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Immunological insights into equine responses against *Strongylus vulgaris*

Ex vivo studies using equine intestinal organoids and blood mononuclear cells

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Cover: *Strongylus vulgaris* third stage larvae
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Abstract

Anthelminthic drugs have controlled parasitic infections in veterinary species for decades, but today this use is questioned due to concerns about antimicrobial resistance. For most microorganisms, a feasible alternative is vaccination but for parasites, commercial vaccines are scarce. This is partly due to the complex life cycles of most helminths together with their immunomodulatory capacity. In the horse, *Strongylus vulgaris* has recently gained attention due to its re-emergence in Sweden. The overall aim of the present thesis was therefore to establish *ex vivo* methods to study responses against *S. vulgaris* that can guide formulation of a future vaccine. In the search for relevant antigens, protocols for generating defined larval (L) stages were established. These *S. vulgaris* preparations, alone or in combination with an adjuvant, were evaluated for their cytokine-inducing capacity. All larval preparations induced a Th2 profile in equine PBMC characterised by up-regulation of IL-4, IL-5, IL-9, IL-13 and TSLP. The L4 stage skewed this response by also up-regulating IFN-γ. The presence of a novel saponin adjuvant affected the Th2 profile induced by the L3 stage of *S. vulgaris*. To study responses to *S. vulgaris* at the site of infection, equine intestinal stem cells were differentiated into intestinal organoids. From these, monolayer cultures were established that displayed both genetic and functional similarities with the equine intestine, expressing pro-inflammatory cytokine genes at exposure to TLR-agonists. These organoid monolayers were applicable for generation of *ex vivo* co-cultures with equine PBMC and *S. vulgaris* larvae. Overall, this thesis provides new insights into the biology of *S. vulgaris* infection and *ex vivo* methods that will aid in the development of a future vaccine.

Keywords: *Strongylus vulgaris*, enteroids, equine, PBMC, *ex vivo*, parasite

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Sammanfattning

Anthelmintika har under en lång tid varit ett effektivt sätt att bekämpa parasitinfektioner hos djur. På grund av risken för utveckling av läkemedelsresistens har användningen av anthelmintika numera ifrågasatts. Det rimliga alternativet för att kontrollera de flesta mikroorganismer är genom vaccin, men mot parasiter finns det väldigt få kommersiella vaccin. Detta beror dels på parasiters komplexa livscykler och dels på deras förmåga att manipulera immunförsvaret. *Strongylus vulgaris* har på senare tid blivit uppmärksammad med anledning av en ökad förekomst i Sverige. Målet med denna avhandling var därför etablera metoder för att studera immunreaktioner mot *S. vulgaris* som kan vägleda framtida formuleringar av ett vaccin. För att identifiera relevanta antigen etablerades metoder för odling av olika *S. vulgaris* larvstadier. Dessa larvpreparationer, ensamma eller i kombination med ett adjuvans, utvärderades för deras cytokininducerande förmåga. Alla larvstadier inducerade en Th2 profil i ekvina mononukleära blodceller (PBMC) genom upp-reglering av gener för IL-4, IL-5, IL-9, IL-13 and TSLP. L4 stadiet skiljde sig däremot från de tidigare larvstadierna genom att även uppreglera IFN-γ. Närvaron av ett saponinbaserat adjuvans påverkade Th2 profilen inducerat av *S. vulgaris* L3. För att studera rektioner mot *S. vulgaris* i tarmen etablerades cellkulturer från stamceller isolerade från hästens tarm, s.k. organoider. Från dessa utvecklades s.k. monolayers bestående av ett enskiktat lager av celler vilka visade både genetiska och funktionella likheter med hästens tarm, samt uttryckte gener for pro-inflammatoriska cytokiner vid exponering för TLR-agonister. Dessa monolayers kunde samodlas med ekvina PBMC och *S. vulgaris* larver. Sammanfattningsvis har denna avhandling genererat nya insikter om immunsvaret vid *S. vulgaris* infektion samt *ex vivo* metoder som kan användas i utvecklingen av ett framtida vaccin.

Nyckelord: *Strongylus vulgaris*, enteroider, häst, PBMC, parasit, *ex vivo*

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Dedication

To my equine teachers
Happy, Gullan, Charter, Pelle, Sita, HopSun and Smalltown Gangster

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. **Hellman S**, Tydén E, Hjertner B, Nilsfors F, Hu K, Morein B, Fossum C (2021). Cytokine responses to various larval stages of equine strongyles and modulatory effects of the adjuvant G3 in vitro. Parasite Immunology 43(1):e12794.

II. **Hellman S**, Hjertner B, Morein B, Fossum C (2018). The adjuvant G3 promotes a Th1 polarizing innate immune response in equine PBMC. Veterinary Research, 49(1):108.

III. **Hellman S** (2021). Generation of equine enteroids and enteroid-derived 2D monolayers that are responsive to microbial mimics. Veterinary Research 52(1):108.

The contribution of Stina Hellman to the papers included in this thesis was as follows:

I. Planned the study together with co-authors. Performed the laboratory experiments and analyzed the data. Wrote the manuscript with support from the co-authors.

II. Planned the study together with co-authors. Performed the laboratory experiments and analyzed the data. Wrote the manuscript with support from the co-authors.

III. Planned the study, performed the laboratory experiments, analyzed the data and wrote the manuscript.

Related publication

Ahlberg V, Hjertner B, Wallgren P, **Hellman S**, Lövgren Bengtsson K, Fossum C (2017). Innate immune responses induced by the saponin adjuvant Matrix-M in specific pathogen free pigs. Vet Res 48(1):30.

Abbreviations

2D	Two-dimensional
3D	Three-dimensional
AMM	Alternatively activated macrophages
APC	Antigen presenting cell
CD	Cluster of differentiation
cDNA	Complementary DNA
ConA	Concanavalin A
COVID19	Coronavirus disease of 2019
CTL	Cytotoxic T lymphocyte
CTR	C-type lectin receptor
DAMP	Damage-associated molecular pattern
DC	Dendritic cell
ELISA	Enzyme-linked immunosorbent assay
ES	Excretory-secretory
EPG	Eggs per gram
ExL3	Exsheated L3
Fc	Fragment crystallisable
FliC	Recombinant flagellin protein encoded by the fliC gene
GALT	Gut-associated lymphoid tissue
GI	Gastro-intestinal
H1N1	Hemagglutinin Neuraminidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cell
ISCOM	Immunostimulating complexes

J-chain	Joining chain
L	Larval stage
LPS	Lipopolysaccharide
MALT	Mucosa-associated lymphoid tissue
M cell	Microfold cell
MD-2	Myeloid differentiation factor 2
MHCII	Major histocompatibility complex II
miRNA	Micro RNA
NK	Natural killer
NLR	Nod-like receptor
Pam3CSK4	Synthetic triacetylated lipoprotein
PAMP	Pathogen associated molecular pattern
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
pIgR	Polymeric immunoglobulin receptor
PMA	Phorbol myristate acetate
Poly I:C	Polyinosinic:polycytidylic acid
PRR	Pattern recognition receptor
Quil A	*Quillaja saponaria*
qPCR	Quantitative polymerase chain reaction
RT-PCR	Real-time polymerase chain reaction
sIgA	Secretory immunoglobulin A
TGF	Transforming growth factor
TEER	Trans epithelial electrical resistance
Th	T helper
TNF	Tumor necrosis factor
TLR	Toll-like receptor
Treg	T regulatory
SAWA	Soluble adult worm antigen
SvSXP	*S. vulgaris* SXP protein

1. Introduction

Helminth parasite infections are widespread among all animal species affecting animal health and performance resulting in negative consequences for the agricultural sector (Foster & Elsheikha, 2012). The discovery of broad-spectrum anthelminthic compounds in the 1960s revolutionized both animal and human health. For the first time in history, parasite infections could be controlled leading to a reduced morbidity and mortality from parasitic disease. In the equine sector, anthelminthic treatments several times per year were originally aimed to control the large strongyle parasite *Strongylus vulgaris*. This treatment regime was extremely effective in reducing worm burdens, making *S. vulgaris* an uncommon equine parasite (Kaplan & Nielsen, 2010). Unfortunately, the frequent usage of anthelminthic drugs backfired as drug resistance emerged in other equine parasite species. Today, resistance against anthelminthic drugs is a recognized problem in the equine roundworm *Parascaris univalens* and small strongyles *Cyathostominae* spp. (reviewed by Raza et al, 2019).

To slow down the development of anthelmintic resistance, the European Union (2001/82/EG) issued treatment restrictions in 2007. In the Nordic countries, this was applied in practice by so-called "targeted selective treatment", by determining strongyle fecal egg counts (EPG) for all horses and only treat those exceeding a predetermined threshold. The aim were to achieve a reduction of overall egg shedding, while leaving a proportion of the herd untreated, which lowers anthelminthic treatment intensity and reduces selection pressure for development of anthelmintic resistance. However, reports from Sweden and Denmark show that the prevalence of *S. vulgaris* has increased the last years, coinciding with the new treatment

regime (Nielsen et al, 2012; Tydén et al., 2019) despite advices about improved pasture management routines (Hedberg-Alm et al, 2020).

With no novel anthelminthic drugs in sight (Nixon et al, 2020), alternative methods to control *S. vulgaris,* such as vaccination, are therefore warranted. Indeed, before the "era of anthelminthics", efforts were made to develop vaccines against a variety of helminths for several animal species, including the horse (Bain, 1999). The early attempts to produce vaccines against *S. vulgaris* used irradiated larval preparations as antigen (Klei et al, 1982; Monahan et al, 1994; Swiderski et al, 1999). These vaccines gave partial protection but were not further progressed when anthelminthic oral pastas became commercially available.

It is generally accepted that making vaccines against helminths is troublesome. Typically, helminths establish long-term chronic infections that cannot be resolved by the host's immune defense. This evasion is at least partly thought to rely on the helminth's ability to modulate the host's immune response. Three major obstacles for successful development of helminth vaccines can be summarized: (1) identification of relevant antigens from the parasite; (2) identification of the host's protective mechanisms, and (3) development of the appropriate stimuli needed to mobilize these pathways while at the same time overcoming the intrinsic immune regulatory capabilities of the parasite. Thus, knowledge about the biology of infection for the parasite of choice needs to be deepened to select relevant antigen(s) and adjuvant for vaccine formulation. Furthermore, a suitable *ex vivo* model is needed for initial screening of the proposed components.

2. Background

2.1 Helminths and the immune defense

Parasitic helminths comprise the three taxonomic groups nematodes (roundworms), trematodes (flukes) and cestodes (tapeworms). They all differ in host and tissue specificity with different invasion routes and life-cycle pathways, making them a very heterogeneous group of organisms. However, the gastro-intestinal (GI) nematodes are similar in that they progress from eggs through multiple developmental stages, commonly referred to as "larval (L) stages" until becoming adult worms. The various developmental stages are separated by molting events when the cuticle surrounding the larval body is released. These shifts between larval stages are typically fast and often accompanied by migrations through different organs and tissues (Wiedemann & Voeringer 2020). In addition, molecules produced and released by helminths referred to as excretory-secretory (ES) products are immunomodulatory, meaning that they can interfere with the host's immune response (Maizels et al, 2018).

Most knowledge of nematode immunity comes from rodent laboratory models and the effector mechanisms necessary for elimination of GI nematodes of large animal species, such as the horse, have only been partly delineated (Foster & Elsheikha, 2012; Grencis 2015). Because of the migrating life cycle of *S. vulgaris*, the host's immune system is likely to be exposed to various antigen setups, representing different larval stages in different immune compartments including blood and intestinal mucosa. Below, I will give a brief overview of immune mechanisms generally involved in recognition, processing and elimination of GI helminths.

2.1.1 Innate immune recognition

When a pathogen first infects a host, two types of alarm signals can trigger the immune defense that either sense molecular structures originating from the pathogen (pathogen associated molecular patterns; PAMPs) or from damaged tissue (danger associated molecular patterns; DAMPs). The PAMPs and DAMPs are recognized by pattern recognition receptors (PRRs) expressed by innate immune cells such as dendritic cells (DCs), monocytes/macrophages, mast cells, granulocytes, natural killer (NK) cells and innate lymphoid cells (ILCs), but also by other cell types such as epithelial cells (reviewed in Kumar et al, 2011; Constant et al, 2021). There are several types of PRRs with different cellular localizations recognizing a vast array of conserved motifs expressed by microorganisms (Li and Wu, 2021). Although, there is no PAMP uniform for helminths, multiple types of PRRs can recognize helminth molecules. Of these, Toll-like receptors (TLRs), C-type lectin receptors (CTRs) and NOD-like receptors (NLRs) have most frequently been associated with helminth recognition (Perrigoue et al, 2008; Motran et al, 2018). The invasive nature of most GI nematodes also results in release of DAMPs from injured tissue. Recognition of PAMPs or DAMPs by PRRs rapidly trigger induction of inflammatory mediators such as cytokines and chemokines, which initiate and coordinate the ensuing immune response.

Cytokines are small secretory proteins that regulate most elements of the immune system and play a fundamental role in initiation of immune responses. Some cytokines have pleiotropic effects while others only induce a limited set of actions. Chemokines are cytokines able to act chemotactic for immune cells. Thus, chemokines are especially important to attract immune cells to the site of infection but also essential for cell trafficking at homeostasis. The kind of cytokines and chemokines that are elicited in response to a PAMP or DAMP influences the ensuing immune response (Borish & Steinke, 2003). Traditionally, helminth infections are associated with secretion of the cytokines interleukin-(IL)-4, IL-5, IL-9, IL-13 along with the alarmins IL-25, IL-33 and thymic stromal lymphopoietin (TSLP) that all together support the initiation of a T helper cell type 2 (Th2) response (discussed in 2.1.6).

2.1.2 Induction of acquired immunity

To initiate an antigen-specific immune response, the pathogen needs to be detected, engulfed and presented by professional antigen presenting cells (APCs). The most efficient APCs are the DCs, although other cell types such as macrophages and B-cells also can act as APCs. Interesting out of a parasite perspective are the eosinophilic granulocytes that also seem to be able to present antigens, although much less effective compared to DCs (Makepeace et al, 2012). Once a DC is activated, it migrates to the draining lymph node where it presents antigen peptides on MHC class II molecules to naïve CD4+ T helper (Th) cells. Activation and priming of naïve T cells requires three signals: (1) the antigen presented on MHCII is recognized by the T cell receptor (TCR); (2) co-stimulatory molecules on the DC bind to activation/deactivation receptors on the T cell; (3) priming cytokines guide differentiation to particular Th subsets specialized to induce different effector functions. However, T cell receptor engagement in the absence of signals 2 or 3 induces tolerance, and prolonged cytokine stimulation as well as cytokine pre-exposure can exhaust and/or impair the T cell response (Sckisel et al, 2015). Thus, persistent infections are likely to modulate the host's immune response, hindering development of an antigen-specific immune response, as seen for most helminth infections (Hewitson & Maizels, 2014). If, however, successfully primed, Th cells will migrate towards the B cell zone to aid B cell differentiation into plasma cells and antibody class switching.

2.1.3 Antibody-mediated immunity

Helminth infections in mice are typically associated with elevated serum levels of the Th2 associated antibody subclasses IgE and IgG1 (Sorobetea et al, 2018). However, studies in sheep reveal that other IgG subclasses as well as IgA are induced at mucosal surfaces by different larval stages of GI helminths (Aboshady et al, 2020). In horses, early characterizations of equine IgG identified the five subclasses IgGa, IgGb, IgGc, IgG(T) and IgG(B). In current literature, seven equine IgG subclasses, designated IgG1-IgG7 are recognized. The former IgGa now corresponds to IgG1 and IgG2, IgG(T) corresponds to IgG3 and IgG5, IgGb corresponds to IgG4, and IgGc corresponds to IgG6 and IgG7 (Wagner, 2006). In addition, equine IgM, IgA and IgE have been described, which along with some of the seven IgG

subclasses can be quantified using a multiplex assay (Keggan et al, 2013). Although studies on antibody responses to equine parasites are limited, both *S. vulgaris* and cyathostomins, as well as the protozoan parasite *Theileria equi*, have been associated with increased levels of IgG(T) in serum (Dowdall et al, 2002; Mealey et al, 2012; Andersen et al, 2013a) indicating that this subclass (IgG3/IgG5 in present nomenclature) is induced by extracellular pathogens. A role for IgE at GI nematode infection in horses has been suggested (Pittaway et al, 2014) although not yet extensively evaluated in this context. Equine IgE is however known to mediate type 1 hypersensitivity reactions to *Culicoides* midge bites (Schaffartzik et al, 2012).

The majority of plasma cells residing in the intestinal mucosa express IgA. Class switching to IgA is supported by multiple factors, but the most important is transforming growth factor (TGF)-β. This cytokine enables development of antigen-specific IgA, propagation of long-lived plasma cells and memory B cells (Lycke & Bemark, 2017). IgA antibodies that are secreted into the intestinal lumen occur in dimers paired by the J-chain protein. J-chain ligation to the polymeric Ig receptor (plgR) expressed on the basolateral surface of the epithelium mediates uptake and transport into the intestinal lumen. In the lumen, the secreted IgA (sIgA) can bind to antigens on both commensals and pathogens, to neutralize or inhibit their functions as well as facilitate antigen transport and presentation by mucosal DCs (Bunker & Bendelac, 2018). As in other mammal species, sIgA is the dominating isotype in nasopharyngeal secretions and saliva of horses (Palm et al, 2016; Schnabel et al, 2017). Molecular characterization of the equine IgA system, including J-chain and pIgR, show both genetic and functional similarities with human IgA (Lewis et al, 2010). In addition, there is a high level of homology between human and equine FcαRI (Morton et al, 2005) suggesting that equine IgA can mediate cellular effector responses similar to those described in man. Yet, equine IgA has mostly been studied in the context of respiratory infections (e.g. Crouch et al, 2005; Bordin et al, 2014; Bannai et al, 2021) and there is a limited knowledge about IgA at GI infection in the horse. However, a recent pilot study found a positive correlation between strongyle EPG and sIgA in feces (Żak et al, 2021), indicating a role of IgA at strongyle infection.

