Immunoregulation by *Trichinella spiralis*: benefits for parasite and host

Cover photograph
This painting represents the Universe according to the Incas. It is found in Cusco (Peru), in the Coricancha (former Sun and Moon Temple) now Santo Domingo Monastery. It was painted by Miguel Aráoz Cartagena

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Carmen Aranzamendi Esteban and Enrique Aranzamendi Esteban

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Immunoregulation by *Trichinella spiralis*: benefits for parasite and host

Immunoregulatie door *Trichinella spiralis*: voordelen voor parasiet en gastheer

Proefschrift

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Carmen Rosa Aranzamendi Esteban

Promotoren:	Prof. dr. W. van Eden
	Prof. dr. V.P.M.G. Rutten
	Prof. dr. C.J.P. Boog
Co-promotor:	Dr. E. Pinelli Ortiz

Para mis dos amores
Floris & Sebastian

Contents

Chapter 1

General introduction

Partly published in:

Helminths: Immunoregulation and Inflammatory Diseases—Which side are *Trichinella* spp. and *Toxocara* spp. on?

Carmen Aranzamendi, Ljiljana Sofronic-Milosavljevic and Elena Pinelli

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and

***Toxocara* infection and its association with allergic manifestations**

Elena Pinelli and Carmen Aranzamendi

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Helminths

Helminths are complex eukaryotic organisms with large genomes and complex multistage life cycles that involve several hosts [1]. They are comprised of three broad taxa, the Nematodes (round worms, including the model organism *Caenorhabditis elegans*), Trematodes (flukes), and Cestodes (tapeworms), each separated by approximately 500 million years of evolution. While taxonomically distant, the parasitic species induce common immunological features which are likely to have co-evolved under similar selective pressure from the immune system of the host [2]. Soil-transmitted helminths (*Ascaris lumbricoides, Trichuris trichiura* and hookworm) and schistosomes are the most common helminths worldwide and their distribution is determined by climate, hygiene and diet [3]. The World Health Organization estimates that 2 billion people around the world are currently infected with the above mentioned helminths. Besides, most of the cases occur in areas of poverty in low-income countries in the tropics and subtropics where the highest prevalence is in children between the ages of 5 and 14 [3].

It has become clear in the past years that certain helminths can induce an immunoregulatory network which suppresses the host immune response against these parasites. The induced inmmunesuppression benefits parasite survival and in part it is beneficial for the host since inflammatory diseases such as allergy and autoimmune diseases are also suppressed. Understanding the mechanisms involved in the helminth-induced immunoregulation is essential for the development of alternative treatments for these immune disorders which are increasing worldwide.

Immunity to helminths

Immune responses induced by helminths are predominantly of the Th2 type involving the cytokines interleukin-3 (IL-3), IL-4, IL-5, IL-9, IL-10 and IL-13. These cytokines mediate immune responses typically characterized by increased levels of circulating IgE antibodies, eosinophils, basophils and mast cells [2]. During infection, the immune system is exposed to different helminth-derived molecules, including proteins, lipids, and glycoconjugates (proteins or lipids modified by glycans), present at the surface of the worms as excretory-secretory (ES) products [4]. These molecules target host pathways to shift the host immune response from an inflammatory towards an anti-inflammatory type of response by modifying dendritic cell (DC) function and downregulating adaptive immune responses through the induction of regulatory T (Treg) cells, that produce anti-inflammatory cytokines such as IL-10 and TGF-β and/or other cells such as alternatively activated macrophages (AAM) and regulatory B cells [1, 2].

Dendritic cells

Dendritic cells (DC) are sentinels on alert for possible danger signals to immediately activate local innate immune cells and subsequently, after antigen presentation, initiate the proper adaptive immune responses. The interaction with DC determines the function and cytokine production of lymphocytes [5]. DC are located throughout the body forming a complex network that allows them to communicate with different populations of lymphocytes. Different subsets may have distinct locations, where they acquire antigens to be transported to the draining lymph nodes for T cell priming [6] . DC as well as other innate immune cells possess various families of Pattern Recognition Receptors (PRR) such as Toll Like Receptors (TLR), NOD-Like Receptors, RIG-like receptors, and the C-type Lectin Receptors (CLR) that allow them to recognize a great variety of pathogen-associated molecular patterns (PAMP). As a consequence of the pathogen recognition via various PRR, DC receive signals that are subsequently translated into production of molecules that polarize to different types of responsiveness like Th1-, Th2-, Th17- or Treg related [7]. After pathogen recognition via various PRR, DC produce molecules that induce polarization of different types of responsiveness such as Th1-, Th2-, Th17- or Treg related. The response of DC to pathogens is mediated in large part via TLR, with input from other PRR resulting in changes in gene expression that leads to DC maturation. Maturation of these cells refers to a transition from a resting state into a more dynamic state in which the cells present antigen in the context of MHC, express co-stimulatory molecules such as CD40, CD80 and CD86 and secrete a broad spectrum of cytokines and chemokines [8]. TLR-mediated responses are controlled mainly by the MyD88-dependent pathway, which is used by all TLR except TLR3, and the TRIF-dependent pathway, which is used by TLR3 and TLR4 [9]. TLR have been implicated in the recognition of helminth products by DC. For instance, LNFPIII and ES-62, phosphorylcholine-containing proteins secreted by the nematode *Acanthocheilonema viteae*, condition DC to induce Th2 responses through TLR4 [10]. Likewise, monoacetylated phosphatidyl serine lipids from schistosomes specifically instruct DC to preferentially induce IL-10-producing Treg cells in a TLR2-dependent fashion [11]. This was also demonstrated in TLR2-deficient mice that showed a reduced number of $CD4^+CD25^+$ Treg cells and immunopathology during schistosomiasis [12]. CLR also play an important role in the sensing of helminth glycans by DC. For example, studies using Schistosomal antigens suggest that helminth glycans may be the conserved molecular pattern that instructs DC via CLR to drive Th2-polarized responses [13]. Other recent studies demonstrate that the host-like glycan antigens expressed by many helminths are recognized by DC via lectin receptors [4]. Schabussova *et al.*(2007) found that blood

group-like glycans from *Toxocara canis* bind the lectin DC-SIGN [14], which may enable the activation of signal transduction pathways involving Raf-1 and subsequent modulation of DC maturation resulting in skewing towards a Th2 responses [15].Lewis X antigen, a host-like glycan expressed on the surface of schistosomes in all life stages and present in secreted products such as the soluble egg antigens (SEA), also binds to DC-SIGN [16].

DC maturation is considered to be essential for DC to be able to induce T-cell responses. However, it has become clear that DC responding to helminth products do not mature in the conventional way upon encountering parasitic antigens but acquire a semi-matured status and are still capable of inducing T-cell polarization. For example, SEA suppresses LPS-induced activation of immature murine DC, including MHC class II, costimulatory molecule expression, and IL-12 production. This was a result of increased LPS-induced production of IL-10 by SEA [17]. Another clear example is ES-62, which inhibits the proliferation of $CD4^+$ T cells and conventional B2 cells *in vivo*, and reduces IL-4 and IFN-γ production [18]. Pre-treatment of DC and macrophages with ES-62 also inhibits their ability to produce IL-12p70 in response to LPS [19]. In another study, a mixture of high molecular weight components from *Ascaris suum* was found to reduce the expression of MHCII, CD80, CD86, and CD40 molecules on mouse $CD11c^+$ DC and subsequently hampered T cell proliferative responses *in vitro*. This inhibitory effect was abolished in IL-10-deficient mice [20]. *Fasciola hepatica* tegumental antigen alone did not induce cytokine production or cell surface marker expression on murine DC however; it significantly suppressed cytokine production and cell surface marker expression in DC matured with a range of TLR and non-TLR ligands [21]. These and other studies clearly indicate that helminth products fail to induce conventional DC maturation but skew the immune responsiveness towards Th2 or regulatory responses.

Regulatory T cells

Treg cells control peripheral immune responses and are likely to play a central role in autoimmune, infectious, allergic, and asthmatic diseases. Three phenotypes of Treg have been described to date, categorized according to their origin, function, and expression of cell surface markers: natural Treg cells ($CD4^+CD25^+Foxp3^+$) and inducible Treg cells that include the IL-10-producing Tr1 cells and the $Foxp3^+$ T cells induced in the periphery [22]. In spite of the complexity of regulatory cell types, $CD4^+CD25^+Foxp3^+$ Treg are the most prominent population of immunoregulatory cells operating during helminth infections described to date [23].

Several studies provided evidence of Treg cell activity during helminth infections eg. IL-

10 and TGF-ß were shown to mediate hyporesponsiveness observed in peripheral blood mononuclear cells from individuals with generalized onchocerciasis caused by the infective larvae of the filarial nematode *Onchocerca volvulus* [24]. In a study in filariasis patients, lymphedema was associated with a deficiency in the expression of Foxp3, GITR, TGF-β, and CTLA-4, known to be expressed by Treg cells [25] while in children infected with intestinal nematodes (*Ascaris lumbricoides* and *Trichuris trichiura*) high levels of IL10 and TGF-β were found and generalized T cell hyporesponsiveness [26, 27]. Likewise, schistosome-infected individuals in Kenya and Gabon had higher $CD4^+CD25^+$ and $CD4^+CD25^+Foxp3^+$ T cell levels compared with uninfected individuals [28]. In Indonesia, Treg cells from geohelminth-infected individuals were more effective at suppressing proliferation and IFN- γ production by effector T cells in response to malaria antigens and BCG than Treg cells from healthy individuals [29] .

Various studies have been performed in animal models. In mice, $CD25^+$ Treg cells restrained the pathology towards the eggs during schistosome infection [12] and towards *Trichuris muris* in the gut [30]. Moreover, depletion of $CD25^+$ Treg cells resulted in enhanced immunity to filarial nematodes (*Litomosoides sigmodontis*) in mice when combined with antibodies to GITR or CTLA-4, two surface markers characteristic for Treg cells [31]. Generation of Treg cells with elevated expression of Foxp3 has also been demonstrated. For instance, infection of BALB/c mice with *Brugia pahangi* third-stage larvae (L3) resulted in expansion of a population of $CD4^+CD25^+$ T cells that was highly enriched in Foxp3 and IL-10 gene expression [32]. Another filaria, *Brugia malayi*, was found to induce the expression of members of the TGF-β and TGF-β receptor superfamilies. This worm secretes TGH-2, a homologue of host TGF-β which can bind to the mammalian TGF-β receptor and promote the generation of regulatory T cells, as has been found for mammalian TGF-β [33]. In addition, a significant increased expression of Foxp3 and regulatory effector molecules such as TGF-β, CTLA-4, PD-1 and ICOS was found in infected individuals with live microfilariae of *B. malayi* [34]. Induction of Treg cells was also demonstrated to be necessary to establish a chronic *L. sigmodontis* infection since depletion of Treg cells in susceptible mouse, cleared infection [35]. In chronic infection with the natural intestinal parasite of mice *Heligmosomoides polygyrus*, it was established that levels of Foxp3 expression within the $CD4^+$ T cell population of the mouse mesenteric lymph nodes were significantly increased and that purified $CD4^+CD25^+$ Treg cells possess suppressive activity *in vitro* [36, 37].

Apart from being an advantage for the parasites, induction of a regulatory network can be beneficial for the host in some situations as well. Helminth infections have shown to

suppress allergic and autoimmune responses. It has been reported that helminth infections may contribute to a decreased incidence of type I diabetes. Infection with *L. sigmodontis* or injection of *L. sigmodontis* antigens prevented diabetes in NOD mice. In this study, protection was associated with increased Th2 responses and Treg cell numbers [38]. Likewise, infection with *S. mansoni* also protected against diabetes in NOD mice where transfer of $CD25^+$ cells from infected mice protected against disease [39]. In a mouse model of OVA-induced colitis, it was demonstrated that *H. polygyrus* infection reduced colitis. In this case, infection increased IL-4 and IL-10 production and after blocking these two cytokines, restoration of IL-17 production *in vitro* was observed [40]. Allergic responses are also suppressed by helminth infections and it will be described later.

Thus, it is clear that promoting the induction and expansion of Treg responses result in downregulation of Th2 effector responses against the parasites increasing, in this manner, not only the chances of parasite survival but also favoring the host.

Other regulatory cells

Helminth infections have also been associated with expansion of populations of immunoregulatory cells other than Treg cells, like alternatively activated macrophages (AAM) and regulatory B cells.

