm+ HPV+ in comparison to being hookworm+ HPV-.

Table 14: Logistic regression analysis assessing the association between immune parameters measured in the FRT and being either hookworm+ HPV+ (n=11) or hookworm+ HPV- (n=24).

	Univariate		Multivariate	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
MIP-1α	0.992 (0.973 - 1.012)	0.431		
SDF1α	1.000 (1.000 - 1.001)	0.534		
IL-1β	1.000 (1.000 - 1.000)	0.397		
IL-2	1.029 (1.006 - 1.053)	**0.014**		
IL-4	1.059 (1.005 - 1.115)	**0.031**		
IL-5	1.028 (1.006 - 1.050)	**0.012**	1.028 (1.006 - 1.050)	**0.012**
IP-10	1.000 (0.998 - 1.001)	0.899		
IL-6	1.000 (0.998 - 1.001)	0.732		
IL-8	1.000 (1.000 - 1.000)	0.407		
Eotaxin	1.431 (1.029 – 1.989)	**0.033**		
IL-12	1.046 (0.992 - 1.104)	**0.098**		
IL-13	1.049 (0.995 - 1.106)	**0.076**		
RANTES	0.994 (0.962 - 1.027)	0.714		
IFN-g	1.004 (0.994 - 1.015)	0.410		
GM-CSF	1.006 (0.999 - 1.013)	**0.102**		
TNF-α	1.031 (0.997 - 1.066)	**0.077**		
MIP-1β	1.000 (0.999 - 1.001)	0.701		
MCP-1	1.000 (0.999 - 1.001)	0.483		
GRO-α	1.000 (1.000 - 1.000)	0.245		
IL-18	1.000 (1.000 - 1.000)	0.315		
IL-10	0.922 (0.777 - 1.093)	0.347		
Eotaxin-2	0.997 (0.991 - 1.004)	0.408		
Eotaxin-3	1.010 (0.895 - 1.141)	0.869		
IL-17	1.003 (0.991 - 1.016)	0.629		
IL-21	1.011 (0.995 - 1.028)	**0.174**		
IL-22	1.007 (0.998 - 1.016)	**0.139**		
IgG1	1.000 (1.000 - 1.000)	0.304		
IgG2	1.000 (1.000 - 1.000)	0.669		
IgG3	1.000 (1.000 - 1.000)	0.770		
IgG4	1.000 (1.000 - 1.000)	0.292		
IgE	0.999 (0.998 - 1.001)	0.297		
IgA	1.000 (1.000 - 1.000)	**0.112**		
IgM	1.000 (1.000 - 1.000)	0.852		
IgG4/IgE ratio	1.001 (1.000 - 1.001)	0.565		

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

7.10 VF MIP-1α and IgG4/IgE ratio are positively associated with being hookworm+ HPV-

Next, hookworm+ HPV- and hookworm- HPV+ groups were each compared to the uninfected group to determine if there were differences in associations with VF immune mediators.

In a univariate analysis, SDF1α (OR 1.000, 95% CI 0.999 - 1.000, p=0.079), IL-8 (OR 1.000 95% CI 1.000 - 1.000, p=0.075), MIP-1β (OR 1.000 95% CI 0.999 - 1.000, p=0.097), IgG1 (OR 1.000, 95% CI 1.000 - 1.000, p=0.121) and the IgG4/IgE ratio (OR 1.000, 95% CI 1.000 - 1.001, p=0.097) were positively associated with being hookworm- HPV+, while IP-10 (OR 0.998, 95%CI 0.996 - 1.000, p=0.073), MCP-1 (OR 0.999, 95% CI 0.999 - 1.000, p=0.164) and eotaxin-2 (OR 0.998, 95% CI 0.997 - 1.000, p=0.134) were negatively associated with being hookworm- HPV+ in comparison to being hookworm- HPV- (**Table 15**). However, no analytes were significantly associated with being hookworm- HPV+ in comparison to being hookworm- HPV- after adjusting for multiple comparisons and controlling for potential confounders.

A univariate analysis testing the association between immune mediators and being hookworm+ HPV- or uninfected revealed that MIP-1α (OR 1.003, 95% CI 0.999 - 1.008, p=0.119), SDF1α (OR 1.000, 95% CI 1.000 - 1.000, p=0.165), IL-10 (OR 1.027, 95% CI 0.994 - 1.061, p=0.108), IgG1 (OR 1.000, 95% CI 1.000 - 1.000, p=0.102), IgA (OR 1.000, 95% CI 1.000 - 1.000, p=0.193) and the IgG4/IgE ratio (OR 1.000, 95% CI 1.000 - 1.001, p=0.013) were positively associated with having only hookworm infection in comparison to having no hookworm and no HPV infection (**Table 16**). Eotaxin-2 (OR 0.999, 95% CI 0.997

- 1.000, p=0.126) and IL-22 (OR 0.994, 95% CI 0.986 - 1.002, p=0.161) levels were negatively associated with being hookworm+ HPV- in comparison to being hookworm- HPV- (**Table 16**).

After adjusting for multiple comparisons in a multivariate analysis, the IgG4/IgE ratio (aOR 1.000, 95% CI 1.000 - 1.001, p=0.013) was positively associated with being hookworm+ HPV- in comparison to being hookworm- HPV- (**Table 16**). After controlling for potential confounders, MIP-1α (aOR 1.006, 95%CI 1.000 - 1.011, p=0.042) and the IgG4/IgE ratio (aOR 1.001, 95% CI 1.000 – 1.001, p=0.014) were positively associated with being hookworm+ HPV- in comparison to being uninfected (**Table 17**).

Table 15: Logistic regression analysis assessing the association between immune parameters measured in the FRT and being either hookworm-, HPV+ (n=24) or hookworm-, HPV- (n=54).

	Univariate		Multivariate	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
MIP-1α	1.000 (0.994 - 1.006)	0.930		
SDF1α	1.000 (0.999 - 1.000)	**0.079**		
IL-1β	1.000 (1.000 - 1.000)	0.516		
IL-2	1.001 (0.996 - 1.006)	0.694		
IL-4	0.997 (0.984 - 1.010)	0.634		
IL-5	1.000 (0.993 - 1.007)	0.985		
IP-10	0.998 (0.996 - 1.000)	**0.073**		
IL-6	1.000 (1.000 - 1.000)	0.904		
IL-8	1.000 (1.000 - 1.000)	**0.075**		
Eotaxin	0.935 (0.812 - 1.076)	0.345		
IL-12	0.999 (0.990 - 1.007)	0.727		
IL-13	1.007 (0.989 - 1.025)	0.459		
RANTES	0.987 (0.955 - 1.019)	0.420		
IFN-g	1.000 (0.997 - 1.002)	0.767		
GM-CSF	1.001 (0.998 - 1.003)	0.611		
TNF-α	1.001 (0.997 - 1.004)	0.752		
MIP-1β	1.000 (0.999 - 1.000)	**0.097**		
MCP-1	0.999 (0.999 - 1.000)	**0.164**		
GRO-α	1.000 (1.000 - 1.000)	0.221		
IL-18	1.000 (1.000 - 1.000)	0.732		
IL-10	0.964 (0.892 - 1.043)	0.366		
Eotaxin-2	0.998 (0.997 - 1.000)	**0.134**		
Eotaxin-3	0.968 (0.878 - 1.066)	0.508		
IL-17	0.998 (0.993 - 1.004)	0.594		
IL-21	0.999 (0.995 - 1.003)	0.590		
IL-22	0.999 (0.995 - 1.003)	0.554		
IgG1	1.000 (1.000 - 1.000)	**0.121**		
IgG2	1.000 (1.000 - 1.000)	0.996		
IgG3	1.000 (1.000 - 1.000)	0.261		
IgG4	1.000 (1.000 - 1.000)	0.400		
IgE	1.000 (0.999 - 1.000)	0.354		
IgA	1.000 (1.000 - 1.000)	0.405		
IgM	1.000 (1.000 - 1.000)	0.531		
IgG4/IgE ratio	1.000 (1.000 - 1.001)	**0.097**		

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

Table 16: Logistic regression analysis assessing the association between immune parameters measured in the FRT and being either hookworm+ HPV- (n=24) or hookworm- HPV- (n=54).

	Univariate		Multivariate	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
MIP-1α	1.003 (0.999 - 1.008)	**0.119**		
SDF1α	1.000 (1.000 - 1.000)	**0.165**		
IL-1β	1.000 (1.000 - 1.000)	0.819		
IL-2	1.000 (0.995 - 1.005)	0.914		
IL-4	0.999 (0.989 - 1.008)	0.780		
IL-5	0.999 (0.991 - 1.007)	0.722		
IP-10	1.000 (0.999 - 1.000)	0.506		
IL-6	1.000 (0.999 - 1.000)	0.463		
IL-8	1.000 (1.000 - 1.000)	0.541		
Eotaxin	0.965 (0.885 - 1.052)	0.415		
IL-12	0.999 (0.991 - 1.007)	0.781		
IL-13	1.005 (0.988 - 1.023)	0.459		
RANTES	1.001 (0.985 - 1.016)	0.922		
IFN-g	0.999 (0.995 - 1.002)	0.536		
GM-CSF	1.000 (0.998 - 1.002)	0.999		
TNF-α	1.000 (0.996 - 1.004)	0.979		
MIP-1β	1.000 (1.000 - 1.000)	0.374		
MCP-1	1.000 (1.000 - 1.000)	0.743		
GRO-α	1.000 (1.000 - 1.000)	0.220		
IL-18	1.000 (1.000 - 1.000)	0.307		
IL-10	1.027 (0.994 - 1.061)	**0.108**		
Eotaxin-2	0.999 (0.997 - 1.000)	**0.126**		
Eotaxin-3	0.960 (0.867 - 1.063)	0.433		
IL-17	0.995 (0.987 - 1.003)	0.237		
IL-21	0.994 (0.984 - 1.005)	0.292		
IL-22	0.994 (0.986 - 1.002)	**0.161**		
IgG1	1.000 (1.000 - 1.000)	**0.102**		
IgG2	1.000 (1.000 - 1.000)	0.376		
IgG3	1.000 (1.000 - 1.000)	0.254		
IgG4	1.000 (1.000 - 1.000)	0.786		
IgE	1.000 (0.999 - 1.000)	0.689		
IgA	1.000 (1.000 - 1.000)	**0.193**		
IgM	1.000 (1.000 - 1.000)	0.629		
IgG4/IgE ratio	1.000 (1.000 - 1.001)	**0.013**	1.000 (1.000 - 1.001)	**0.007**

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

Table 17: Multiple logistic regression analysis testing the association between immune parameters measured in the FRT and being either hookworm+ HPV- (n=24) or hookworm- HPV- (n=54) after adjusting for sociodemographic factors.

	aOR (95% CI)	p value
MIP-1α	1.006 (1.000 - 1.011)	**0.042**
IgG4/IgE ratio	1.001 (1.000 – 1.001)	**0.014**

p-value threshold is 0.05. Significant values are in bold.

7.11 VF MCP-1 and IL-1β are positively associated with being hookworm+ HPV- in comparison to being hookworm- HPV+

Finally, associations between immune parameters and being hookworm+ HPV- or hookworm- HPV+ were assessed.

Univariate analysis was first performed, here, IP-10 (OR 1.002, 95% CI 0.999 - 1.004, p=0.193), IL-8 (OR 1.000, 95% CI 1.000 - 1.000, p=0.155), MIP-1β (OR 1.001, 95% CI 1.000 - 1.001, p=0.126), MCP-1 (OR 1.001, 95% CI 1.000 - 1.002, p=0.092), IgG4 (OR 1.000, 95% CI 1.000 - 1.000, p=0.144) and IgE (OR 1.000, 95% CI 1.000 - 1.000, p=0.157) were found to be positively associated with being hookworm+ HPV-, while IL-22 (OR 0.993, 95% CI 0.983 - 1.003, p=0.181) was found to be negatively associated with being hookworm+ HPV- in comparison to being hookworm- HPV+ (**Table 18**).

Adjustment for multiple comparisons in a multivariate regression analysis showed MCP-1 (aOR 1.002, 95% CI 1.000 - 1.003, p=0.027) to be positively associated with, and IL-22 (aOR 0.968, 95% CI 0.939 – 0.998, p=0.034) to be negatively associated with being hookworm+ HPV- in comparison to being hookworm- HPV+ (**Table 18**). After correcting for potential confounders, MCP-1 (aOR 1.002, 95% CI 1.000 - 1.003, p=0.010) and IL-1β (aOR 1.001, 95% CI

1.000 – 1.002, p=0.037) were positively associated with being hookworm+ HPV- in comparison to being hookworm- HPV+ (**Table 19**).

Table 18: Logistic regression analysis assessing the association between immune parameters measured in the FRT and being either hookworm+ HPV- (n=24) or hookworm- HPV+ (n=24).