2.1.4 Cell mediated immunity

The T helper cells can be further divided into subsets based on their cytokine profile and transcription factors. In mice, Th1 and Th2 cells were first discerned based on their ability to produce IL-2 and IFN-γ or induce switch to IgG1 and IgE at *in vitro* stimulations (Mosmann et al, 1986). In accordance, human Th1 cells producing IL-2 and IFN-γ could be discriminated from Th2 cells producing IL-4 and IL-5 at *in vitro* stimulation with antigens from *Mycobacterium tuberculosis* and *Toxocara canis*, respectively (Del Prete et al, 1991). From these early studies, Th1 cells producing IFN-γ are regarded as protective against infection by virus, intracellular bacteria and protozoa, while Th2 cells producing IL-4 are supposed to contribute to the protection against helminths.

Since the first description of Th1 and Th2 cells, other Th subsets have been identified such as T regulatory cells (Treg) that can control immune reactions by secretion of the immunosuppressive cytokines TGF-β and IL-10 (Josefowicz et al, 2012). Th17 cells are characterized by a high production of IL-17 and shown to be protective against some extracellular bacteria and fungi but also associated with autoimmune disease (Harrington et al, 2006). In the horse, similar Th subsets have been identified mainly characterized by their cytokine profile (Wagner et al, 2010) or their transcriptional profile (Fedorka et al, 2021).

The more recently described Th9 subset develops in the presence of TGF-β and IL-4 and its effector functions have been associated with allergy and autoimmune disease as well as with helminth immunity (Kaplan, 2013; Licona-Limón et al, 2017). Cytokines from activated Th cells can also differentiate CD8+ T cells to cytotoxic T cells (CTLs). However, CTLs are mostly associated with intracellular infection and assumed to play a little role at GI helminth infection (Sorobetea et al, 2018).

The Th1, Th2 and Th17 cells seem to have their innate lymphoid cell (ILC) counterparts referred to as ILC1s, ILC2s or ILC3s (reviewed by Vivier et al, 2018; Panda & Colonna, 2019). These cells lack antigen-specific receptors but are activated via receptors for alarmins, neurotransmittors and components from nutrients and microorganisms. The type 2 innate lymphoid

cells (ILC2s) have emerged as a major producer of type 2 cytokines, predominantly IL-5 and IL-13, in response to GI helminths (Neill et al, 2010; Price et al, 2010). In contrast to Th2 cells, ILC2s do not require antigen presentation, but are rapidly activated by cytokines and DAMPs, predominantly by the alarmins IL-33, IL-25 and TSLP released from parasite-infected tissue. In response to the alarmins, ILC2s produce IL-5 and IL-13 that support recruitment of eosinophils, Th2 cell differentiation and tissue repair (Lloyd & Snelgrove, 2018). Moreover, ILCs express MHCII and can interact with naïve T cells *in vitro* and promote Th2 polarization *in vivo* (Mirchandani et at, 2014; Oliphant et al, 2014). The maintenance of ILC2s is further supported by IL-2 secreted by Th2 cells suggesting a mutual cooperation between Th cells and ILC2s during the immune response (Mirchandani et at, 2014). Thus, ILC2s are not only important for initiating local responses; they also seem to play a central part in bridging the innate and adaptive immune response together to support development and maintenance of a Th2 response.

Another link between the innate and adaptive immune response seem to be formed by the gamma-delta T cells (γ/δ T) that shape the cytokine profile according to the type of infectious agent they encounter (Ferrik et al, 1995). The γ/δ T cells are rarely found in lymphoid organs. Instead, they reside in barrier surfaces of many peripheral tissues, such as the intestinal mucosa, where they play a key role in immune surveillance (Suzuki et al, 2020). A role for γ/δ T cells at helminth infection was demonstrated by Inagaki-Ohara et al (2011) showing that the γ/δ T cells are an important source of IL-13, necessary for expulsion of the murine GI helminth *Nippostrongylus brasiliensis*.

Both ILCs and γ/δ T cells are mainly residing in the mucosal tissue. In fact, the intestine harbor the largest population of immune cells anywhere in the body (as reviewed in humans by Mörbe et al, 2021). Resident immune cells are scattered in the lamina propria and epithelium or reside in gut associated lymphoid tissues (GALT). The GALT includes infiltrates of the lamina propria, isolated lymphoid follicles, Peyer's patches and mesenteric lymph nodes. The Peyer's patches are organized clusters of lymphoid follicles that are important sites for initiation of antigen-specific immune responses. The epithelial monolayer lining the lymphoid follicles differ from

the normal epithelium that it consists of enterocytes interspersed with specialized epithelial cells called microfold (M) cells. A limited presence of mucus secreting cells, like goblet cells, makes the follicle-associated epithelium directly exposed to luminal contents. In the absence of damage to the epithelium, the M cells serve as a gateway for antigen transport from the intestinal lumen to the lymphoid follicle. Luminal antigens can also be captured via other routes, e.g. through internalization in goblet cells (McDole et al, 2012) or by transepithelial macrophages or DCs (Rescigno et al, 2001).

In horses, the mucosal associated lymphoid tissue (MALT) has been less studied in the gut compared to the respiratory tract. Unique for horses, as well as for some ruminants and pigs, is the long oval shape of the Peyer's patches in the small intestine. In the horse, the Peyer's patches develop during gestation, reaching a size of 20-35 cm in newborn foals. The size of the Peyer's patches increases with age and remain in constant numbers for the first couple of years before gradually disappearing in the elderly horse (Liebler-Tenorio & Pabst, 2006).

2.1.5 Equine PBMC

Peripheral blood mononuclear cells (PBMC) are commonly used to assess immune capacity in human and veterinary medicine. Isolation of PBMCs by density gradient centrifugation generates a mix of immune cells including a low proportion of monocytes, DCs, ILCs and NK cells, the majority being T and B-lymphocytes and thus reflecting aspects of a systemic immune response. In parasitological research, ovine and caprine PBMCs have been used to study immune reactions to worm antigens (e.g. Wang et al, 2019; Jacobs et al, 2020; Naqvi et al, 2020) and equine PBMCs isolated from *S. vulgaris* infected horses were used to study the re-call response to *S. vulgaris* antigens (Dennis et al, 1993).

Several reagents for phenotyping of equine PBMC (eqPBMC) are available but overall the reagent toolbox is very limited compared to both mice, humans and many other animal species (Kydd et al, 1994; Lunn et al, 1998; Flaminio et al 2007; Ibrahim et al, 2007). Notably, several important T-cell markers e.g. discriminating α/βTCR and γ/δTCR, are missing. However, transcriptional analysis of both α/βTCR and γ/δTCR have

previously identified these cells in eqPBMCs (Tschetter et al, 1997). For equine T cells, only antibodies to equine CD3, CD4, CD8a and CD8b have been developed (Lunn et al, 1998). All three DC subsets, DC1, DC2 and plasmacytoid DCs (pDCs) have previously been confirmed in eqPBMC where pDCs were shown to produce large amounts of IFN-α upon TLR9 activation (Ziegler et al, 2016).

As an alternative, cytokine profiles elicited at antigen exposure can be used to characterize the T cell response. Such functional studies of eqPBMC are at large comparable to other mammals, as demonstrated by the gene- and microRNA profiles induced by lipopolysaccharide from *E. coli* in eqPBMC (Pacholewska et al, 2017; Parkinsson et al, 2017). However, there seem to be some species-specific differences in the reactivity to TLR stimulation making it problematic to translate results obtained from other species directly to the horse (Figueiredo et al, 2009; Wattrang et al, 2012; Vendrig et al, 2013; Pacholewska et al, 2017).

Recently, the cellular landscape of eqPBMC was mapped using a single cell RNA sequencing approach (Patel et al, 2021). This transcriptional profiling of CD3+ lymphocytes confirmed different clusters of CD4+ and/or CD8+ expressing cells as well as indicated the presence of equine γ/δ T cells and NK cells (Patel et al, 2021). Unexpectedly, the majority of B cells in the eqPBMCs were T-bet+ memory B cells. This is a B cell subpopulation that in humans has been associated with both protective and pathogenic immune responses (Knox et al, 2019), but the role of equine T-bet+ B cells remain to be elucidated. Moreover, the study demonstrated that several immune cell subpopulations, such as different subtypes of monocytes and DCs, were analogous to those described in human and mouse (Patel et al, 2021).

2.1.6 Effector mechanisms against GI helminths

A Th2 response results in physiological changes in the intestine characterized by increased gut contractility, goblet- and tuft cell hyperplasia, faster epithelial cell turnover and increased mucus production that together facilitate helminth expulsion from the intestinal lumen. This response is commonly referred to as the “weep and sweep response” (Sorobetea et al,

2018). Essential for initiation of this response are the until recently neglected tuft cells (Schneider et al, 2019) and the newly described ILC2s (Vivier et al, 2018). Tuft cells are epithelial cells that can sense unknown helminth PAMPs and respond with production of TSLP and IL-25. These cytokines, together with the alarmin IL-33 that are released by damaged epithelial cells, activate ILC2s resident in the underlying mucosa. In return, ILC2s produce large amounts of type 2 cytokines, such as IL-13, that further activates the tuft cells. Interaction of IL-13 with the IL-4α receptor affects stem cell differentiation and promotes tuft cell hyperplasia, thus creating a positive feedback loop between tuft cell-produced IL-25 and ILC2-produced IL-13 (von Moltke et al, 2015; Gerbe et al, 2016). Together with IL-4, mainly released by Th2 cells, IL-13 mediates goblet cell hyperplasia and increased mucus production important for helminth expulsion (Gerbe et al, 2016).

One of the early-recruited cell population that is a hallmark for helminth infection is the eosinophilic granulocyte. The presence of IL-5 can activate eosinophils to release granules containing a cocktail of toxic compounds. This degranulation can however be mediated through multiple pathways such as PRR ligation, cytokine activation, or Fc receptor engagement (Makepeace et al, 2012). Similar mechanisms are effectuated by basophils and mast cells that release massive amounts of chemical mediators upon ligation of IgE to the high-affinity FcεRI. The role of mucosal mast cells and IgE in host protection against GI helminths has however been debated as not all experimental models demonstrate a protective role for mast cells (reviewed by Grencis, 2015). Regardless, mast cells, basophils and eosinophils are early sources of IL-4 at helminth infection making them important for initiating and maintaining Th2 responses (Anthony et al, 2007; Makepeace et al, 2012).

Helminth infection are also known to cause differentiation of “alternatively activated macrophages” (AAMs). These AAMs contribute to helminth resistance by controlling pro-inflammatory- and Th1 reactions, promoting Th2 responses and supporting wound healing (Kreider et al, 2007). In addition, AAMs can together with neutrophils, DCs and Th2 cells contribute to the formation of granulomas, shown to be essential for protection against the murine GI nematode *H. polygyrus* (Anthony et al,

2006). However, granuloma formation enables the parasite to "hide" and can thereby contribute to persistence of the infection.

The production of excretory/secretory (ES) products also enables helminths to interfere with the host's immune response creating a skewed immune phenotype unable to clear the infection. ES products can be both by-products from the helminth's metabolism or actively secreted products that facilitate the helminth's establishment and survival in the infected tissue. ES products comes in various forms and can consist of various types of molecules, commonly proteins, lipids or glycoproteins. Most studied are the protein modulators, which are known to act on every phase of the immune response, from antigen recognition to effector mechanisms. Many ES proteins enhance Treg responses, skew the expression of Th polarizing cytokines or reduce the Th proliferation (reviewed by Maizels et al 2018). The recent discovery of helminth excreted extracellular vesicles (EVs) has further expanded the repertoire of immune modulatory ES products. The excretion of EVs provide an opportunity for helminths to deliver fragile material into host cells, often micro RNA, (miRNA) that e.g. can interfere with expression of immune related genes (Tritten & Geary, 2018). Several studies have demonstrated that induction of an antibody response that block ES proteins results in a higher expulsion rate and/or reduced egg burden (Maizels et al, 2018). Accordingly, EVs have been used as antigen in experimental vaccines against the murine nematodes *Heligosomoides polygyrus* and *Trichuris muris*, and the trematodes *Opisthorchis viverrini* and *Echinostoma caproni* generating EV-specific antibodies and reduced egg burden after challenge (Trelis et al, 2016; Coakley et al, 2017; Shears et al, 2018; Chaiyadet et al, 2019).

2.2 *Strongylus vulgaris*

Strongylus vulgaris is considered to be the most pathogenic parasite of horses. This parasite belongs to the superfamily Strongyloidea containing equine nematodes of the intestinal tract, including *Cyathostominae spp*. (small strongyles), *Strongylus edentatus* and *Strongylus equinus* (Nielsen & Reinemeyer, 2018). The members of this superfamily are alike in several aspects, e.g. they share a similar morphology, they have a direct life cycle,

the infective stage is the L3 stage and the only method to differentiate between the species is through fecal culture and subsequent morphological assessment of the larvae or by real-time PCR (Nielsen et al, 2008; Kaspar et al, 2017). Moreover, the cyathostomins are highly abundant in the horse population resulting in that *S. vulgaris* most often occurs in co-infection with cyathostomins.

2.2.1 Life cycle

The life cycle of *S. vulgaris* consists of a free-living and a parasitic phase (Figure 1). Eggs are shed on the pasture through faeces of infected horses. First stage larvae (L1) hatches from the eggs, develop to L2 and then to infective L3 stage larvae. At the free-living stage, the L3 are encapsulated in their L2 cuticle that serves as a protection against environmental conditions. When a horse ingests L3, the L2 cuticle is cast off in the alimentary tract, in a process called exsheatment. The exsheated L3s (exL3) penetrate the mucosa and submucosa of the distal small intestine, cecum and/or colon. After two to three days in the submucosa, the exL3 moults into a fourth stage (L4). The L4 stage of *S. vulgaris* penetrates the arterioles in the submucosa, reaches the mesenteric arteries and continue their migration through the arterial system, against the blood flow, until reaching the cranial mesenteric artery about 14 days post infection. There, the L4s continue to grow for three to four months before they molt into the fifth stage (L5) that finally migrates back to the large intestine. When the pre-adult L5 larvae reaches the large intestine, the larvae are encapsulated in small nodules in the intestinal wall. Eventually these nodules rupture to release adult larvae into the intestinal lumen where the larvae need six to eight more weeks to sexually mature and produce eggs. Together, these extensive migrations can take between 6-7 months (McGraw & Slocombe, 1976). This long pre-patent period complicates the diagnosis of *S. vulgaris* infection as most available diagnostic tests for *S. vulgaris* infection are based on cultivation of L3 from excreted eggs (Andersen et al, 2013a).

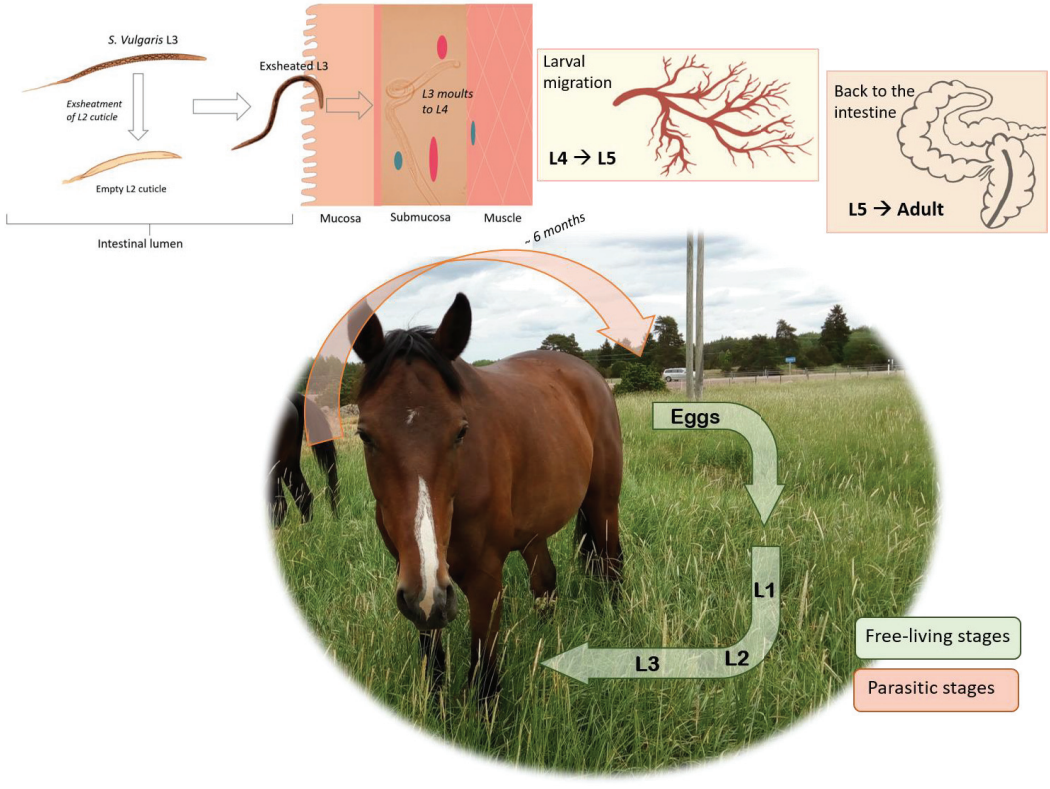


Figure 1. Life cycle of *S. vulgaris*.

2.2.2 Pathogenesis

The pathology of *S. vulgaris* is related to the larval migration and has been described in detail by Duncan & Pirie (1975). Notably, already at two days post infection haemorrhagic foci can be observed in the intestinal mucosa. After a few days more, a local inflammatory response occurs in the submucosa. This response is characterised by infiltration of neutrophils, lymphocytes, macrophages, and eosinophils and is coincident with L4 maturation. During the next following weeks, signs of infarction, thrombosis and thickening of the mesenteric arteries can be observed. At this early phase of infection, tortuous fibrin tracts are found in the intimal space of the arteries. The late L4 and L5 stages are associated with lesions and clot

formation in the cranial mesenteric and ileo-cecal-colic arteries (Duncan & Pirie, 1975). Thus, the larval migration of *S. vulgaris* can cause severe damage to the blood vessels supplying the intestine, potentially resulting in life-threatening non-strangulating intestinal infarction (Pihl et al, 2018).