Signals encountered during migration by developing macrophages determine their function at sites of inflammation or infection. Among these signals, cytokines are responsible for the development of highly divergent macrophage phenotypes: classically activated macrophages and AAM [41]. AAM are triggered by Th2 responses. These AAM have been found to help dampening immune responses in schistosomiasis and to inhibit the development of severe hepatosplenic disease. The protective action of AAM depended on expression of arginase-1 (Arg-1), because it was shown that mice with macrophages lacking Arg-1 failed to suppress Th2 responses, egg-induced inflammation and developed fibrosis [42]. In addition, macrophages from mice lacking the cationic amino acid transporter 2 (Cat2) that regulates L-arginine transport into cells, had increased Arg-1 activity and reduced Th2 activity. Another product of AAM, resistin like molecule-α (RELMα /Fizz1/Retnla), helps to control inflammation in response to *S. mansoni*. Mice lacking RELMα developed severe egg-induced inflammation in the lung and liver that was associated with an enhanced Th2 response and could be reversed by treating the mice with recombinant RELMα [43]. Prieto-Lafuente *et al.* found that the homologues of the mammalian cytokine macrophage migration inhibitory factor (MIF) expressed by *Brugia malayi* synergized with IL-4 to induce the development of suppressive AAM *in vitro* [44].

One pathway for this effect may be through the MIF-mediated induction of IL-4R expression on macrophages, amplifying in this way, the potency of IL-4 itself. Thus, in a Th2 environment, MIF may prevent the classical activation of macrophages. AAM might function primarily to regulate immune responses and to facilitate tissue repair rather than to promote parasite killing [45].

B cells possess a variety of immune functions, including production of antibodies, presentation of antigens, and production of cytokines. IL-10-producing regulatory B cells have great potential to regulate T-cell-mediated inflammatory responses [5] and showed to downmodulate experimental autoimmune encephalomyelitis, collagen-induced arthritis, and inflammatory bowel disease [46]. In addition, in mouse models of chronic parasitic inflammation, such as chronic schistosomiasis, IL-10-producing B cells were also reported to be associated with protection against anaphylaxis and allergic asthma [47, 48]. Moreover, *H. polygyrus*-infected mice generate regulatory B cells that can downmodulate both allergy and autoimmunity in an IL10 independent manner [49].

The recent findings mentioned above on the generation and interactions of regulatory cells different from Treg cells provide a framework that may open novel opportunities for the treatment of inflammatory diseases, such as allergic asthma.

Immune response in allergy

Allergy is a hyperreactivity of the immune system in response to innocuous antigens (allergens). Allergy can be IgE-mediated and non-Ig-E mediated. IgE-mediated allergy such as asthma, rhinitis and eczema, is strongly associated with atopy. Atopy is the genetic predisposition to become sensitized and produce high levels of allergen-specific IgE antibodies in response to environmental allergens [50]. In the group of non–IgE-mediated allergy, the inflammation can be mediated by allergen-specific lymphocytes, as in allergic contact dermatitis, or by antibodies of the IgG isotype, as in anaphylaxis [50].

It is estimated that over 20% of the world's population suffers from IgE-mediated allergic diseases [51]. Asthma is reported by the World Health Organization (WHO) to affect both children and adults. It is estimated that approximately 300 million people of all ages and all ethnic backgrounds suffer from asthma [52]. For many years it has been reported that allergic diseases including asthma are increasing in Western countries [53, 54]. However, international surveys completed by the International Study of Asthma and Allergy in Childhood (ISAAC) carried out in 2007 report on a decrease of asthma symptoms in Western countries. In contrast, the prevalence of asthma symptoms has increased in

regions such as Africa, Latin America and parts of Asia where prevalence was previously low [55]. Although asthma symptom prevalence is no longer increasing in most Western countries, its global burden continues to rise.

Allergic reactions that are IgE-mediated are initiated when allergen is taken up by antigen presenting cells, such as DC, that process the allergen and present peptide fragments in the context of MHC class II to T cells, inducing a Th2 type of immune response. Allergen-specific B cells that take up allergen through the cell-surface immunoglobulin receptor may also initiate these allergic reactions. Activation of the allergen-specific Th2 cells leads to secretion of interleukin (IL)-4, IL-13 and subsequent class- switching of B cells to IgE synthesis. In addition, basophils secrete high levels of IL-4, IL-13 after activation and are suggested to play a role in polyclonal amplification of IgE production and in the differentiation of Th2 cells [56]. The binding of IgE to mast cells via high-affinity Fc receptors and subsequent cross linking of receptor-bound IgE by allergen triggers the release of pro-inflammatory mediators, responsible for the typical allergic reactions. [57].

In allergic asthmatic patients, exposure to allergen leads to an early-phase reaction that involves IgE-mediated degranulation of mast cells and subsequent constriction of the airway smooth muscle. This is followed 4–18 hours later by the late-phase reaction, which is characterized by recruitment of eosinophils and T cells [58]. Th2 cells mediate IgE synthesis via IL-4, eosinophilic inflammation via IL-5 and the recruitment and increase of mast cells via IL-9, which, together with IL-13, contribute to airway hyper-responsiveness (AHR) and other clinical features of allergic disease [59]. Although lung DC are sufficient to initiate and maintain the adaptive Th2 cell responses to inhaled allergens [60], it is now known that the epithelial cells and basophils play a central role. Studies have shown that the house dust mite (HDM) Der p 1 allergen activates airway epithelial cells through protease-activated receptor 2, C-type lectin receptors and Toll-like receptors leading to the production of thymic stromal lymphopoeitin (TSLP), granulocyte-macrophage colony stimulating factor, and IL-33 [61]. TSLP induces immediate innate immune functions in DC leading to chemokine-driven recruitment of Th2 cells and eosinophils to the airways. Epithelial cells produce CCL20 and IL-25 to further attract innate immune cells and Th2 cells to the lungs. TSLP and IL-33 induce DC migration to the mediastinal lymph nodes and stimulate the functions of mast cells and basophils. Induction of DC maturation by TSLP in the absence of IL-12 induces expression of OX40L, the ligand for the cell survival factor OX40, OX40-OX40L interactions are critical for the ability of the DC to drive Th2 polarization. In addition to its effects on DC, TSLP can also activate mast cells and basophils to produce IL-4 for Th2 cell differentiation [61, 62].

In conclusion, effector Th2 cells control the features of asthma in combination with mediators released by eosinophils, mast cells and basophils.

Helminth infections, allergy and the factors involved

The original *hygiene hypothesis* proposed that the lack of childhood infections results in a weaker Th1 cell responsiveness, allowing the expansion of the Th2 cell responses towards environmental allergens. However, studies with helminths known to induce Th2 type of immune reactivity showed that infection with these pathogens can also protect against allergic diseases [63]. It has now become clear that the interaction between helminth infection and allergy often involves Treg cells, induced by contact between host and infectious agents like helminths early in life [64]. The existence of Treg cells has changed the concept of the *hygiene hypothesis* based on their role in dampening both Th1 and Th2 effectors responses [22].

The inverse association between helminth infections and allergy has been reported extensively. Van den Biggelaar *et al.* showed that chronic infection with *Schistosoma haematobium* in an endemic area in Gabon was negatively associated with skin-test reactivity to HDM [65]. In addition, schistosome-specific IL-10 production was significantly higher in infected children and negatively associated with the outcome of skin-test reactivity to mite, suggesting an important role for this cytokine in suppressing atopy [65]. An important role for IL-10 was also found in another study where anti-helminthic treatment of *Schistosoma mansoni*-infected patients with asthma resulted in down-modulation of the Der p 1-specific IL-10 production *in vitro*[66]. In Brazil, Medeiros *et al.* have reported that the frequency of positive skin reactions to HDM antigens in subjects with history of wheezing was significantly lower in a *S. mansoni* endemic area than in a non-endemic area [67]. Similar studies in Ethiopia showed that hookworm infection protects against wheeze in atopic individuals and to a lesser extent, *Ascaris lumbricoides* infection [68]. A recent study has shown that infected individuals in an area endemic for *Brugia malayi* had a significantly reduced risk for atopic reactivity to cockroach [69].

The association between helminths and allergy is, however, not always consistent. In fact, infections with geohelminths have actually been found in some studies to be a risk factor for allergy. Cooper *et al.* suggest that high infection prevalence with geohelminths may confer protection against allergic disease whereas low prevalence infections are associated with increased risk for allergy [70]. Figure 1 summarizes findings on the different effects of helminths on allergy. For instance, the prevalence of skin test reactivity to seven allergens was significantly lower among children that had heavy *Trichuris trichiura*

infections compared to children with light or no infection [71]. In a cross-sectional study of 2,164 children in China, the association between *A. lumbricoides* and asthma was investigated. Infection with this nematode was associated with increased risk of asthma, increased skin test reactivity, and increased airways responsiveness. Here, it was found that the intensity of *Ascaris* infection was light to moderate in the majority of the children studied [72]. Similar findings were obtained in a study in Brazil [73] however, in a study with Cuban children that had low prevalence and intensities of infection, no association between *A. lumbricoides* infection and asthma or positive skin prick test was found [74]. In a nested case-control study drawn from a survey of 7,155 children (1 to 4 years old) from urban and rural areas of Jimma, Ethiopia it was found that wheezing was significantly more prevalent in urban than rural children, and was less prevalent in those infected with *Ascaris*, particularly in those with high intensity of infection [75]. A meta-analysis study analysed the effects of parasite infection intensity of *A. lumbricoides*, *T. trichuria*, and hookworm on asthma and wheeze. Results from this study disclosed no effect of *T. trichuria*, non-significant reductions in risk at higher levels of infection with *A. lumbricoides*, and significant dose-related reductions in risk of both asthma and wheeze with hookworm infection [76].

Induction of specific host immune regulatory mechanisms may be partly determined by host genetics and environmental factors. A study with British asthmatics and Chinese with *Ascaris* infection has revealed that STAT6 haplotypes associated with asthma in the United Kingdom are associated with resistance to *Ascaris* in China [77]. Similarly, the IL-13 promoter allele-1055TT has been associated with increased risk of asthma in Europe [78] and low *Schistosoma haematobium* burden in Mali [79]. Although a network of genetic factors may exist for determining a disease or susceptibility to infection, the effects may be visible only when certain environmental factors are present. Africans in rural areas seem to suffer less from allergies while people of African ancestry living in affluent countries have higher prevalence and severity of allergic symptoms than natives of these host countries [80].

Animal models offer a great opportunity to analyse the interaction between helminths and allergic diseases. Using a well-defined model for allergic airway inflammation, employing ovalbumin-OVA as allergen, it has been shown that chronic, but not acute, schistosome infections can suppress allergic airway inflammation in a dose-dependent manner. In this study, IL-10 was shown to play a central role in suppressing allergic airway inflammation after the adoptive transfer of splenocytes from chronically infected mice [48]. Another study showed that suppression of allergen-induced airway eosinophilia and reduction of

eotaxin production were not observed in IL-10 deficient mice infected with *Nippostrongylus brasiliensis* in comparison to control mice, suggesting that infection with this parasite suppresses the development of allergen induced airway eosinophilia, an effect potentially mediated by IL-10 [81]. *Heligmosomoides polygyrus* was also used as an experimental animal model to study the interaction between helminths and airway allergy. In this study the effect of Th2 cells induced by this gastrointestinal nematode, on experimentally airway allergy induced by OVA and HDM Derp1 was investigated. Infiltration of inflammatory cells in the lungs induced by both allergens was suppressed in infected mice compared to uninfected controls. Suppression was reversed in mice treated with antibodies to CD25. Most notably, suppression was transferable with mesenteric lymph node cells (MLNC) from infected animals to uninfected sensitized mice. MLNC from infected animals were found to have elevated numbers of $CD4^+CD25^+Foxp3^+$ T cells producing TGF-β and IL-10. These data support the argument that helminth infections elicit a Treg cell population able to down-regulate allergen induced lung pathology *in vivo* [64]. Thus helminths can modulate allergy-induced inflammatory responses by affecting different types of innate and adaptive immune cells, thereby suppressing the host immune responses through the development of Treg cells and other regulatory cells such as AAM and regulatory B cells (Fig. 1). A positive association between allergy and helminth infection has been reported from an experimental murine model of *T. canis* infection, that resulted in exacerbation of experimental airway inflammation [82], confirming findings from epidemiological studies [83]. In non-human primates, it has been shown that infection with *A. suum* resulted in AHR and eosinophilia [84, 85]. The effect of this worm infection on an ongoing experimental allergic asthma remains to be investigated.