	Univariate		**Multivariate**	
	OR (95% CI)	**p value**	**OR (95% CI)**	**p value**
MIP-1α	1.004 (0.997 - 1.011)	0.267		
SDF1α	1.000 (1.000 - 1.001)	0.428		
IL-1β	1.000 (1.000 - 1.000)	0.618		
IL-2	0.990 (0.993 - 1.007)	0.246		
IL-4	1.021 (0.976 - 1.069)	0.365		
IL-5	0.997 (0.982 - 1.011)	0.639		
IP-10	1.002 (0.999 - 1.004)	**0.193**		
IL-6	1.000 (0.999 - 1.000)	0.734		
IL-8	1.000 (1.000 - 1.000)	**0.155**		
Eotaxin	1.215 (0.878 – 1.682)	0.241		
IL-12	1.012 (0.961 - 1.065)	0.655		
IL-13	0.995 (0.956 - 1.036)	0.808		
RANTES	1.013 (0.978 - 1.050)	0.456		
IFN-g	0.998 (0.991 - 1.005)	0.523		
GM-CSF	0.998 (0.994 - 1.003)	0.503		
TNF-α	0.995 (0.981 - 1.008)	0.439		
MIP-1β	1.001 (1.000 - 1.001)	**0.126**		
MCP-1	1.001 (1.000 - 1.002)	**0.092**	1.002 (1.000 - 1.003)	**0.027**
GRO-α	1.000 (1.000 - 1.000)	0.988		
IL-18	1.000 (1.000 - 1.000)	0.466		
IL-10	1.090 (0.964 - 1.187)	0.206		
Eotaxin-2	1.000 (0.797 - 1.003)	0.907		
Eotaxin-3	0.982(0.878 - 1.100)	0.758		
IL-17	0.993 (0.981 - 1.005)	0.258		
IL-21	0.995 (0.985 - 1.006)	0.359		
IL-22	0.993 (0.983 - 1.003)	**0.181**	0.968 (0.939 – 0.998)	**0.034**
IgG1	1.000 (1.000 - 1.000)	0.742		
IgG2	1.000 (1.000 - 1.000)	0.706		
IgG3	1.000 (1.000 - 1.000)	0.985		
IgG4	1.000 (1.000 - 1.000)	**0.144**		
IgE	1.000 (1.000 - 1.000)	**0.157**		
IgA	1.000 (1.000 - 1.000)	0.539		
IgM	1.000 (1.000 - 1.000)	0.405		
IgG4/IgE ratio	1.001 (1.000 - 1.001)	0.597		

For univariate analysis, p-value threshold is 0.2. For multivariate analysis, p-value threshold is 0.05. Significant values are in bold.

Table 19: Multiple logistic regression analysis testing the association between immune parameters measured in the FRT and being either hookworm+ HPV- (n=24) or hookworm- HPV+ (n=24) after adjusting for sociodemographic factors.

	aOR (95% CI)	**p value**
MCP-1	1.002 (1.000 - 1.003)	**0.010**
IL-1β	1.001 (1.000 – 1.002)	**0.037**

p-value threshold is 0.05. Significant values are in bold.

7.12 Discussion

We detected elevated Type 2 (IL-4, IL-5 and IL-13) and Type 1 (TNF-α, IL-2 and IL-12) cytokines and eotaxin in the VF of HPV and hookworm co-infected women in comparison to uninfected women. Among women with only HPV or hookworm infection, IL-13 was the only Type 2 cytokine elevated in the VF, with TNF-a and IL-12 also elevated in both groups in comparison to the uninfected women. IL-2 and GM-CSF were increased in the HPV only group while IL-10 was increased in the hookworm only group. When the women in our study were grouped into either hookworm only, HPV only or HPV and hookworm co-infected categories, we found modest differences in the immune response elicited in the FRT following either hookworm or HPV infection. Importantly, we observe what appears to be a synergistic effect of hookworm and HPV infection that results in a significantly elevated Type 2 immune signature in co-infected women (IL-4, IL-5, IL-13 and eotaxin) that is not apparent in women with only hookworm or only HPV infection, where IL-13 was the only Type 2 cytokine elevated in the VF. Furthermore, the Th2/Th1 cytokine ratio was elevated in the HPV and hookworm co-infected group in comparison to the HPV only group. As elevated Type 2 immunity is associated with a poor HPV prognosis (Bais *et al.*, 2007; Peghini *et al.*, 2012), the raised Type 2 immunity we observe in the FRT of hookworm and HPV co-infected women in our study could have implications for outcome of HPV infection in the long term, as discussed in chapter 6.

In preclinical helminth-virus co-infection studies, Type 2 responses were associated with impaired immunity to viral infection, for example, IL-4 and STAT6 signalling and active diminishing of IFNγ responses induced by *H. polygyrus* and

S. mansoni infection resulted in reactivation of murine gamma herpesvirus from latency (Reese *et al.*, 2014). Furthermore, Type 2 signalling, M2 macrophage differentiation, and subsequent impairment of virus specific CD8+ T cell responses resulted in elevated viral loads in mice co-infected with *T. spiralis* and murine norovirus (Osborne *et al.*, 2014). In the FRT, *N. brasiliensis* induced Type 2 immunity (characterised by IL-5 production and ILC2 and eosinophil accumulation) resulted in inflammation induced damage in the mucosa that worsened the outcome of HSV-2 infection (Chetty *et al.*, 2021). IL-5 and eosinophil depletion rescued co-infected mice from exacerbated HSV-2 pathology (Chetty *et al.*, 2021).Consistent with these studies, we report elevated Type 2 responses and an elevated Th2/Th1 ratio in hookworm and HPV co-infected women in comparison to women with only HPV infection, furthermore, in chapter 3, hookworm infection was shown to be positively associated with HPV intensity (a semi-quantitative measure of HPV viral load). Our findings suggest that being hookworm co-infected and associated Type 2 skewed immunity induced in the FRT can potentially impact the host's ability to control HPV infection.

Type 2 mediated disruption of membrane barrier integrity and microbial translocation in the gut led to collapse of systemic virus specific CD8+ T cell responses to West Nile Virus in mice co-infected with *H. polygyrus bakeri* (Desai *et al.*, 2021). In addition, dysbiosis in the FRT induced IL-33 production and the subsequent ILC-2 and eosinophil accumulation resulted in supressed immunity to HSV-2. Investigating the impact of helminth infections that do not colonise the genital tract on the FRT microbiome is worthwhile to understand whether this potentially contributes to the increased susceptibility to HPV infection at the

unrelated mucosa. Furthermore, permeability assays coupled with blocking of Type 2 signalling need to be performed *in vitro* or in mouse models to determine how the elevated Type 2 signature we observe in the FRT impacts mucosal integrity in the FRT and subsequent risk of HPV, given that HPV infection requires a breach in the FRT mucosa.

Helminth infection on its own alters the cytokine milieu in the unrelated FRT. Whether this is linked to the increased risk of HPV infection that we observe in our study needs to be explored further. A study by Jacobs et al reports contrasting findings on how helminths influence HPV infection (Jacobs *et al.*, 2018). Here, treatment of cervical cancer cell lines with *N. brasiliensis* somatic antigen resulted in impaired HPV pseudovirion uptake due to degradation of cell surface vimentin on HeLa cells following *N. brasiliensis* antigen treatment. Vimentin is a restriction enzyme that is involved in HPV pseudovirion uptake by HeLa cells (Schäfer *et al.*, 2017). This appears to suggest that helminth products may negatively influence HPV infection. Thus, another mechanism, such as the skewing of the host immune response towards a Type 2 phenotype, could be involved in increasing risk of HPV among helminth-infected women that we and others (Gravitt *et al.*, 2016) report.

Finally, when we tested associations between immune molecules and Infection status of the women, hookworm and HPV co-infection was positively associated with VF GM-CSF levels and the IgG4/IgE ratio, while hookworm only infection was associated with VF MIP-1α, MCP-1 and IL-1β levels and the IgG4/IgE ratio. The logistic regression analysis reveals associations between few immune mediators and HPV or hookworm infection status, thus women with only

hookworm, only HPV or hookworm and HPV co-infection cannot be distinguished based on immune marker expression in the VF. Furthermore, associations between the infection status and the immune mediators in the VF could be masked by other factors not controlled for in the analysis such as use of hormonal contraceptives (women in our study reported use of contraceptives but did not specify the type of contraceptives used), stage of the menstrual cycle and other vaginal infections.

In summary, hookworm and HPV co-infected women maintain a prominent Type 2 response in the FRT and a higher Th2/Th1 ratio, that coupled with a higher HPV intensity suggests that these women are at risk for adverse outcomes of HPV infection. Hookworm infection status needs to be factored into HPV control programs to ensure better outcomes for women living in hookworm endemic areas.

Chapter 8: B cell, memory T cell and T follicular helper cell (Tfh) frequencies are elevated in the lymph nodes draining the female reproductive tract of Nippostrongylus brasiliensis infected mice

8.1 Introduction

Th2 cells are important for control of intestinal nematodes as demonstrated in mouse models of infection with *H. polygyrus* and *N. brasiliensis* (Finkelman *et al.*, 1997; Thawer *et al.*, 2014), though they also contribute to tissue damage in the lung following *N. brasiliensis* infection (Mearns *et al.*, 2008). In *N. brasiliensis* infected mice, worm expulsion occurs between 9-14 days after infection (Camberis, Le Gros and Urban, 2003) and IL-13 and signalling via IL-4Ra and STAT-6 are required for resolution of infection (Urban *et al.*, 1998). Th2 cytokines induced following helminth infection promote goblet cell hyperplasia which together with increased epithelial cell turnover and smooth muscle contractility facilitate worm clearance (Ishikawa, Wakelin and Mahida, 1997; Zhao *et al.*, 2003; Cliffe *et al.*, 2005; Horsnell *et al.*, 2007).

B cells also play a role in helminth infection, and are required for clearance of *H. polygyrus*, evidenced by impaired worm expulsion in B cell deficient *H. polygyrus* infected mice (Liu *et al.*, 2010). B cells though not required for clearance of primary *N. brasiliensis* infection (Liu *et al.*, 2010), were demonstrated, through adoptive transfer of antigen experienced B cells into B cell deficient mice, to enhance worm expulsion during secondary infection that was dependent on IL-4ra signalling and IL-13 production (Horsnell *et al.*, 2013).

B cell antibody responses to enteric helminth infection in mouse models are primarily of the IgG1 and IgE isotypes, and in humans IgG4 and IgE (Harris and Gause, 2011; McSorley and Maizels, 2012). In the *N. brasiliensis* model, Th2 derived IL-4 is critical in promoting antibody class switch to IgG1 and IgE in B cells of infected mice (Lebrun and Spiegelberg, 1987; Turqueti-Neves *et al.*, 2015). In addition to Th2 cells, Tfh cells localize to germinal centres within secondary lymphoid organs where they interact with B cells, they also produce IL-4 following infection with *Leishmania* parasites and helminths (King and Mohrs, 2009; Reinhardt, Liang and Locksley, 2009; Zaretsky *et al.*, 2009; Liang *et al.*, 2012). Furthermore, Tfh cell derived IL-4 was demonstrated to promote IgE class switching in draining lymph nodes of *H. polygyrus bakeri* infected mice (Meli *et al.*, 2017). Nb infection can also alter B cell responses to unrelated pathogens, for example vaccine responses against salmonella are impaired in Nb infected mice (Bobat *et al.*, 2014).

Whether adaptive immunity in the unrelated female reproductive tract (FRT) is impacted by helminth infection is not well understood. We wanted to test, using the *N. brasiliensis* model, whether helminth infection induces B and T cell changes in the FRT. We did this by comparing B and T cell populations in lymph nodes that drain the FRT, i.e., the iliac lymph nodes (ILN), of *N. brasiliensis* infected and uninfected mice. We also assessed B cell function by comparing proportions of antibody producing cells between *N. brasiliensis* infected and uninfected mice. As described in previous chapters, understanding the impact of helminth infections that do not colonize the FRT on vaginal immunity provides useful information on how this can alter the response to subsequent FRT infections.

8.2 B cells frequencies are significantly elevated in the ILN of N. brasiliensis infected mice seven days post infection (dpi)

Given that *N. brasiliensis* infection was previously demonstrated to alter immunity in the unrelated FRT, with increased eosinophilia, IL-33, IL-5 and ILC-2 (Chetty *et al.*, 2021), we wanted to expand these findings and investigate how the adaptive immune response in the FRT would be impacted following infection. We first measured B cells frequencies in lymph nodes draining the FRT, iliac lymph nodes (ILN). For comparison, we also measured responses in the lymph nodes draining sites that are colonised by the parasite, the mesenteric lymph nodes (MLN) and the mediastinal lymph nodes (MST), that drain gut and lungs, respectively.

Mice were synchronised with Depo-Provera treatment on day zero to synchronise oestrus cycles, seven days later they were infected sub-cutaneously with *N. brasiliensis* and killed seven days post-infection for immunological analysis (**Figure 22A**).

The proportion of total B cells was significantly higher in the ILN of *N. brasiliensis* infected in comparison to uninfected mice (median 59% vs 38%, p=0.02). We did not find any significant differences in the proportion of plasma cells (**Figure 22C**), plasmablasts (**Figure 22D**) and follicular B (FOB) cells (**Figure 22D**) in the ILN of *N. brasiliensis* infected in comparison to uninfected mice, where there appeared to be a decline in the proportion of plasma cells and follicular B cells, though this was not significant. These findings are contrary to the increased

frequency of total B cells that we observe in the ILN of *N. brasiliensis* infected mice.

When the B cells were further categorised based on expression of IgD and IgM, we observed a trend towards elevated IgD-IgM+ cell frequencies in the ILN of *N. brasiliensis* infected mice in comparison to uninfected mice (median 4.13% vs 3.07%, p=0.06; **Figure 22F**). Furthermore, B cells categorised based on CD21 and CD23 expression showed a trend towards elevated CD21+ CD23- cell frequencies in *N. brasiliensis* infected in comparison to uninfected mice (median 5.84% vs 4.46%, p=0.73; **Figure 22G**) though the increase was not significant. These findings show that the increased B cell proportions in the ILN of *N. brasiliensis* infected mice was in part due to expansion of IgD-IgM+ and CD21+ CD23- B cells. Other B cell subsets that we did not phenotype in our panel may also be contributing.