2.2.3 Immune response

Early studies show that the immune response against migratory *S. vulgaris* larvae is characterized by recruitment and activation of eosinophils and neutrophils (Turk & Klei, 1984; Dennis et al, 1992; Dennis et al, 1993; Monahan et al, 1994) as well as production of antibodies (Klei et al, 1983; Swiderski et al, 1999). Increased levels of IgM, IgGa, IgGb, IgG(T) and IgA have been recorded at *S. vulgaris* infection (Swiderski et al, 1999). Out of these, IgGa, IgG(T) and IgA have been associated with immune protection (Swiderski et al, 1999; Edmonds, 2001).

Several early studies especially highlighted eosinophils as very important for *S. vulgaris* resistance. This activation was demonstrated by a higher frequency of eosinophils expressing Fc and complement receptors in *S. vulgaris* infected horses compared to that in uninfected horses (Dennis et al, 1988). Another study showed that eosinophils, but not neutrophils, from *S. vulgaris* infected ponies were able to kill *S. vulgaris* larvae in an antibody dependent manner *in vitro* (Klei et al, 1992). In addition, cytokines chemotactic for eosinophils were found in supernatants from cultures of PBMC obtained from *S. vulgaris* immune ponies, but not in cultures of PBMC obtained from naïve ponies, at *in vitro* re-stimulation (Dennis et al, 1993). In accordance, gene expression analysis of cytokines in PBMC from experimentally infected horses revealed an up-regulation of IL-5 two weeks post infection (Swiderski et al, 1999).

2.2.4 Vaccination trials

In the 1980s and 90s, experimental immunizations using attenuated *S. vulgaris* L3 were conducted by researchers at the University of Kentucky, USA. *S. vulgaris* L3 were attenuated using Cobalt^{60} radiation and different doses were tested on pony foals by oral inoculations (Klei et al, 1982). Overall, immunization with radiation-attenuated *S. vulgaris* L3 reduced both the larval burdens and clinical signs at challenge infections (Klei et al, 1982; Monahan et al, 1994; Swiderski et al, 1999). The immunized ponies did not

develop thrombosis or arteritis but instead showed signs of periportal fibrosis in the intestinal submucosa indicating that the protective immune response was directed against the late L3 or L4 stage of larval development. Intramuscular injection of crude soluble homogenates from larval (L3 and L4) preparations together with a Ribi adjuvant induced higher pre-challenge antibody titres compared to the recipients of attenuated larvae, but did not generate immune protection. On the contrary, parental immunization with somatic extracts seemed to sensitize these ponies resulting in a heightened response to challenge (Monahan et al, 1994).

The partial protection from oral immunization with attenuated L3s was associated with activation of eosinophils and production of *S. vulgaris*-specific antibodies reinforcing that this combined response is important for protection (Dennis et al, 1988; Klei et al, 1992; Monahan et al, 1994). The levels of antibodies specific for a soluble adult worm antigen (SAWA) from *S. vulgaris*, was estimated in vaccinates and non-vaccinates following challenge (Dennis et al, 1992). Both vaccinates and non-vaccinates produced similar levels of SAWA-specific IgM, IgGa and IgGb while only the vaccinates produced IgG(T) following challenge. In addition, the levels of IgA increased in both experimental groups after challenge but reached higher levels in the non-vaccinates. Transcriptional analysis of cytokine genes indicated an increase in IL-4 and IL-5 and a decrease in IL-2 and IFN-γ expression in the vaccinated group following challenge. A similar response was recorded for non-vaccinates but appeared to be temporarily delayed compared to vaccinated ponies (Swiderski et al, 1999).

Despite these promising results, the emergence of efficient and easily administrated anthelminthic drugs in the 1980-90s made development of parasite vaccines less prioritized, resulting in that the *S. vulgaris* vaccine project in Kentucky ceased in the late 90s and changed focus to improve the diagnostics of *S. vulgaris*.

2.2.5 SvSXP

For the purpose of developing an immunodiagnostic assay indicating prepatent *S. vulgaris* infection, a protein expressed by migrating *S. vulgaris* larvae, designated SvSXP, was identified (Andersen et al, 2013b). By screening a larval *S. vulgaris* cDNA library using hyperimmune serum raised

against *S. vulgaris* ES antigens, SvSXP was identified as the only positive clone, indicating that SvSXP is an immunodominant protein (Andersen et al, 2013a; 2013b). Sequence analysis revealed that SvSXP is homologous to the SXP/RAL-2 group of proteins that exists in a broad range of nematodes. Interestingly, some of the SXP/RAL-2 protein family members have been evaluated as vaccine candidates against several nematode species, such as *Ascaris suum*, *Brugia malayi* and *Ancylostoma caninum* (Wang et al, 1997; Tsuji et al, 2001; Fujiwara et al, 2007; Wei et al, 2017).

A recombinant SvSXP protein is currently used as antigen in an enzyme-linked immunosorbent assay (ELISA) to measure SvSXP-specific IgG(T) antibodies in the bloodstream of horses (Andersen et al, 2013b; Nielsen et al, 2014; Nielsen et al, 2015; Hedberg-Alm et al, 2020). In a cohort of naturally infected 2-year-old horses, ELISA-positive IgG(T) titers lasted up to 5 months after anthelminthic treatment (Nielsen et al, 2015). In foals, positive ELISA values were not obtained until 3-5 months of age, indicating a protective role of maternal antibodies (Nielsen et al, 2014).

Except for its potential in diagnostics, the immunogenicity of SvSXP makes it an interesting candidate to explore as a vaccine antigen. Within this thesis project, studies on immunomodulatory effects of the recombinant SvSXP protein are initiated (Addendum) along with studies on a novel saponin adjuvant formulation.

2.3 Saponin adjuvants

Adjuvants are components capable of enhancing and/or shaping antigen-specific immune responses. Traditionally, vaccines have been composed of live attenuated or inactivated microorganisms, which by their heterogeneous structure naturally contain both antigen epitopes and multiple PAMPs that activate the immune system. However, many modern vaccines built on purified protein antigens lack these inherent adjuvant properties, making them less immunogenic. Therefore, protein or subunit vaccines need to be formulated with an adjuvant to induce an immune response that is potent enough to give a long-lasting protection (Reed et al, 2013). Except for strengthening the immunogenicity of an antigen, adjuvants can also be used

to broaden the immune profile, e.g. against viruses with high antigenic drift, or to counteract undesirable immune modulatory capacities, e.g. by parasite antigens (Reed et al, 2013; Zawawi & Else 2020).

In veterinary medicine, several types of vaccine adjuvants are used. These includes aluminium salts, emulsions, carbomers, liposomes and immune stimulating complexes (ISCOMs), that all have different compositions, shapes and mode of actions. Some mainly serve as delivery agents (e.g. emulsions) while others also have immune stimulatory properties (e.g. ISCOMs). In some vaccines, adjuvants are combined with PRR agonists that, through induction of cytokines, help guide the immune response in a desired direction (Reed et al, 2013; Burakova et al, 2017). In the present thesis, the immunomodulatory effects of a novel particulate adjuvant referred to as "G3" was evaluated *in vitro* using eqPBMC. The cytokine inducing capacity of G3, alone or together with TLR-agonists or *S. vulgaris* larvae, was assessed using qPCR and ELISA (paper I and II). The G3 adjuvant is a descendant to the ISCOMs, which is why I will give a brief description of ISCOMs as well as G3.

2.3.1 ISCOMs

The original ISCOM consisted of purified *Quillaja* saponin (Quil A), cholesterol, phospholipids and viral envelope proteins that were formulated into three-dimensional 40 nm cage-like structures (Morein et al, 1984). This composition was one of the first vaccine formulations that delivered a subunit protein antigen to the cytosol with subsequent induction of CTLs (Jones et al, 1988; Takahashi et al, 1990). Some years later, it was demonstrated that the antigen does not need to be physically integrated in the ISCOM particle but can simply be mixed with the ISCOM-Matrix before administration (Lövgren-Bengtsson & Sjölander, 1996). The immune modulatory capacity of the ISCOM-Matrix was fine-tuned by varying the ratios of the purified saponin subfractions QHA and QHC generating MatrixA and MatrixC, respectively, and was manufactured as Matrix-M™ by Isconova AB, Uppsala. The combination of these immune-stimulatory components with a structure and size optimal for antigen delivery is known to induce a balanced Th1/Th2 response with long-lasting antibody production (Lövgren Bengtsson et al., 2011). Matrix-M is currently used in

a number of veterinary vaccines, including the equine Influenza vaccine (Equilis® Prequenza), and in the recently licensed vaccine against Strangles (Strangvac) from Intervacc AB, Stockholm.

The adjuvant technology of Matrix-M is today hold by the US company Novavax (https://www.novavax.com/our-unique-technology#matrix-m-adjuvant-technology) and applied in phase 3 studies of experimental vaccines against seasonal influenza, Respiratory Syncytial Virus and Coronavirus (https://www.novavax.com/our-pipeline). The Swedish government has recently signed a pre-purchase agreement with Novavax for 2.2 million doses of their subunit COVID19 vaccine (NVX-CoV2373).

A later adaptation of the ISCOM-technology is the "third generation formula", here referred to as the G3 adjuvant. The G3 adjuvant has been supplied by MoreinX AB (Uppsala, Sweden) as Adjuvaq-V100^{TM} but is now licensed to Brenntag Biosector A/S (Frederikssund, Denmark) that has marketed the product as NanoQuil® (Croda Denmark A/S, Frederiksund, Denmark). The G3 adjuvant formula resembles that of ISCOMs but differ in that it lacks phospholipids, which makes it easy to produce at a lower cost. The G3 nanoparticle is around 20 nm and has a high bioavailability that makes it useful for both oral and parental administration (Patent no. WO 2013/051994). The G3 formula allows for incorporation of other immune stimulating molecules making it possible to "tailor-make" the vaccine formula to induce a desired immune response. This versatility was demonstrated by incorporation of a diterpene in the G3 formula, which enhanced and broadened the immune response to H1N1 influenza virus strains in mice (van de Sandt et al, 2014). Immunization trials in mice combining influenza virus antigens with either Adjuvaq-V100 or aluminum hydroxide resulted in comparable levels of antigen specific IgG1 (Th2 associated subclass) but substantially higher level of antigen specific IgG2a (Th1 associated subclass) compared to alum (Hjertner et al, 2018a). A similar Th1 profile was recorded in cultures of porcine PBMCs after *in vitro* exposure to the G3 adjuvant (Hjertner et al, 2018b).

2.4 Modelling the equine intestine

To allow *in vitro* studies of helminth antigens in combination with adjuvant formulations, improved culture systems reflecting the natural route of infection are urgently needed. At present, there is a lack of such *in vitro/ex vivo* models that can more closely replicate the interactions of parasites with its host at mucosal surfaces. In this context, stem cell-derived organoid cultures has emerged as a new *ex vivo* tool that can potentially be used to study host-parasite interactions (Duque-Correa et al, 2020). In addition, methods to co-culture immune cells with organoid cells are starting to emerge (Noel et al, 2017; Spalinger et al, 2020; Staab et al, 2020). In the present thesis, I have used equine intestinal organoids (paper III) to study reactions to the G3 adjuvant, TLR-agonists and preparations of *S. vulgaris* larvae *ex vivo*.

2.4.1 The equine intestine

Compared to other herbivore species, the horse's GI tract is unique by its highly developed large hindgut. The major function of the horse's colon and caecum is to ferment dietary fibers from forage, which is dependent on an active and well-functioning microflora with fibrolytic activity (Costa & Weese, 2012). External factors such as GI nematode infection, dietary changes or antibiotic treatment can lead to microbial dysbiosis (Peachey et al, 2019; Collinet et al, 2021a, 2021b) likely affecting the local immune response. For example, treatment with a broad-spectrum antibiotic resulted in loss of commensals and bloom of potential pathobionts affecting immune homeostasis as shown by increased concentrations of sIgA in feces and decreased TNF-α levels in serum samples (Collinet et al, 2021a).

The intestinal epithelium in most species express a variety of PRRs enabling recognition of both commensal and pathogenic microorganisms (Andrews et al, 2018). However, studies on the expression of PRRs in the equine intestine is limited (Vendrig & Fink-Gremmels, 2012; Werners & Bryant, 2012). Expression of TLR4, along with the co-receptors MD-2 and CD14 has been tested and confirmed in tissue sections from equine ileum, dorsal colon and rectum (Fossum et al, 2012; Olofsson et al, 2015). In the same study, cytokine expression of IL-12p40, IL-17A and IL-23p19 was

recorded in seven tissue sections of the equine intestine, from duodenum to rectum, as well as in rectum biopsies from both healthy horses and horses with clinical signs of equine inflammatory bowel disease (Hjertner et al, 2013; Olofsson et al, 2015). Furthermore, the cytokine expression patterns in the equine intestinal mucosa at cyathostomin infection has been evaluated in tissue sections from the large intestine (Davidson et al, 2002, 2005; Steuer et al, 2020).

2.4.2 The intestinal epithelium

The lack of equine specific reagents has limited detection of different cell lineages present in the equine small and large intestine. However, each known type of epithelial cell in the small intestine has also been identified in equine intestinal tissue using cross-reactive antibodies or transmission electron microscopic imaging (Gonzales et al, 2015).

The intestinal mucosal surface is composed of a single layer of columnar epithelial cells overlain by a mucus layer that physically separates the epithelium from direct contact with the microbiota. The epithelial cells are linked by tight junctions, adherence junctions and desmosomes that regulate barrier integrity and permeability. Most cells of the intestinal epithelium are involved in water and nutrient absorption but some are also important for immune regulation. The epithelium of the small intestine contains regular small cavities, called crypts of Lieberkühn, which contains stem cells interspersed by Paneth cells. The small intestine further contains villi that protrude from the epithelia to maximize absorption of nutrients. As the stem cells divide, progenitor cells progress through the crypt-villus axis and eventually differentiate to either absorptive enterocytes (dominant cell type) or to more specialized cell types, such as enteroendocrine-, goblet- or tuft cells (Gonzales et al, 2015). When they reach the tip of the villi, normally after 5-6 days, the cells go into a form of detachment-induced cell death referred to as anoikis (Figure 2).

The epithelial turnover requires a balanced regulation between cellular proliferation and cellular losses to remain in homeostasis. When the epithelial integrity is challenged, e.g. by invading parasites, the rate of epithelial turnover can however change. In fact, most GI helminths disrupts

the epithelial barrier at some point during infection by mechanisms not well understood for most helminth species. As a result, the intestinal integrity becomes impaired causing an increase in intestinal permeability. To compensate, the speed of epithelial turnover often increases at helminth infection (McKay et al, 2017).

2.4.3 Intestinal organoids

Organoids are stem cell-derived three-dimensional cultures that resembles the architecture and cell composition of the organ of origin. Small intestinal organoids, referred to as enteroids, were first generated from murine crypts cultured in the presence of intestinal niche growth factors supported by extracellular matrix components (Sato et al, 2009). Since then, the enteroid technology has become standardized in mice and humans, and are now beginning to be explored for veterinary species, including the horse (Bartfield, 2016; Steiler Stewart et al, 2017; Kar et al, 2021). The culture system utilizes the inherent ability of the intestinal epithelia to self-renew and differentiate to specialized intestinal cell types. Accordingly, enteroids contain most cell lineages of the small intestine, such as stem cells, enterocytes, enteroendocrine cells, goblet cells, paneth cells and tuft cells, reflecting important aspects of the original architecture and function of the intestinal epithelia (Figure 2). Enteroid cultures have a wide range of applications and have been used to model gastrointestinal infections, gut-microbiota interactions and inflammatory diseases (Bartfield, 2016).

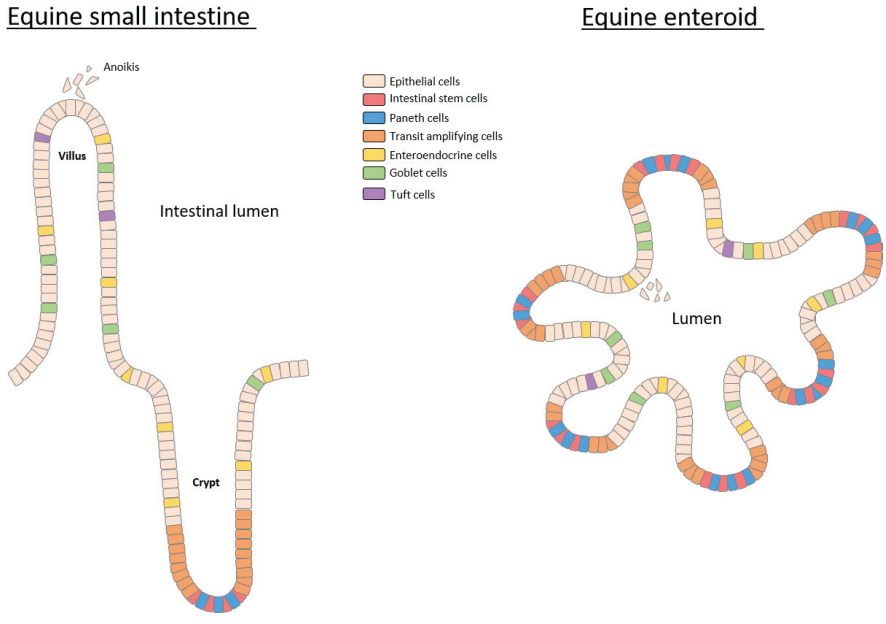


Figure 2. Illustration of the epithelium in the equine small intestine and enteroid cultures. The composition of cell lineages and their distribution along the epithelium of the small intestine are reflected in the equine enteroids.

