In the last decade, a new therapy for several inflammatory disorders, such as inflammatory bowel disease (IBD) and multiple sclerosis (MS), has emerged that utilizes the immunomodulatory effects of helminths. Clinical trials have shown that treatment of patients with these parasitic worms leads to reduced inflammatory phenotypes, or even remission of the disease (1–4). To evade the host immune system, helminths produce specific products that skew these immune responses. In general, helminths skew the immune response towards a T helper 2 (Th2) or regulatory T cell (Treg) phenotype by suppressing the production of pro-inflammatory mediators, such as cytokines and chemokines, produced by antigen presenting cells (APCs). In addition, the altered surface expression of maturation markers on APCs results in immune suppressive or regulatory signaling to T cells. These characteristics make helminths very attractive candidates in the treatment of several autoimmune diseases, which are generally characterized by inflammatory Th1 and Th17 responses. The ingestion of live helminth eggs is not considered to be appealing to patients; therefore, treatment with helminth-derived products or mimics thereof may be a good alternative. In this thesis, the effects of helminth products Trichuris suis soluble products (SPs) and Schistosoma mansoni soluble egg antigens (SEA) in the innate immune response have been investigated.

1. The use of helminth products for protection in inflammatory diseases

As described in detail in chapter 1, several clinical trials with different models have shown that helminth infection is beneficial in the treatment of inflammatory diseases. However, the effects of products isolated from or secreted by helminths in the treatment of these diseases is less clear. For example, one study showed that although mucosal immune responses were affected, S. mansoni SEA was not able to improve clinical signs of DSS colitis in mice, whereas infection with S. mansoni cercariae did attenuate DSS-induced colitis (5). In contrast, treatment with Schistosoma japonicum SEA did ameliorate the severity and progression of experimental autoimmune encephalomyelitis (EAE), an animal model for multiple sclerosis (MS), has emerged that utilizes the immunomodulatory effects of helminths. Clinical trials have shown that treatment of patients with these parasitic worms leads to reduced inflammatory phenotypes, or even remission of the disease (1–4). To evade the host immune system, helminths produce specific products that skew these immune responses. In general, helminths skew the immune response towards a T helper 2 (Th2) or regulatory T cell (Treg) phenotype by suppressing the production of pro-inflammatory mediators, such as cytokines and chemokines, produced by antigen presenting cells (APCs). In addition, the altered surface expression of maturation markers on APCs results in immune suppressive or regulatory signaling to T cells. These characteristics make helminths very attractive candidates in the treatment of several autoimmune diseases, which are generally characterized by inflammatory Th1 and Th17 responses. The ingestion of live helminth eggs is not considered to be appealing to patients; therefore, treatment with helminth-derived products or mimics thereof may be a good alternative. In this thesis, the effects of helminth products Trichuris suis soluble products (SPs) and Schistosoma mansoni soluble egg antigens (SEA) in the innate immune response have been investigated.

2. Helminth products in the intestinal immune system

The ability of helminth products to suppress inflammatory diseases indicates that they are able to induce changes in one or more host immune cell types and thereby alter the immune response that is generated. In helminth therapy regimens, helminth eggs are ingested and transported through the intestinal tract where larvae hatch and come in contact with the host’s immune system. The intestinal lining consists mainly of intestinal epithelial cells (IECs), which may react to contact with helminths. It has previously been shown in porcine T. suis infection models that larvae were able to migrate within the IEC layer, allowing the passage of T. suis products in vitro. In vivo, T. suis products reduce the secretion of TNFα by LPS-stimulated macrophages in both M1 and M2 macrophages. The secretion of IL-6 is significantly reduced upon co-stimulation with T. suis products. In addition, the expression of IL-12 (IL-12p70) in IECs is reduced when T. suis products are present (6). These data support the concept of Th2 polarization when T. suis products are present. The ability of helminth products to suppress inflammatory diseases indicates that they are able to induce changes in one or more host immune cell types and thereby alter the immune response that is generated. In helminth therapy regimens, helminth eggs are ingested and transported through the intestinal tract where larvae hatch and come in contact with the host’s immune system. The intestinal lining consists mainly of intestinal epithelial cells (IECs), which may react to contact with helminths. It has previously been shown in porcine T. suis infection models that larvae were able to migrate within the IEC layer, allowing the passage of T. suis products in vitro. In vivo, T. suis products reduce the secretion of TNFα by LPS-stimulated macrophages in both M1 and M2 macrophages. The secretion of IL-6 is significantly reduced upon co-stimulation with T. suis products. In addition, the expression of IL-12 (IL-12p70) in IECs is reduced when T. suis products are present (6). These data support the concept of Th2 polarization when T. suis products are present.