Total B cell and plasma cell proportions were not significantly increased in the MLN and MST of *N. brasiliensis* infected mice in comparison to uninfected mice (**Figure 22B** and **Figure 22C**). Plasmablast proportions were however higher in the MLN of *N. brasiliensis* infected mice in comparison to uninfected mice (median 12.3% vs 4.78%, p=0.02; **Figure 22D**), and follicular B cell proportions were decreased in the MLN and MST of *N. brasiliensis* infected mice (FOB cells MLN: median 82.5% vs 92.2%, p=0.02; FOB cells MST: median 82.3% vs 94.3%, p=0.02; **Figure 22E**).

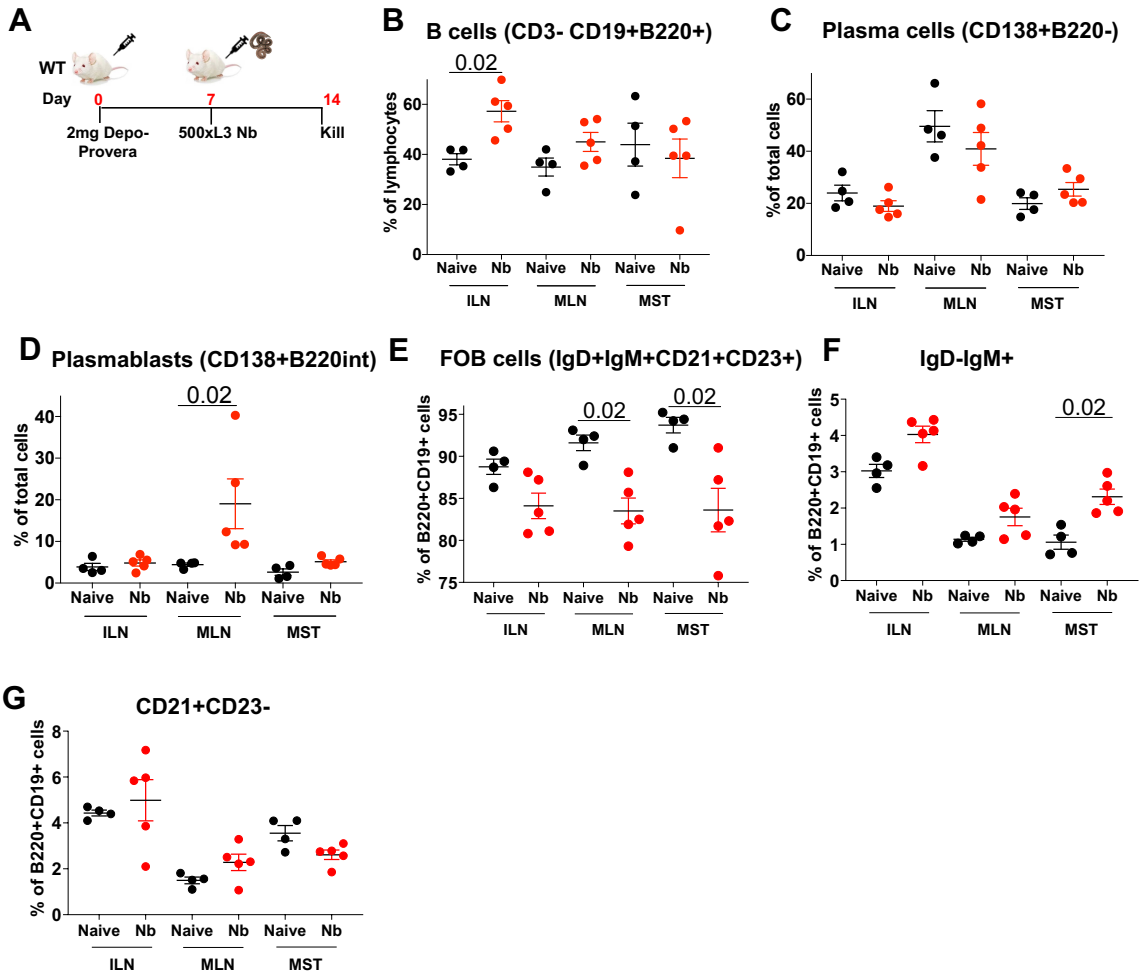


Figure 22: B cell frequencies are elevated in the ILN of *N. brasiliensis* infected mice. Depo-Provera treatment and Nb infection timeline (A). B cell (B), plasma cell (C), plasmablast (D), Follicular B (FOB; E), IgD-IgM+ (F) and CD21+CD23- (G) cells in the iliac (ILN), mesenteric (MLN) and mediastinal (MST) lymph nodes (LN) of naïve and *N brasiliensis* (Nb) infected mice. Data are representative of two experiments naïve n=4, Nb n=5. Mann Whitney U test was used to compare naïve and Nb infected groups.

8.3 IgG1+ B cell frequencies are significantly elevated in the ILN of N. brasiliensis infected mice seven dpi

We next assessed B cell class switching by measuring intracellular IgG1 and IgG2a expression in B cells within lymph nodes of *N. brasiliensis* infected and compared this to uninfected mice. IgG1 antibodies are associated with Type 2 immunity such as that induced during helminth infection, while IgG2a antibodies are associated with Type 1 immunity (Snapper and Paul, 1987; Finkelman, Holmes and Paul, 1990). Representative flow cytometry plots showing IgG1 and IgG2a in total B cells and follicular B cells are shown in **Figure 23A.**

Total IgG1+ B cell frequencies were increased in the ILN of *N. brasiliensis* infected mice (median 2.04% vs 0.73%, p=0.03; **Figure 23B**), while total IgG2a+ B cell proportions did not differ between *N. brasiliensis* infected and uninfected mice **(Figure 23C**).

IgG1+ and IgG2a+ plasma cell and plasmablast proportions in the ILN, did not differ between *N brasiliensis* infected and uninfected mice (**Figure 23D-G**). On the other hand, IgG1+ and IgG2a+ FOB cell proportions were increased in the ILN of infected mice in comparison to uninfected mice (IgG1 median 2.87% vs 0.73%, p=0.02; **Figure 23H** and IgG2 median 0.27% vs 0.73%, p=0.09, p=0.02; **Figure 23I**).

Total IgG1+ B cell frequencies were elevated in the MLN and MST of *N. brasiliensis* infected mice (IgG1+ B cells MLN: median 5.56% vs 1.22%, p=0.03; IgG1+ B cells MST: median 13.40% vs 0.41%, p=0.02; **Figure 23B**). Total IgG2a+ B cell frequenies in the MST did not differ between *N. brasiliensis*

infected and uninfected mice (**Figure 23C**), though these cells expanded in the MLN of *N. brasiliensis* infected mice (median 4.69% vs 0.45%, p=0.03; **Figure 23C**).

IgG1+ and IgG2a+ plasma cell proportions in the MLN and MST did not differ between *N. brasiliensis* infected and uninfected mice (**Figure 23D and Figure 23E**), IgG1+ plasma cell proportions were increased in the MST (median 80.1% vs 4.98%, p=0.02; **Figure 23D**) but not MLN of *N. brasiliensis* infected mice. IgG1+ and IgG2a+ FOB cell frequencies were increased in the MST but not MLN of infected mice (IgG1+ FOB cells MST: median 27.5% vs 1.48%, p=0.02; **Figure 23H** and IgG2a+ FOB cells MST: median 1.19% vs 0.15%, p=0.02; **Figure 23I**).

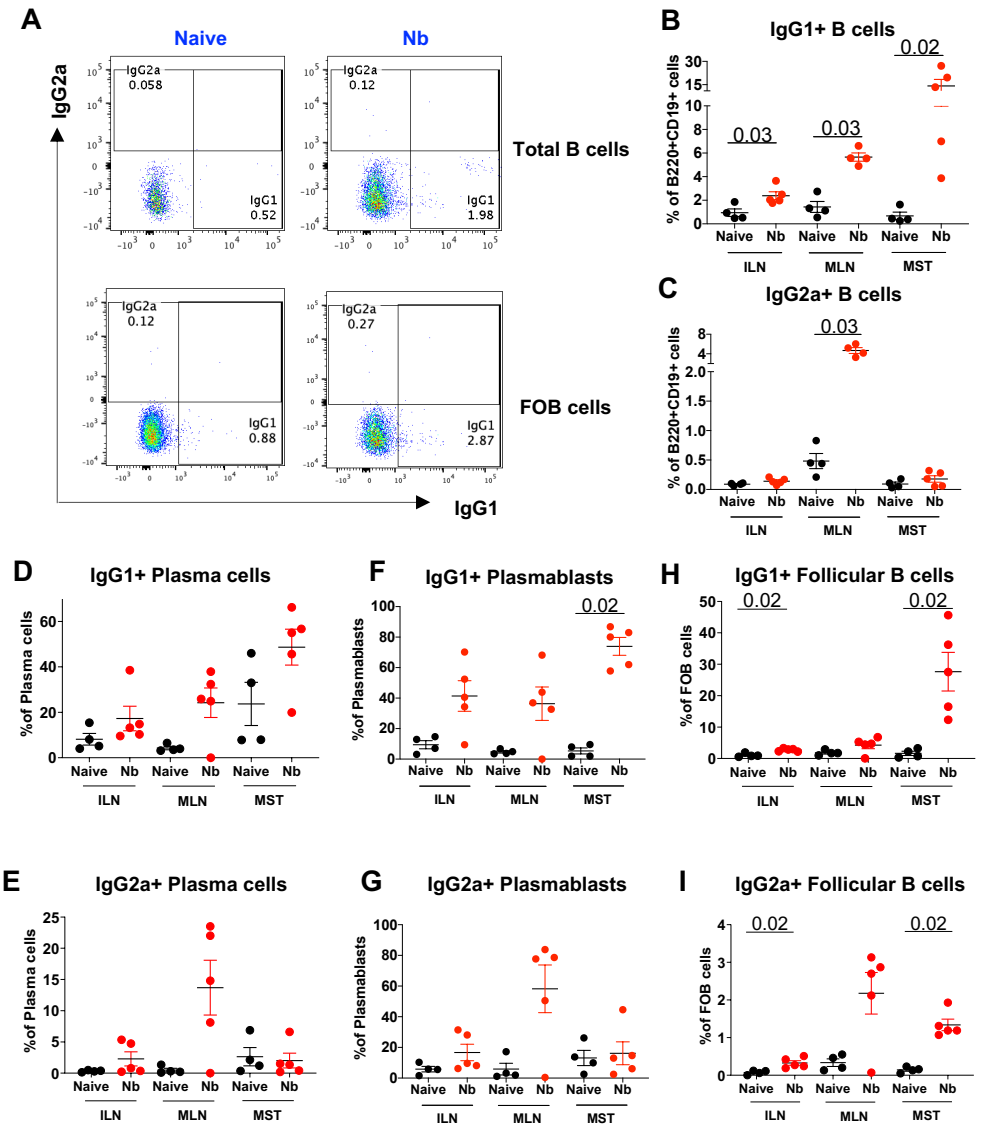


Figure 23: IgG1 + B cells and FOB cell frequencies are elevated in the ILN of *N. brasiliensis* infected mice. Representative flow cytometry plots showing IgG1 and IgG2a+ B cells and Follicular B cells in the ILN of naïve and *N. brasiliensis* infected mice (A). Graphs showing IgG1+ (B) and IgG2+ (C) B cells, IgG1+ (D) and IgG2+ (E) plasma cells, IgG1+ (F) and IgG2+ (G) plasmablasts and IgG1+ (H) and IgG2+ (I) FOB cells, in the iliac (ILN), mesenteric (MLN) and mediastinal (MST) lymph nodes (LN) of naïve and *N brasiliensis* (Nb) infected mice. Data are representative of two experiments naïve n=4, Nb n=5. Mann Whitney U test was used to compare naïve and Nb infected groups.

8.4 Effector CD4+ T cell and Tfh cell frequencies are significantly elevated in the ILN of N. brasiliensis infected mice seven dpi

We then measured T cells in the ILN. There were no significant differences in total CD4+ T cell frequencies between *N. brasiliensis* infected and uninfected mice, there however appeared to be a trend towards lower CD4+ T cell frequency in infected mice though this was not significant **(Figure 24A**).

We also analysed Tfh and memory CD4 T cells, representative flow cytometry plots showing Tfh (CXCR5+PD1+), effector memory (CD44+CD62L-) and naïve (CD62L+CD44-) CD4 T cells in naïve and *N. brasiliensis* infected mice are presented in **Figure 24B**.

Tfh cell (CXCR5+PD1+) frequencies were increased in the ILN of *N. brasiliensis* infected mice (median 22.3% vs 8.48%, p=0.02; **Figure 24C**). Tfh cells are T cells essential for germinal center maintenance and provide B cell help result affinity maturation of the B cells they interact with (Crotty, 2014). We also observed increased effector T cell (CD62L-CD44+; **Figure 24D**) and decreased naïve T cell proportions (CD62L+CD44-; **Figure 24E**) in the ILN of *N. brasiliensis* infected mice in comparison to uninfected mice.