One limitation with the 3D enteroid model is its enclosed morphology, with the apical surface of the epithelia facing inwards and the basolateral surface facing outwards. This makes it difficult to access the enteroid lumen and expose the apical surface to experimental treatments. This is especially problematic when it comes to large complex helminths that, depending on the species and life cycle stage, simply cannot fit into the 3D enteroid. However, there are several strategies to gain access to the apical surface of the epithelum, e.g. by microinjection, “inside-out enteroids” or enteroid-derived 2D monolayers, as recently demonstrated in studies of enteropathogenic bacteria (Co et al, 2019; Geiser et al, 2021; Samperio Ventayol et al, 2021). In fact, most infection studies using enteroids are on bacteria, viruses or protozoa, and very little is published on helminths (Duque-Correa et al, 2020). A few studies have used enteroids to study

epithelial responses to ES products (Eichenberger et al, 2018; Chandra et al, 2019; Luo et al, 2019) and some studies have indirectly investigated the effect of helminth-induced immune responses on the intestinal epithelium. For example, the role of IL-13 in tuft cell activation was partly demonstrated in murine enteroids (von Moltke et al, 2016; Luo et al, 2019). To my knowledge, there is currently only one paper describing direct interactions of living helminths with enteroid cells (Smith et al, 2021). However, new adaptations of enteroid culture methods such as enteroid-derived 2D monolayers or inside-out enteroids open up for future studies of host-helminth interactions. In addition, methods to co-culture enteroid cells with immune cells allows for studies of cross-communication between different cell populations adding on new dimensions to the enteroid model system (Noel et al, 2017; Spalinger et al, 2020; Staab et al, 2020).

3. Aim and objectives

To develop a vaccine against *S. vulgaris*, it is essential to understand how the infective stage larvae interacts with its host. To balance possible immunomodulatory capabilities of helminth antigens, it might be necessary to define appropriate adjuvant formulations. The overall aim of this thesis was therefore to set up *ex vivo* models and establish methods to study immune responses against *S. vulgaris* in the horse.

The specific objectives were to:

- Establish methods to generate defined larval stages of *S. vulgaris*
- Map the cytokine response to these larval stages
- Map the cytokine response to a recombinant protein from *S. vulgaris* (SvSXP)
- Evaluate innate immune responses to the G3 adjuvant
- Establish methods to generate cultures of equine enteroids and enteroid-derived 2D monolayers
- Apply these enteroid models to study host-parasite interactions at the site of infection
- Study how the infective stage of *S. vulgaris* affects the intestinal epithelium in enteroid-derived 2D monolayers, alone or in co-culture with eqPBMC

4. Comments on material and methods

This thesis was built upon *ex vivo* experiments using larval cultures and equine primary cells. Here, I will comment on the methods used. For detailed descriptions, I refer to the individual papers I-III.

4.1 Blood donor horses

Blood was collected from Swedish warmblood horses (Paper I, n = 12; Paper II, n =11 and Addendum, n = 10) housed at the Department of Clinical Sciences at SLU or in private stables. These mares and geldings, aged between 5 and 21 years, were regularly vaccinated against tetanus and influenza and regarded as healthy by their owners. Parasite diagnostics for all horses were performed every spring. This included tapeworm and strongyle egg counts (EPG) followed by larval culture or qPCR for detection of *S. vulgaris*. Horses with an EPG >200 and/or confirmed as *S. vulgaris* positive were treated with ivermectin. The blood sampling was approved by the Ethical Committee for Animal Experiments, Uppsala, Sweden (C.214813/14; 5.8.18-15533/2018 and 5.8.18-08784/2020).

4.2 Parasite material

Fecal samples collected from private horses with a diagnosed *S. vulgaris* infection were kindly provided from other projects on *S. vulgaris* (Tydén et al, 2019; Hedberg Alm et al, 2020). Since *S. vulgaris* almost always occur in co-infection with cyathostomins, horses with a high proportion of *S. vulgaris* as determined by microscopy or diagnostic qPCR were selected. From these

fecal samples, third stage larvae (L3) were generated by coproculture and collected using the inverted petri-dish method that is routinely used at diagnosing equine strongyles (Van Wyk & Mayhew, 2013). All *S. vulgaris* L3s were identified morphologically according to Bevilaqua et al (1993). In addition, *S. vulgaris* DNA was confirmed by qPCR analysis of samples collected from the same larval batches (National Veterinary Institute, Uppsala, Sweden). To obtain single species samples, *S. vulgaris* L3s were collected under the microscope using a 10 µL pipette. The L3s were stored in tap water at +4 ºC until use. In our hands, the cyathostomin and *S. vulgaris* L3s could be preserved in water for up to 6 and 10 months, respectively, as the L3s at this stage still are covered by their protective L2 cuticle and relying on stored nutrients.

4.3 *S. vulgaris* larval cultures

To study immune reactions to the early larval stages of *S. vulgaris*, a protocol for molting of *S. vulgaris* from L3 to L4 (Chapman et al, 1994) was adopted and slightly modified. Since the L3s were generated from fecal cultures, the larvae were decontaminated by incubation in PBS containing antibiotics, antifungals and the endotoxin-neutralizing agent polymyxin B. Bacterial cultures and endotoxin tests showed no microbial growth and less than 0.70 IU/mL endotoxin after 24 h decontamination. Despite the low level of endotoxin in the L3 preparations, parallel cultures with PBMCs exposed to 1 IU endotoxin/mL were included as controls (paper I).

When indicated, the L2 cuticle was exsheated from the L3s by incubation in sodium hypochlorite (detailed in Paper I). Without its protective L2 cuticle, the exsheated L3s (exL3s) became more fragile and sensitive to the environment and care was taken to ensure that the exL3s were put into culture immediately after exsheatment. The exL3s were cultured in an optimized medium “cKW2” known to trigger L3 development and molting to the L4 stage (Chapman et al, 1994). As noted by Farrar & Klei (1985), the morphology of the late exL3 is almost identical to the early L4, making it difficult to distinguish between the two larval stages before and after molting. However, all larvae molted almost simultaneously at day 5 of culture. The cuticle release progressed over several hours with cuticle casts remaining visible in the culture medium for at least 24 h after shedding.

Therefore, molted larvae devoid of their L2 and L3 cuticle are in the present thesis referred to as "*S. vulgaris* L4" according to previous definitions (Farrar & Klei, 1985; Chapman et al, 1994; Monahan et al, 1994). The procedure for generation of L3, exL3 and L4 stages of *S. vulgaris* is illustrated in figure 3.

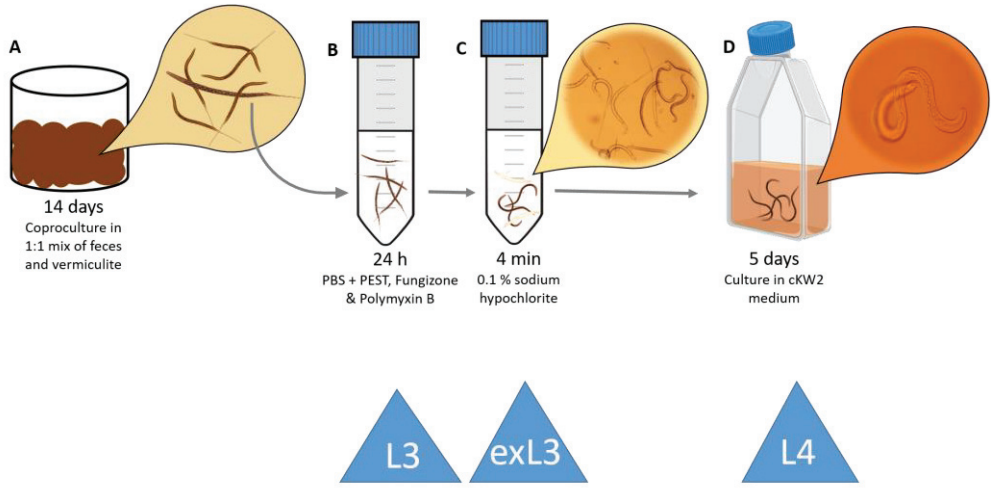


Figure 3. Scheme for generating different larval stages of *S. vulgaris*. L3 larvae were generated from fecal cultures (**A**) and decontaminated in PBS containing antimicrobials and antimycotics (**B**). Decontaminated L3s were exsheated in hypochlorite to obtain exL3s (**C**). ExL3s were cultured in cKW2 medium to trigger molting to L4 (**D**).

To evaluate the cytokine response to surface antigens on the three larval stages of *S. vulgaris*, a procedure for attenuation by UV irradiation of larvae was set up (Paper I). Due to the limited access to *S. vulgaris* larvae, cyathostomin L3s were initially used to optimize these conditions (time and dose as specified in Paper I) giving intact but non-motile larvae. These conditions were then applied to the *S. vulgaris* L3, exL3 and L4s preparations.

4.4 Cultures of equine PBMC

4.4.1 Culture conditions

Cytokine induction by the G3 adjuvant, TLR agonists and attenuated *S. vulgaris* larvae (papers I and II) were studied in cultures of eqPBMCs. In addition, eqPBMCs were used in co-culture experiments with enteroid derived 2D monolayers cultured in transwell inserts (see 4.5.5). Before seeding the eqPBMcs, cell viability was estimated by counting in Trypan blue, and confirmed to be >95 %. Occasional staining with Türks reagent showed a minor contamination with erythrocytes. Cells to be harvested for transcriptional analysis (qPCR) were cultured in 6-well plates at a concentration of 5-10 x 10^6 cells/mL and cells used for protein quantification (ELISA) were cultured in round bottomed 96-well plates at a concentration of 4 x 10^6 cells/mL in agreement with previous studies using eqPBMCs (Wattrang et al, 2005, 2012; Olofsson et al, 2016). The periods of stimulation for eqPBMCs used in transcriptional studies and for protein quantification were 18-20 h and 48 h, respectively.

4.4.2 Cell induction experiments

Based on serial dilutions of the G3 adjuvant (Paper II), a concentration of 5 µg G3/mL was used throughout the Thesis and Addendum. To study effects of G3 in combination with other immune stimulatory compounds, the TLR5 agonist FliC and TLR2/1 agonist Pam3CSK4 were chosen. In other animal species, FliC and Pam3CSK4 enhance the immunogenicity of viral, bacterial and protozoan vaccines (Mizel & Bates, 2010; Jayakumar et al, 2011; Caproni et al, 2012). To date, only a few studies are available on effects of FliC and Pam3CSK4 in equine cells (Figueiredo et al, 2009; Leise et al 2010; Irvine et al, 2013; Kwon et al, 2011).

To study immune reactions to strongyle larvae, eqPBMC were exposed to UV attenuated *S. vulgaris* L3, exL3 and L4 as well as to cyathostomin L3s. The polysaccharide chitin, known to be abundant in parasitic nematodes, and suggested to play a key role in recruitment and activation of innate immune cells (Perrigoue et al, 2008), was included as a control (Paper I).

4.4.3 Set up of intracellular cytokine staining and cell surface phenotypic characterization of equine IFN-γ producing cells (Addendum)

Intracellular staining for IFN-γ was performed on eqPBMC cultured in the presence of 5 µg G3/mL, 0.5 µg Pam3CSK4/mL or in the combination of G3 and Pam3CSK4. EqPBMC grown in medium alone or in medium with 0.05 µg PMA/mL and 1 µg Ionomycin/mL were used as negative and positive controls, respectively. For each type of culture, approximately 1.5 x 10^6 eqPBMCs/mL were grown in 2 mL RPMI without phenol-red in non-stick tubes at culture conditions previously described (Papers I and II). Initially, the total culture time was varied from 24-48 h and two secretion blockers (Brefeldin A and Monensin) as well as the time for their inclusion was tested. From these experiments the conditions for the final phenotypic characterization of IFN-γ producing cells was determined as specified below and the positive control was changed to ConA (2.5 µg/mL) to facilitate the gating procedures.

The cultures were first incubated for 12 h at 37 º C and thereafter stimulated with 5 µg G3/mL and Pam3CSK4 0.5 µg/mL (G3+Pam), 2.5 µg/mL ConA or in plain growth medium for another 12 h. The secretion blocker Brefeldin A was included the whole stimulation time. After stimulation, the PBMCs were washed in staining buffer consisting of PBS without Mg^{2+} and Ca^{2+}, 1 % fetal calf serum (FCS), and 0.09 % sodium azide and stained with LIVE/DEAD Green (fixable dead cell stain kit, Invitrogen) and monoclonal antibodies to equine CD4, CD8α and CD8β (Table 1). Fixation and intracellular staining for equine IFN-γ (Table 1) were subsequently performed using the BD Cytofix/Cytoperm Plus Fixation/Permeabilization Kit according to the manufacturer's instructions.

Flow cytometry was performed using a BD FACSVerse™ (BD Biosciences), equipped with 488 nm blue, 633 nm red and 405 nm violet lasers and results were analyzed using the FACSDiva (BD Biosciences) software. Single-stained compensation controls and fluorescence minus one (FMO) negative controls were included in the assays. Samples were recorded for 5 min at a low flow rate. Titrations of all antibodies were performed to determine optimal labelling conditions prior to the experiment.

Table 1. Fluourchrome conjugated antibodies for phenotypic identification of equine IFN-γ producing cells

Antibody	Clone	Specificity	Fluorochrome	Company
CD4-PECy7	HB61A	Equine CD4	R-phycoerythrin-cyanine 7[a]	Kingfisher Biotech
CD8α-RPE	CVS21	α-chain of equine CD8	R-phycoerythrin[b]	BioRad
CD8β-PerCPCy5.5	HT14A	β-chain of equine CD8	Peridinin chlorophyll-cyanine 5.5[b]	Kingfisher Biotech
IFN-γ-PF647P	MT116	Equine IFN-γ	PromoFluor-647P[b]	Mabtech

[a] Fluorochrome conjugated using Lightning-Link® conjugation kits (Abcam) according to the manufacturer's protocol.
[b] Fluorochrome conjugated by manufacturer.

4.4.4 SvSXP

A recombinant *S. vulgaris* protein SvSXP expressed in *E. coli* was kindly provided by Assoc Prof Martin Nielsen at Gluck Equine Research Center, University of Kentucky, USA. Before use, the SvSXP protein was purified from endotoxin using Pierce high capacity endotoxin removal resin and measured using the Pierce LAL chromogenic endotoxin quantification kit (Life Technologies) according to the manufacturer's instruction. The purified SvSXP protein was thereafter used as re-call antigen in cultures of eqPBMC obtained from horses with a known history of previous exposure to *S. vulgaris* and in equine enteroid cultures. Various concentrations of recombinant SvSXP were co-cultured with eqPBMC in 6-well plates at the same conditions as described in Paper I and II. The cytokine inducing concentration of SvSXP was tested in four separate experiments using either 0.25, 0.5 and 0.75 µg SvSXP/mL or 1.0, 1.5 and 2.0 µg SvSXP/mL. The horses were categorized based on their records for the last four years regarding parasite burden (EPG and *S. vulgaris* detection as described in 4.1) provided by the horse owners. The "low exposure" group includes horses (n = 3) with only negative test results for *S. vulgaris*, the "previous confirmed" group includes horses (n = 5) previously tested positive one or multiple times but negative for *S. vulgaris* at the time of blood sampling, and the "confirmed" group includes horses (n = 3) positive for *S. vulgaris* at the time of sampling.

After 20 h exposure to SvSXP in PBMC obtained from horses in the various groups, the eqPBMC were analyzed for their expression of cytokine genes (IFN-γ, IL-1β, IL-2, IL-4, IL-5, IL-8, IL-9, IL-10, IL-13, IL-17A, IL-33 and TSLP) using methods described in paper I and II. This data set is far from complete, but preliminary results are given in Addendum (5.3.2) and referred to in the Discussion.

4.5 Enteroids

4.5.1 Generation and culture of equine enteroids

Equine enteroids were generated from crypts isolated from equine mid-jejunum (Figure 4). To avoid excessive cell death, tissue sections were immediately put in ice-cold PBS containing antimicrobials, antifungals and an apoptosis blocker (Rho kinase inhibitor Y27632) and processed within one hour of collection. Isolation of crypts and the subsequent culture were performed in accordance with a previously published protocol for equine enteroids (Steiler Stewart et al, 2018).

To enable three-dimensional growth, the crypts were cultured in Matrigel, which is a medium generated from mouse sarcoma cells containing extracellular matrix components such as laminin, collagen type IV, entactin/nidogen and heparin sulfate proteoglycans as well as a variety of growth factors (Kleinman et al, 1986; Kleinman & Martin, 2005). In the equine enteroids however, a Matrigel with low concentrations of growth factors (GFR membrane matrix, Corning) was used to generate a more defined culture medium. The growth factors included in the equine enteroid medium were Wnt3a, R-spondin, Noggin, EGF and CHIR99021 that stimulates the Wnt signaling pathway essential for cell proliferation and differentiation of intestinal epithelial cells. In addition, the Rho kinase inhibitor Y-27632 prevents anoikis induced cell death, the p38 inhibitor SB202190 prevents apoptosis as well as enhance proliferation and differentiation, and the TGF-β receptor I inhibitor LY2157299 reduce apoptosis and promotes stem cell survival (Merker et al, 2016). Together, these factors support initiation, propagation and long-term maintenance of enteroid cultures.

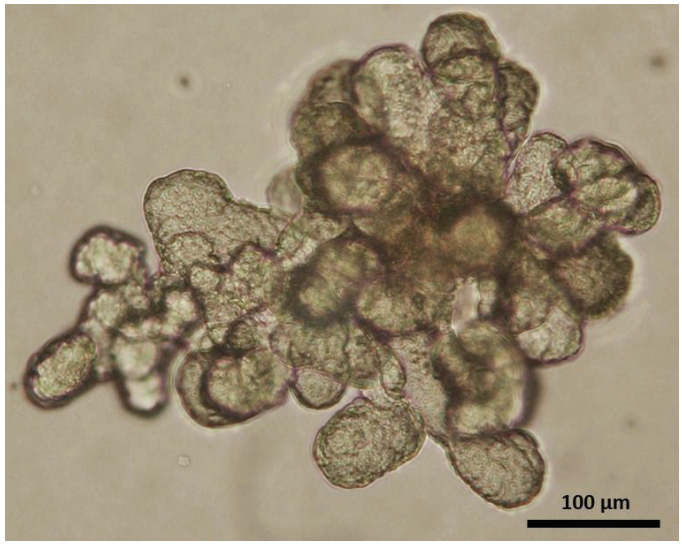


Figure 4. Equine enteroid after seven days of culture.