3. Helminth products suppress inflammatory Th1/Th2 responses

Although the cause of the anti-inflammatory effect of helminth products remains unknown, it has been suggested that it involves an exaggerated response to one or more inflammatory agents. Studies in ulcerative colitis (UC) patients have shown that the...
increased expression of TLR4 and uncontrolled inflammatory responses in the disease impaired regeneration of the gut mucosa, leading to more mucosal damage (21). Accordingly, therapy using TLR4 antagonists shows promising results in animal models of several inflammatory diseases (22). For inflammation in the brain, it has been shown that pertussis toxin (PTX), the agent generally used to induce EAE, binds to TLR4, thereby leading to the surface expression of P-selectin, which aids in the extravasation of immune cells into brain tissues (23). These detrimental effects of TLR4 in inflammatory diseases as well as the efficacy of TLR4 antagonists in animal models of these diseases indicates that dampening of TLR4 responses is an attractive target for their treatment. The results shown in chapter 3 and chapter 7 indicate that the immune suppressive effects of the helminth products used was the strongest when using TLR4 stimulation. Therefore, results were further focused on the effects of helminth product in TLR4-stimulated cells. Lipopolysaccharide (LPS) derived from Escherichia coli is the most commonly used in vitro ligand of TLR4. LPS interaction with TLR4 results in a pro-inflammatory immune response via the use of several signaling cascades. The TLR4 pathway consists of two main pathways: MyD88-dependent signaling occurs when TLR4 is expressed on the cell surface, whereas endosomal TLR4 interaction with LPS results in initiation of the TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent signaling cascade (24). TRIF-dependent signaling leads to the secretion of type I interferons, which have antiviral and antibacterial properties. The Myeloid differentiation primary response gene 88 (MyD88)-dependent signaling cascade splits into the nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) and the mitogen activated protein kinase (MAPK) pathways, both of which induce inflammation resulting from the production of inflammatory factors including TNFα, IL-6, IL-12 and CCL5 (25, 26). Although helminths are found throughout several evolutionary phyla and differ in their habitat, life cycles and make up, they show surprisingly similar immune modulatory effects on human hosts. However, the mechanisms used to achieve immune suppression may differ. In an in vitro TLR4-stimulated DC model we have described effects of T. suis products and S. mansoni SEA on pro-inflammatory cytokine and chemokine production, co-stimulatory molecule expression and T cell skewing abilities. In chapter 4, we show that DCs stimulated with S. mansoni SEA suppress inflammatory cytokine production and allogeneic mixed lymphocyte reactions (MLRs). In addition, SEA induces the expression of Suppressor of Cytokine Signaling 1 (SOCS1) as well as SH2-containing protein tyrosine phosphatase 1 (SHP1). SOCS1 participates in the negative regulation of TLR4 signaling by interacting with Interleukin-1 receptor-associated kinase (IRAK) and NF-κB, promoting their degradation and thereby preventing their prolonged activation. SOCS1 directly binds to the p65/RelA subunit of NF-κB, enhancing its ubiquitination and subsequent proteolysis, resulting in a reduced activation of NF-κB. In addition, SOCS1 targets MyD88 adaptor-like (Mal), downregulation of which would constrain the complete MyD88-dependent signaling pathway (27). SHP1 interacts with IRAK1 in the TLR4 signaling pathway, leading to reduced signaling of the NF-κB and MAPK cascades, thereby limiting the transcription of pro-inflammatory genes (28). Deficient SHP1 expression in macrophages has been observed in MS patients, leading to an enhanced inflammatory phenotype (29). In conclusion, induction of SOCS1 and SHP1 by S. mansoni SEA will limit the MyD88-dependent signaling pathway (Figure 2), thereby