Similar to what we observe in the ILN, no significant change in total T cell proportions were detected in the MLN and MST of *N. brasiliensis* infected and uninfected mice (**Figure 24A**). However, we detect an expansion of Tfh (CXCR5+PD1+; median 24.1% vs 6.27%, p=0.02; **Figure 24D**) and effector memory (CD62L-CD44+; median 26.1% vs 8.87%, p=0.02; **Figure 24D**) T cell proportions and a reduction in the naïve cell proportions (CD62L+CD44-; median

13.9% vs 49.1%, p=0.02; **Figure 24E**) in the MST of *N brasiliensis* infected mice. There was a trend towards increase in Tfh cell and effector memory CD4 T cell proportions and a decrease in naïve T cell proportions in the MLN of infected mice, however, this was not statistically significant.

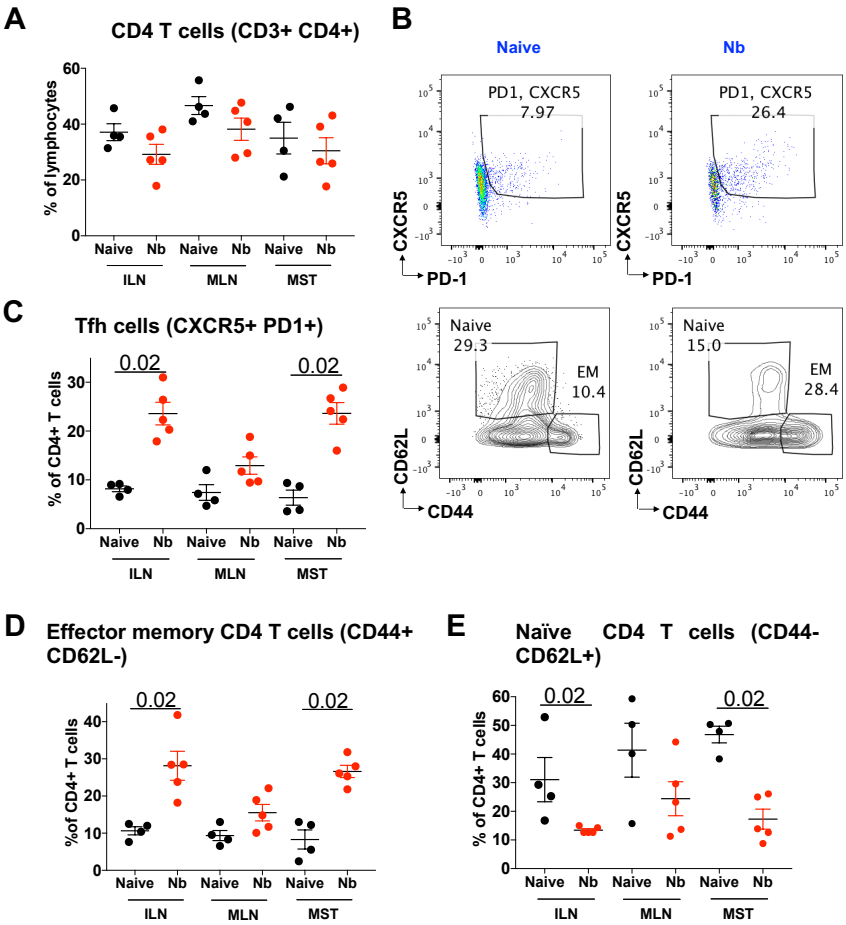


Figure 24: T follicular helper (Tfh) and effector memory T cell frequencies are elevated in the ILN of *N. brasiliensis* infected mice. Representative flow cytometry plots showing Tfh cells and effector memory and naive cells in the ILN of naïve and *N. brasiliensis* infected mice (A). Graphs showing Total CD4+ T cells (B) Tfh cells (C) effector memory T cells (D) and naïve T cells (E) in the iliac (ILN), mesenteric (MLN) and mediastinal (MST) lymph nodes (LN) of naïve and *N brasiliensis* (Nb) infected mice. Data are representative of two experiments naïve n=4, Nb n=5. Mann Whitney U test was used to compare naïve and Nb infected groups.

8.5 Discussion

We observed elevated B cell proportions in the iliac lymph nodes (ILN) draining the FRT of WT Balb/c mice. Plasma cells, follicular B cells or plasmablasts, however were not altered in the ILN of *N. brasiliensis* infected in comparison to uninfected mice. We did however observe a trend towards increased IgD-IgM+ and CD21+CD23- B cell frequencies, these cells display a marginal zone like phenotype. Marginal zone-like cells in lymph nodes have previously been described as being $B220^{+}$ $CD1d^{hi}CD23^{lo}$ and IgM^{hi}, IgD^{lo} (Palm, Friedrich and Kleinau, 2016) and were demonstrated to have a higher proportion of memory cells than follicular B cells. Therefore, our findings indicate that a portion of the B cells that are increased in the ILN of N brasiliensis infected mice could be antigen experienced B cells that have encountered *N. brasiliensis* antigen in circulation, however, this requires confirmation by staining B cells for memory markers such as PDL-2, CD73, CD80, used to define memory B cell subsets (Tomayko *et al.*, 2008; Zuccarino-Catania *et al.*, 2014).

We observed an increase in total IgG1 antibody producing B cell proportions in the ILN following *N. brasiliensis* infection, with IgG1+ cells in the FOB cell subset being significantly increased in infected mice. We also observed an increase in Tfh cell frequencies in the ILN of *N. brasiliensis* infected in comparison to uninfected mice. Following helminth infection, IL-4 influences B cell antibody class switch to IgE and IgG1 (Turqueti-Neves *et al.*, 2015), classical Type 2 associated antibodies. Furthermore, Tfh cells were reported to be the main source of IL-4 that induces B cell class switching to IgG1 in germinal centers of the draining lymph nodes of helminth infected mice (King and Mohrs, 2009). Our

data therefore suggests that there is Type 2 associated B cell polarisation occurring in the germinal centers in the ILN following *N. brasiliensis* infection, driven by Tfh cells.

In humans, B cells in germinal centers were shown to constitutively express IL-4 (Johansson-Lindbom and Borrebaeck, 2002). In addition, in mice B cell help was shown to be important in enhancing Th2 responses in *N. brasiliensis* infection through IL-13 production and TLR mediated interactions with T cells (Horsnell *et al.*, 2013). Thus, B cells could also be contributing to Th2 polarization within germinal centers in lymph nodes draining the FRT. Further work is required in understanding the extent to which the T cell response in FRT and its draining lymph nodes is impacted by helminth induced B cells, for example by measuring cytokine production from T cells in B cell deficient mice.

While we don't find a significant change in total T cell proportions in the ILN of *N. brasiliensis* infected in comparison to uninfected mice, we detect differences in the functional T cells detected in infected mice as evidenced by increased proportions of effector memory and Tfh CD4+ T cells and a concomitant decline in naïve CD4+ T cell proportions. Previously, expansion of Th2 cells induced following *N. brasiliensis* infection in the lung was detected using IL-4/eGFP reporter mice, these cells displayed an activated phenotype, being CD62L low and CD44 high (Seidl, Panzer and Voehringer, 2011). In addition, *N. brasiliensis* was shown to induce IL-4 producing Tfh in mediastinal lymph nodes following infection (Liang *et al.*, 2012). Our findings therefore suggest that antigen-experienced T cells, that are potentially of Th2 or Tfh lineage, can be detected in increased proportions in the FRT draining lymph nodes of *N. brasiliensis* infected

mice. Restimulation of lymph node cells with *N. brasiliensis* antigen and measurement of cytokines would provide more information on the antigen specificity and lineage of these effector memory CD4 T cells.

We also observed increased IgG1+ B cell, IgG1+ FOB cell, Tfh cell and effector memory T cell proportions in the MST of *N. brasiliensis* infected mice. These findings are consistent with previous reports that show the lung as an important site for both primary (Harvie *et al.*, 2010) and secondary (Thawer *et al.*, 2014) responses to *N. brasiliensis* infection. We do not find significant differences in proportions of Tfh cells, effector memory CD4 T cells and naïve CD4 T cells in the MLN of naïve and *N. brasiliensis* infected mice, though the gut is a site of infection for the parasite. There are however trends towards increased Tfh cell and effector memory CD4 T cell proportions and decreased naïve cell proportions in infected mice.

In conclusion, our data showing increased B cell, Tfh cell and effector memory CD4 T cell proportions in the ILN of *N. brasiliensis* infected mice demonstrate that helminth infection skews the immune response in the FRT. Furthermore, the increased IgG1+ B cells and FOB cell proportions in the ILN of *N. brasiliensis* infected mice that we report indicates that the response is Type 2 associated, though further experiments (i.e., cytokine analyses) are required to determine if the increased effector T cells we observed are Th2 cells. Results presented in this chapter are consistent with previous findings showing increased Type 2 immunity in the FRT and draining lymph nodes of *N. brasiliensis* infected mice (Chetty *et al.*, 2021), where the consequence of helminth induced Type 2 signalling was impaired control of subsequent FRT infection with HSV-2. The

mechanisms through which helminths modulate immunity at distal sites they do not colonise have not been elucidated, however it is hypothesised that helminths excretory secretory products transported in circulation from sites of infection may be contributing to the widespread immune modulation (Zarek and Reese, 2021; Vacca and Le Gros, 2022).

Chapter 9: Nippostrongylus brasiliensis infection prior to HSV-2 vaccination results in decreased effector memory CD4 T cell frequencies in lymph nodes draining the female reproductive tract and increased genital pathology following HSV-2 challenge

9.1 Introduction

HSV-2 infection occurs in the mucosa of the anogenital tract (Whitley, 2012). Early in infection HSV-2 virions migrate via axons to dorsal root ganglia of the nervous system where they establish latent infection (Cunningham *et al.*, 2006). The human immune system can contain HSV-2 infection, however, reactivation from spinal neurons results in chronic persistent infection, that increases the risk of pathology in infected individuals (Jaishankar and Shukla, 2016).

Mouse models have provided useful insight into primary as well vaccine mediated immune responses to HSV-2 (Parr and Parr, 2003). The key role played by CD4+ and CD8+ T cells in protection against HSV-2 was demonstrated by increased viral titres and disease severity in HSV-2 vaccinated, T cell depleted mice following challenge with lethal dose virus (Parr and Parr, 1998). In wild type CD4 competent mice, HSV-2 vaccination and subsequent viral challenge resulted in expansion of memory CD4+ T cells in the FRT (King, Parr and Parr, 1998; Morrison, 2008; Odegard *et al.*, 2016). CD4+ T cell mediated protection was IFN-γ dependent, as mice depleted of IFN-γ or IFN-γ producing CD4+ T cells displayed increased viral shedding (Parr and Parr, 1999) and impaired viral clearance from the FRT (Milligan and Bernstein, 1997) following HSV-2

challenge. Furthermore, In a primary infection model, CD4+ T cells were shown to be required for recruitment and priming of HSV-2 specific CD8+ T cells in the FRT (Nakanishi *et al.*, 2009; Kumamoto *et al.*, 2011).

During primary HSV-2 infection, viral clearance from the female genital tract was dependent on IFN-γ production and cytolytic activity of CD8 T cells (Dobbs *et al.*, 2005; Nelson *et al.*, 2011). Furthermore, following vaccination, topical chemokine application in the FRT resulted in recruitment of memory CD8+ T cells that protected mice (via IFN-γ production) from severe disease in comparison to mice that did not receive chemokine treatment (Shin *et al.*, 2016).

Interestingly, T cell-deficient immune mice still displayed some level of protection against HSV-2 such as resistance to epithelial infection (albeit lower than in wild type mice) and protection from severe neurological illness (Parr and Parr, 1998). This suggests that other immune mechanisms such as B cell and antibody responses were also required in protective immunity against HSV-2.

The role of B cells in immunity to HSV-2 was demonstrated in B cell-deficient (μMT and Igh-6tm 1Cgn) mice that displayed higher vaginal viral titres than wild type mice following HSV-2 challenge (Dudley, Bourne and Milligan, 2000; Parr and Parr, 2000). Moreover, vaccinated J_HD mice (B cell deficient) had increased neuronal viral titres in comparison to wild type mice following HSV-2 challenge (Iijima and Iwasaki, 2016). Passive administration of immune serum to vaccinated B cell-deficient mice significantly lowered viral titres (Dudley, Bourne and Milligan, 2000; Morrison, Zhu and Thebeau, 2001) and resulted in virus resolution kinetics similar to that in mice with intact B cells (Dudley, Bourne and Milligan, 2000). Consistent with these findings, passive administration of IgG

from immunized mice resulted in lower viral titres and reduced disease severity in mice challenged with HSV-2, while IgG isolated from immunized mice showed *in-vitro* neutralization activity against HSV-2 isolates (Parr and Parr, 1998). Passive transfer of antibodies (McDermott, Brais and Evelegh, 1990) or immune serum (Morrison, Zhu and Thebeau, 2001), however, failed to protect mice against HSV-2 infection in the vaginal epithelium.

Antibody responses elicited by HSV-2 are primarily IgG and IgA responses (McDermott, Brais and Evelegh, 1990; Milligan and Bernstein, 1995b). In both serum and cervico-vaginal fluid, IgG responses were the first to develop and of the highest magnitude in response to vaccination with a thymidine kinase deficient mutant HSV-2 strain (Milligan and Bernstein, 1995a). The IgA response appeared a few days after the IgG response and was of lower magnitude (Milligan and Bernstein, 1995b). Following HSV-2 challenge, IgG and IgA responses increased in magnitude in both serum and vaginal lavage of vaccinated mice (Milligan and Bernstein, 1995b; Oh *et al.*, 2019). IgG+ B cells also expanded rapidly in the vagina of HSV-2 vaccinated mice post viral challenge (Oh *et al.*, 2019).