4.5.2 Establishment of enteroid-derived 2D monolayer cultures

To enable easy access to the apical surface of the epithelium, a protocol for two-dimensional cultures of porcine enteroids was adapted (van der Hee et al, 2018). To prevent detachment-induced anoikis, the wash medium was supplemented with Y-27632. The cell viability after washing was between 60-90 % in the different experiments, estimated by Trypan blue staining. The 2D monolayers were cultured on 24-well flat-bottomed culture plates or on 12-well transwell inserts. To facilitate cell attachment and monolayer growth, plates and inserts were pre-coated with Matrigel as previously elaborated in several studies (e.g. Liu et al, 2018; van der Hee et al, 2018; Altay et al, 2019).

The transwell culture system is built up by an upper and lower compartment separated by a permeable membrane for culture of epithelial monolayers (Figure 5b). In the following text I will refer to the upper compartment as the “apical surface” and the lower compartment as the

"basolateral surface". In these cultures, transepithelial electrical resistance (TEER) was monitored as a measurement of the barrier integrity.

4.5.3 Exposure of enteroid monolayers to microbial mimics

The same TLR-agonists used to stimulate eqPBMC (Paper II) were used to assess the functionality of the equine 2D monolayers (Paper III). The TLR agonists used were a recombinant flagellin protein from *Salmonella Typhimurium*, FliC (TLR5), lipopolysaccharide (LPS) from *E. coli* O111:B4 (TLR4), the synthetic lipopeptide Pam3CSK4 that mimics the acetylated aminoterminus of bacterial lipopeptides (TLR2/1) and Poly I:C, a double stranded RNA analogue (TLR3) mimicking viral infections. Together, these TLR agonists represent an array of microorganisms supposed to trigger immune-regulatory reactions in the intestinal epithelia (Andrews et al, 2018) and were thus considered appropriate to test the responsiveness of the equine monolayers.

4.5.4 Exposure of enteroid monolayers to *S. vulgaris* in the presence of Th polarizing cytokines

To study interactions of *S. vulgaris* with the intestinal epithelium under the influence of Th polarizing cytokines, the basolateral surface of enteroid monolayers cultured on transwell inserts were exposed to IL-4 and IL-13 or IFN-γ (equine yeast-derived recombinant proteins, Kingfisher Biotech, Inc). After 72 h, approximately 20 living exsheated *S. vulgaris* L3 larvae were added to the apical surface of each well and incubated for another 20 h (Figure 5c). As controls, *S. vulgaris* were added to the apical surface of enteroid monolayers not exposed to cytokines and enteroid monolayers were exposed to cytokines but not *S. vulgaris*. In parallel, monolayers cultured in plain growth medium were included as controls. All experimental treatments were performed in triplicates and pooled at harvest. The methods used for transwell culture, cell harvest, RNA isolation, cDNA synthesis and transcriptional analysis of cell lineage markers were performed as detailed in paper III. Preliminary results from this experiment are given in Addendum (5.6) and referred to in the Discussion.

4.5.5 Exposure of enteroid monolayers to *S. vulgaris* in co-culture with eqPBMC

To further expand the equine enteroid 2D model, a co-culture system of enteroid monolayers and eqPBMC was set up using transwell inserts (Figure 5d). Cultures of eqPBMC were established using previously described methods (Papers I and II) and incubated overnight in the presence of 5 µg/mL G3, 2 µg/mL SvSXP or in plain growth medium. Approximately 2 x 10^6 PBMCs were transferred to the basolateral surface of confluent enteroid monolayers. These co-cultures were thereafter challenged with approximately 20 living exsheated *S. vulgaris* L3s per well, added to the apical surface of the enteroid monolayer. After 20 h incubation the enteroid monolayers and the eqPBMCs were harvested separately and processed for transcriptional analysis of cytokine genes performed as described in papers I, II and III. In addition, TEER was measured (described in Paper I) in enteroid monolayers after 20 h exposure to *S. vulgaris* exL3 or G3. Preliminary results are given in Addendum (5.6) and referred to in the Discussion.

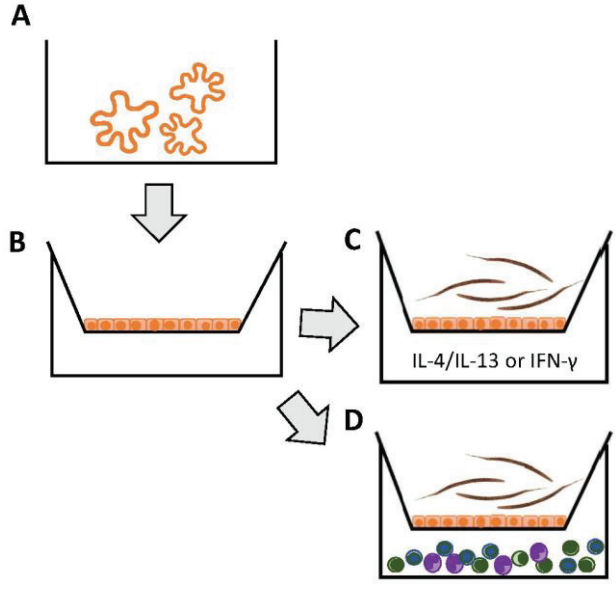


Figure 5. Scheme for enteroid monolayer experiments. Equine enteroids (**A**) were dissociated to single cells and cultured as monolayers on transwell inserts (**B**). *S. vulgaris* exL3s were added to the apical surface of enteroid monolayers cultured in the presence of Th polarizing cytokines at the basolateral surface (**C**) or in co-culture with eqPBMC (**D**).

4.6 Gene expression analysis

4.6.1 RNA isolation and quality control

RNA from the PBMC and enteroid cultures was extracted using a combination of the Trizol reagent and the column-based E.Z.N.A Total RNA Kit as previously described by Wikström et al (2011). By using spin columns, the risk of Trizol contamination is reduced which can otherwise impact RNA quantification on spectrophotometers and interfere with the subsequent cDNA synthesis. However, for some of the samples with low RNA yield, the 260/280 ratios, measured by a Nanodrop spectrophotometer, were below the recommended interval of 1.8-2.0, indicating the presence of contaminants. Despite this, cDNA was successfully synthesized from these samples, and showed no discrepancy in the expression of reference genes. In addition, the quality of the RNA was estimated in a selection of eqPBMC samples (Paper I and II) using capillary gel electrophoresis (Experion). All samples tested had an RNA quality index (RQI) ≥9.3 indicating intact RNA of good quality.

4.6.2 Synthesis of cDNA

To control for genomic DNA contamination, all samples were DNAse treated prior to cDNA synthesis. To confirm removal of genomic DNA, an assay targeting a product lacking introns was performed on non-reverse transcribed control samples as previously described (Hjertner et al, 2013). The cDNA synthesis was performed using the GoScript Reverse transcription system kit using 1.0 µg RNA per reaction (Paper I). For samples with a lower yield, a smaller amount of RNA, between 0.4-1.0 µg, was used (Paper II). Regardless, equal amounts of RNA were used within each individual horse to facilitate comparisons between treatments.

4.6.3 Assays for qPCR

Primer pairs for a panel of equine cytokines, representing Th1, Th2, Th17, Treg and Th9 responses, and TLR receptors were used to assess immune reactions to experimental treatments in eqPBMCs and enteroid-derived 2D monolayers. A gradient qPCR was run to estimate the optimal assay-specific annealing temperature and primer concentration for each assay. The assay-specific efficiency was thereafter estimated by running a serial dilution of

reference cDNA. For assays targeting pro-inflammatory cytokines and TLR receptors, the reference cDNA was PMA/Ionomycin stimulated eqPBMC. For assays targeting interferons, a reference cDNA made from eqPBMC stimulated with ODN2216 was used. The cut-off for an acceptable assay was set to >90 <105 % efficiency and r^2> 0.9.

The enteroids and enteroid-derived 2D monolayers were characterized by their expression of cell lineage markers. A panel of primer pairs for LGR5 (stem cells), SOX9 (stem and progenitor cells), PCNA (proliferative cells), EPCAM (epithelial cells), LYZ (paneth cells), CGA (enteroendocrine cells), MUC2 (goblet cells) and DCLK1 (tuft cells) were tested and optimized for their assay specific annealing temperature and primer concentration using qPCR. By running serial dilutions of reference cDNA made from sections of equine intestinal segments (Hjertner et al, 2013), all assays except LGR5 and DCLK1 were optimized with an efficiency >90 % and r^2>0.9. The LGR5 gene is exclusively expressed by intestinal stem cells and is therefore an important marker for characterization of enteroids (Sato et al, 2009). However, the basal LGR5 expression in the equine enteroids as well as in the reference cDNA was very low, hindering efficiency estimations by serial dilution. The tuft cell marker DCLK1 was also expressed at low levels in both enteroids and reference cDNA, complicating efficiency estimations. Therefore, annealing temperature and primer concentration for DCLK1 were determined by gradient qPCR analysis only.

4.6.4 Normalization of gene expression

To enable normalization of the gene expression data, a variety of reference genes were evaluated for their expression stability (geNorm software, qBase^{PLUS}) using a representative collection of samples from eqPBMC and equine enteroids. Ranked by a gene stability parameter and a coefficient of variation, RPL32 and SDHA were selected for normalization of eqPBMC samples whereas GAPDH, HPRT and SDHA were selected for normalization of enteroid material. This corresponds with previous data on the equine intestine, where the same set of reference genes were identified as most stably expressed in the equine jejunum (Hjertner et al, 2013).

5. Results

5.1 Cytokine responses to different larval stages of *S. vulgaris* (Paper I)

At invasion of the intestinal mucosa and during migration in the mesenteric arterial system, the *S. vulgaris* larvae undergo several molts. To study how these different larval stages influence the cytokine response at an early phase of infection, methods for generating defined larval stages of *S. vulgaris* were established and the preparations tested in cultures of eqPBMC (Paper I). Procedures for decontamination, culture and attenuation by UV-irradiation were elaborated using L3 stage larvae of the related species cyathostomins and thereafter applied to *S. vulgaris*.

When tested in cultures of eqPBMC, L3s from both cyathostomins and *S. vulgaris* induced gene expression for the Th2 polarizing cytokines IL-4, IL-5, and IL-13 but not for IL-1β, IL-6, IL-8, IL-9, IL-10, IL-12p40, IL-17A, IL-23p19 or TGF-β. Molting of *S. vulgaris* from the L3 to L4 stage did not affect the expression of IL-4 and IL-13 but was accompanied by a shift to high expression of IL-5, IL-9 and TSLP. Unexpectedly, the L4s also induced high levels of IFN-γ indicating a shift from a Th2 to Th1 response (Figure 6). Thus, the L4 stage appears to induce a different cytokine profile compared to earlier larval stages.

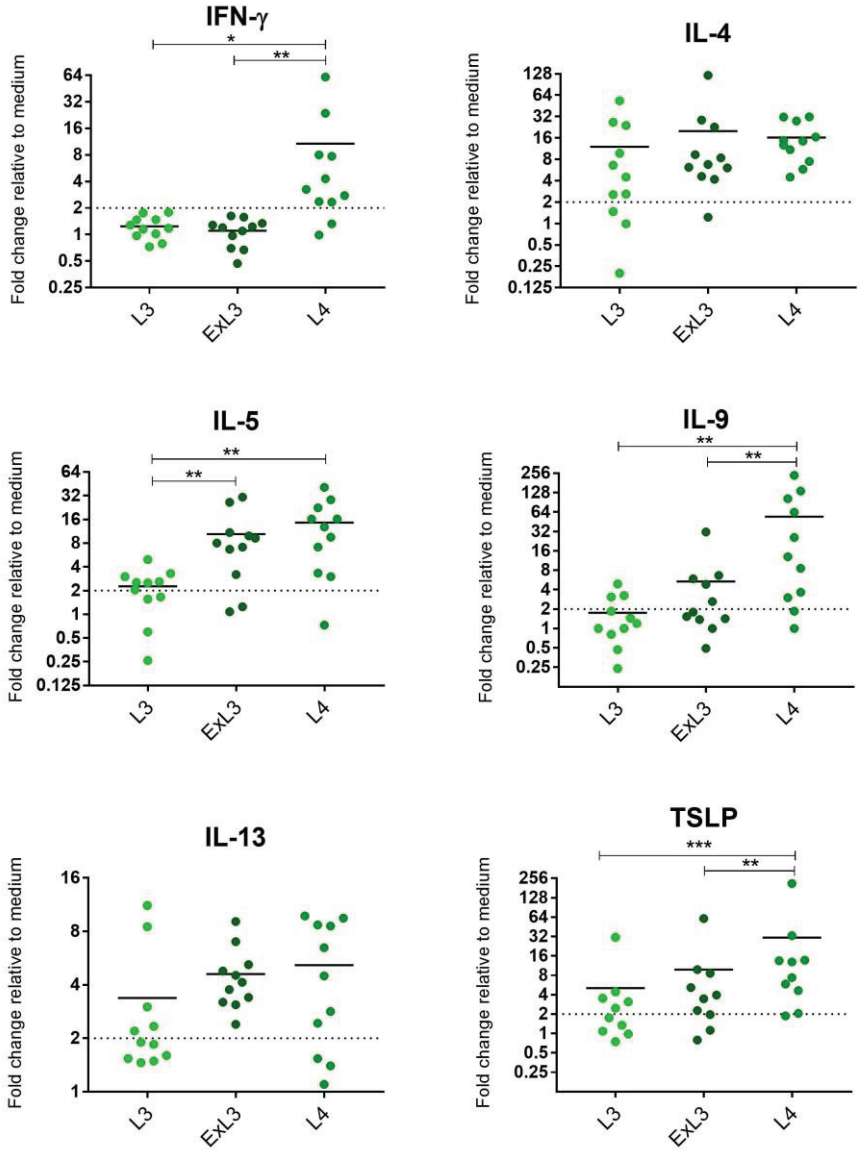


Figure 6. Relative expression of IFN-γ, IL-4, IL-5, IL-9, IL-13 and TSLP in eqPBMC after exposure to defined larval stages of *S. vulgaris*. The eqPBMCs were cultured in the presence of UV irradiated L3, exsheated L3 or L4 of *S. vulgaris* followed by transcriptional analysis of cytokine genes. The figure is adapted from Paper I.

5.2 Cytokine responses to the G3 adjuvant (Paper I, II)

The immune stimulatory capacity of the G3 adjuvant was studied in cultures of eqPBMC (Paper II). Transcriptional analysis of cytokine genes showed that G3 alone induces a response mainly characterized by up-regulation of the pro-inflammatory cytokines IL-1β, IL-6, IL-8 and the Th1-associated cytokines IFN-γ, IL-12p40 and IL-23p19. The Th2 associated cytokines IL-4 and IL-13 as well as the Treg cytokines IL-10 and TGF-β were in general not differentially expressed (Paper II). This immune profile, summarized in Figure 7 is coherent with the results found in Paper I, where eqPBMC were stimulated with 5 µg G3/mL, alone or in combination with strongyle larvae.

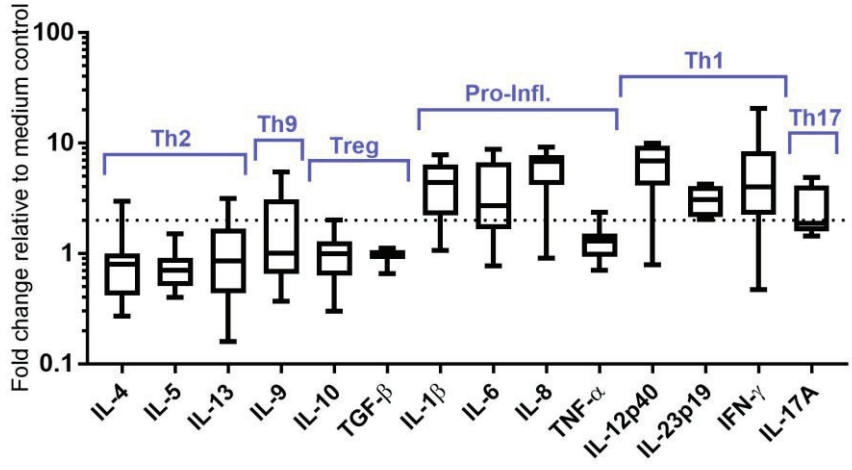


Figure 7. The cytokine profile induced by the G3 adjuvant (5µg/mL) in cultures of eqPBMC. The figure shows gene expression data summarized from Paper I and II.

5.3 Cytokine responses to the G3 adjuvant in combination with TLR-agonists or strongyle larvae (Paper I, II)

Effects of G3 on the cytokine response to microbial PAMPs was studied in eqPBMC exposed to G3 in combination with TLR-ligands (Paper II) or UV-attenuated *S. vulgaris* or cyathostomin L3s (Paper I). Both the TLR2/1 and TLR5 agonists Pam3CSK4 and FliC, respectively, induced similar cytokine

profiles with up-regulation of the genes encoding IL-1β, IL-6, IL-8 and IL-10, but at different magnitudes. This response was partly counteracted by the G3 adjuvant, most evidently by reducing the FliC and Pam3CSK4 induced production of IL-10 (Fig. 4, Paper II). Interestingly, G3 seemed to act in synergy with Pam3CSK4 by enhancing the production of IFN-γ whereas G3 combined with FliC increased the gene expression of IL-8 (Fig 3, Paper II). In agreement, the Th2 polarizing effect by *S. vulgaris* and cyathostomin L3s, was counteracted by the G3 adjuvant that reduced the expression of IL-4, IL-5 and IL-10 while in parallel enhanced the expression of IFN-γ (Fig. 4, Paper I). Thus, the G3 adjuvant appears to promote a Th1 polarizing immune response in eqPBMC, both by favouring IFN-γ production and by reducing production of IL-10 induced by co-delivered TLR agonists or UV-attenuated strongyle larvae.