Taking all these studies together, it is clear that there are several factors that may influence the association between helminth infections and allergic manifestations [82, 86, 87]. These include: (1) The helminth species involved: studies with different helminths suggest that depending on the pathogen, infection can either lead to protection or exacerbation of allergies. (2) Definitive vs. accidental host: It is likely that helminth parasites of humans have evolved with their host and have developed strategies to survive without causing much damage. This probably does not happen in an accidental host where the parasite cannot develop to the adult stage as occur with *T. canis*. (3) Host genetics: Individuals who are genetically susceptible to allergic disease may be genetically more resistant to worm infection or low worm burden.(4) Sporadic vs. chronic infection: chronic infections appear to result in immunosuppression not only against the parasite but also against other inflammatory diseases such as allergies whereas sporadic or transient infections may enhance allergic manifestations. (5) Intensity of infection: high parasite burden

may induce a suppressive type of immune response compared to light infections. (6) Timing of infection in relation to allergen exposure: for certain geohelminths infection in thefirst years of life is crucial in order to induce the type of immune response required for protection against allergic diseases.

All the mentioned factors may influence whether helminth infections protect or exacerbate allergic manifestations. Pinelli *et al*. have previously shown that infection with *T. canis*, exacerbates experimental allergic airway inflammation [82]. Since humans are accidental hosts for *Toxocara* infections, we became interested in determining the effect on allergic manifestations by a helminth that not only infects humans but also mice and completes its life cycle in a single host (definitive host). For this reason, we chose *Trichinella spiralis* for the studies described in this thesis.

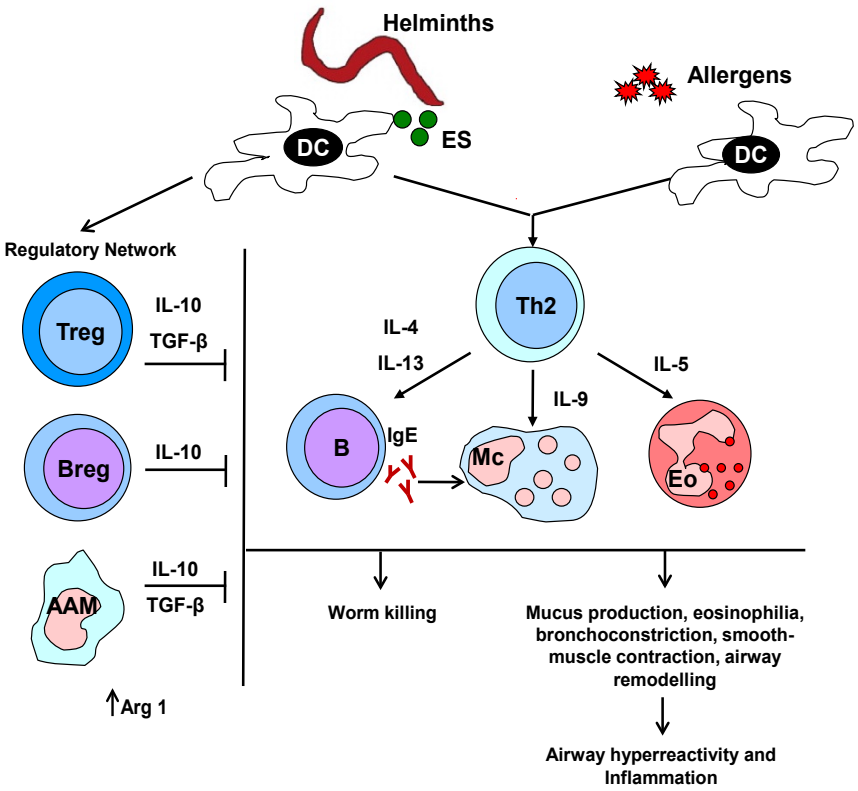

Fig. 1. Mechanisms by which helminths or their products could inhibit allergic responses. Helminth-induced regulatory network such as regulatory T cells (Treg), regulatory B cells (Breg) and alternatively activated macrophages (AAM) create an immunosuppressive environment that together with cytokines IL-10 and TGF-β and expression of arginase 1 (Arg 1) interfere with allergic effector mechanisms. B: B cells, Mc: macrophages, Eo: eosinophils, ES: excretory-secretory products

Trichinella spiralis

Trichinellosis is a parasitic infection caused by a nematode belonging to the genus *Trichinella*. The severity of the clinical course depends on parasitic factors such as the species involved, the number of living larvae ingested, and host factors such as sex, age, ethnic group, and immune status [88]. *Trichinella spiralis* is the most pathogenic specie for humans. This helminth completes its cycle in one single host where it survives for many years and modulation of the host immune responses at all phases of infection is most likely a pivotal strategy for survival [89].

Life cycle

Infection occurs when raw meat contaminated with L1 larvae of *T. spiralis* is ingested. The intestinal or acute phase is initiated when the larvae are freed from the tissue by the action of pepsin and hydrochloric acid in the stomach. Then, once in the small intestine, the larvae molt four times in a 30-hour period, transforming into the adult worm [89]. Five days after mating, the female adult releases newborn larvae (NBL), which measure 0.08 mm long by 7 μm in diameter (Fig. 2). These larvae cross the intestinal epithelia and enter into either the mesenteric lymphatics or the blood circulation, in which they are transported to skeletal muscle [89]. Larvae initiate the muscle phase of infection when they individually invade the muscle cell transforming this cell in a nurse-cell which supports growing and development of the larva [90].The nurse cell becomes surrounded by a collagenous wall, that provides protection to the parasite. [91]. Once the parasite completes its development in the muscle, it usually remains alive and infectious for months to years [92].

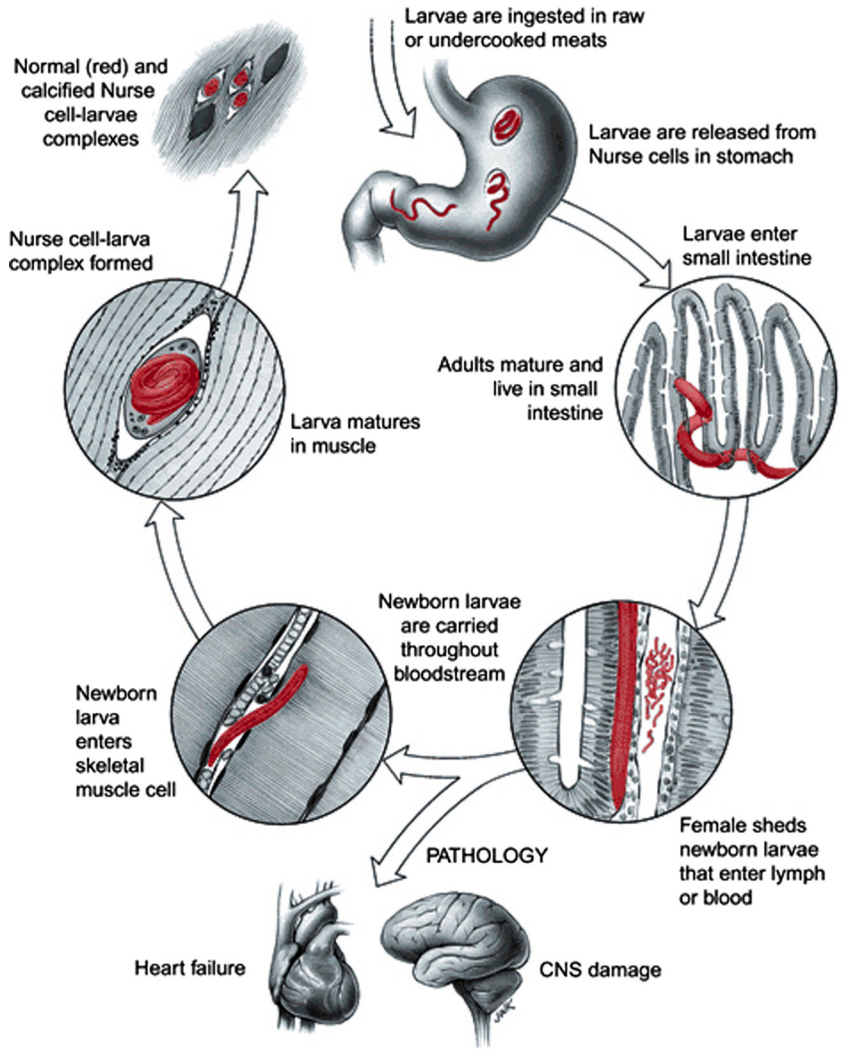

Fig. 2. Life cycle of *T. spiralis* [1]. After ingestion of infected undercooked meat, the *Trichinella* larvae are exposed to gastric acid and pepsin in the stomach. The larvae are released from the cysts and invade the small intestine where they develop into adult worms. About one week after infection, the female worms release newborn larvae that migrate to skeletal muscles where they encyst. Depending on the *Trichinella* species and the parasite load, infection can lead to pathology. [1] From Despommier D., Gwadz R.G, Hotez P., Knirsch C., 2005. *Trichinella spiralis*. In: Parasitic diseases. Apple Trees Productions L.L.C., New York.

Clinical manifestations

Low grade infection with *Trichinella* parasites is usually asymptomatic. However ingestion of undercooked meat infected with *Trichinella spiralis* may result, first in an acute or intestinal phase followed by a muscle or chronic phase of infection. During the acute phase of infection the following clinical manifestation may occur: upper abdominal pain, diarrhoea or constipation, vomiting, malaise, and low-grade fever that can persist for 1 to 3 weeks. [93, 94].

After migration of the newborn larvae, new signs and symptoms develop. A mild to moderate chronic infection can produce muscle pain, fever, swelling of the face, headache, chills, itchy skin or rash, cough, diarrhoea, constipation. These symptoms may occur within 2 weeks after eating infected meat and can last up to 8 weeks [93]. In moderate to severe infection, symptoms due to invasion of muscle cells increase during the third week, and oedema of the face, eyelids, hands, and feet becomes a prominent feature. If the infection is heavy, individuals may have trouble coordinating movements, and have heart and breathing problems [94]. Although rare, death can occur in severe cases. For mild to moderate infections, most symptoms disappear within a few months.

Diagnosis of Trichinella infection

According to the European Centre for Disease Control, a case definition for human trichinellosis is based on three criteria [94]: (1) clinical findings (at least three of the following six: fever, muscle soreness and pain, gastrointestinal symptoms, facial oedema, eosinophilia, and subconjunctival, subungual, and retinal haemorrhages); (2) laboratory findings (at least one of the following two laboratory tests: demonstration of *Trichinella* larvae in tissue sections derived from muscle biopsies and demonstration of a *Trichinella*-specific antibody response by indirect immunofluorescence, ELISA, or Western blot; and (3) epidemiological data (identification of source and origin of infection and outbreak studies).

Definitive laboratory diagnosis consists in finding the nurse cell containing the *Trichinella* larva in muscle biopsy by microscopy examination or detecting the *Trichinella*-specific DNA by PCR. These methods have a disadvantage because it requires surgical intervention and the sensitivity of the diagnosis depends on the parasite load and the amount of muscle sample tested.

Antibody detection tests are useful adjuncts to diagnosis starting on about day 12 after infection. By 14 days, most patients will suffer from clinical symptoms and will seek medical assistance. Immunofluorescence-based assays and ELISA to detect IgG

antibodies may be positive and remain positive for years after infection. The sensitivity of the IgG-ELISA reaches 100% on day 50. The test remains positive for more than 2 years in 88% of infected people. Responses by other immunoglobulins like IgA and IgE are similar as to that of IgG, but tests to detect these antibodies have lower sensitivity [93]. Although ELISA is the most commonly used serological test to diagnose trichinellosis, it has not been standardized, and most of the commercial ELISA kits for human serology are unreliable [95]. One reason for this unreliability is the use of excretory-secretory (ES) products from the *T. spiralis* muscle larvae that has serious disadvantages. The preparation of the antigen is laborious and requires the use of laboratory animals. Furthermore, the quality of the antigen may be affected by micro-environmental factors during culture of the animal-derived larvae [96], resulting in standardization problems. Finally, it may give rise to cross-reactivity to other antigenically related parasites [97]. Replacement of the *Trichinella* ES with synthetic antigens that have sufficient sensitivity and specificity could solve these problems. So far, an ELISA using the synthetic glycan tyvelose (3, 6-dideoxy-D-arabinohexose) has been used for diagnosis of human trichinellosis [98], but this glycan is not useful because it is not commercially available and its synthesis is complex.