With respect to IgG isotypes, IgG2a and IgG1 were the main IgG isotypes elicited in serum following HSV-2 vaccination (Gyotoku, Ono and Aurelian, 2002; Awasthi *et al.*, 2019), while IgG2b, IgG2c, IgG1 and IgA were the predominant isotypes detected in the vaginal lumen of vaccinated mice post viral challenge (Oh *et al.*, 2019).

Taken together, these studies demonstrate that both T and B cells are required in immunity to HSV-2 in mouse models, with T cell derived IFN-γ and IgG and

IgA from B cells paying a critical role in protection. Currently, no information exists about the effect of underlying helminth infection on primary and vaccine-mediated immunity to HSV-2. Therefore, in this study, we will assess how B and T cell immunity elicited in a mouse model of HSV-2 vaccination and challenge is influenced by underlying *N. brasiliensis* infection and whether this also impacts pathology resulting from HSV-2 infection in the genital tract.

9.2 B cell populations in ILN of HSV-2 vaccinated mice with prior N. brasiliensis infection are not significantly altered following subsequent HSV-2 challenge

Having detected changes in B cell subsets in the ILN induced by primary *N. brasiliensis* infection in Chapter 7, we wanted to assess the potential impact of helminth infection on vaccine mediated B cell responses to a viral infection of the FRT, HSV-2. The timeline of vaccination and infection is summarised in **Figure 25A**. Mice oestrus cycles were first synchronized with Depo-Provera on day zero, seven days later mice were infected with 500 *N. brasiliensis* L3 larvae. Seven days after *N. brasiliensis* infection mice were vaccinated with $1x10^6$ PFU of formalin inactivated (FI) HSV-2 strain G, three weeks after the first vaccine, mice were given a booster dose of $1x10^6$ PFU FI HSV-2. Two weeks after the booster, mice were challenged intravaginally with $5x10^5$ PFU of HSV-2 strain G.

The test groups included in this study were: *N. brasiliensis* infected, HSV-2 vaccinated; HSV-2 vaccinated with no prior *N. brasiliensis* infection; naïve, unvaccinated; *N brasiliensis* infected and naïve mice, hereafter referred to as NbHSV-2 (v), HSV-2 (v), HSV-2 only, Nb and naïve, respectively in this chapter. NbHSV-2 (v), HSV-2 (v) and HSV-2 only mice were all challenged with HSV-2 while Nb and naïve mice were neither vaccinated nor challenged with HSV-2.

Following HSV-2 challenge, we found no significant differences in the proportion of ILN B cells (**Figure 25B**), plasma cells (**Figure 25C**), plasmablasts (**Figure 25D**), follicular B (FOB) cells (**Figure 25E**), IgD-IgM+ (**Figure 25F**), and CD21+CD23- (**Figure 25G**) cells between NbHSV-2 (v) and HSV-2 (v) mice.

There however appeared to be a trend towards decreased proportions of these B cell subsets (with the exception of FOB cells) in the ILN of NbHSV-2 (v) in comparison to HSV-2 (v) mice, though this was not statistically significant.

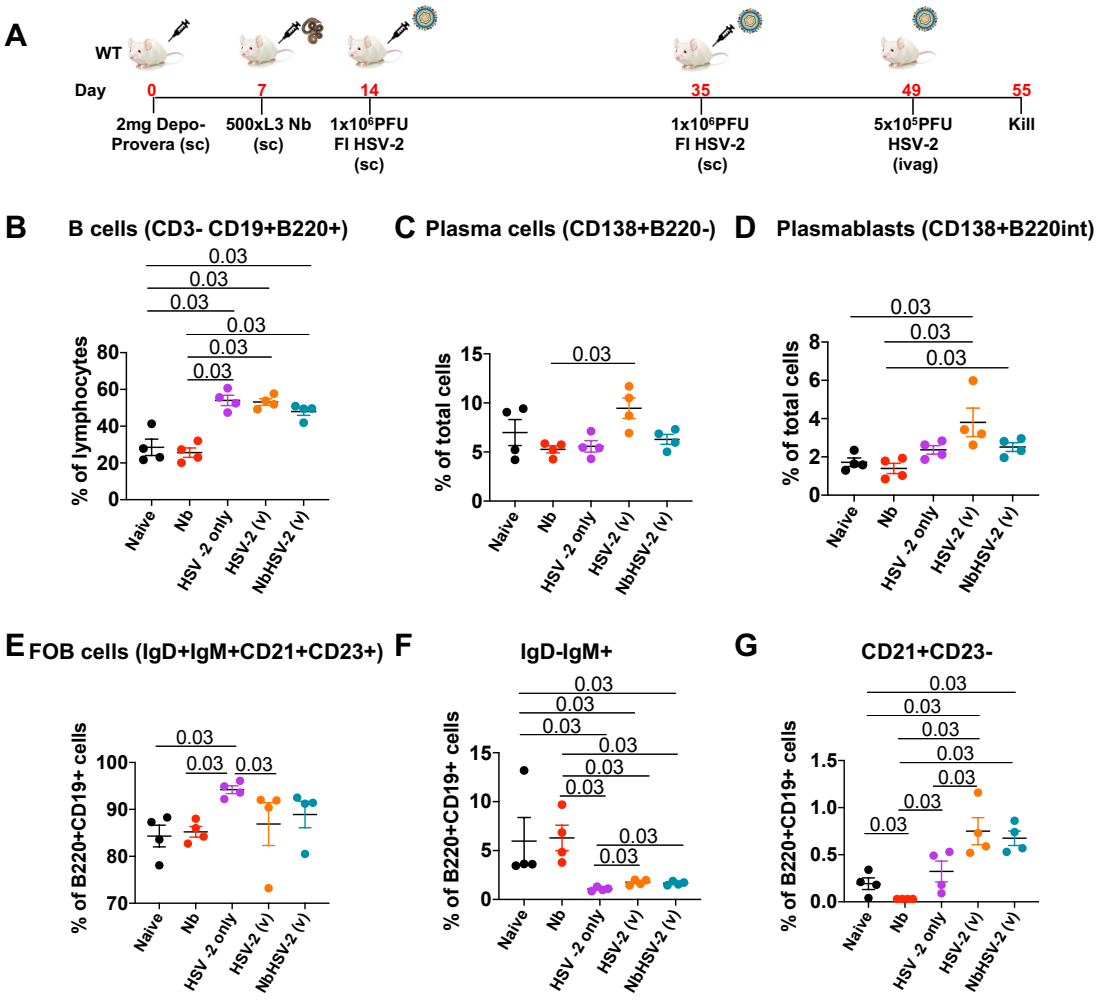


Figure 25: B cell responses in mice previously infected with *N. brasiliensis* (Nb) and vaccinated with HSV-2 are not significantly diminished in comparison to Nb uninfected HSV-2 vaccinated mice following HSV-2 challenge. Depo-Provera treatment, Nb infection, HSV-2 vaccination and HSV-2 challenge timeline (A). Graphs showing B cell (B), plasma cell (C), plasmablast (D), Follicular B (FOB; E), IgD-IgM+ (F) and CD21+CD23- (G) cell in the iliac (ILN) of naïve, *N brasiliensis* infected (Nb), HSV-2 infected (HSV-2 only), HSV-2 vaccinated and infected (HSV-2 (v)) and *N brasiliensis* infected, HSV-2 vaccinated and infected (NbHSV-2 (v)) mice. Data are representative of two experiments, n=4 for all groups. Mann Whitney U test was used to compare groups.

B cell frequencies were significantly increased in the ILN of HSV-2 only, HSV-2 (v) and NbHSV-2 (v) mice when compared to naïve mice (HSV-2 only vs naïve: median 54.05% vs 24.45%, p=0.03; HSV-2 (v) vs naïve: median 52.85% vs 25.45%, p=0.03; NbHSV-2 (v) vs naïve: median 49.55% vs 24.45%, p=0.03; **Figure 25B**).

Plasma cell proportions did not differ significantly between naïve mice and HSV-2 only, HSV-2 (v) and NbHSV-2 (v) mice (**Figure 25C**). Plasmablast proportions, on the other hand, were increased in the ILN of HSV-2 (v) but not NbHSV-2 (v) mice in comparison to naïve mice after HSV-2 challenge (HSV-2 (v) vs naïve: median 3.31% vs 1.61, p=0.03; **Figure 25D**).

Follicular B cell proportions were increased in the ILN of HSV-2 only mice in comparison to naïve mice (HSV-2 only vs naïve: median 94.25% vs 85.45%, p=0.03; **Figure 25E**), but not in the ILN of HSV-2 (v) and NbHSV-2 (v) mice in comparison to naïve mice (**Figure 25E**).

IgD-IgM+ B cell frequencies were decreased in the ILN of HSV-2 only mice as well as HSV-2 (v) and NbHSV-2 (v) mice in comparison to naïve mice (HSV-2 only vs naïve: median 1.05% vs 3.62%, p=0.03; HSV-2 (v) vs naïve: median 1.79% vs 3.62%, p=0.03; NbHSV-2 (v) vs naïve: median 1.64% vs 3.62%, p=0.03; **Figure 25F**). The frequencies of these cells were however increased in the ILN of HSV-2 (v) and NbHSV-2 (v) mice in comparison to HSV-2 only mice (HSV-2 (v) vs HSV-2 only: median 1.79% vs 1.05%, p=0.03; NbHSV-2 (v) vs HSV-2 only: median 1.64% vs 1.05%, p=0.03; **Figure 25F**).

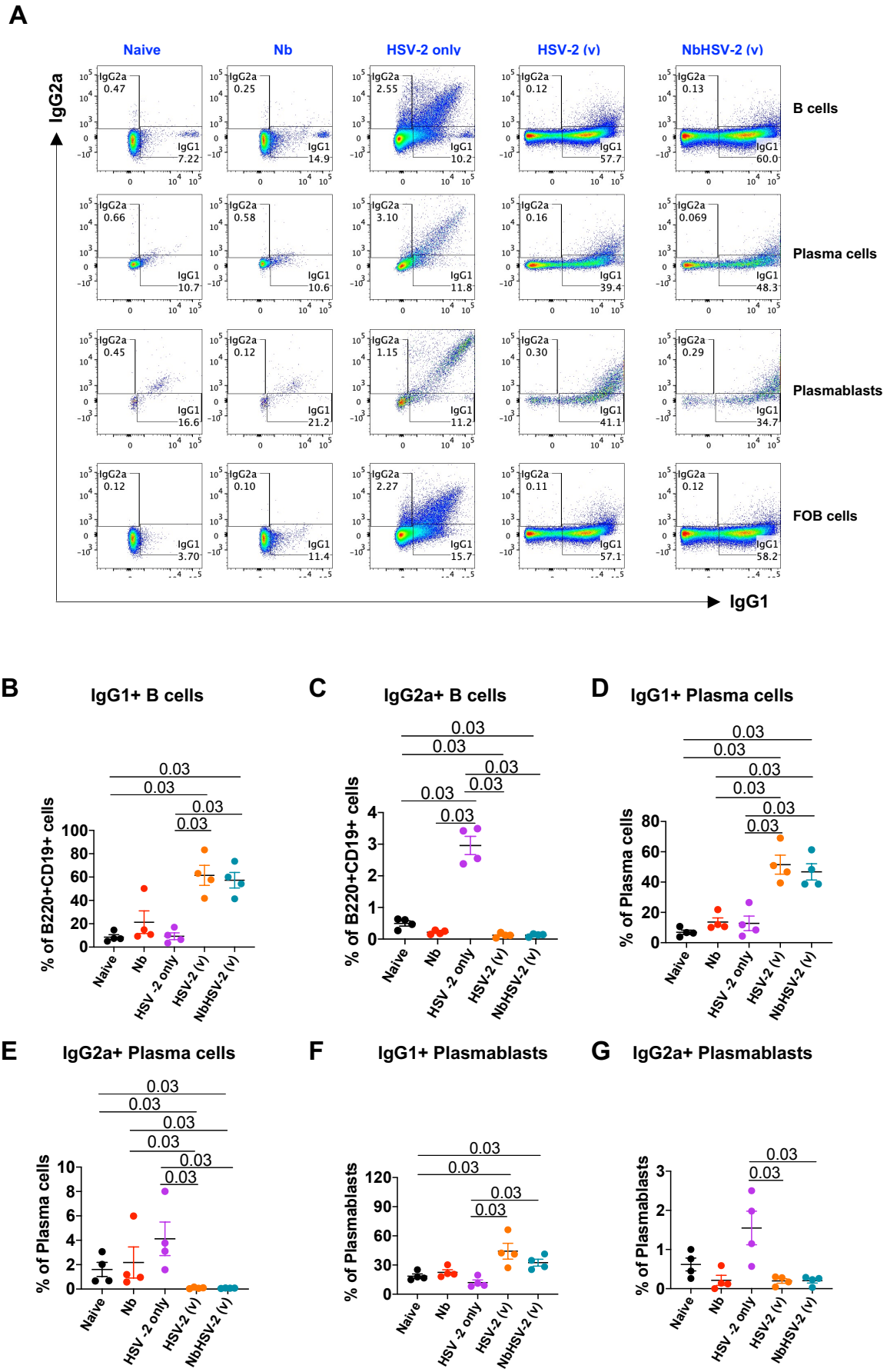
CD21+CD23- B cell proportions were increased in the ILN of both HSV-2 (v) and NbHSV-2 (v) mice in comparison to naïve mice (HSV-2 (v) vs naïve: median

0.66% vs 0.20%, p=0.03; NbHSV-2 (v) vs naïve: median 0.66% vs 0.20%, p=0.03; **Figure 25G**), and in HSV-2 (v) in comparison to HSV-2 only mice (HSV-2 (v) vs HSV-2 only: median 0.66% vs 0.34%, p=0.03; **Figure 25G**).