5.3.1 Phenotypic characterization of IFN-γ producing cells (Addendum)

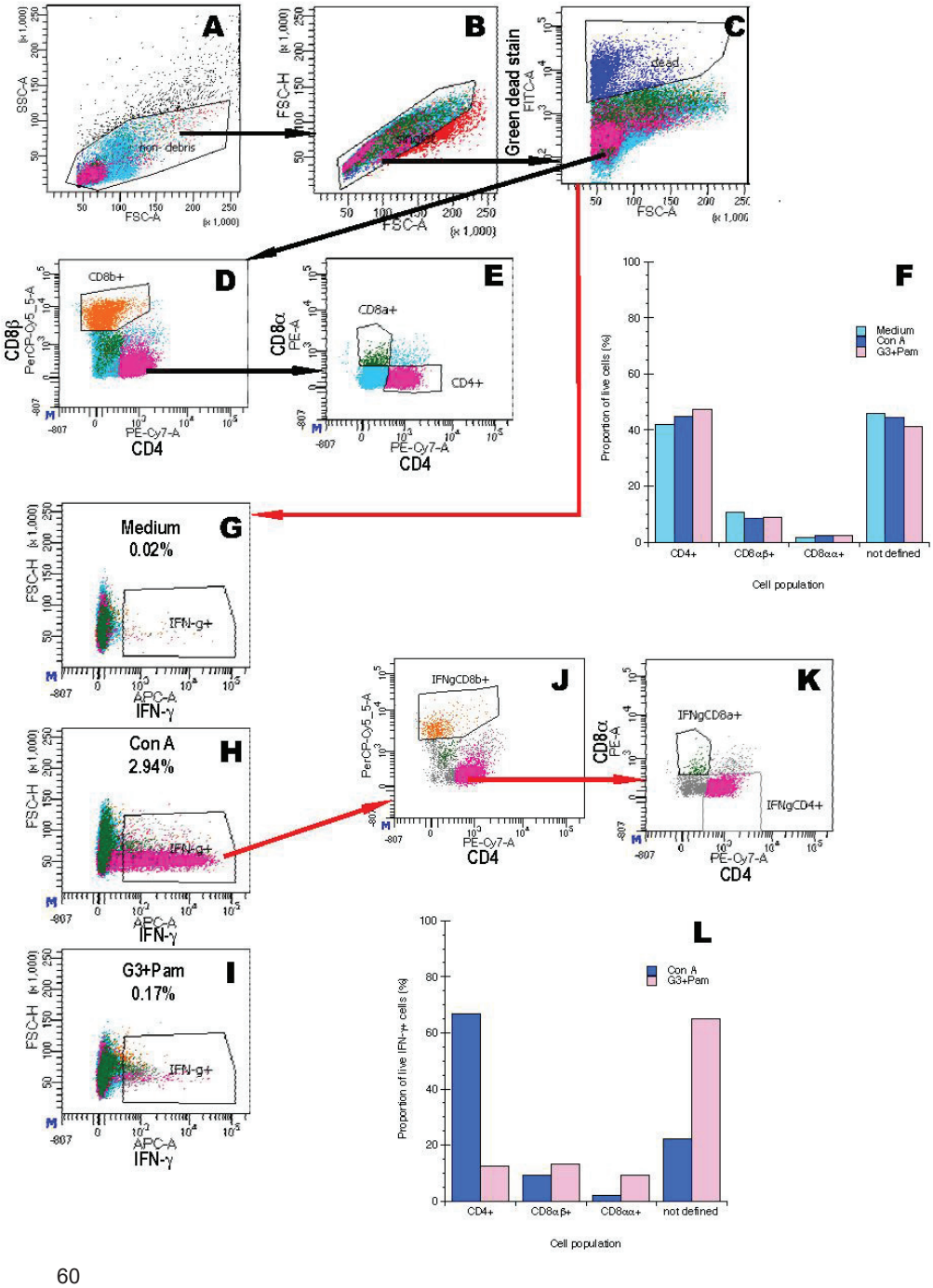
Both the original ISCOM formulation and the ISCOM-Matrix have been shown to induce IFN-γ producing cells in horses vaccinated against equine influenza virus (Paillot et al, 2008; Paillot & Prowse, 2012). Therefore, attempts were made to characterize the cells that produced IFN-γ in response to the G3 adjuvant and/or Pam3CSK4 explaining the indicated synergy effect (Fig.4, Paper II). As expected, a great individual variation was observed between horses in their response to PMA/Ionomycin (ranging from 10.6 to 37.7 % IFN-γ+ cells, n = 8). All horses showed a low, but varied spontaneous IFN-γ production (0.14 ± 0.11 % IFN-γ+ cells, n = 8). The proportion of cells detected as IFN-γ positive also varied within groups after incubation in G3 (0.27± 0.18%, n=8), Pam3CSK4 (0.54 ± 0.38 %, n = 7) or the combination of G3 and Pam3CSK4 (0.72 ± 0.41 %, n= 8). Thus, this preliminary data do not support any synergistic effect between G3 and Pam3CSK4. Instead, it appears that G3 and Pam3CSK4 initiates IFN-γ production in two different or partly overlapping cell populations.

In order to further characterize the cells producing IFN-γ in response to the combination of G3 + Pam3CSK4, a panel of antibodies detecting equine CD4, CD8α, CD8β and intracellular production of IFN-γ were tested using multicolor flow cytometry (Figure 8). As controls, eqPBMC cultured in plain growth medium or stimulated with ConA were used. Preliminary results

from one blood donor horse show that CD4, CD8α and CD8β are expressed at similar levels in ConA, G3+Pam3CSK4 and non-stimulated cells (Figure 8F). Stimulation with ConA increased the fraction of IFN-γ producing cells from 0.02 % in the medium control to 2.94 % in ConA stimulated cells (Figure 8G-H). A large proportion these (~66 %) were CD4+ cells (Figure 8L). Exposure to G3+Pam3CSK4 resulted in a slight increase (0.17 %) in IFN-γ producing cells compared to the medium control (Figure 8I). In contrast to ConA, a large proportion (~ 65 %) of the G3+Pam3CSK4 stimulated cells were CD4-, CD8α- and CD8β negative. In addition, the fraction of cells positive for CD8α but negative for CD4 and CD8β (thus, classified as CD8αα) was larger in G3 + Pam3CSK4 (10%) compared to ConA stimulated cells (2 %; Figure 8L). Thus, from these preliminary data it appears as a large proportion of the cells that produce IFN-γ in response to G3 + Pam3CSK4 are negative for CD4, CD8α and CD8β indicating that other cell types than T cells are predominantly activated by this adjuvant combination. However, additional experiments are needed to characterize these cells.

Figure 8 (figure on next page). Gating strategy for flow cytometry and results from phenotypic identification of IFN-γ producing eqPBMC from one healthy blood donor horse after 12 h stimulation in cell culture. Identification of CD4+, CD8αβ+ and CD8αα+ cells, respectively, on these cells through FSC/SSC characteristics, singlet gating and using CD4-PE/Cy7, CD8β-PerCp/Cy5.5, CD8α-PE and IFN-γ-PF647P. From the gate excluding debris in initial dot-plot in **A** gating through FSC-H vs FSC-A was performed in **B** to exclude large aggregates. From this gate, live cells were gated through exclusion of events stained with Green dead stain in **C**. From the live gate CD8β+ cells (i.e. CD8αβ+) were identified in **D**. Subsequently CD4+ and CD8α+ (i.e. CD8αα+) cells were identified from the CD8β- cells in **E**. **F** shows results phenotyping the whole live cell population in PBMC cultures in plain growth medium or in medium with the indicated inducers. **G**, **H** and **I** shows gating of IFN-γ+ cells from the whole live PBMC population in cultures in plain growth medium in **G**, stimulated with ConA in **H** and Stimulated with G3+Pam in **I**. The proportion of IFN-γ+ out of the whole live cell population is indicated in the respective dot-plot. From the live IFN-γ gate CD8β+ cells (i.e. CD8αβ+) were identified in **J**. Subsequently CD4+ and CD8α+ cells were identified from the CD8β- cells in **K**. **L** shows results phenotyping the live IFN-γ+ cells in cultures stimulated with the indicated inducers (in medium cultures fewer than 100 IFN-γ+ events were identified

and further phenotyping was therefore not meaningful). All dot-plots except **G** and **I** show results from ConA stimulated cultures.



5.3.2 Cytokine responses to SvSXP (Addendum)

The cytokine response to the recombinant SvSXP protein was studied in cultures of PBMC isolated from horses with an ongoing or known history of *S. vulgaris* infection (description in 4.4.4). The eqPBMCs were stimulated with 0.25, 0.5 and 0.75 µg SvSXP/mL or 1.0, 1.5 and 2.0 µg SvSXP/mL for 20 h followed by transcriptional analysis of cytokine genes. Preliminary data, from a yet incomplete set of horses, indicate that the SvSXP protein induce expression of the genes encoding IFN-γ, IL-2 and IL-17A in eqPBMC from horses in all three groups of horses (Figure 9A-C). Expression of IFN-γ and IL-2, but not IL-17A, appeared to be induced in a dose-dependent manner. With this limited data set, it was not possible to estimate differences in the cytokine expression between the three groups. The genes for IL-1β, IL-4, IL-8, IL-10, IL-33, TNF-α and TSLP were not differentially expressed whereas the genes encoding IL-5 and IL-9 were occasionally slightly up-regulated.

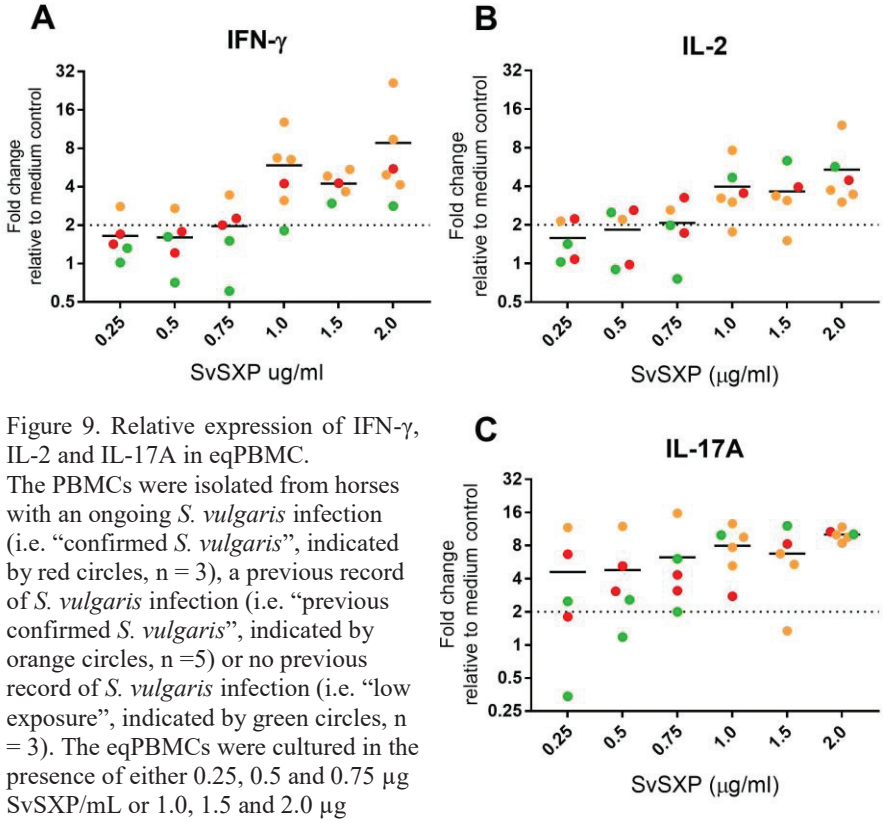


Figure 9. Relative expression of IFN-γ, IL-2 and IL-17A in eqPBMC.
The PBMCs were isolated from horses with an ongoing *S. vulgaris* infection (i.e. "confirmed *S. vulgaris*", indicated by red circles, n = 3), a previous record of *S. vulgaris* infection (i.e. "previous confirmed *S. vulgaris*", indicated by orange circles, n =5) or no previous record of *S. vulgaris* infection (i.e. "low exposure", indicated by green circles, n = 3). The eqPBMCs were cultured in the presence of either 0.25, 0.5 and 0.75 µg SvSXP/mL or 1.0, 1.5 and 2.0 µg SvSXP/mL as indicated in the graph.

5.4 Generation of equine enteroids and enteroid-derived 2D monolayers (Paper III)

Equine enteroid cultures were established from crypts isolated from equine mid-jejunum (Paper III). The enteroid cultures could be passaged, frozen and thawed multiple times with >80 % recovery. To enable easy access to the apical surface of the epithelium, the enteroids were dissociated into single cells and seeded on transwell inserts or on standard flat-bottom culture plates pre-coated with Matrigel. At a seeding concentration of 4-5 x 10^4 cells/cm^2, more than 90 % confluence was reached after three days culture. The monolayers remained intact for up to 8 days on the flat-bottomed culture plates and up to 13 days on the transwell inserts. TEER analysis of the transwell cultures showed that the monolayer integrity was stabilised after one week of culture and remained so for another week, with average TEER values of 860±160 Ω per cm^2. Transcriptional analysis of cell lineage markers confirmed the presence of epithelial cells, stem cells, Paneth cells, proliferative cells, enteroendochrine cells, goblet cells and tuft cells in both enteriods and enteroid-derived 2D monolayers. However, the transfer from 3D enteroids to 2D monolayers slightly modified the relative expression levels of these markers, possibly indicating a decrease of goblet- and paneth cells in the monolayers. Furthermore, both enteroids and monolayers expressed the genes encoding TLR2 and TLR5, but not TLR4.

5.5 Cytokine responses to TLR-agonists in enteroid-derived 2D monolayers

To assess the responsiveness of the enteroid-derived 2D monolayers to microbial stimuli, monolayers were exposed to the TLR agonists Pam3CSK4 (TLR2/1), Poly I:C (TLR3), LPS (TLR4) or FliC (TLR5) for 20 h followed by transcriptional analysis of cytokine genes (Paper III). No morphological changes were observed after exposure to Pam3CSK4, LPS or FliC. However, exposure to the TLR3 agonist poly I:C resulted in disruption of the monolayer with a high number of floating cells in the culture medium. In accordance, poly I:C was the only TLR agonist that induced gene expression of the alarmins TGF-β and IL-33. In addition, poly I:C induced high levels

of IFN-β mRNA as well as the pro-inflammatory cytokines TNF-α and IL-8. Induction of TNF-α and IL-8 was also recorded in the monolayers exposed to Pam3CSK4 and LPS whereas monolayers exposed to FliC only up-regulated TNF-α. (Figure 10).

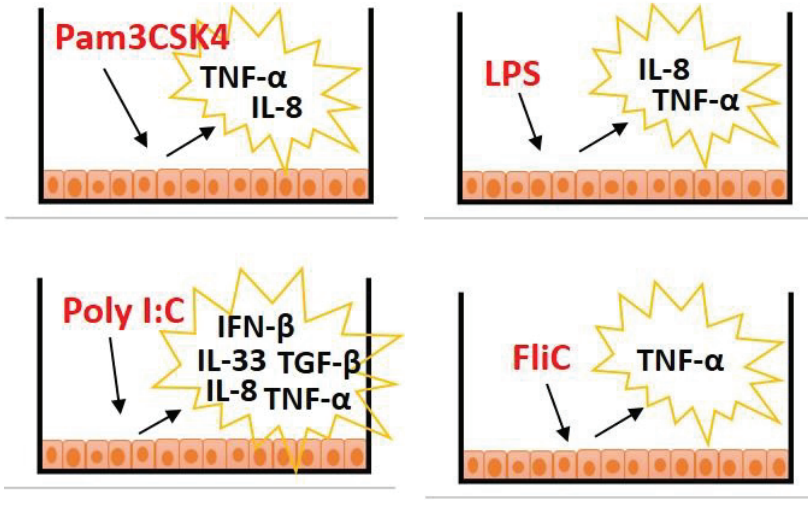


Figure 10. Cytokine responses to TLR-agonists in equine enteroid-derived monolayers. Both Pam3CSK4 (TLR2/1) and LPS (TLR4) induced gene expression of IL-8 and TNF-α, while FliC (TLR5) only induced TNF-α. Exposure to poly I:C resulted in up-regulation of IFN-β, IL-8, IL-33, TNF-α and TGF-β. The illustration is adapted from Paper III.

5.6 Exposure of enteroid monolayers to *S. vulgaris* in the presence of Th polarizing cytokines (Addendum)

To study interactions of *S. vulgaris* larvae with the intestinal epithelium, exsheated *S. vulgaris* L3s were added to the apical surface of enteroid monolayers cultured on transwell membranes in plain growth medium or pre-exposed to the Th1 polarizing cytokine IFN-γ or the Th2 polarizing cytokines IL-4 and IL-13 on the basolateral surface. After 20 h exposure to *S. vulgaris* exL3s, the enteroid monolayers were analyzed for their expression of cell lineage markers. The enteroid monolayers cultured in the presence of IL-4 and IL-13 slightly up-regulated the goblet- and tuft cell markers MUC2 and DCLK1, respectively. Apical exposure to *S. vulgaris* exL3s further up-regulated MUC2 and DCLK1 in IL-4 and IL-13 primed monolayers. In contrast, exposure to *S. vulgaris* exL3s on monolayers cultured in the absence of IL-4 and IL-13 did not seem to affect the expression of MUC2 or DCLK1. Furthermore, culture of monolayers in the presence of IFN-γ appeared to increase the expression of the Paneth cell marker LYZ (Figure 11). Thus, these preliminary data indicate that the equine enteroid-derived monolayers are responsive to the cytokine milieu created at the basolateral surface, affecting the cellular composition and the response to stimuli at the apical surface.