![Figure 1](image_url)
Chapter 6

Reducing the production of pro-inflammatory cytokines and chemokines. The induction of SOCS1 and SHP1 expression is not a mechanism generally used by helminths, as *T. suis* products did not induce these negative regulators of TLR4 signaling. To determine the effects of *T. suis* on TLR4 signaling, an Illumina array was done on DCs treated with LPS with and without *T. suis* products. Chapter 6 shows that this resulted in an induction of RAS-related protein Rab7b, which has been shown to be involved in TLR4 trafficking. The exact mechanism used by Rab7b remains unclear, as one research group showed that expression of Rab7b resulted in TLR4 transport from the endosome to the trans-Golgi network (TGN), whereas another group found that Rab7b-dependent transport of TLR4 resulted in endosome to lysosome transport, leading to degradation of TLR4 (Figure 3B) (30, 31). DC stimulation with *T. suis* products strongly reduced TLR4 surface expression levels (chapter 6), however, the total amount of TLR4, i.e. the combined presence of TLR4 both inside the cell and on the surface remained unaltered. This indicates that TLR4 is internalized, but not degraded. Therefore, our data indicate that Rab7b interferes with the recycling of TLR4 to the cell surface and is not broken down. The simultaneous upregulation of Rab7b levels and relocation of TLR4 in response to *T. suis* and the previously described role of Rab7b in TLR4 trafficking indicates that the *T. suis*-induced Rab7b expression could be responsible for the reduced surface expression of TLR4.

Whereas the reduced surface expression of TLR4 induced by *T. suis* products after 24h stimulation could account for the strong reduction of cytokine secretion upon pretreatment of *T. suis* products 24h before LPS is given (chapter 6), it cannot account for the observed fast reduction of cytokine secretion in LPS-stimulated DCs. The pro-inflammatory cytokines TNFα and IL-6 are suppressed by *T. suis* products as soon as 2h after stimulation (chapter 5), at a time point before *T. suis* products have had sufficient time to reduce TLR4 surface expression. In addition, chapter 6 shows that mRNA and protein levels of key genes in the cascades induced upon TLR4 stimulation were reduced after 1-2h upon costimulation with *T. suis* products (Figure 3A). Therefore, mechanisms other than TLR4 internalization must also play a role. The fast suppression of mRNA levels of MyD88 and phosphorylation of pIRAK1 indicates that negative regulation of MyD88 signaling may occur at the start of the pathway, perhaps via negative regulators of the TLR4 pathway. Another possibility would be that TLR4 signaling on the cell surface is inhibited, thereby reducing MyD88-dependent signaling. However, since we have shown that *T. suis* products do not bind to TLR4 in HEK293 cells, *T. suis* products would have to use an indirect mechanism for TLR4 inhibition, possibly via interaction with C-type lectin receptors (CLRs), see further in 4.2.

Chapter 5 shows that LPS-induced TNFα mRNA and protein levels in DCs are reduced in response to *T. suis* products. However, the suppression of TNFα was much more pronounced on the level of secretion than on mRNA expression level. Therefore, *T. suis* products appear to be able to interfere with either protein secretion pathways or translation. mRNA translation could be altered in many different ways, including the induction of specific microRNAs (miRNAs), the induction of translational repression or aggregation of messenger ribonucleoproteins (mRNP) (32). For other helminths, including *S. mansoni* and *T. spiralis* it was shown that they themselves are able to produce miRNAs (33, 34). In conclusion, the work described here provides the first insights into the ability of *T. suis* products to suppress immune responses as well as into some of the mechanisms used to achieve immune suppression, however, several currently unknown mechanisms used by *T. suis* products remain to be determined.
4.1 The role of helminth glycosylation in immune suppression