B cell responses were not significantly different between Nb and naïve mice (**Figure 25B-F**), except for CD21+CD23- B cell frequencies which were decreased in the ILN of Nb in comparison to naïve mice (Nb vs naive: median 0.03% vs 0.20%, p=0.03; **Figure 25G**).

9.3 Antibody producing B cells in ILN are not significantly altered by underlying N. brasiliensis infection following HSV-2 vaccination and subsequent viral challenge

Next, IgG1 and IgG2a producing B cells in the ILN were quantified to determine if B cell function was altered by *N. brasiliensis* infection prior to HSV-2 vaccination in mice that were later challenged with HSV-2. Representative flow cytometry plots showing IgG1 and IgG2a expression within the different B cell subsets phenotyped are shown in **Figure 26A**. We did not observe any significant differences in the proportions of IgG1+ and IgG2a+ cells within the total B cell (**Figure 26B and Figure 26C**), plasma cell (**Figure 26D and Figure 26E**), plasmablast (**Figure 26F and Figure 26G**) and follicular B cell (**Figure 26H and Figure 26I**) compartments between NbHSV-2 (v) mice and HSV-2 (v) mice, though there was a trend towards fewer antibody producing B cells in NbHSV-2 (v) mice.


A
Naive
Nb
HSV-2 only
HSV-2 (v)
NbHSV-2 (v)
IgG2a
IgG1
B cells
Plasma cells
Plasmablasts
FOB cells
B
IgG1+ B cells
% of B220+CD19+ cells
C
IgG2a+ B cells
% of B220+CD19+ cells
D
IgG1+ Plasma cells
% of Plasma cells
E
IgG2a+ Plasma cells
% of Plasma cells
F
IgG1+ Plasmablasts
% of Plasmablasts
G
IgG2a+ Plasmablasts
% of Plasmablasts
0.03
Naive
Nb
HSV -2 only
HSV-2 (v)
NbHSV-2 (v)

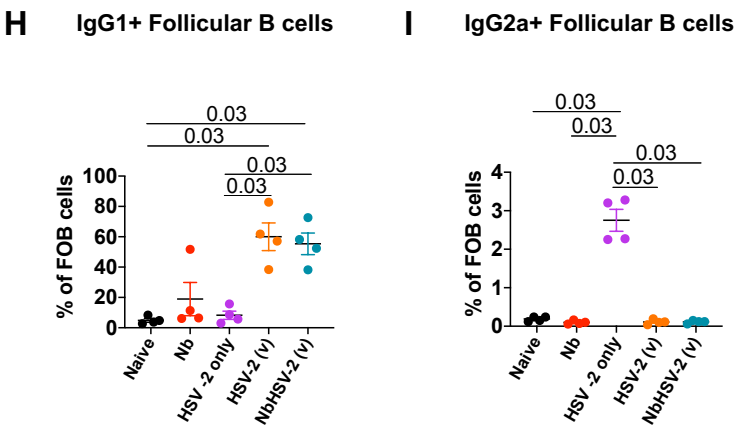


Figure 26: IgG1+ B cell responses are increased and IgG2a responses decreased in the ILN mice vaccinated with HSV-2 following HSV-2 challenge. Representative flow cytometry plots showing B cell, plasma cell, plasmablast and FOB cell IgG1 and IgG2a (A). Graphs showing IgG1+ (B) and IgG2+ (C) B cells, IgG1+ (D) and IgG2+ (E) plasma cells, IgG1+ (F) and IgG2+ (G) plasmablasts and IgG1+ (H) and IgG2+ (I) FOB cells in the iliac (ILN) of naïve, *N brasiliensis* infected (Nb), HSV-2 infected (HSV-2 only), HSV-2 vaccinated and infected (HSV-2 (v)) and *N brasiliensis* infected, HSV-2 vaccinated and infected (NbHSV-2 (v)) mice. Data are representative of two experiments, n=4 for all groups. Mann Whitney U test was used to compare groups.

When compared to naïve mice, total IgG1+ B cell proportions in the ILN of HSV-2 (v) and NbHSV-2 (v) mice were significantly increased (HSV-2 (v) vs naïve: median 60.4% vs 7.32%, p=0.03; NbHSV-2 (v) vs naïve: median 57.1% vs 7.32%, p=0.03; **Figure 26B**). IgG1+ B cell frequencies were however not increased in the HSV-2 only group in comparison to the naïve group but were lower in comparison to the HSV-2 (v) and NbHSV-2 (v) groups (HSV-2 only vs HSV-2 (v): median 8.35% vs 60.4%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 8.35% vs 57.1%, p=0.03; **Figure 26B**).

In contrast, total IgG2a+ B cell proportions were decreased in the ILN of HSV-2 (v) and NbHSV-2 (v) mice in comparison to naïve mice (HSV-2 (v) vs naïve: median 0.12% vs 0.54%, p=0.03; NbHSV-2 (v) vs naïve: median 0.14% vs 0.54%, p=0.03; **Figure 26C**). Total IgG2a+ B cell frequencies were however elevated in the ILN of HSV-2 only mice in comparison to naïve, HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 only vs naive: median 2.99% vs 0.54%, p=0.03; HSV-2 only vs HSV-2 (v): median 2.99% vs 0.12%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 2.99% vs 0.14%, p=0.03; **Figure 26C**). These results suggest that the FI HSV-2 vaccine induced a IgG1 rather than IgG2 response, in contrast to direct challenge of unvaccinated mice, which favoured an IgG2a over an IgG1 response.

A similar pattern of increased IgG1 but not IgG2a following FI HSV-2 vaccination was observed in the other B cell subsets investigated. IgG1+ plasma cell frequencies were increased in the ILN of HSV-2 (v) and NbHSV-2 (v) mice in comparison to naïve mice (HSV-2 (v) vs naïve: median 48.8% vs 6.53%, p=0.03; NbHSV-2 (v) vs naïve: median 43.5% vs 6.53%, p=0.03; **Figure 26D**). IgG1+ plasma cell proportions did not differ in the ILN of HSV-2 only mice in comparison to naïve mice and were fewer in the ILN of HSV-2 only in comparison to HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 only vs HSV-2 (v): median 10.12% vs 48.8%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 10.12% vs 43.5%, p=0.03; **Figure 26D**). IgG2a+ plasma cell frequencies were decreased in the ILN of the HSV-2 (v) and NbHSV-2 (v) groups in comparison to the naïve group (HSV-2 (v) vs naïve: median 0.08% vs 1.36%, p=0.03; NbHSV-2 (v) vs naïve: median 0.08% vs 1.36%, p=0.03; **Figure 26E**) and increased in the ILN of HSV-2 only mice in comparison HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 only vs HSV-2 (v): median

3.44% vs 0.08%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 3.44% vs 0.08%, p=0.03; **Figure 26E**).

IgG1+ plasmablast frequencies were increased in the ILN of HSV-2 (v) and NbHSV-2 (v) mice in comparison to naïve mice (HSV-2 (v) vs naïve: median 47.15% vs 17.15%, p=0.03; NbHSV-2 (v) vs naïve: median 31.45% vs 17.15%, p=0.03; **Figure 26F**). IgG1+ plasmablasts did not expand in the ILN of HSV-2 only mice in comparison to naïve mice (**Figure 26F**) and were detected at decreased frequency in the ILN of HSV-2 only mice in comparison to HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 only vs HSV-2 (v): median 10.12 vs 41.75%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 10.12% vs 31.45%, p=0.03; **Figure 26F**). IgG2a+ plasmablast proportions were however higher in the ILN of HSV-2 only mice in comparison to HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 only vs HSV-2 (v): median 1.57% vs 0.23%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 1.57% vs 0.26%, p=0.03; **Figure 26G**).

IgG1+ follicular B cell frequencies were increased in the ILN of HSV-2 (v) and NbHSV-2 (v) mice in comparison to naïve mice (HSV-2 (v) vs naïve: median 59.45% vs 4.27%, p=0.03; NbHSV-2 (v) vs naïve: median 55.3% vs 4.27%, p=0.03; **Figure 26H**). There were no differences in IgG1+ follicular B cell proportions between HSV-2 only mice and naïve mice (**Figure 26H**) in the ILN. IgG1+ follicular B cell proportions were however lower in the ILN of HSV-2 only mice in comparison to HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 only vs HSV-2 (v): median 7.15% vs 59.45%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 7.15% vs 55.3%, p=0.03; **Figure 26H**).

IgG2a+ follicular B cell proportions were increased in the ILN of HSV-2 only mice in comparison to naïve, HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 only vs naïve: median 2.74% vs 0.2%, p=0.03; HSV-2 only vs HSV-2 (v): median 2.74% vs 0.1%, p=0.03; HSV-2 only vs NbHSV-2 (v): median 2.74% vs 0.12%, p=0.03; **Figure 26I**).

IgG1 and IgG2a responses did not differ significantly between Nb and naïve mice (**Figure 26B-I**).

9.4 Effector memory CD4 T cell frequencies are decreased in ILN of HSV-2 vaccinated mice with prior N. brasiliensis infection following viral challenge

We next examined CD4 and CD8 responses in the ILN of HSV-2 vaccinated mice following viral challenge to determine if they were impacted by *N. brasiliensis* infection prior to HSV-2 vaccination. There were no differences in total CD4+ T cell, CD8+ T cell, Tfh cell, naïve CD4 T cell, effector memory CD8 and naïve CD8 T cell frequencies between NbHSV-2 (v) and HSV-2 (v) mice (**Figure 27A-B, Figure 27D, Figure 27F-H**).

Of note, effector memory CD4+ T cell proportions were decreased in the ILN of NbHSV-2 (v) in comparison to HSV-2 (v) mice (NbHSV-2 (v) vs HSV-2 (v): median 28.5% vs 32.25%, p=0.03; **Figure 27E**). Furthermore, effector memory CD4+ T cell proportions were also lower in the ILN of HSV-2 only mice in comparison to HSV-2 (v) mice (NbHSV-2 (v) vs HSV-2 (v): median 27.3% vs 32.25%, p=0.03); **Figure 27E)** but were comparable between NbHSV-2 (v) mice and HSV-2 only mice (**Figure 27E**).

Interestingly, in comparison to naïve mice, we detected a decline in CD4+ T cell proportions in both HSV-2 (v) and NbHSV-2 (v) mice (HSV-2 (v) vs naïve: median 27.55% vs 45.65%, p=0.03; NbHSV-2 (v) vs naïve: median 27.3% vs 45.65%, p=0.03; **Figure 27A**). The decline was also apparent in HSV-2 only mice (HSV-2 only vs naïve: median 26.4% vs 45.65%, p=0.03).