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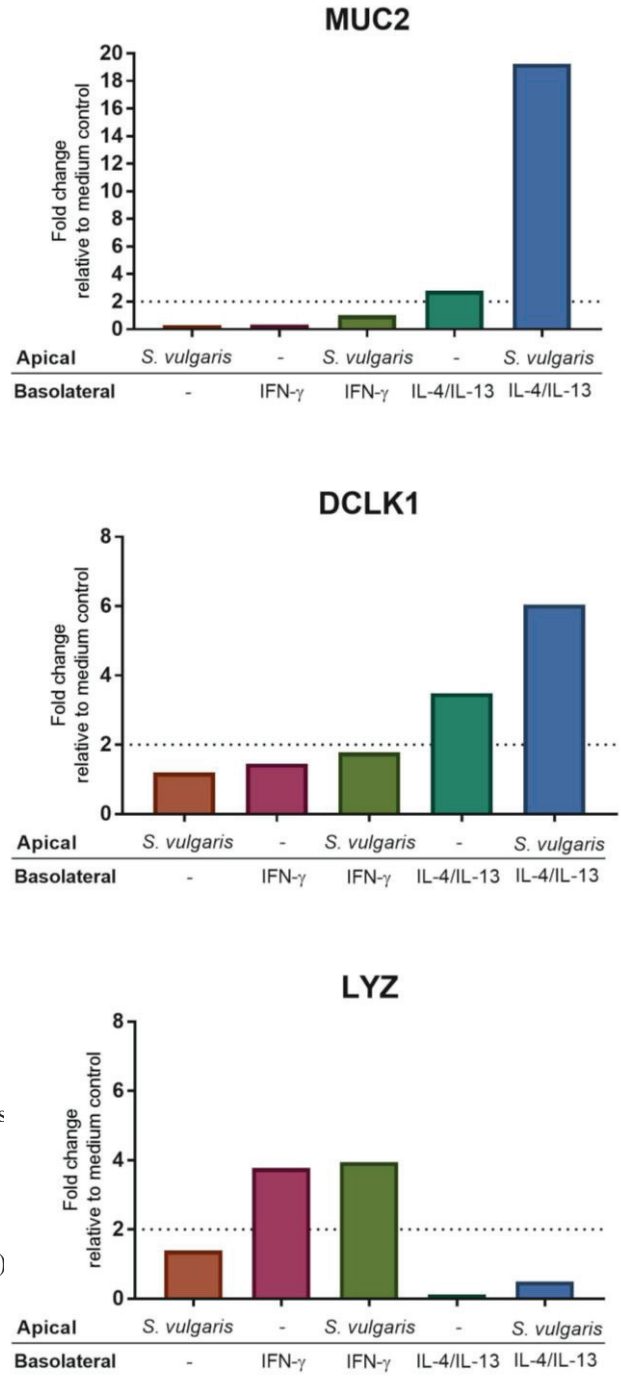

Figure 11. Relative gene expression of cell lineage markers for goblet cells (MUC2), tuft cells (DCLK1) and Paneth cells (LYZ) in transwell cultures of equine enteroid-derived 2D monolayers basolaterally primed with IFN-γ or IL-4 and IL-13 after 20h apical exposure to *S. vulgaris* exL3s. The gene expression was normalized to the geometric mean for the reference genes (GAPDH, SDHA and HPRT) and calibrated to that in the medium control.

5.7 Exposure of enteroid monolayers to *S. vulgaris* at co-culture with eqPBMC

To study interactions between the intestinal epithelium and immune cells at *S. vulgaris* infection, a co-culture model was developed from equine enteroid-derived 2D monolayers and eqPBMCs (Figure 5D). Enteroid monolayers were cultured on transwell membranes in connection with eqPBMCs cultured in the basolateral compartment and exposed to *S. vulgaris* exL3s on the apical surface of the monolayer. After 20 h *S. vulgaris* exposure, transcriptional analysis of cytokine genes was conducted in the enteroid monolayers and in the eqPBMCs. The preliminary results show that the only gene induced in the monolayers by *S. vulgaris* exposure was IL-8. The genes encoding IL-5, IL-33 and TGF-β were not differentially expressed while expression of IFN-γ, IL-4 and IL-13 were undetectable in the monolayers. In the eqPBMCs, an up-regulation of the gene encoding IL-5 was recorded while the other cytokine genes tested (IFN-γ, IL-1β, IL-4, IL-5, IL-8, IL-10, IL-13 IL-17A and TGF-β) were not differentially expressed (Figure 12). Furthermore, the genes encoding IL-9, TNF-α and TSLP were occasionally detected in both monolayer and eqPBMC cultures but with very high Cq values complicating fold change calculations.

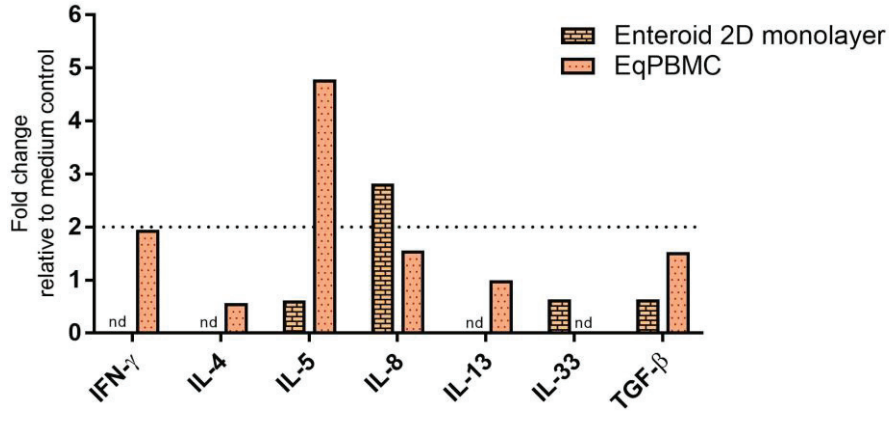

Figure 12. Relative expression of cytokine genes in co-cultures of enteroid monolayers and eqPBMCs challenged with *S. vulgaris* exL3s. The gene expression was normalized to the geometric mean for the reference genes (GAPDH, SDHA and HPRT) and calibrated to that in the medium control.

To test the effect of the G3 adjuvant or SvSXP protein in the co-culture system, eqPBMCs were primed with 5 µg/mL G3 or 2 µg/mL SvSXP for 18 h prior to the co-culture experiment. In G3-primed eqPBMCs, the up-regulation of IL-5 appeared to be higher compared to that in SvSXP-primed and non-primed eqPBMCs after apical exposure to *S. vulgaris* exL3s (Figure 13a). In the enteroid monolayers in co-culture with G3-primed or non-primed eqPBMCs, a slight up-regulation of the gene encoding IL-8 was indicated after exposure to *S. vulgaris* exL3s (Figure 13b). Furthermore, TEER analysis indicate that neither the *S. vulgaris* exL3s nor the G3 adjuvant affected the monolayer integrity during this 20 h incubation (Figure 14). However, additional experiments are needed to confirm these results.

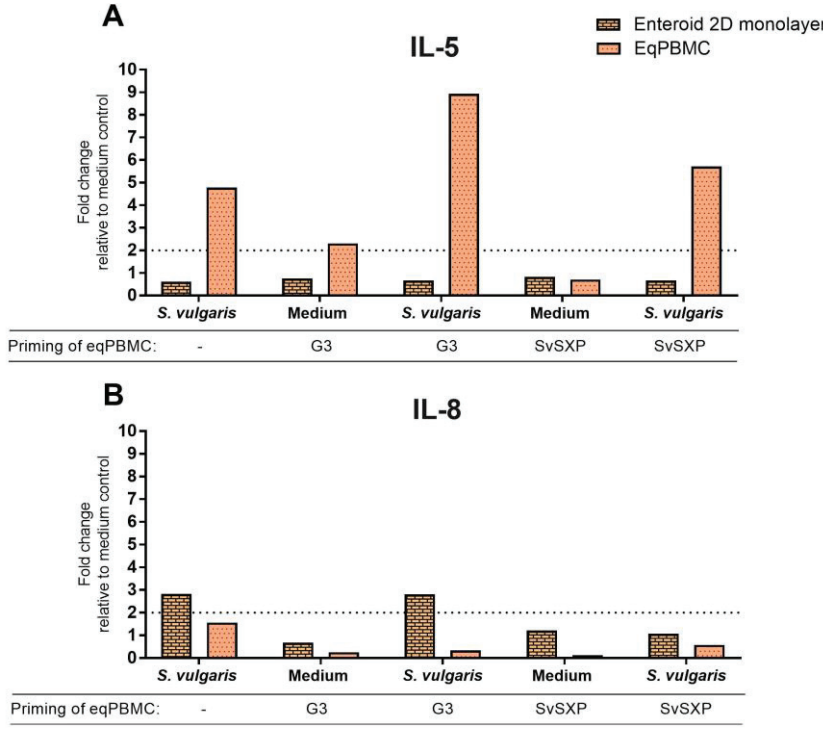

Figure 13. The relative expression of IL-5 (**A**) and IL-8 (**B**) in co-cultures of enteroid monolayers and eqPBMC after apical exposure to *S. vulgaris* exL3s. Non-exposed monolayers are indicated as "medium".

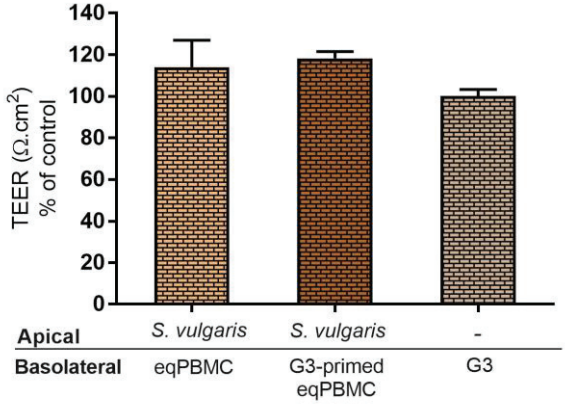

Figure 14. Transepithelial electrical resistance (TEER) in equine enteroid-derived monolayers in co-culture with non-primed eqPBMCs or eqPBMCs primed with 5 µg/mL G3 or basolaterally exposed to 5µg G3/mL. The co-cultures were apically exposed *S. vulgaris* exL3s and TEER was measured after 20 h incubation. The results are given as mean values ± SD for three technical replicates.

6. Discussion

Concerns about antimicrobial resistance combined with an increased prevalence of *S. vulgaris* reported in Sweden motivates development of new prophylactic tools to control this parasite. Initial immunization studies using orally administrated irradiated *S. vulgaris* L3 demonstrated that a protective immunity against *S. vulgaris* can be achieved by vaccination (Klei, 2000). Acquiring large amounts of live helminths is however impractical and ethically problematic as it would require the maintenance of experimentally infected "donor" horses. A more feasible option would therefore be a subunit vaccine based on recombinant *S. vulgaris* proteins. Such a vaccine requires identification of relevant vaccine antigens as well as an appropriate adjuvant that can increase and prolong the immunogenicity of the vaccine. To do this, we need a better understanding of the protective mechanisms against *S. vulgaris*. The initial studies on *S. vulgaris* performed in Kentucky indicate that immune protection is correlated with Th2 cytokines such as IL-5 and production of antibodies, predominantly IgG(T), that are recognizing the early larval stages of *S. vulgaris*. It is widely acknowledged that Th2 responses are necessary for immune protection against most helminth species, although this dogma was recently challenged by Perera & Ndao (2021). Assembled data in this review suggest that for some helminth species Th1-driven mechanisms may be important to combat juvenile larvae at initial infection while Th2-driven mechanisms are necessary for expulsion of adult worms. Regardless, the intrinsic ability of helminths to manipulate the host's immune response needs to be defined and potentially counteracted to achieve immune protection. It is therefore essential to understand the biology of the host-parasite relationship during *S. vulgaris* infection.

Research into helminths and immunology has traditionally relied on the use of animals. While studies in large animals is expensive and demand plenty of resources, murine models are cheaper and easier to manage. However, the murine models cannot always fully recapitulate immune responses in out-bred large animal species (Guzman & Montoya, 2018; Bush et al, 2020). Adding the fact that most helminths are host-specific motivates development of *ex vivo* systems based on cells or tissues isolated from the target species. Naturally, *ex vivo* models cannot replace the complexity of a living organism but they can be useful as an initial screening tool to build theories that later can be tested and confirmed in the living animal. In this thesis, I have used cultures of eqPBMC and equine enteroids to model both systemic and local reactions to infective stage *S. vulgaris* larvae. In addition, the immunomodulatory capacity of the novel adjuvant G3 together with *S. vulgaris* preparations was evaluated.

There is currently an incomplete knowledge of the host-parasite interactions and immune mechanisms conferring protection to *S. vulgaris*. Considering its complex life cycle comprising several larval molts, *S. vulgaris* likely express a plethora of antigenic components at each larval stage. To study how different larval stages of *S. vulgaris* influence the immune response, eqPBMC were cultured in the presence of UV-attenuated L3s, exsheated L3s (exL3s) or L4s. Transcriptional analysis of cytokine genes indicate that the L3 and exL3 stage induce a typical helminth associated Th2 response characterized by up-regulation of IL-4, IL-5, IL-9, IL-13 and TSLP. Although the L4s also induced these cytokines, this stage appear to skew the Th2 profile by inducing the Th1 polarizing cytokine IFN-γ. As the L4 is the life cycle stage when *S. vulgaris* first encounter peripheral blood, this observation may be important to understand the initial response to this parasite. While development of Th2 responses is considered essential for immune protection against helminths, Th1 responses have been associated with establishment of chronic infections. Studies in mice demonstrate that primary infection with *T. muris* induce a pro-inflammatory response with production of Th1 cytokines, such as IL-12 and IFN-γ, inhibiting activation of protective Th2 responses (e.g. Else et al, 1992; Bancroft et al, 1994; Humphreys et al, 2004). In addition, the murine GI nematode *H. polygyrus* was shown to induce IL-1β early during infection inhibiting expression of IL-25 and IL-33 allowing establishment of chronic

infection (Zaiss et al, 2013). Thus, these murine studies indicate that helminth antigens can alter polarization of the Th profile by induction of pro-inflammatory and Th1 polarizing cytokines to promote establishment in the host. It is therefore tempting to speculate that the IFN-γ induction by *S. vulgaris* L4, observed in the present study, contributes to *S. vulgaris* establishment in the equine host. Reports from other helminth species that also spend parts of their life cycle in peripheral blood, such as *Fasciola hepatica, Schistosoma mansoni, Brugia malayi* and *Trichinella spiralis*, indicate that a mixed Th1/Th2 response is necessary for immune protection (reviewed by Perera & Ndao, 2021). Indisputable, further studies are necessary to scrutinize the role of IFN-γ during the initial response to *S. vulgaris*.

Interestingly, the preliminary results indicate that the recombinant *S. vulgaris* SXP protein (SvSXP) protein also induce gene expression of IFN-γ as well as IL-17A and IL-2 in eqPBMC. The SvSXP protein was identified using migratory larvae at the L5 stage (Andersen et al, 2013b) and its expression by earlier stages remains to be confirmed. However, other SXP/RAL-2 proteins homologous to SvSXP have been localized to the worm intestine, hypodermis, cuticle and excretory glands as well as found among ES-products (Tsuji et al, 2001; Caballero et al, 2008), suggesting that SvSXP may be an excretory protein. Several proteins from the SXP/RAL-2 family have besides being studied as vaccine antigens or diagnostic markers also been identified as allergens in humans (García-Mayoral et al, 2014; Ahumada et al, 2020). A potential allergenic activity of some SXP/RAL-2 proteins might complicate their use as vaccine antigens and further stress the importance of inducing a balanced Th1/Th2 response to the antigen.

Many of the keys to designing an effective vaccine against *S. vulgaris* are likely to be found by studying its life cycle. During the early phase of infection, the *S. vulgaris* L3s and early L4s reside in the intestinal mucosa before beginning the migratory route through the mesenteric arterial system. A vaccine against *S. vulgaris* should optimally target these early larval stages to stop migration into the small blood vessels of the intestinal mucosa. To get a better understanding how *S. vulgaris* interacts with its equine host, it is therefore essential to study reactions to *S. vulgaris* also at the site of entry. For that purpose, equine enteroid cultures were generated from adult stem

cells isolated from equine jejunum. To allow *S. vulgaris* interaction studies with enteroid cells, 2D monolayer cultures were established from the equine enteroids. Both the enteroids and the enteroid-derived 2D monolayers contained cell lineages normally found in the equine small intestine (Gonzalez et al, 2015), such as intestinal stem cells, progenitor cells, Paneth cells, enteroendocrine cells and goblet cells. In addition, tuft cells were detected using doublecortin-like kinase 1 (DCLK1) which is a commonly used marker to identify tuft cells. In the healthy intestine, DCLK1 is uniquely expressed by a majority of tuft cells (Gerbe et al, 2009; Middelhoff et al, 2017) but can also be selectively expressed by several kinds of cancer stem cells (Cao et al, 2020). Although, the role of tuft cells in the equine intestine remains to be elucidated, expression of DCKL1 was recently confirmed in different sections of the equine intestine by transcriptome analysis (Coleman et al, 2020). Recent studies in mice demonstrate that intestinal tuft cells play an important role in the defense against helminths. Tuft cells are an important source of IL-25 that drives the tuft-ILC2s signaling feedback loop in which ILC2s are activated to produce Th2 cytokines, such as IL-5, IL-9 and IL-13. The presence of IL-13 skews the differentiation pattern of the epithelium towards goblet- and tuft cells resulting in a rapid increase of these cell types during helminth infection (Schneider et al, 2019). In the horse, goblet cell hyperplasia has been described in horses with cyathostomin infection showing that expression of MUC2 and IL-13 in the large intestine and peripheral blood, respectively, can be correlated to worm burdens (Steuer et al, 2020). In the present thesis, increased expression of MUC2 and DCLK1 was indicated in enteroid-derived 2D monolayers after basolateral exposure to IL-4 and IL-13. Apical challenge with *S. vulgaris* exL3 on monolayers primed with IL-4/IL-13 further increased the expression of these cell lineage markers indicating that helminth-induced goblet- and tuft cell hyperplasia can be replicated in the present *ex vivo* model.