Helminths and their products are heavily glycosylated with both host-like and helminth-type glycans. It has been suggested that helminth glycosylation plays an important role in the formation of host immune responses against helminths (35). For S. mansoni, SEA it has been shown that meta-periodate treatment, resulting in functional removal of glycan structures, severely reduced the ability of SEA to induce Th2 responses in murine models (36, 37). However, the role of glycans of many other helminths and the effects on the inflammatory capacity of APCs is still incompletely understood.

Chapter 4 describes the role of SEA glycans in inflammatory responses induced in human DCs. SEA is able to suppress TLR4-induced pro-inflammatory cytokine signaling in DCs and reduce Toll-like receptor 4 (TLR4) expression (38). In addition, chapter 5 it is shown that glycans present in S. mansoni SEA were able to alter TLR2-induced cytokine secretion (42). The mannose receptor (MR) and DC-specific ICAM3-grabbing non-integrin (DC-SIGN) can alter the secretion of pro-inflammatory cytokines, such as IL-10 and IL-12, in human DCs (39). To date, only a few glycans have been shown to inhibit TLR signal transduction. These are apparently not broken down by the RNase activity of the MR, although SEA-dependent induction of SOCS1 and SHP1 does not require RNase activity of the MR (43). Now that it has been shown that TLR4 products are recognized by the MR and DC-SIGN, leading to the internalization of SEA antigens (47–49). The glycan LDN, which is present on S. mansoni, is recognized by the MR. In contrast, fucosylated LDN structures have been shown to bind with MGL and DC-SIGN and high mannose structures can interact with DC-SIGN and DC-SIGN in a manner that is independent of their structural presentation (48).

4.2 Involvement of CLRs

CLRs are receptors known to internalize soluble glycoconjugates. In addition, several CLRs have been shown to be able to induce intracellular signaling cascades, thereby enhancing or suppressing TLR signaling pathways (39, 40). For example, glycans can interact with DC-specific ICAM3-grabbing non-integrin (DC-SIGN) can alter the secretion of pro-inflammatory cytokines, depending on the glycans that bind the receptor (41). Furthermore, the human CLR macrophage galactose lectin (MGL) is able to alter TLR2-induced cytokine secretion (42). The mannose receptor (MR) does not contain a signaling motif and has not directly been shown to be able to signal. However, MR ligation has been shown to lead to intracellular signaling events, either directly via MR or via ligand binding to another intracellular receptor after uptake by MR (43).

Although glycans of Trichuris muris, a family member of T. suis, have been shown to interact with the MR (44), the ability of T. suis products to interact with CLRs has not been described. In chapter 5 it is shown that glycans present in T. suis are able to interact with the CLRs MR, DC-SIGN and MGL. Binding assays using plant lectins indicated that within T. suis products high amounts of terminal α-linked N-acetylgalactosamine (α-GalNAc) structures, which have been shown to bind to MGL, and oligo mannose structures, that potentially could interact with the MR and DC-SIGN, were present. Glycan structures commonly found on S. mansoni glycoconjugates include high mannose structures, Lewis X (LeX), Lac-di-Nac (LDN) and several types of fucosylated LDN structures (45). Recently we showed that S. mansoni cercarial products can interact with DC immunoceptor (DDIR) (46). In addition, S. mansoni SEA has been shown to interact with the CLRs MR, MGL and DC-SIGN, leading to the internalization of SEA antigens (47–49). The glycan LDN, which is present on S. mansoni, is recognized by MGL. In contrast, fucosylated LDN structures have been shown to interact with MGL and DC-SIGN. S. mansoni LeX and high mannose structures can interact with DC-SIGN and the MR, possibly depending on their structural presentation (48).