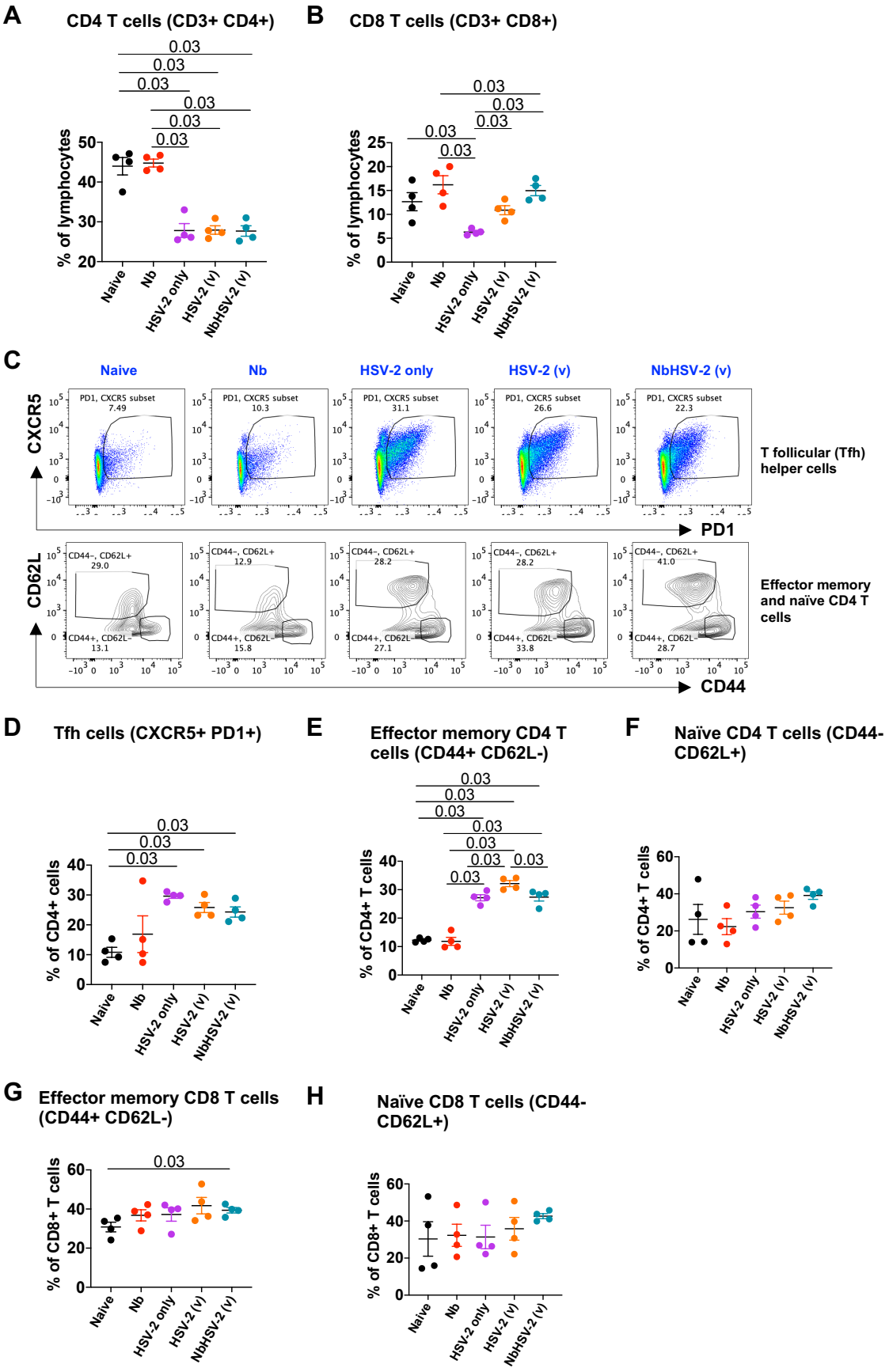

A
CD4 T cells (CD3+ CD4+)
% of lymphocytes
B
CD8 T cells (CD3+ CD8+)
% of lymphocytes
Naive
Nb
HSV-2 only
HSV-2 (v)
NbHSV-2 (v)
0.03
C
CXCR5
PD1
PD1, CXCR5 subset
7.49
10.3
31.1
26.6
22.3
T follicular (Tfh) helper cells
CD62L
CD44
CD44−, CD62L+
29.0
12.9
28.2
28.2
41.0
CD44+, CD62L−
13.1
15.8
27.1
33.8
28.7
Effector memory and naïve CD4 T cells
D
Tfh cells (CXCR5+ PD1+)
% of CD4+ cells
E
Effector memory CD4 T cells (CD44+ CD62L-)
% of CD4+ T cells
F
Naïve CD4 T cells (CD44-CD62L+)
% of CD4+ T cells
G
Effector memory CD8 T cells (CD44+ CD62L-)
% of CD8+ T cells
H
Naïve CD8 T cells (CD44-CD62L+)
% of CD8+ T cells

Figure 27: Effector memory CD4 T cell frequencies are decreased in the ILN of mice infected with *N. brasiliensis* prior to HSV-2 vaccination when challenged with HSV-2. Graphs showing total CD4+ (A) and CD8+ (B) T cells in the iliac (ILN) of naïve, *N brasiliensis* infected (Nb), HSV-2 infected (HSV-2 only), HSV-2 vaccinated and infected (HSV-2 (v)) and *N brasiliensis* infected, HSV-2 vaccinated and infected (NbHSV-2 (v)) mice. Representative flow cytometry plots showing Tfh cells and effector memory and naive CD4 T cells in the ILN of naïve, Nb, HSV-2 only, HSV-2 (v) and NbHSV-2 (v) mice (B). Graphs showing Tfh (D), effector memory CD4 (E) Naïve CD4 (F) effector memory CD8 (G) and naïve CD8 (H) cells in the ILN of naïve, Nb, HSV-2 only, HSV-2 (v) and NbHSV-2 (v) mice. Data are representative of two experiments, n=4 for all groups. Mann Whitney U test was used to compare groups.

CD8+ T cell frequencies were not significantly lower in HSV-2 (v) and NbHSV-2 (v) mice when compared to naïve mice, though there was a trend towards reduction of these cells in the vaccinated groups (**Figure 27B).** HSV-2 only mice, however, had decreased CD8+ T cell frequencies in the ILN in comparison to naïve mice (HSV-2 only vs naïve: median 6.19% vs 12.6%, p=0.03; **Figure 27B**). Additionally, CD8+ T cell frequencies were increased in the ILN of HSV-2 (v) and NbHSV-2 (v) mice in comparison to HSV-2 only mice (HSV-2 (v) vs HSV-2 only: median 10.85 vs 6.19%, p=0.03; NbHSV-2 (v) vs HSV-2 only: median 14.70% vs 6.19%, p=0.03; **Figure 27B**).

T follicular helper (Tfh) cell frequencies were increased in the ILN of HSV-2 only, HSV-2 (v) and NbHSV-2 (v) in comparison to naïve mice (HSV-2 only vs naïve: median 29.85% vs 10.22%, p=0.03; HSV-2 (v) vs naïve: median 24.9% vs 10.22%, p=0.03; NbHSV-2 (v) vs naïve: median 23.6% vs 10.22%, p=0.03;

Figure 27D). There was no difference in Tfh cell proportions between HSV-2 only, HSV-2 (v) and NbHSV-2 (v) mice (**Figure 27D**).

Similar to Tfh cells, effector memory CD4 T cells expanded in the ILN of HSV-2 only, HSV-2 (v) and NbHSV-2 (v) in comparison to naïve mice (HSV-2 only vs naïve: median 27.3% vs 12.2%, p=0.03; HSV-2 (v) vs naïve: median 32.25% vs 12.2%, p=0.03; NbHSV-2 (v) vs naïve: median 28.5% vs 12.2%, p=0.03; **Figure 27E**). Effector memory CD8 T cell proportions were increased in NbHSV-2 (v) in comparison to naïve mice (NbHSV-2 (v) vs naïve: median 39.6% vs 31.8%, p=0.03; **Figure 27G**)**.** Effector memory CD8 T cell proportions were however not increased in the ILN of HSV-2 only and HSV-2 (v) mice in comparison to naïve mice.

Naïve CD4 and naïve CD8+T cell proportions did not differ between HSV-2 only, HSV-2 (v) and NbHSV-2 (v) mice or in comparison to naïve mice (**Figure 27F and Figure 27H**), while T cell responses were not significantly different between Nb and naïve mice in the ILN (**Figure 27A-B, Figure 27D, Figure 27F-H**).

9.5 Prior N. brasiliensis infection decreases vaccine mediated protection from pathology following HSV-2 challenge

Vaginal pathology following HSV-2 challenge was scored for each of the treatment groups up to day 6 post infection when the mice were killed, results are summarised in **Figure 28**. NbHSV-2 (v) mice displayed elevated pathology in comparison to HSV-2 (v) mice on days two to six post HSV-2 challenge. In addition, HSV-2 (v) mice displayed significantly lower pathology than HSV-2 (unvaccinated) mice, days two to six post HSV-2 challenge. NbHSV-2 (v) mice,

on the other hand, displayed a lower level of vaccine mediated protection from pathology following HSV-2 infection, as pathology was only significantly lower than that in unvaccinated mice on days four and five post HSV-2 challenge.

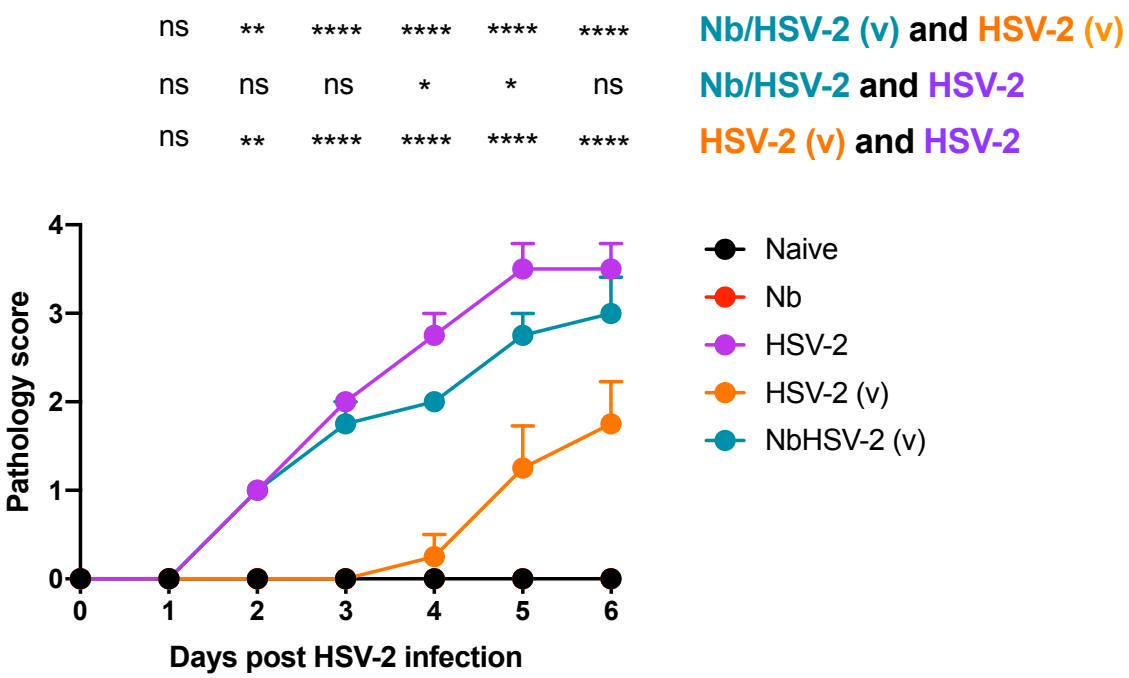


Figure 28: Mice infected with *N. brasiliensis* prior to HSV-2 vaccination display increased pathology in comparison to *N. brasiliensis* uninfected mice following HSV-2 challenge. Graph showing pathology scores of the external vagina of naïve, *N brasiliensis* infected (Nb), HSV-2 infected (HSV-2 only), HSV-2 vaccinated and infected (HSV-2 (v)) and *N brasiliensis* infected, HSV-2 vaccinated and infected (NbHSV-2 (v)) mice. Data are representative of two experiments, n=4 for all groups. Two-way analysis of variance (ANOVA) test was used to compare groups with Bonferroni correction for multiple comparisons. P value summary, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$

9.6 Discussion

We found no significant alteration of HSV-2 vaccine mediated B cell responses in the ILN of mice infected with *N. brasiliensis* prior to HSV-2 vaccination and subsequently challenged with lethal dose HSV-2 in comparison to mice that were not infected with *N. brasiliensis* before vaccination. Effector memory CD4+ T cell proportions were however decreased in the ILN of mice infected with *N. brasiliensis* before HSV-2 vaccination in comparison to mice with no prior helminth infection.

As expected, and consistent with previous reports (Milligan and Bernstein, 1995b; Oh *et al.*, 2019), B cells expanded in the ILN of HSV-2 vaccinated mice in comparison to uninfected mice, we also observed a similar expansion in the ILN of mice infected with lethal dose HSV-2 in a primary infection. These findings suggest that vaccination with FI HSV-2 did not confer an advantage to immunized mice with respect to increase in B cells in the ILN. However, with respect to functionality, frequencies of antibody producing (IgG1+) B cells, plasmablasts and follicular B cells were all significantly elevated in vaccinated mice in comparison to mice that received only primary HSV-2 challenge.

We observed an expansion of IgG1+ but not IgG2a+ B cells in the ILN following HSV-2 vaccination in both HSV-2 (v) and NbHSV-2 (v) mice. Even though we used a different HSV-2 strain for vaccination, our results are consistent with a previous study that reported detection of IgG1, IgG2b, IgG2c and IgA in the vaginal lumen but not IgG2a following HSV-2 vaccination and subsequent viral challenge (Oh *et al.*, 2019).

Consistent with previous studies (King, Parr and Parr, 1998; Morrison, 2008; Odegard *et al.*, 2016), HSV-2 vaccination in our study resulted in expansion of effector memory CD4+ T cells in the ILN following HSV-2 challenge, however, a smaller proportion of these cells were detected in mice that were infected with *N. brasiliensis* before HSV-2 vaccination. Given the important role of IFN-γ producing CD4+ T cells in protective immunity against HSV-2 (Milligan and Bernstein, 1997; Parr and Parr, 1999), the finding that effector memory CD4+ T cell proportions are reduced in the ILN of mice that were *N. brasiliensis* infected prior to HSV-2 vaccination suggests that vaccine mediated CD4+T cell immunity to HSV-2 is impaired by underlying helminth infection. We did not measure virus titres in vaginal lavage to determine if reduced effector memory CD4 T cells translated into loss of viral control and future experiments are required to probe this further.

A type 2 immune environment in the FRT, induced by dysbiosis, results in defective recruitment of effector memory CD4 T cells to the vagina in mice challenged with HSV-2 (Oh *et al.*, 2016). In addition, in Chapter 7, we demonstrated that effector memory CD4+ T cells expand in the ILN following *N. brasiliensis* infection, these cells are likely to be Th2 cells that could suppress Type 1 immunity in the ILN of infected mice. Furthermore, a recent publication from our lab demonstrated that *N. brasiliensis* induced a Type 2 cytokine milieu in the FRT, with elevated IL-33 and IL-5 being detected in the vaginal tract of infected mice (Chetty *et al.*, 2021). Furthermore, following HSV-2 challenge, mice with underlying *N. brasiliensis* infection displayed increased pathology in comparison to mice with no prior helminth infection. Based on these findings, we can interpret our data to mean that the Type 2 environment created in the ILN

by helminth (*N. brasiliensis*) infection impairs recruitment of effector memory CD4+ T cells to the FRT draining lymph nodes following HSV-2 vaccination and subsequently results in impaired expansion of these cells following a secondary challenge that was accompanied by impaired protection against HSV-2 induced pathology in the FRT.