It is well established that the interplay between the intestinal epithelium and resident immune cells are important for initiating immune responses against helminth infections (Inclan-Rico & Siracusa, 2018). The intestinal epithelium can respond to helminth PAMPs by cytokine and/or chemokine production or respond to tissue damage caused by helminth invasion by production of alarmins. The function of the enteroid monolayers was therefore further assessed by analyzing their expression of cytokine genes at

exposure to defined TLR agonists mimicking bacterial and viral PAMPs. Apical exposure to TLR2/1, 3, 4 and 5 agonists triggered gene expression of pro-inflammatory cytokines and the viral mimic poly I:C (TLR3) was the only one to induce IFN-β indicating that the equine enteroid monolayers can respond to microbial structures in a similar fashion as expected *in vivo* (Andrews et al, 2018). When challenged with *S. vulgaris* exL3s, no differential cytokine expression was recorded in the enteroid monolayers. However, *S. vulgaris* challenge on enteroid monolayers in co-culture with eqPBMC induced a slight up-regulation of IL-8 in the enteroid cells, but not in the eqPBMC. On the contrary, up-regulation of IL-5 was recorded in the eqPBMCs, but not in enteroid cells, suggesting a cross-communication between the enteroid monolayer and eqPBMCs at *S. vulgaris* challenge. Although methods to co-culture enteroid cells with immune cells are emerging (e.g. Staab et al, 2020; Schreurs et al, 2021), the use of such co-cultures in studies of host-microbe interactions are hitherto few and have mainly been focused on bacterial infections in humans and mice (e.g. Noel et al, 2017; Schulte et al, 2020). However, the transwell co-culture system established in the present thesis using eqPBMCs and equine enteroid monolayers could be a valuable tool in future studies of host-GI parasite interaction in the horse.

Except for immunological studies, transwell systems have been widely used to study changes of epithelial barrier integrity at exposure to external factors, such as microorganisms, feed components or medical compounds (Srinivasan et al, 2015). Such a culture system was for example demonstrated by Noel et al (2017) that evaluated barrier integrity and cytokine production to different *E. coli* strains in human enteroid-macrophage co-cultures. Given the invasive nature of most helminth species, transwell culture of enteroid monolayers are not only interesting in terms of co-culture with immune cells but may also be used to elucidate the mechanisms involved in tissue invasion. In the present thesis, the epithelial barrier integrity, estimated by TEER, remained unchanged after 20 h exposure to living *S. vulgaris* exL3s indicating no disruption of the monolayer during this short incubation time. Since the mechanism(s) of larval penetration through the intestinal mucosa by *S. vulgaris* is unknown, appropriate culture conditions for triggering larval invasion is yet to be defined.

Early vaccination trials using attenuated *S. vulgaris* L3 or soluble somatic extracts indicate that the antigen should preferentially be administrated via the oral route (Monahan et al, 1994; Edmonds, 2001). A major challenge with orally administered vaccines is to compose an immunogenic formulation, especially when it comes to non-living or subunit antigens. The default mode in the intestine to these types of antigens is tolerance or ignorance. To break the tolerogenic milieu, an adjuvant suitable for administration at mucosal surfaces needs to be included in the vaccine formula. While adjuvants based on toxins from *Cholerae* and *E. coli* are most commonly used in mucosal vaccines for humans (Lavelle & Ward, 2021), ISCOMs have been successfully included in a number of experimental mucosal vaccines, either standalone (e.g. Kazanji et al, 1994; Coulter et al, 2003; Crouch et al, 2005; Zhang et al, 2014) or formulated with toxoid compounds (Lycke, 2004). In the horse, a "systemic prime/mucosal boost strategy" using an ISCOM-based intranasal booster generated protection against equine influenza (Crouch et al, 2005). Furthermore, ISCOMs have successfully been included in oral experimental vaccines against protozoan parasites (Kazanji et al, 1994; Zhang et al, 2014). The efficacy of ISCOMs at mucosal surfaces is considered to rely on its ability to carry and deliver protein antigens to APCs (Mowat et al, 1999). A similar mode of action is plausible for the G3 adjuvant that can be formulated to incorporate additional molecules. Preliminary results from phenotyping eqPBMCs stimulated with the G3 adjuvant and the TLR2/1 agonist Pam3CSK4 suggests that the G3 adjuvant, similar to ISCOMs, may target APCs. Furthermore, the evaluation of cytokine responses to the G3 adjuvant in cultures of eqPBMC indicates that G3 pre-dominantly induces a Th1 polarizing profile characterized by induction of IL-12p40 and IFN-γ. A Th1 skewed cytokine profile was also observed in porcine PBMCs after *in vitro* exposure to the G3 adjuvant (Hjertner et al, 2018b), and inclusion of the G3 adjuvant in an experimental influenza vaccine generated a mixed Th1/Th2 response in mice (Hjertner et al, 2018a).

How an adjuvant should be formulated to optimally induce immune protection against helminth antigens in the mucosa is not entirely clear. Suggestively, a mucosal adjuvant should include components that enable uptake across the mucosal barrier, e.g. by targeting M-cells. This will facilitate antigen presentation by mucosal DCs that can in turn enhance T

cell priming and development of long-lasting plasma cells and memory B cells (Gerdts et al, 2005; Lycke, 2012). Indeed, methods to differentiate M cells in enteroid cultures are emerging (Kanaya et al, 2018), possibly enabling *ex vivo* screenings of adjuvant compounds aimed to target M cells in the future. Moreover, as mucosal DCs play a central role in priming immune responses, targeting specific mucosal DC subsets, such as $CD103^+$ DCs, could direct systemic responses towards mucosal sites (Ruane & Lavelle, 2011; Lycke, 2012). In this context, bacterial flagellin has gained attention for its ability to target immune cells of the intestinal mucosa (reviewed by Cui et al, 2018). Systemic immunization with FliC, isolated from *Salmonella enterica serovar Typhimurium*, were shown to drive TLR5-dependent recruitment of $CD103^+$ DCs in the mesenteric lymph nodes resulting in an increased local differentiation of Treg cells and class switching to IgA (Flores-Langarica et al, 2012). In the present thesis, the immunomodulatory effect of the G3 adjuvant in combination with FliC was studied in cultures of eqPBMC. The results indicate that the G3 adjuvant can enhance the FliC-induced gene expression of IL-8 while simultaneously reduce the gene expression of IL-10. A similar effect on the IL-10 gene expression was recorded in eqPBMC after combining G3 with Pam3CSK4. As many helminths are known to manipulate the host's immune response to produce IL-10, this feature makes the G3 adjuvant an interesting candidate adjuvant for use in equine vaccines with prospects of evading antigenic undesired immune reactions. The inactivated larval preparations of *S. vulgaris* generated in the present thesis did not induce gene expression of IL-10 in eqPBMCs. However, as most immunomodulatory actions by helminths are mediated through ES-products (Maizels et al, 2018), an induction of IL-10 at *S. vulgaris* infection should not be ruled out. The Th1 polarizing capacity of the G3 adjuvant also occurred in the presence of *S. vulgaris* L3s, as shown by up-regulation of IFN-γ and down-regulation of IL-4 and IL-5. Together, these *in vitro* data suggest that the G3 adjuvant can balance Th2 polarizing immune responses towards a mixed Th1/Th2 profile in the horse.

Taken together, work within this thesis has extended previous knowledge on the biology of *S. vulgaris* infection and advanced the *ex vivo* tools for further studies on GI pathogens in the horse and/or initial screening of vaccine components.

7. Future perspectives

- As *S. vulgaris* also invades the submucosa of the large intestine, future studies should focus on generation of equine colonoids.
- Since the mechanism(s) of larval penetration through the intestinal mucosa is unknown, future studies should elucidate these mechanisms.
- To aid targeting the intestinal mucosa, targeting molecules such as FliC will be considered in future vaccine designs against *S. vulgaris*.
- The candidate vaccine components identified in this thesis should be tested for their immunogenicity *in vivo*.

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Popular science summary

The equine large strongyle (*Strongylus vulgaris*) is considered to be the most dangerous parasite of horses. Recent reports of an increased prevalence of *S. vulgaris* in Sweden along with concerns about drug resistance motivates development of a vaccine to control this parasite.

Formulation of vaccines against intestinal worms have always been a challenge. Gastro-intestinal (GI) worms commonly progress from eggs through multiple developmental stages, referred to as "larval (L) stages" until becoming adult worms. The various developmental stages are separated by molting events when the cuticle surrounding the larval body is released. These shifts between larval stages are typically fast and accompanied by migrations through different organs and tissues. In addition, molecules produced and released by helminths referred to as excretory-secretory (ES) products are immunomodulatory, meaning that they can interfere with the host's immune response. Rapid shifts between larval stages along with the parasite's inbuilt capacity to manipulate immune responses makes it difficult for the immune system to mount a response needed to develop immunological memory. To guide the immune response on the right path, immune stimulatory substances, called adjuvants, can be included in the vaccine formula to increase vaccine efficiency.

A better understanding how GI worms interact with the host's immune system and how unwanted immunological reactions can be balanced are critical to develop effective vaccines against GI worms. To address these issues, immune reactions to *S. vulgaris* larvae and a novel adjuvant called "G3" were studied in laboratory cell culture models built up by immune cells isolated from equine blood (PBMC) and stem cells isolated from the intestine (enteroids).

Immune reactions to *S. vulgaris* larvae and/or the G3 adjuvant were mainly estimated by qPCR analysis of cytokine genes. Cytokines are small signaling proteins that play an essential role in initiation of immune responses. By measuring cytokine genes, we can in theory get a good idea how certain stimuli act on immune cells and what the ensuing immune response will be like.

Enteroids are three-dimensional lab-grown "miniature intestines" resembling the composition and function of the real intestine. To enable studies of host-parasite interactions in the equine intestine, methods to culture enteroid cells on a two-dimensional plane, as a "monolayer", were established. The equine enteroids and enteroid monolayers were evaluated with regards of their cell composition and responsiveness to bacterial or viral mimics. The equine enteroid monolayers displayed genetic and functional similarities with the equine intestine making it a suitable laboratory model for studies of host-GI parasite interactions in the horse.

It is well established that the interplay between the cells of the intestinal barrier and underlying immune cells are important for initiating immune responses against worm infections. Therefore, the enteroid culture system was further expanded using enteroid monolayers co-cultured with equine PBMCs. A simulated infection with *S. vulgaris* exL3s indicate a cross-communication between the enteroid monolayer cells and the equine PBMCs. Thus, this co-culture model can therefore be used to generate additional knowledge how immune cells reacts to intestinal parasites during the early phase of infection.

Horses are infected by *S. vulgaris* by ingesting infective third stage larvae (L3) on pasture. During the first phase of infection, the larvae penetrate the intestinal wall and mature to the fourth larval stage (L4) before starting its migration through the blood vessels of the intestine. To study how these early life cycle changes influence the immune response, methods for culture of *S. vulgaris* from the third to the fourth stage were established, generating three *S. vulgaris* preparations defined as "L3", "exsheated L3" (exL3) and "L4". Cultures of equine PBMC were thereafter exposed to larvae weakened by UV-irradiation and analyzed for their expression of cytokine genes. The

results show that both *S. vulgaris* L3 and exL3 induced gene expression of cytokines that is typically associated with worm infection. The L4 stage, however, induced a skewed cytokine profile compared to the earlier stages indicating that the developmental stages in the life cycle of *S. vulgaris* may generate different immune responses. This diversity depending on the life cycle stage will likely have implications for the choice of components to include in a future vaccine.

To increase vaccine efficiency, an adjuvant is commonly included in the vaccine formula. Our studies on the he G3 adjuvant show an up-regulation of cytokine genes not typically associated with a parasitic response. However, the G3 adjuvant were shown to balance the response by other immunostimulatory compounds and *S. vulgaris* L3s. This feature makes G3 is an interesting candidate adjuvant for use in equine vaccines with prospects of balancing undesired immune responses.

To sum up, this thesis adds on new knowledge about the diversity of the cytokine response to different larval stages of *S. vulgaris*, which is important to understand the initial response to this parasite. Establishment of equine enteroids and enteroid monolayers brings new possibilities to study interactions between *S. vulgaris* and the equine intestine and can in future studies be used for initial screenings of vaccine candidates.

Populärvetenskaplig sammanfattning

Den stora blodmasken (*Strongylus vulgaris*) anses vara hästens farligaste parasit. Rapporter om en ökad förekomst av *S. vulgaris* i Sverige samt risken för resistensutveckling mot avmaskingsmedel motiverar utvecklingen av ett vaccin för att bekämpa denna parasit.

Framställning av vacciner mot inälvsmaskar har alltid varit en utmaning. Inälvsmaskar utvecklas vanligtvis från ägg genom flera utvecklingsstadier, kallade ”larv (L) stadier” tills de blir vuxna maskar. Mellan de olika utvecklingsstadierna släpper larven sitt yttre hölje vilket ofta åtföljs av migrationer genom olika organ och vävnader. Dessutom kan larver utsöndra så kallade ”ES-produkter” som kan påverka värddjurets immunförsvar. Snabba skiften mellan olika larvstadier tillsammans med parasitens inbyggda förmåga att vilseleda värddjurets immunsvar gör det svårt formulera ett effektivt vaccin mot inälvsmaskar. För att leda immunförsvaret på rätt väg kan ett immunstimulerande ämne, ett så kallat adjuvans, tillsättas för att öka vaccineffekten.

En bättre förståelse för hur inälvsmaskar interagerar med värddjurets immunförsvar och hur oönskade immunologiska reaktioner kan balanseras är avgörande för att utveckla effektiva vacciner mot inälvsmaskar. För att studera hur *S. vulgaris* interagerar med hästens immunförsvar, användes cellodlingsmodeller uppbyggda av immunceller från hästens blod (mononukleära blodceller) och stamceller isolerade från hästens tarm (enteroider) för att studera immunreaktioner mot *S. vulgaris* larver och ett nytt adjuvans kallat ”G3”.

Immunreaktioner mättes framförallt genom qPCR-analys av cytokiner. Cytokiner är små signalproteiner som spelar en viktig roll vid initiering av ett immunsvar. Genom att mäta uttryck av gener som kodar för cytokiner kan vi i teorin få en uppfattning om hur vissa stimuli verkar på immunceller och hur det efterföljande immunsvaret kan komma att se ut.

Enteroider är tredimensionella labb-odlade ”miniatyrtarmar” som liknar den verkliga tarmens sammansättning och funktion. För att kunna studera interaktioner mellan *S. vulgaris* och hästens tarm etablerades metoder för att odla enteroida celler som ett s.k. monolayer, d.v.s. ett enskiktat lager av celler. Enteroiderna och enteroida monolayers utvärderades med avseende på deras uppsättning av celltyper och förmåga att inducera cytokiner vid stimulering med strukturer typiska för bakterier och virus. De enteroida monolayerna visade både genetiska och funktionella likheter med hästens tarm vilket gör dem till en lämplig laboratoriemodell för att studera inälvsparasiter i häst.

Det är välkänt att samspelet mellan tarmcellerna och underliggande immunceller är viktigt för att initiera ett immunsvar mot inälvsmaskar. Därför utvecklades det enteroida odlingssystemet ytterligare genom att samodla enteroida monolayer med ekvina immunceller. En simulerad infektion med infektiösa *S. vulgaris* larver indikerade att tarmcellerna kommunicerar med immuncellerna genom produktion av cytokiner. Denna samodlingsmodell skulle därför kunna användas för att generera ytterligare kunskap om hur immunförsvaret reagerar mot inälvsparasiter under det tidiga infektionsskedet.

Hästar infekteras av *S. vulgaris* när de får i sig infektiösa tredje stadiets larver (L3) på ett smittat bete. Under den första infektionsfasen penetrerar larverna tarmväggen och mognar till det fjärdelarvstadiet (L4) innan de börjar migrera genom tarmens blodkärl. För att studera hur dessa tidiga livscykelförändringar påverkar immunsvaret, etablerades metoder för odling av *S. vulgaris* larver från det tredje till fjärde larvstadiet. Detta genererade tre *S. vulgaris* preparat definerade dom ”L3”, ”exL3” och ”L4”. Ekvina immunceller exponerades därefter för dessa larvpreparationer försvagade genom UV-bestrålning. Resultaten visar L3 och exL3 stadierna av *S. vulgaris* inducerar cytokiner som är typiskt associerade med maskinfektion.

Till skillnad från de tidigare stadierna, inducerade L4 stadiet en avvikande cytokinprofil vilket antyder att olika utvecklingsstadier av *S. vulgaris* kan ge upphov till olika immunsvar. Dessa olikheter i immunsvar beroende på livscykelstadie kommer sannolikt att påverka valet av komponenter som bör inkluderas i ett framtida vaccin mot *S. vulgaris*.

För att öka vaccineffektiviteten brukar vanligtvis ett adjuvans tillsättas. Våra studier på G3 adjuvanset visar på en uppreglering av cytokiner som vanligtvis inte brukar associeras med tarmparasiter. Dock kunde G3 adjuvanset balansera cytokinsvaret mot andra immunstimulerande ämnen och *S. vulgaris* larver. Denna egenskap gör G3 adjuvanset intressant att fortsätta utvärdera för användning i hästvacciner där ett oönskat immunsvar behöver balanseras.

Sammanfattningsvis bidrar arbetet inom denna avhandling till ny kunskap om cytokinsvaret mot olika larvstadier av *S. vulgaris*, vilket är viktigt för att förstå hur immunförsvaret reagerar vid ett tidigt infektionsskede. Etablering av nya cellodlingssystem baserat på hästens tarmceller ger nya möjligheter att studera interaktioner mellan *S. vulgaris* och hästens immunförsvar i tarmen och kan i framtida studier användas för inledande screening av vaccinkandidater.

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Acta Universitatis Agriculturae Sueciae

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Concerns about antimicrobial resistance together with an increased prevalence of the equine parasite *Strongylus vulgaris* reported in Sweden motivates development of new prophylactic vaccines to control this parasite. This thesis established *ex vivo* methods to study reactions to *S. vulgaris* and/or vaccine candidates at the site of infection. Overall, the work within this thesis has generated new insights into the biology of *S. vulgaris* infection and generated methods that will aid in the development of a future vaccine.

Stina Hellman received her graduate education at the Department of Biomedical Sciences and Veterinary Public Health. Her undergraduate degree was received from The Swedish University of Agricultural Sciences and Uppsala University.

Acta Universitatis Agriculturae Sueciae presents doctoral theses from the Swedish University of Agricultural Sciences (SLU).

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