Immunity to Trichinella infection

Immune responses against *T. spiralis* are characterized by the induction of a mixed Th1 and Th2 response. A dominant Th1 type of immune response is induced at the beginning of the intestinal phase. A protective Th2 type of response, responsible for the parasite expulsion, becomes dominant when dissemination of the NBL takes place [99, 100].
During chronic muscle infection, parasite-specific IgG1, IgG2 and IgE levels increase significantly which is associated with a strong Th2 response [101]. Several groups have investigated the mechanisms underlining the Th2 responses by which T cells regulate inflammation during chronic infection. For instance, cells recovered from cervical lymph nodes of C57BL/10 or C57BL/6 mice bearing muscle larvae produce IL-5, IL-10, IL-13 and IFN-γ after stimulation with somatic larval antigens [102, 103]. In humans, blood mononuclear cells recovered over a year after *Trichinella britovi* infection and produce significant quantities of IFN-γ, IL-10 and IL-5, and retain the ability to proliferate in response to larval antigens for as long as 3 years after infection [104]. Pinelli *et al*. found that the humoral immune response (IgG, IgM, IgA) against *Trichinella* antigens can persist up to 15 years after the initial infection with *T. britovi* [105]. In relation to the local immune response in muscle, it was demonstrated by Beiting *et al.* that IL-10 is critical in

the control of initial inflammation during development of *T. spiralis* [106]. Here, wild-type and IL-10$^{-/-}$ mice were infected by injecting NBL intravenously, in this way there was not interference of the immune response induced by the intestinal phase. The absence of IL-10 was most evident 20 days after muscle infection, when an exaggerated inflammatory response around nurse cells was observed in IL-10$^{-/-}$ mice compared to C57BL/6 wild-type mice. Thus, IL-10 limits local inflammation during the early stages of muscle infection but chronic inflammation is controlled by a Th2 response characterized by a strong IgG1 response to tyvelose-bearing glycoproteins that are produced only by mature first-stage larvae [106]. In addition, Beiting *et al*. also showed that T effector cells-derived IL-10 limits acute myositis, IFN-γ levels and local iNOS production [102]. During the life cycle of *T. spiralis*, surface and ES products from the adult parasite are involved in acute inflammatory responses while during the chronic phase only ES products from the muscle-larvae L1 (ES-L1) are responsible for molecular cross-talking with the host [100]. These ES products have a clear effect on the host's immune response such as reduction of the inflammation provoked by the invasion of muscle cells, modulation of the immune response in order to protect both the parasite and the host and at the same time they participate in orchestrating the biological process of host's cell remodelling [100]. The ES products contain cystatins, serpins, glycans, mucins, lectins or cytokine homologs that could influence antigen processing, presentation and subsequent T-cell polarization [107].

Studies that focus on the effect that *T. spiralis* ES products have on DC have been described. For instance, using antigens from all three life stages (adult, NBL and muscle larvae-L1) Ilic *et al.* showed that parasite antigen-stimulated DC from Dark Agouti (DA) rats and C57BL/6 mice exhibited incomplete maturation [108, 109]. None of the parasite antigens showed any effect on the up-regulation of MHC II on rat DC, but they provoked up-regulation of CD86 and ICAM-1 as well as increased production of IL-10 and decreased production of IL-12p70 [108]. Results obtained in studies using mouse DC showed moderate up-regulation of MHC II, significant up-regulation of CD80 and CD86, non-significant up-regulation of OX40L and ICAM-1 and elevated production of both pro- and anti-inflammatory cytokines [109]. In spite of the immature state of DC, they possess the capacity to present antigens to T cells and to produce cytokines. Ilic *et al.* also showed *in vitro* that DC from DA rats primed with *T. spiralis* antigens from different life stages induced increased production of IL-4, IL-10 and TGF-β and decreased production of IFN-γ in CD4^{+} T cells while C57BL/6 mouse DC induce elevated production of both Th2 (IL-4, IL-9, IL-13 and IL-10) and Th1 (IFN-γ) cytokines by naïve T cells [109]. However, an increase in the population of Foxp3^{+} T regulatory cells was not observed which is contrary to findings related to other helminths [109]. Gruden-Movsesijan *et al.*

found that priming naïve T cells *in vitro* with rat DC pulsed with ES-L1 induced strong Th2 polarization, accompanied by elevated production of regulatory cytokines IL-10 and TGF-β, but no increase in the population of $CD4^+CD25^+Foxp3^+$. While injection of rats with ES-primed DC resulted in a mixed Th1/Th2 cytokine response, dominated by the Th2 type, elevated levels of IL-10 and TGF-β and a significant increase in the population of $CD4^+CD25^+Foxp3^+$ cells in the spleen [110].

As described previously, helminth parasites can influence the outcome of autoimmune and allergic diseases. In relation to *T. spiralis*, a limited number of experiments in animal models of human autoimmune diseases and allergic asthma have been performed. Khan *et al.* showed that *T. spiralis* infection reduces the severity of dinitrobenzenesulphonic acid (DNBS)-induced colitis in C57BL/6 mice [111] while Motomura *et al.* demonstrated that in addition to the protection exerted by the actual infection, rectal submucosal administration of *T. spiralis* crude muscle larvae antigen previous to the induction of colitis can also protect against this autoimmune disease [112]. *T. spiralis* infection also ameliorated the development of autoimmune diabetes in NOD mice [113] and modulated severity of disease in the experimental model of multiple sclerosis named experimental autoimmune encephalomyelitis (EAE) in Dark Agouti rats in a dose-dependent manner [114]. Severity of EAE as judged by lower maximal clinical score, cumulative disease index, duration of illness and the number of mononuclear cells infiltrating the spinal cord in infected animals were reduced in comparison to the uninfected EAE-induced group. In a following study, it was found that in infected-EAE rats the increased production of IL-4 and IL-10 mediated the reduction of IFN-γ and IL-17 production in draining lymph nodes. The decreased production of these cytokines coincided with alleviation of the disease. The authors suggest that the enhanced production of IL-4 correlates with a Th2 polarized immune response with strong activation of regulatory mechanisms. Transfer of T cell-enriched splenic cells from *T. spiralis*-infected rats into rats in which EAE was induced, caused amelioration of EAE. This population of cells contained a higher proportion of $CD4^+CD25^+Foxp3^+$ regulatory cells and produced a high level of IL-10 when compared with spleen cells from uninfected rats [115]. Soluble products from *T. spiralis* used for treating murine EAE resulted in significant suppression of EAE symptoms [116].

In conclusion, *T. spiralis* induces a mixed Th1/Th2 type of response during the initial phase of infection followed by a dominant Th2 type of response as well as the engagement of a regulatory network. So far, there is evidence on the immunomodulatory effect of *T. spiralis* on the host's immune system, however the mechanisms involved in this immunomodulation and the consequences to unrelated antigens are still undefined and need further investigation.

Aims and outline of this thesis

Several helminths and their products have been shown to modulate the immune response in order to survive in their host. By doing so, the hosts may also benefit due to concomitant reduction of immune pathology associated with allergies and autoimmune diseases. The mechanisms involve in immunomodulation differ nevertheless among helminths since each of these parasite species has evolved its own surviving strategy. Therefore, studying molecules and mechanisms involved in the modulation of the host immune responses by different helminths is necessary for a better understanding especially in the context of developing new strategies for treatment of allergies and autoimmune diseases.

The studies described in this thesis contribute to our knowledge on immunomodulation by helminth antigens, particularly, on the induction of regulatory T cells *in vitro* and *in vivo*. The aim of this thesis was to investigate the mechanisms by which *T. spiralis* and its secreted products modulate the host immune response, and its effect in experimental allergic asthma. In addition, as a spin-off from this study we used the knowledge on detection, identification and synthesis of molecules from this parasite to develop new tools for the serodiagnosis of *T. spiralis* infection.

In chapter 2, we aimed at determining the effect of the *T. spiralis* excretory-secretory (TspES) antigens on DC maturation. We performed *in vitro* experiments using murine bone marrow-derived DC (BM-DC) and found that TspES by itself did not have an effect on maturation of BM-DC. To determine whether TspES had any effect on matured BM-DC we used LPS, a well-known DC maturation stimulus, which was used in combination with TspES. We found that TspES suppress DC maturation induced by the smooth (S)-form of *E. coli* LPS. These findings suggested that one of the mechanisms by which TspES suppress DC maturation is by interacting with TLR4 or the molecules triggered after TLR4 engagement by LPS. We also tested the rough (R)-form of *Neisseria meningitidis* LPS and found that TspES does not suppress DC maturation induced by this LPS. These findings indicated that the suppressive effect of TspES might depend on the bacterial source of LPS.

In chapter 3, we investigated this effect further by using LPS from other bacteria and, also aimed at determining the effect of TspES on T cell activation and the induction of Treg cells. To this end, in addition to using the previously described LPS, we tested the LPS R- form of *E. coli* and the S-form of *Salmonella minnesota.* Both bacteria are members of the *Enterobacteriaceae* family. We found that TspES suppress DC maturation induced by both the S-form and R-form of enterobacterial LPS, but not by the R-form of *N.*

meningitidis, as mentioned above. This may suggest that *Trichinella*-secreted products suppress DC maturation induced only by LPS (independent of the S or R-form) derived from enterobacteria. During migration through the intestinal wall, *Trichinella* larvae may drag bacteria on their surface. These larvae will migrate further in the blood stream towards the muscles. Suppressing the effect of LPS from the dragged bacteria may be a strategy to avoid a septic shock which could kill the host. Using a panel of TLR ligands, we found that the suppressive effect of TspES on DC maturation is restricted to TLR4 and that these helminth products interfere with the expression of several genes related to the TLR-mediated signal transduction pathways. Using splenocytes derived from OVA-TCR transgenic D011.10 mice, we showed that TspES induce the expansion of $CD4^+CD25^+$ $Foxp3^+$ Treg cells *in vitro* in a TGF-β-dependent manner.

After the *in vitro* studies on the mechanisms involved in immunoregulation induced by *T. spiralis* antigens, in chapter 4, we investigated whether *T. spiralis* infection had any effects on allergic asthma. We combined the OVA–induced murine experimental allergic airway inflammation (EAAI) and the murine model for trichinellosis. We examined the *in vivo* effect of the acute (intestinal) and chronic (muscle) phases of *T. spiralis* infection on EAAI. In addition, the involvement of regulatory T cells in this process was investigated. We found that *Trichinella* infection resulted in significant suppression of OVA-specific IgE levels, Th2 cytokine production, eosinophils in BAL and lung airway inflammation, indicating that *Trichinella* infections suppressed EAAI. Our data showed that the chronic as well as the acute phase of *Trichinella* infection protects against EAAI. This protective effect was found to be stronger during the chronic phase of infection and to be associated with increased numbers of $CD4^+CD25^+Foxp3^+$ Treg cells in the spleen. The Treg cells induced after *T. spiralis* infection were shown to have suppressive activity and adoptive transfer of $CD4^+$ T cells from spleen of chronic infected mice containing elevated numbers of Treg cells resulted in partial protection against EAAI, indicating a possible role for Treg cells in suppression of EAAI during *T. spiralis* infection.

Among the molecules responsible for the immunomodulation of the host immune response by helminths, certain glycans have been suggested to play an essential role. These glycans are also linked to the generation of host antibody responses during infection which make them excellent targets for diagnostic purposes. In chapter 5, we examined the potential use of parasite glycan antigens for serodiagnosis of *T. spiralis* infection. We show that specific parasite glycan antigens can be identified by using sera of infected patients and glycan arrays. In addition, we demonstrated that an ELISA based on neoglycoconjugates has a high sensitivity for serodiagnosis of trichinellosis, indicating the potential value

of the glycan microarray technology for developing serodiagnostic assays of parasitic infections.

Finally, in chapter 6, the findings from the studies described in this thesis are summarized and discussed.