In chapter 4, a role for the MR is described in S. mansoni SEA-dependent induction of SOCS1 and SHP1. These negative regulators of TLR signaling are induced by SEA in a glycan-dependent manner. Blocking of SEA binding to the MR using synthetic compound Allyl-D-Mannoside (AllMan) or specific anti-MR antibodies largely prevented the induction of SOCS1 and SHP1 mRNA expression levels. In contrast, blocking of the mannose-binding receptor DC-SIGN did not affect the ability of S. mansoni SEA to induce SOCS1 and SHP1 expression. Therefore, it is specifically the binding of S. mansoni SEA to the MR that is able to induce signaling events that finally result in SOCS1 and SHP1 induction. It has previously been described that omega-1, a LeX-containing glycoprotein with RNase activity found in S. mansoni SEA, is able to be internalized via the MR on DCs and induce Th2 skewing (50). This Th2 skewing was thought to be the result of a general reduction in protein synthesis, as omega-1 would be able to break down RNA following internalization by the MR. However, we showed here that S. mansoni SEA is also able to induce mRNA expression levels and protein levels of factors that play an important role in T cell proliferation. These are apparently not broken down by the RNase activity of omega-1. Thus, we propose that SEA uptake by the MR results in signaling events either by signaling of the MR via a currently unknown mechanism, or via MR ligand endocytosis followed by interaction of the ligand with a different receptor present in the endosome and induce the transcription of negative regulatory genes.

Figure 3. T. suis-induced effects on TLR4. Toll-like receptor 4 (TLR4) signaling results in transcription of pro-inflammatory genes. A. Trichuris suis products directly suppress the TLR4 signaling cascade in several places. All factors indicated in yellow were significantly suppressed by T. suis. B. TLR4 is continuously shuttled between the cell surface and the endosomal compartments. Ras related protein Rab7b has been shown to be involved in intracellular trafficking of TLR4, resulting in either lysosomal TLR4 degradation or recycling in the trans-golgi network (TGN). T. suis products are shown to induce the expression of Rab7b in human dendritic cells and reduce surface expressed TLR4. Since intracellular TLR4 is not broken down, T. suis-induced Rab7b appears to result in TLR4 recycling to the TGN.
5. Treatment of inflammatory diseases

Although further clinical trials will have to be performed, current research done on helminths and inflammatory diseases indicates that helminth therapy may be an effective treatment for inflammatory disorders. Side effects of helminth infection can be prevented by the use of helminths that do not infect humans. The most and most promising results were found in clinical trials for IBD and MS using porcine helminth T. suis ova (TSO) (1-4). In addition, it has been reported that helminths Enterobius vermicularis and Necator americanus are beneficial in the treatment of IBD (51, 52). The use of helminth products in the treatment of inflammatory disorders would prevent the necessity of infection patients with a helminth. However, before these products can be used, further research is required. In order to interest pharmaceutical companies and commercialize therapy using helminth infection, a range of experiments remains to be performed (53). Most of these prerequisites also apply to the use of helminth products. For example, for optimal comparison treatment with helmins or helminth products should be directly compared to conventional therapies in the treatment of IBD and MS. In addition, patients generally show reluctance to ingest live worms, which can be overcome by the use of helminth products.

The determination of the efficacy of helminth products in inflammatory diseases can be approached in different manners. Establishing the mechanisms used by helminth products to suppress immune responses may determine specific receptors and pathways involved. For several helminth products it has been shown that they interact with CLR's and that helminth glycan(-like) structures play a substantial role in immune suppression. Therefore, determination of the CLR(s) or other glycan-binding receptors required for immune suppression will human. This may help to further establish the mechanisms used. To that end, knock out mice for different mannose-binding or GalNAc-binding receptors could be used, which would indicate what type of helminth glycan(-like) structures play a role in immune suppression. In addition, these data provides some preliminary leads that could be followed for the use of helminth products in the treatment of human inflammatory disorders.

References


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