In summary, results presented in this chapter demonstrate that FI HSV-2 vaccination induced expansion of effector memory CD4+ T cells in HSV-2 vaccinated mice after viral challenge that was not otherwise induced by primary viral challenge and that these cells were diminished in mice infected with *N. brasiliensis* prior to HSV-2 vaccination. Furthermore, there was a trend towards decreased B cell proportions in mice with underlying helminth infection even though this was not statistically significant. Importantly, HSV-2 vaccinated mice with underlying *N. brasiliensis* infection displayed increased pathology in comparison to *N. brasiliensis* uninfected mice following HSV-2 challenge. These findings underscore the importance of taking into account helminth infection in designing HSV-2 vaccination strategies for women living in helminth endemic regions.

Chapter 10: Discussion

We report a 20.5% prevalence of the hookworm *Ancylostoma duodenale* infection in our study which is higher than hookworm prevalence estimates for SSA, 13.6% (Pullan *et al.*, 2014), and countries in West Africa, 12.1% in Ghana (Adu-Gyasi *et al.*, 2018), and 16.8% in Nigeria (Yaro, Kogi and Luka, 2018). The higher hookworm prevalence we observe in our cohort could be site specific. Furthermore, we recruited participants entirely from a rural area in Togo. Warm tropical climate and rural settings with potentially limited access to proper sanitation are linked to increased risk of helminth infections (Loukas *et al.*, 2016).

Similar to the hookworm prevalence, approximately 1/5 of the participants in our study (19.4%) were infected with HPV. This is lower than the HPV prevalence reported in some cohorts in SSA, ranging from 24-53% (Bruni *et al.*, 2010; Mbatha *et al.*, 2017; Ferré *et al.*, 2019; Krings *et al.*, 2019; Kuassi-Kpede *et al.*, 2021). The difference in prevalence between our study and the aforementioned studies could be attributed to factors such as younger age of study participants and inclusion of high risk participants and HIV infected participants in these studies, all of which are associated with increased risk of HPV infection (Bruni *et al.*, 2010; Veldhuijzen *et al.*, 2011; Roset Bahmanyar *et al.*, 2012; McDonald *et al.*, 2014).

Hookworm infection is identified as an independent risk factor for HPV infection in our cohort, with hookworm infected participants being 2 times more likely to be HPV positive. This is consistent with data from the study by Gravitt et al that identifies elevated risk of HPV among women with soil-transmitted helminth

infections (Gravitt *et al.*, 2016). We also found that HPV risk was positively associated with the number of sexual partners and negatively associated with condom use, consistent with other studies that found unprotected sex and multiple sexual partners to be risk factors for HPV infection (Roset Bahmanyar *et al.*, 2012; Tran *et al.*, 2015). Though women in our cohort employed harsh vaginal cleaning techniques, these practices did not associate with risk of HPV infection.

In our cohort, in addition to elevated Type 1 cytokines (IL-2, TNF-α and IL-12), we observe a Type 2 immune signature in the FRT of hookworm infected women with elevated IL-4, IL-5, IL-13, Eotaxin and IL-10 in addition to an elevated IgG4/IgE ratio. The IgG4/IgE ratio is a common feature of helminth infection where IgG4 production is promoted over the strongly anti-parasitic IgE (Palmer, Bradley and Bundy, 1996; Turner *et al.*, 2005; Figueiredo *et al.*, 2012). We hypothesise that this Type 2 immunity could be contributing to increased risk of HPV among hookworm infected women.

Notably, the Type 2 immune signature we observe in the FRT of hookworm infected women is not detected in the plasma. Previous studies report both Type 1 and Type 2 immune responses in circulation and in the small intestine mucosa in hookworm infected individuals (Quinnell *et al.*, 2004; Gaze, McSorley, Daveson, Jones, Jeffrey M Bethony, *et al.*, 2012). These studies report immune responses in the context of antigen stimulation which is likely why higher systemic immune responses to hookworm are observed in comparison to what we find in our study. In agreement with our findings, baseline IL-4 and IL-13 responses to hookworm were low, while IL-5 was undetectable when measured

directly *ex vivo* (Gaze, McSorley, Daveson, Jones, Jeffrey M. Bethony, *et al.*, 2012).

We also observe elevated Type 1 (IL-2, TNF-α and IL-12) and Type 2 (IL-4, IL-5 and IL-13, eotaxin) immune responses in the FRT of HPV infected women. Vaginal IL-10 levels and Th2/Th1 ratios were however not elevated in HPV infected women. Higher vaginal IL-10 and Th2/Th1 ratios are linked to late stage HPV associated cervical lesions (Bais, Beckmann, Ewing, Marinus J.C. Eijkemans, *et al.*, 2007; Peghini *et al.*, 2012; Berti *et al.*, 2017). Our data therefore suggests that HPV infections among the women in our cohort had not developed to advanced lesions. This however requires cytological examination to determine conclusively (Nayar and Wilbur, 2015). The elevated Type 2 immunity we detect in the FRT of HPV infected women however still indicates a potential risk for development of severe cervical lesions as Type 2 immune skewing is associated with progression of HPV associated lesions in the cervix (Clerici *et al.*, 1997; Al-Saleh *et al.*, 1998; Peghini *et al.*, 2012).

Notably, in the hookworm and HPV co-infected women, elevated Type 2 immunity (IL-4, IL-5, IL-13 and eotaxin) in the FRT is pronounced. This appears to be a synergistic effect of hookworm and HPV co-infection as the increase is not apparent for women with only hookworm or only HPV infection (with IL-13 as the only Type 2 cytokine elevated in the FRT). Furthermore, in the hookworm and HPV co-infected women, the ratio of Th2/Th1 cytokines in the FRT was elevated in comparison to the HPV only group. Elevated Type 2 immunity is associated with poor HPV infection outcomes such as progression of cervical lesions or cervical cancer (Bais, Beckmann, Ewing, Marinus J. C. Eijkemans, *et*

al., 2007; Peghini *et al.*, 2012). Thus, this data suggests that hookworm and HPV co-infection and the prominent Type 2 cytokine milieu in the FRT induced as a result could be a risk factor for progression of HPV associated disease. Furthermore, the positive association between hookworm infection and the intensity of HPV infection suggests that hookworm infection could be promoting viral persistence and replication and we postulate that this could be related to Type 2 immune signalling in the FRT.

Previous studies in helminth and virus co-infection models have indeed demonstrated that Type 2 immunity is associated with a loss in viral control. IL-4 and STAT6 signalling and suppression of IFN-γ responses following *S. mansoni* and *H. polygyrus* infection resulted in reactivation of latent murine gamma herpesvirus (Reese *et al.*, 2014). In addition, membrane barrier integrity disruption induced by Type 2 signalling in the gut resulted in microbial translocation and subsequent collapse of systemic virus specific CD8+ T cell responses to West Nile Virus in *H. polygyrus bakeri* co-infected mice that was accompanied by increased viral burdens (Desai *et al.*, 2021).

The mechanism by which hookworm infection increases risk of HPV still needs to be elucidated. In contrast to our findings, Jacobs et al report impaired HPV pseudovirion uptake in cervical cancer cell lines treated with *N. brasiliensis* somatic antigen that resulted in degradation of cell surface vimentin on HeLa cells (Jacobs *et al.*, 2018). Vimentin is a restriction enzyme that mediates HPV pseudovirion uptake by HeLa cells (Schäfer *et al.*, 2017). This suggests that helminth products impair HPV infection of target cells. We hypothesize that another mechanism, such as Type 2 signalling, could be involved in increasing

risk of HPV among helminth-infected women that we and others (Gravitt *et al.*, 2016) report. Experiments in mouse models or relevant *in vitro* assays to determine the impact of Type 2 immunity on membrane barrier integrity that can potentially provide easy entryway for HPV infection at barrier surfaces would be useful. Furthermore, blocking Type 2 signalling in a mouse model to determine if this impacts HPV infectivity would also provide information on whether Type 2 signalling is a definitive mediator of increased HPV infectivity.

Evidence of helminth induced Type 2 immune skewing in the FRT is further confirmed in the mouse model. We demonstrate that IgG1+ B cell, IgG1+ follicular B cell, effector memory CD4 T cell and Tfh cell frequencies were increased in the lymph nodes draining the FRT (ILN) of *N. brasiliensis* infected mice in comparison to uninfected mice. IgG1 antibodies are Type 2 associated and are induced following helminth infection as a result of the influence of IL-4 on B cells (Turqueti-Neves *et al.*, 2015). Tfh have also been demonstrated to produce IL-4 that influences B cell antibody class switching to IgG1 in germinal centers following helminth infection (King and Mohrs, 2009). In addition, the effector memory CD4 T cells whose proportions were increased in the ILN of *N. brasiliensis* infected mice are likely to be Th2 cells. Th2 cells with an activated phenotype, CD62L low, CD44 high (detected using IL-4/eGFP reporter mice), have previously been reported to expand in the lungs following *N. brasiliensis* infection (Seidl, Panzer and Voehringer, 2011).

We further demonstrate in the mouse model that hookworm infection impacts vaccine mediated CD4 T cell responses to a viral infection of the FRT, HSV-2. In our study, effector memory CD4 T cell frequencies were decreased in the ILN of

mice that were infected with *N. brasiliensis* prior to HSV-2 vaccination and challenged with lethal dose HSV-2 in comparison to HSV-2 vaccinated and challenged mice with no underlying helminth infection. Effector memory CD4 T cells expand in the FRT following HSV-2 vaccination (King, Parr and Parr, 1998; Morrison, 2008; Odegard *et al.*, 2016) and IFN-γ producing CD4+ T cells play an important role in protective immunity against HSV-2 (Milligan and Bernstein, 1997; Parr and Parr, 1999).

Previously, defective immunity to HSV-2 was demonstrated in mice that developed Type 2 immunity in the FRT following induced dysbiosis (Oh *et al.*, 2016). In these mice, Type 2 immunity resulted in suppression of inflammation in the FRT that led to impaired recruitment of HSV-2 specific effector memory CD4 T cells. Furthermore Chetty et al demonstrate that *N. brasiliensis* infection results in elevated Type 2 cytokines, IL-33 and IL-5, and eosinophilia in the FRT of mice (Chetty *et al.*, 2021). Mice with underlying *N. brasiliensis* infection subsequently displayed increased pathology following HSV-2 challenge that was alleviated following IL-5 and eosinophil depletion (Chetty *et al.*, 2021). These data, in addition to the elevated effector memory CD4 T cells we detected in the ILN of N. *brasiliensis* infected mice (that are likely to be Th2 cells), could be interpreted to mean that a Type 2 environment induced in the ILN by *N. brasiliensis* infection impairs effector memory CD4 T cell recruitment following HSV-2 vaccination and their expansion following secondary viral challenge.

In the mouse model, progesterone treatment was administered to synchronise the mice and promote HSV-2 infectivity in the FRT (Marshak, Dong and Koelle, 2014). The stage of the oestrus cycle in mice can influence immunity in the

female reproductive tract both at baseline and in response to mucosal vaccination at distal sites (Gallichan and Rosenthal, 1996; Gockel *et al.*, 2003; Hickey, Fahey and Wira, 2013). It would be worth analysing the effect of the oestrus cycle on *N. brasiliensis* and HSV-2 co-infection in future studies.

The type of controlled environment created in the mouse model through progesterone treatment was not achievable in the human cohort. Furthermore, while study participants reported use of contraceptives, the type of contraception used was not disclosed, therefore the influence of progesterone on HPV infectivity could not be investigated. Interestingly we found contraception to be a risk factor for STI in our cohort (Holali Ameyapoh *et al.*, 2021), however, we found no association between contraceptive use and HPV positivity. This is consistent with the study by Kiweewa et al where HPV infection was not linked to contraceptive use (Kiweewa *et al.*, 2019).

In the context of our clinical study, where hookworm infection is associated with raised Type 2 immunity in the FRT, that is more pronounced in hookworm and HPV co-infected women, the Type 2 cytokine milieu in the FRT could contribute to suppression of effector memory CD4 T cells that are of the Type 1 lineage. While we did not measure CD4 T cells in the cervix, it would be worthwhile to do a cellular analysis to determine the cell types that are potentially altered in HPV and hookworm co-infected women. The higher Th2/Th1 ratio in the FRT of HPV and hookworm co-infected women in comparison to women with only HPV that we detected hints at a potential suppression of Th1 responses in co-infected women. Future studies to investigate the impact of underlying helminth infection

on vaccine responses to HPV would also provide further insight into helminth and HPV interactions.

In our vaccination study, there was a trend towards decreased B cell responses following HSV-2 challenge in HSV-2 vaccinated mice with underlying helminth infection in comparison to helminth uninfected HSV-2 vaccinated mice, these were however not statistically significant.

Overall, in this thesis we demonstrate that Type 2 immune profiles in the FRT are induced following helminth infection in both humans and mice. We also demonstrate an increased risk of HPV in hookworm infected women and a positive association between hookworm infection and HPV intensity that could potentially be linked to Type 2 immune signalling in the FRT. We further demonstrate that vaccine mediated effector CD4 T cell responses to a viral infection of the FRT, HSV-2 are dampened following *N. brasiliensis* infection in a mouse model that is also likely associated with Type 2 immune induction in the FRT. The findings in this thesis highlight a need to consider underlying helminth infections as potential risk factors for STI among women living in helminth endemic regions and can be employed in the design of prevention and control strategies for STI, particularly in LMIC with a large burden of both helminth infections and STI.

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