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Chapter 2

Suppression of dendritic cell maturation by *Trichinella spiralis* excretory/secretory products

Merel Langelaar, Carmen Aranzamendi, Frits Franssen,
Joke van der Giessen, Victor Rutten,
P. van der Ley and Elena Pinelli

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Abstract

Infection with helminths induces a dominant Th2 type of immune response and a regulatory network in the infected host. This immunomodulation is not only parasite specific but it can also influence the immune response to unrelated antigens and other pathogens. In this study, we focus on the effect of *Trichinella spiralis* excretory/secretory antigens (TspES) on dendritic cell (DC) maturation. Bone marrow derived DC from BALB/c mice were incubated *in vitro* with TspES either alone or in combination with lipopolysaccharide (LPS) derived from two different bacteria. As indicators of DC maturation, the cytokine production (IL-1α, IL-6, IL-10, IL-12p70 and TNF-α) and the expression of various surface molecules (MHC-II, CD40, CD80 and CD86) were measured. Results indicate that while TspES alone did not change the expression of the different surface molecules or the cytokine production, it completely inhibited DC maturation induced by *Escherichia coli* LPS (*E.coli* LPS). In contrast, DC maturation induced by LPS from another bacterium, *Neisseria meningitidis,* was not affected by TspES. In conclusion, *T. spiralis* ES antigens lead to suppression of DC maturation but this effect depends on the type of LPS used to activate these cells.

Introduction

Trichinella spiralis (*T. spiralis*) is a zoonotic helminth with worldwide distribution. Humans and other mammals become infected after ingestion of the larval stage present in raw or undercooked meat. The larvae migrate to the intestine of the infected host and mature to the adult reproductive stage, where the parasites mate and newborn larvae (NBL) are released within 6 days after infection. These NBL migrate to skeletal muscle, where they encyst and can remain in a dormant stage for years. Although generally asymptomatic, the infection can result in clinical disease characterized by an acute, followed by a convalescent and eventually chronic phase in which the larvae are still present but there are not necessarily clinical signs [1].

During infection, pathogens and/or their products may interact with dendritic cells (DC) via specific receptors, giving rise to DC maturation which is fundamental for the generation and polarization of the adaptive immune response. These antigen presenting cells are armed to recognize a large variety of antigens from different pathogens through various families of pattern recognition receptors (PRR) such as Toll-like receptors (TLRs) [2, 3].

Infection with helminths is generally associated with suppression of the host immune response to parasite as well as to unrelated antigens, which would benefit parasite survival but can also influence the immune response to other immunopathologies or infections [4-6]. Infection with *T. spiralis* using murine and rat models has been suggested to be associated with immunosuppression [7, 8]. However, the precise mechanisms and molecules involved in this immunoregulation are unknown. In this study we aim at determining the effect of *T. spiralis* ES antigens (TspES) on DC maturation *in vitro*.

Materials and methods

Cell Culture

Bone-marrow derived DC from BALB/c mice were prepared using a modified procedure previously described by Lutz *et al*. [9]. Briefly, cells were incubated in 6- well plates (BD Falcon™, BD Europe, Erembodegem, Belgium) and at day 2 and 4, 20 ng/ml rGM-CSF (Cytocen, Utrecht, The Netherlands) were added. On day 7, cells were incubated with helminth antigens and/or the following TLR ligands: *E. coli* LPS (LPS_{Eco}) O26:B6 (Sigma), or LPS of *Neisseria meningitidis* (LPS_{Neiss}), purified as described previously [10]. In addition to bone marrow-derived DC, we used HEK293 cells transfected with mouse TLR4/MD2/CD14 (Invivogen). Cells ($2x10^5$/ well) were incubated O/N with TspES alone, TspEs in combination with LPS_{Eco} or LPS_{Neiss} or each LPS alone. The production of IL-8 by HEK293 cells in response to the different antigens (or combinations) was measured to analyse the effect of TspES on LPS-induced maturation of these cells.

Trichinella spiralis excretory–secretory (E/S) antigens

The excretory–secretory (E/S) antigen was prepared from *T. spiralis* muscle (TspES) larvae from infected rats, recovered by acid-pepsin digestion and washed and incubated in RPMI medium for 19 hours as described by Gamble [11]. After centrifugation, the supernatant containing the E/S products (TspES) was dialyzed, concentrated and the protein concentration was determined. Endotoxin determination was performed using the QCL-1000 chromogenic LAL Endpoint Assay (Lonza, Basel, Switzerland). The endotoxin value in TspES was below the detection limit of the assay (0.2 EU/ml).

Flow cytometry

Dendritic cells were incubated with TspES alone, TspEs in combination with LPS_{Eco} or LPS_{Neiss} or each LPS alone. After O/N incubation supernatants were harvested for cytokine determination and cells were used for flow cytometry experiments. The effects of the different antigens on the expression of surface molecules on DC were measured using fluorescence-activated cell sorting (FACScan, BD Biosciences, Erembodegem, Belgium) analysis, following standard FACS protocols using monoclonal antibodies directed against the following antigens: CD40, CD80, CD86 and MHC class II, labelled with phycoerythrin (PE) and CD11c labelled with allophycocyanin (APC). All fluorochrome-conjugated antibodies were purchased from BD Biosciences. Propidium iodide (Sigma) was used to determine the viability of the cells. Viable cells were 70-95% CD11c positive.

For analysis, CD11c-positive cells were gated.

Cytokine determination

Interleukin-1α(IL-1α), IL-6, IL-10, IL-12p70 and tumour necrosis factor-α (TNF-α) were measured using ELISA kits (BD OptEIA, BD Biosciences), according to the manufacturer's instructions. The levels of IL-8 in the TLR4/MD2-CD14 HEK293 cell culture supernatants were measured using ELISA PeliPair reagent set (Sanquin, Amsterdam, The Netherlands)

Statistical analysis

Cytokine data are represented as mean ± SD. An unpaired, two-tailed Student's t-test was used to determine significant differences. A probability value $p < 0.05$ was considered statistically significant

Results

Expression of surface molecules on DC after incubation with TspES and LPS derived from *E. coli* and *N. meningitidis*

When DC were incubated with 1 ng/ml LPS_{Eco} or LPS_{Neiss}, the expression of the surface molecules MHC II, CD40, CD80 and CD86 increased, as expected. Incubation of the cells with 5 μg/ml TspES alone did not result in changes in the expression of any of these surface molecules as compared to cells incubated in medium only. However, when cells were incubated with LPS_{Eco} in combination with TspES, the surface molecule expression was reduced to background levels (Table 1). This suppression was observed at concentrations of 5 to up to 0.5 μg/ml of TspES. At a concentration of 0.05 μg/ml TspES, the suppressive effect ranged between 32 and 57% and at a concentration of 0.005 μg/ml the suppressive effect was abolished (Fig. 1). These results indicate that the suppressive effect of TspES on DC maturation is dose dependent.

In contrast, to the findings with LPS_{Eco}, TspES did not affect the expression levels of the surface molecules induced by LPS_{Neiss} (Table 1). Different concentrations of LPS_{Neiss} were used to activate DC, however, under no conditions was the expression of surface molecules significantly reduced by TspES (Fig. 2).

Cytokine production by DC after incubation with TspES and LPS derived from *E. coli* and *N. meningitidis*

Similar to the findings with the expression of surface molecules, TspES alone did not induce DC to produce cytokines. However, the production of all the tested cytokines was reduced to background levels when LPS_{Eco} was combined with TspES. The difference in cytokine production by cells incubated with LPS_{Eco} compared to cells incubated with TspES and LPS_{Eco} was in all cases statistically significant ($p<0.05$). In contrast, no statistically significant differences could be observed between cells incubated with LPS_{Neiss} alone and LPS_{Neiss} in combination with TspES (Fig.3A-E).

Cell death by apoptosis by TspES could not explain the observed effects, since cells incubated with this helminth antigen were highly viable. The percentage of cells, analysed by flow cytometry, that were stained with propidium iodide and annexin-V did not exceed 5% of the total number of cells (data not shown).

IL-8 production by TLR4/MD2/CD14 transfected HEK cells after incubation with TspES and LPS derived from *E. coli* and *N. meningitidis*

TLR4/MD2/CD14 transfected HEK cells incubated with *E. coli* or *N. meningitidis* LPS together with TspES, resulted in comparable findings as when using DC. In other words, TspES suppressed IL-8 production by the transfected HEK cells induced by LPS_{Eco} but no significant inhibition of the IL-8 production was observed when TspES was combined with LPS_{Neiss} (Fig 3F).

Incubation of DC with TspES and LPS								
Surface molecules:	**MHC II**		**CD40**		**CD80**		**CD86**	
	MFI (SD)	% pos (SD)	MFI (SD)	% pos (SD)	MFI (SD)	% pos (SD)	MFI (SD)	% pos (SD)
Medium	106 (79)	49 (12)	40 (1)	21 (1)	83 (9)	41 (3)	41 (19)	22 (7)
TspES	563 (100)	72 (1)	40 (4)	20 (3)	97 (4)	46 (1)	74 (3)	38 (1)
LPS_{Eco}	2036 (326)	90 (1)	119 (7)	68 (2)	261 (23)	71 (3)	357 (86)	75 (4)
LPS_{Eco} +TspES	534 (97)*	72 (3)*	42 (4)*	23 (3)*	103 (13)*	48 (3)*	73 (4)*	38 (2)*
LPS_{Neiss}	952 (345)	87 (2)	142 (11)	74 (1)	323 (165)	68 (6)	378 (129)	76 (3)
LPS_{Neiss} +TspES	1086 (429)	87 (2)	147 (13)	74 (2)	304 (78)	70 (2)	421 (108)	77 (3)

Table 1. Expression of surface molecules on mouse bone marrow-derived DC. Median fluorescence intensity (MFI) and percentages of cells positive (% pos) for the different surface molecules after O/N incubation of the cells in medium only or with *T. spiralis* E/S antigens (TspES), *E .coli* LPS (LPSEco), LPSEco together with TspES, *N .meningitidis* LPS (LPSNeiss) or LPSNeiss together with TspES. Significant differences between MFI and % pos cells incubated with LPS only and cells incubated with LPS together with TspES are indicated with asterix * ($p<0.05$). Values in the table are from one experiment performed in triplicate (+/- SD) that has been repeated at least ten times with similar results.

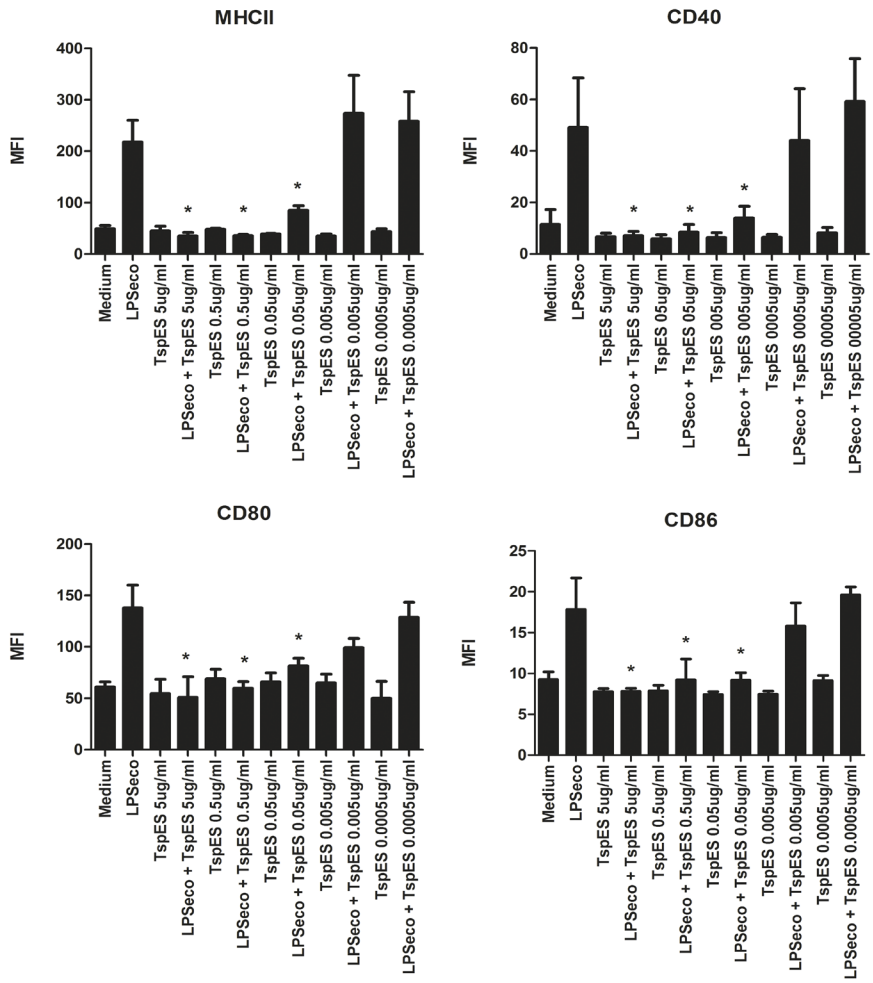

Figure 1. Expression of surface molecules determined by FACS, after O/N incubation of cells in medium only or with *T. spiralis* E/S antigens (TspES) at different concentrations, or with *E. coli* LPS 1 ng/ml (LPSeco) together with different concentrations of TspES. The different surface molecules are indicated at the top of every figure. Error bars represent SD of one experiment carried out in triplicate, representative of three independent experiments. Significant differences between LPSeco and LPSeco + TspES are indicated with asterix * ($p<0.05$).

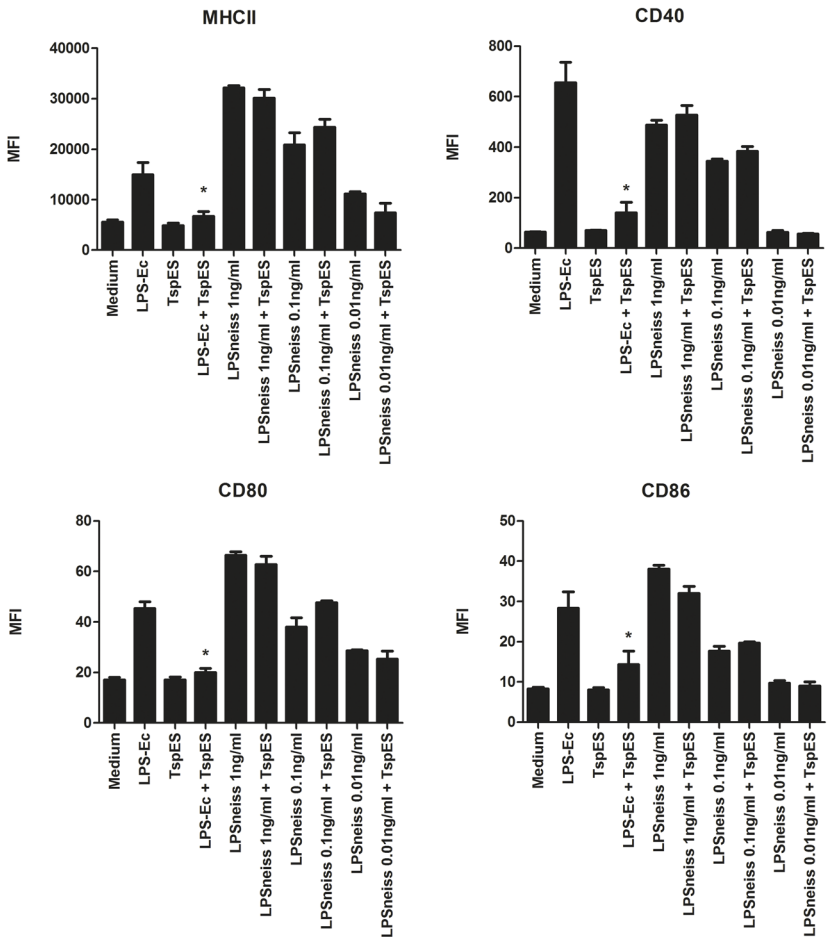

Figure 2. Expression of surface molecules determined by FACS, after O/N incubation of cells in medium only or with *T. spiralis* E/S antigens 5 µg/ml (TspES) or with *N. meningitidis* LPS (LPSneiss) at different concentrations together with TspES 5 µg/ml. The different surface molecules are indicated at the top of every figure. Error bars represent SD of one experiment carried out in triplicate, representative of three independent experiments. Significant differences between LPSeco and LPSeco + TspES or LPSneiss and LPSneiss + TspES are indicated with asterix * ($p<0.05$).

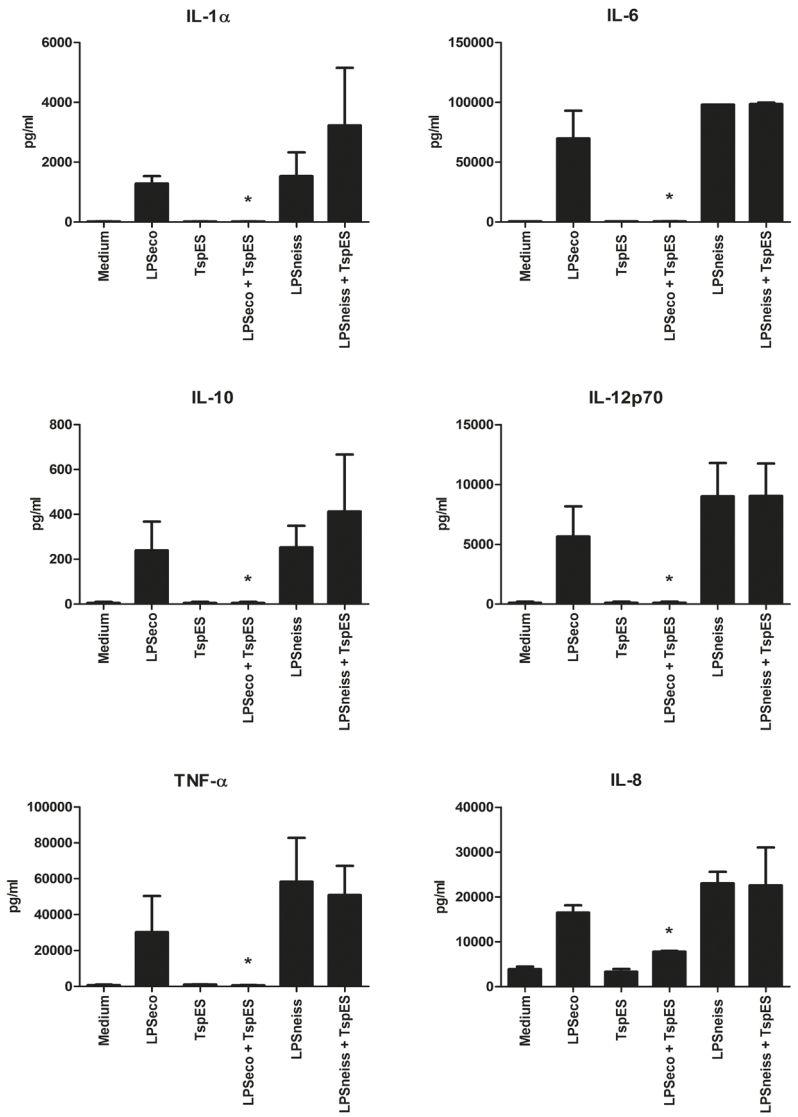

Figure 3. Cytokine production, determined by ELISA, after O/N incubation of cells in medium (Med) only or with *T. spiralis* E/S antigens (TspES), *E. coli* LPS (LPSeco), LPSeco together with TspES, *N. meningitidis* LPS (LPSneiss) or LPSneiss together with TspES. The different cytokines are indicated at the top of every figure. Error bars represent SD of one experiment carried out in triplicate, representative of three independent experiments. Significant differences in cytokine production between cells incubated with LPS only and cells incubated with LPS together with TspES are indicated with asterix * ($p<0.05$). A-E: Cytokine concentrations in supernatant of DC. F: Cytokine concentration in supernatant of TLR4/MD2/CD14-transfected HEK cells

Discussion

Little is known about the mechanisms involved in the effect of *Trichinella* infection on the immune response to other unrelated antigens or pathogens. From murine models there is some evidence indicating that during the early phase of infection with *Trichinella spiralis* the systemic immune response against heterologous antigens is suppressed [7], and severity of EAE in *Trichinella spiralis* infected rats is reduced [8]. Studies carried out *in vitro* have shown that E/S components of *T. spiralis* have a suppressive effect on the function of human neutrophils and murine macrophages [12, 13]. Unlike the findings from our study, Ilic *et al.* [14] report on up-regulation of costimulatory molecules (CD54 and CD86) and production of IL-10 by DC incubated with different types of *Trichinella spiralis* antigens. The differences between these two studies may rely on the nature and concentration of the antigens used. Ilic *et al.* used 100 µg/mL of crude larval antigen; mannose-rich glycoproteins, ES products from adult parasites and soluble extract of newborn larvae whereas we use 5 µg/mL of ES products from muscle larvae. In addition differences in culture conditions including the use of DC derived from mice compared to rats cannot be excluded.

The results obtained with DC were confirmed using HEK293 cells transfected with mouse TLR4/MD2/CD14. *T. spiralis* ES antigens obviously affect the interaction of *E. coli* LPS with TLR4. Involvement of TLR4 in modulation of DC maturation by other helminth antigens has been previously described by Goodridge *et al.* using ES62, a phosphorylcholine-containing glycoprotein secreted by the filarial nematode *Acanthocheilonema viteae* [15] and by others using soluble egg antigens (SEA) derived from *Schistosoma mansoni* [16, 17]. Recently, Segura *et al.* [18] reported on the inhibition of TLR induced cytokine production of DC with *Heligmosomoides polygyrus* (Hp) ES for several TLR ligands. The effect of helminth ES antigen and SEA on DC maturation appeared to be dependent on different TLR, including TLR4.

The differential effect that we observed for TspES suppression on DC maturation by *E. coli* and *N. meningitidis* LPS might reflect the difference in carbohydrate composition of the two molecules. Competition on TLR4 for the glycan binding sites of the *E. coli* but not of the *Neisseria* LPS by TspES may account for the inhibitory effect observed by the former but not by the latter. Another explanation could be involvement of the CD14 molecule within the TLR4/MD2/CD14 complex. LPS in the smooth (S) form, as synthesized by most wild type gram-negative bacteria, such as *E. coli,* requires the CD14 molecule as a co-receptor for TLR4 to activate the signalling pathway in DC [19, 20]. The R-form LPS synthesized by rough (R) mutants of gram-negative bacteria,

which lacks the O-polysaccharide chain that is present in the S- LPS, is capable of DC activation through TLR4 independently of CD14. *Neisseria meningitidis* synthesizes LPS with a highly reduced number of sugar residues, thus resembling R-form LPS [21]. The differential effect that we observed for TspES suppression on DC maturation by *E. coli* and *N. meningitidis* LPS could therefore suggest a role for CD14 in addition to TLR4.

The contrasting effect of TspES on DC activated with two different bacterial LPS, both acting via TLR4, indicate a very specific mechanism of action for this helminth antigen. These results suggest that the immunosuppressive effect of helminth antigens to unrelated antigens and other infections may depend on the helminth species involved [22] but also on the nature of the unrelated antigen or the infectious agent. Future investigations including *in vivo* studies will provide a better insight into the mechanisms involved in immunomodulation by helminth molecules. Finally, identification of the molecules in TspES that exert the strong immunosuppressive effect could have an application in the development of therapeutic agents for the treatment of different immunopathologies and other infections.

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Chapter 3

Trichinella spiralis secreted products modulate DC functionality and expand regulatory T cells *in vitro*

Carmen Aranzamendi, Floris Fransen, Merel Langelaar,
Frits Franssen, Peter van der Ley, Jos P.M. van Putten,
Victor Rutten, Elena Pinelli

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Abstract

Helminths and their products can suppress the host immune response which may benefit parasite survival. *Trichinella spiralis* can establish chronic infections in a wide range of mammalian hosts including humans and mice. Here, we aim at studying the effect of *T. spiralis* muscle larvae excretory/secretory products (TspES) on the functionality of DC and T cell activation. We found that TspES suppress *in vitro* DC maturation induced by both S- and R-form LPS (lipopolysaccharide) from enterobacteria. Using different TLR agonists, we show that the suppressive effect of TspES on DC maturation is restricted to TLR4. These helminth products also interfere with the expression of several genes related to the TLR-mediated signal transduction pathways. In order to investigate the effect of TspES on T cell activation we used splenocytes derived from OVA-TCR transgenic D011.10 that were incubated with OVA and TspES –pulsed DC. Results indicate that the presence of TspES resulted in expansion of $CD4^+CD25^+$ Foxp3+ T cells. These regulatory T (Treg) cells were shown to have suppressive activity and to produce TGF-β. Together these results suggest that *T. spiralis* secretion products can suppress DC maturation and induces expansion of functional Treg cells *in vitro*.

Introduction

Helminths and their products have been shown to suppress the host's immune response by inducing a regulatory network e.g. regulatory T (Treg) cells that dampen parasite specific immune responses and favor parasite survival [1]. This anti-inflammatory property of a number of helminths is not restricted to the parasite itself but may also affect other immune responses and immunopathological disorders of the host [2]. In this regulatory network, dendritic cells (DC) play a pivotal role. DC through several types of pattern recognition receptors such as toll-like receptors (TLR), C-type lectin receptors, RIG-I like receptors and Nod-like receptors may recognize various pathogen-associated molecular patterns. Upon stimulation, DC undergo phenotypic and functional changes that allow them to migrate to lymph nodes and prime appropriate T cell responses [3, 4]. DC can prevent, inhibit or modulate T cell-mediated effector responses by production of anti-inflammatory factors that can result in induction of Treg cells [5].

After ingestion of *T. spiralis* infected meat, the larvae are released in the stomach, migrate to the small intestine where they maturate into adult worms and release newborn larvae that rapidly disseminate throughout the host, and eventually enter skeletal muscle to remain for many years [6, 7]. The host-mediated immune response against *T. spiralis* depends on $CD4^+$ T cells and mast cells that have an essential role in worm expulsion [8]. Interleukin-4 and IL-13 are involved in this protective immune response and there is evidence that when the effects of both of these cytokines are inhibited, worm survival is extended [9]. Other findings indicate that IL-10 and TGF-β control the level of inflammation during the muscle stage of infection [10]. Few studies have suggested that *T. spiralis* can modulate the host immune response, protecting it against other immune pathologies [11-13]. However, little is known about the role of Treg cells induced by this helminth and their products.

Modulation of the immune response by helminths involves the excretory/secretory (ES) products released by these parasites. These products include proteases, protease inhibitors, venom allergen homologues, glycolytic enzymes, lectins, lipids and glycans which together or individually could be potential immunomodulators [14, 15]. There are only few studies on *T. spiralis* products and their effect on the immune response [16, 17]. We have previously shown that ES products from *T. spiralis* (TspES) suppress DC maturation induced by the S-form of *Escherichia* coli LPS but not by the R-form of *Neisseria meningitidis* LPS [16]. Since the S-form of LPS requires CD14 for TLR4 activation whereas the R-form does not [18-20], a possible role for CD14 in this

suppression was suggested.

Here, we aim at studying further the effect of TspES on DC maturation and on T cell activation. We found that the suppressive effect of TspES on DC maturation does not depend on the form of the LPS used, and therefore it is independent of CD14. In addition, we show that the suppressive effect of TspES on DC maturation is restricted to TLR4 and that these helminth products interfere with the expression of several genes related to the TLR-mediated signal transduction pathways. Using splenocytes derived from OVA-TCR transgenic D011.10 mice, we show that TspES induce *in vitro* the expansion of $CD4^+CD25^+$ $Foxp3^+$ Treg cells in a TGF-β-dependent manner. These findings contribute to our understanding on the mechanisms involved in the immunoregulation induced by *T. spiralis*.

Materials and methods

Animals

Six- to 10-week-old BALB/c mice (Harlan, Zeist, The Netherlands) and OVA-TCR transgenic (DO11.10) mice on BALB/c background were housed under specific pathogen-free conditions at the animal care facility of the National Institute for Public Health and the Environment (RIVM). The transgenic mice were kindly provided by Prof. Dr. Willem van Eden from the Utrecht University, the Netherlands. This study was agreed upon by the Committee on Animal Experimentation of the RIVM (Bilthoven, the Netherlands) under permit number 200900205 and 200900192. Animal handling in this study was carried out in accordance with relevant Dutch national legislation, including the 1997 Dutch Act on Animal Experimentation.

Preparation of parasite antigen

Preparation of TspES was performed as previously described by Gamble [21]. Briefly, Wister rats (Harlan, Horst, The Netherland) were infected with 3000 *T. spiralis* RIVM-strain. At 42 days post-infection the muscle larvae were recovered by acid-pepsin digestion, washed and incubated at a concentration of 10^5 larvae per ml, for 19 h at 37°C in 5% CO_2 in RPMI medium supplemented with 1% penicillin/streptomycin. After incubation, the medium was centrifuged and the supernatant containing the secreted products (TspES) was dialyzed and concentrated. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA). The endotoxin level was below the detection limit (0.2 EU/ml) of the QCL-1000 chromogenic LAL Endpoint Assay (Lonza, Basel, Switzerland).

TLR ligands

The TLR ligands: *E. coli* LPS-S-form (TLR4), Pam3CSK4 (TLR1/2), FSL-1 (TLR2/6), Poly I:C (TLR3), flagellin derived from *Salmonella* typhimurium (TLR5), ssRNA40 (TLR7) and ODN1826 (TLR9) were purchased from Invivogen (San Diego, CA, USA). E .coli LPS serotype R15 (R-form) and *S. minnesota* S-form LPS were purchased from Alexis Biochemicals (San Diego, CA, USA). *Neisseria meningitidis* LPS, which in nature exist as a R-form only, was purified from wild type strain H44/76 by a modified hot phenol-water extraction [22] .

Bone marrow dendritic cell culture

Culture of bone marrow-derived dendritic cells (BM-DC) was performed according to the adapted method of Lutz [23]. Briefly, bone marrow cells were collected from euthanized naive mice by flushing femurs and tibiae with sterile phosphate-buffered saline (PBS) (Invitrogen, Carlsbad, CA, USA). Cells were resuspended at 2.5×10^5 cells/ml in complete RPMI-1640 medium (Invitrogen) containing 1% penicillin/ streptomycin, l% glutamine (Gibco-Invitrogen, Grand Island, NY, USA), 50 μM β-mercaptoethanol (Invitrogen), 10% fetal bovine serum (FBS) (Gibco, Invitrogen). Granulocyte–macrophage colony-stimulating factor (GM-CSF) (Cytocen, Utrecht, The Netherlands) at 20 ng/ml was added and the cells were grown in 1 ml per well in 12-well plates (day 0). On day 2, equal amounts of fresh medium (1 ml) and 20 ng/ml GM-CSF were added and on day 4 only 20 ng/ml GM-CSF was added. On day 7, cells were incubated with TspES (5 μg/ml) alone or together with the different LPS forms (1ng/ml) or other TLR ligands (concentrations are indicated in the legends of figure 3) for 24 hours. After incubation, non-adherent cells were harvested for FACS analysis and the supernatants stored at -20^{o}C for cytokine determination.

Culture of TLR4/MD2-CD14 HEK293 cells

Human Embryonic Kidney 293 (HEK) cells stably transfected with mouse TLR4/MD2-CD14 (Invivogen) were cultured in DMEM (Gibco-Invitrogen) containing 10% FBS, 10 μg/ml Blasticidin (Invivogen) and 50 μg/ml Hygromycin B (HygroGold, Invivogen). These cells were incubated with the different LPS forms (1 ng/ml) in the presence or absence of TspES (5 μg/ml). Culture supernatants were collected to measure IL-8 production.

Cytokine gene expression

Total RNA (10 ng) was extracted from DC incubated either with the different LPS forms (1 ng/ml) alone or together with TspES (5 μg/ml) for 6h using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Total RNA was used as a template for the kit Quantifast SYBR Green RT-PCR One-Step (Qiagen). This is a one-step reaction where reverse transcription (cDNA) and PCR take place. The expression of Il1a, Il6, Il10, Il12a and Tnf genes was measured relative to the housekeeping genes Gapdh, Gusb and Hprt1. Primers for RT-PCR analysis were from Qiagen (QuantiTect Primer Assays). Light Cycler 480 (Roche, Almere, The Netherlands) was used for detection. All reactions were performed

according to the manufacturer's instructions.

PCR array for TLR signaling pathways

Total RNA (10 ng) was extracted from DC incubated with either *E. coli* LPS S-form (1 ng/ml) alone or together with TspES (5 μg/ml) for 6h using the RNeasy Plus Mini Kit (Qiagen). For cDNA synthesis, a Reverse Transcriptase 1st-Strand cDNA Synthesis Kit (SABiosciences, Frederick, MD, USA) was used. The cDNA was analyzed using the mouse TLR signaling pathway RT^2 Profiler PCR Array (For more information cat. num. PAMM-18F, SABiosciences) that measures the expression of 84 genes related to TLR-mediated signal transduction (TLRs, effectors and members of the NFKB, JNK/p38, NF/IL6, and IRF signaling pathways downstream of TLR signaling) and 5 house-keeping genes. This PCR array also includes mouse genomic DNA, positive PCR controls and 3 reverse transcription controls. Procedures were performed according to manufacturer's instructions. Briefly, samples were run in the Light Cycler 480, using the following protocol, heat activation 95°C for 10 min, PCR cycles 45 of 15 sec at 95°C, and 1 min at 60°C and melting curves of 15 seconds at 60°C and 1 minute at 95°C. The normalized threshold cycle (Ct) value was obtained for each gene and the ΔCt was determined by subtracting the Ct value of the housekeeping gene, from the Ct value of the gene of interest. The change in the Ct value between control and treated samples for each gene of interest, ΔΔCt was then calculated and the fold change was determined using the formula $2^{-\Delta\Delta Ct}$. Up-regulation of gene expression was considered for values above 2 and down-regulation for values below -2 fold change of gene expression.A complete list of genes analyzed by the array can be found at: http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-018A.html

Culture of spleen cells and TGF-b assay

Splenocytes derived from OVA-TCR transgenic (tg) DO11.10 mice were cultured in 96-well round-bottomed plates (Corning) at 5×10^5 cells/well in complete RPMI-1640 (prepared as mentioned above). TspES-pulsed DC (pulsed for 24 hours) (TspES-DC) or DC in medium only were added to the spleen cells together with 20 μg/ml OVA protein (Endograde, Hyglos GmbH, Germany). After 4 days, supernatants were stored for cytokine analysis and cells were harvested for flow cytometric analysis.

For the TGF-b assay, 100 µg/ml anti-TGF-β1(Sigma, St. Louis, MO) and 100 µg/ml IgG1 isotype control (Sigma) were added to splenocytes incubated with OVA and TspES-DC or DC. Recombinant hTGF-b1 (R&D systems Minneapolis, MN, USA) 2ng/ml was added to splenocytes incubated with DC+OVA as positive control and in the presence of anti-TFG-b1 as negative control. After 4 days, supernatants were stored for cytokine analysis and cells were harvested for flow cytometric analysis

Treg suppression assay

In order to determine whether the Treg cells induced by the *T. spiralis* products have suppressive activity, an *in vitro* suppression assay previously described by Finney *et al* [24] was carried out. Briefly, 5 $x10^4$ $CD4^+CD25^+$ T cells isolated from OVA-TCR tg D011.10 splenocytes, that were previously cultured with TspES-DC +OVA or DC+OVA, were added to 96-well round-bottom plates (Costar, Corning, NY, USA) together with 5 $x10^4$ CFSE-labeled effector cells ($CD4^+CD25^-$) T cells derived from the spleen of naïve BALB/c mice and naïve BM-DC (1 $x10^5$) in the presence of 2.5 µg/ml Concavalin-A (Con-A) during 4 days. $CD4^+CD25^+$ or $CD4^+CD25^-$ T cells were isolated by MACS (Magnetic-activated cell sorting) using the Regulatory T Cell Isolation Kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) according to the manufacturer instructions. The effector cells were labeled at 10^6 cells/ ml in PBS containing CFSE at a concentration of 2 µM for 10 min at 37°C. Cells were then washed repeatedly in complete RPMI 1640 before addition to suppression assays. Proliferation of effector cells was measured by flow cytometry using a FACSCanto II (BD Biosciences, Erembodegem, Belgium). The percentage of cell division was calculated using the FlowJo software (version 7.6, Tree Star Inc, Ashland, OR.).

Flow cytometric analysis

For flow cytometric analysis using BM-DC, the cells were washed in FACS buffer (PBS containing 5% FBS and 5 mM sodium azide) and stained for 30 min at 4°C with the fluorescent-labeled antibodies: anti-CD40-FITC, anti-CD80-PE, anti-CD86-PE, anti-MHCII-FITC and anti-CD11c-APC (eBioscience, San Diego, CA, USA). Cells (10^5 events) were acquired and analyzed on a FACSCanto II. FACS analysis was performed on CD11c positive cells. Data were analyzed using the FlowJo software.

For splenocytes, the cells were first washed in PBS followed by staining for 30 min at 4°C with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen). Next, cells were blocked

with an unconjugated anti-CD32/CD16 for 10 min. at 4°C. Then, anti-CD4-APCH7 and anti-CD25-PerCPCy5.5 (BD Pharmigen) were added to the cells and incubated for 30 min. at 4°C. For intracellular staining anti-FOXP3-APC (Bioscience, San Diego, CA, USA), anti-IL-10-FITC (BD Pharmigen, San Diego, CA, USA) and anti-TGF-β1-2-3-PE (R&D systems Minneapolis, MN, USA) were added for 30 min at 4°C after fixation and permeabilization of the cells according to the manufacturer's recommendation. Cells ($5x10^5$ events) were acquired and analyzed on a FACSCanto II. Data were analyzed using the FlowJo software.

Cytokine determination

The levels of IL-1α, IL-6, IL-10, IL-12p70 and TNF-a in DC culture supernatants and IL-2, IL-4, IL-5, IL-10, IL-17 and IFN-γ in supernatants from spleen cell cultures were measured using Bio-Plex assays (Bio-Rad, Hercules, CA) according to manufacturer's instructions. The samples were analyzed on a Luminex 100 (Luminex, Austin, TX). The levels of IL-8 in the TLR4/MD2-CD14 HEK293 cell culture supernatants were measured using ELISA PeliPair reagent set (Sanquin, Amsterdam, The Netherlands). The levels of free active TGF-β1 in spleen cell culture were measured using ELISA kit Legend Max (Biolegend, San Diego, CA)

Statistical analyses

One-way analysis of variance (ANOVA) was performed followed by the Bonferroni's multiple comparison test to analyze differences in means between different groups of treated cells (GraphPad Prism version 4.00 for Windows, GraphPad Software, San Diego, CA, USA). Data are presented as means +/- SEM, and differences were considered significant at p values of ≤ 0.05.

Results

TspES suppress DC maturation induced by S- and R-form LPS

Since TspES suppress DC maturation induced by *E. coli* LPS (S-form) but not by *N. meningitidis* LPS (R-form), we investigated whether this effect was dependent on the form of LPS used implying a role for CD14. For this purpose, the S-form of *S. minnesota* LPS and the R-form of *E. coli* LPS were also tested. Dendritic cells were cultured for 24 h with the different LPS forms, TspES alone, or the combination of both. The expression of DC maturation markers, cytokine production and gene expression were measured. Consistent with our previous findings, TspES suppressed the expression of the maturation markers and cytokine production induced by E- coli LPS S-form but not *N. meningitidis* LPS R-form (Fig. 1 A, B). These differences were also observed at the cytokine gene expression level (Table 1). The expression of DC maturation markers, cytokine production and cytokine gene expression induced by *S. minnesota* LPS S-form were also suppressed by TspES (Fig. 1 A, B, table 1). Reduced expression of the maturation markers due to the presence of TspES was stronger when the concentration of *S. minnesota* LPS S-form was reduced to 0.1 ng/ml (data not shown). Interestingly, the expression of DC maturation markers, cytokine production and gene expression induced by *E. coli* R-form LPS were also reduced by TspES (Fig. 1 A, B; Table1). The reduced expression of MHCII was stronger when a lower concentration (0.1 ng/ml) of *E. coli* R-form LPS was used (data not shown). These results were confirmed by using HEK cells transfected with the mouse TLR4 receptor complex (TLR4/MD2-CD14). These cells were incubated with the different LPS forms, TspES, or the combination of both. TspES significantly suppressed the production of IL-8 induced by the S- and R-form of *E. coli* and the S-form of *S. minnesota* S-form, but it did not suppress the production of IL-8 induced by *N. meningitidis* LPS R-form (Fig 2). These results suggest that TspES inhibits the response to most LPS variants tested. Remarkably, the response to *Neisseria* LPS was not affected even when LPS concentrations as low as 0.01 ng/ml were used (data not shown).

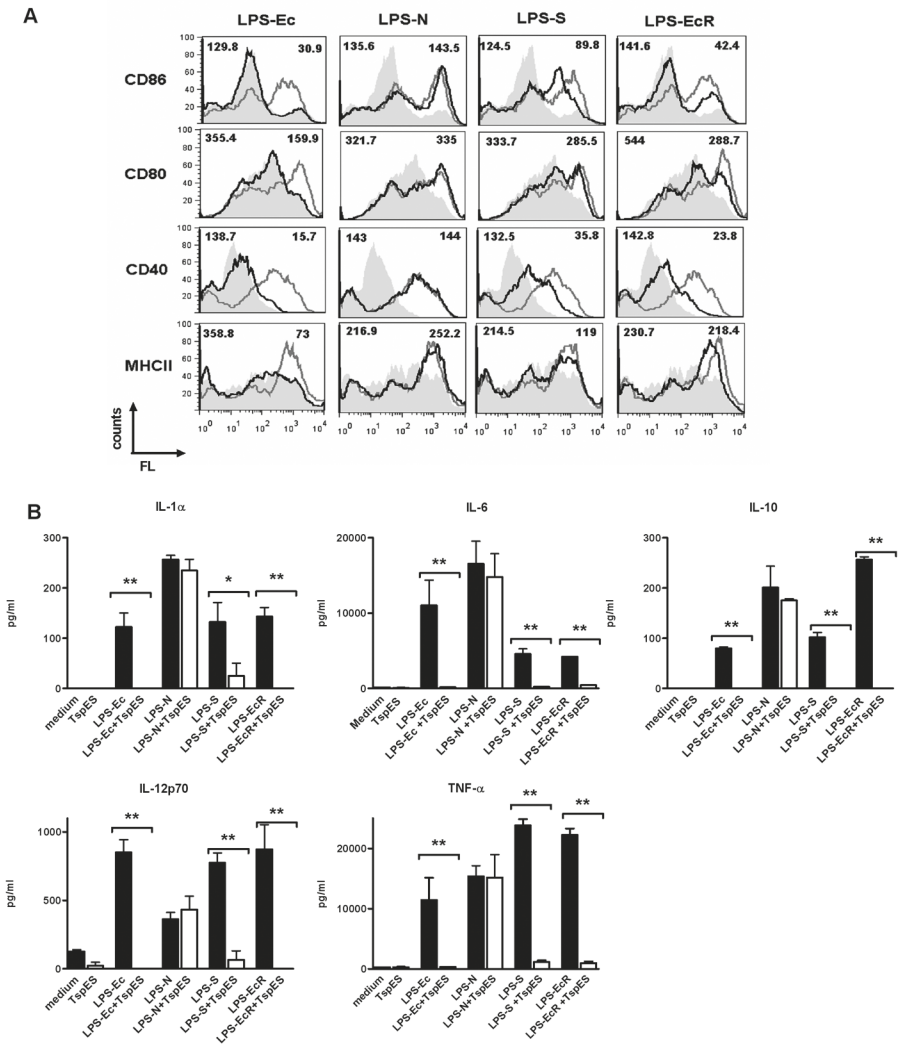

Figure 1. Effect of TspES on DC maturation induced by different S- and R-form LPS. DC were incubated with TspES (5 µg/ml) alone or in combination with different LPS (1 ng/ml) for 24 h. (A) Expression of surface markers on DC incubated in medium only (filled histogram), with LPS (grey line) or with LPS + TspES (black line). Numbers inside each histogram are the MFI (median fluorescence intensity) of DC with LPS alone (left) and LPS + TspES (right). (B) Production of cytokines by DC is represented as mean of triplicate values (pg/ml $^{+}$/- SEM). The data are representative of three independent experiments. Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: *p<0.05, **p<0.01. LPS-Ec: *E. coli* LPS S-form; LPS-S: *S. minnesota* S-form LPS; LPS-EcR: *E. coli* R-form LPS; LPS-N: *N. meningitidis* R-form LPS; FL: fluorescence. TspES: excretory/secretory products from *T. spiralis*

	Il1a	*Il6*	*Il10*	*IL12p35*	*Tnf*
LPS-Ec	657.87	758.32	4.49	128.15	20.21
LPS-Ec+TspES	10.47	6.96	1.52	4.4	1.47
LPS-N	513.18	767.13	10.24	116.84	12.41
LPS-N+TspES	756.57	1241.9	12.50	84.06	18.04
LPS-S	360.37	277.88	3.85	29.21	38.19
LPS-S+TspES	35.88	8.60	1.07	1.43	6.67
LPS-EcR	303.38	177.91	7.08	16.55	15.96
LPS-EcR+TspES	40.13	12.79	2.46	4.62	4.90

Table 1. Fold increase cytokine gene expression of DC stimulated with different types of LPS and TspES. Dendritic cells were incubated with different types of LPS (1 ng/ml) either alone or in combination with TspES (5 µg/ml) for 24 h. The data is shown as fold-increase expression over unstimulated cells. The data are representative of two independent experiments. LPS-Ec: *E. coli* LPS S-form; LPS-S: *S. minnesota* S-form LPS; LPS-EcR: *E. coli* R-form LPS; LPS-N: *N. meningitidis* R-form LPS. TspES: excretory/secretory products from *T. spiralis.*

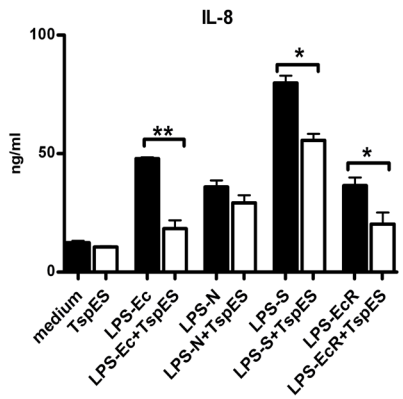

Figure 2. Effect of TspES on IL-8 production by HEK-TLR4/MD2-CD14 cells stimulated with different LPS. HEK cells transfected with mouse TLR4/MD2-CD14 were incubated with TspES (5 µg/ml) alone or in combination with different types of LPS (1 ng/ml) for 24 h. The data represent the mean of triplicate values (ng/ml $^{+}$/- SEM) and are representative of three independent experiments. Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: *p<0.05, **p<0.01. LPS-Ec: LPS-Ec: *E. coli* LPS S-form; LPS-S: *S. minnesota* S-form LPS; LPS-EcR: *E. coli* R-form LPS; LPS-N: *N. meningitidis* R-form LPS. TspES: excretory/secretory products from *T. spiralis*

Suppression of DC maturation by TspES is restricted to TLR4

Since TspES suppresses TLR4-mediated activation of DC, we investigated whether TspES suppress DC activation induced by other TLRs. For this purpose, DC were incubated with the following TLR ligands: Pam3CSK4 (TLR1/2), FSL-1 (TLR2/6), Poly I:C (TLR3), ST-FLA (TLR5), ssRNA40 (TLR7) and ODN1826 (TLR9) in the presence or absence of TspES. Results show that TspES had no significant effect on the expression of CD80, CD86, CD40 and MHCII on DC after incubation with any of the TLR ligands mentioned above (Fig. 3A). Similarly, no effect on the production of IL-1α, IL-6, IL-10, IL-12p70 and TNF-α (Fig. 3B) was observed. In this experiment, *E. coli* LPS was also tested as a positive control. As expected TspES suppressed expression of surface molecules and cytokine production induced by *E. coli* LPS S-form (data not shown). For Poly I:C, only the expression of surface markers is shown since this TLR agonist does not induce measurably amounts of any of the tested cytokines. Titration of the different TLR ligands in combination with TspES was performed. TspES did not suppress DC maturation induced by concentrations of TLR agonist 10 fold lower or higher than the ones indicated in Fig. 3 (data not shown). These results indicate that the suppressive effect of TspES on DC maturation is restricted to TLR4.

TspES alter LPS-induced gene expression involved in the TLR signaling pathway

To investigate the effect of TspES on the expression of different genes associated with the TLR signaling pathway, we used a PCR array that measures: the gene expression of the different TLRs; the adaptors and proteins that interact with the TLRs and effectors and members of the NFKB, JNK/p38, NF/IL6 and IRF family. For this experiment DC were incubated for 6 h with TspES, *E. coli* LPS S-form or the combination of both. No major changes in the expression of genes in DC incubated with TspES only were observed. Out of the 84 genes included in this array 25 were affected by *E. coli* LPS S-form (Fig. 4). The presence of 5 mg/ml TspES interfered with the LPS-mediated expression of genes of the MyD88 dependent and independent signaling pathway as well as genes from the NFkB, JNK/p38, NF/IL6 and IRF downstream pathway. The expression of all of these genes was reduced to background levels in the presence of TspES. As expected, reduced expression was observed for genes encoding for different surface molecules and cytokines. Together these results indicate that TspES interfere with the expression of different LPS-induced genes involved in the TLR signaling pathway.

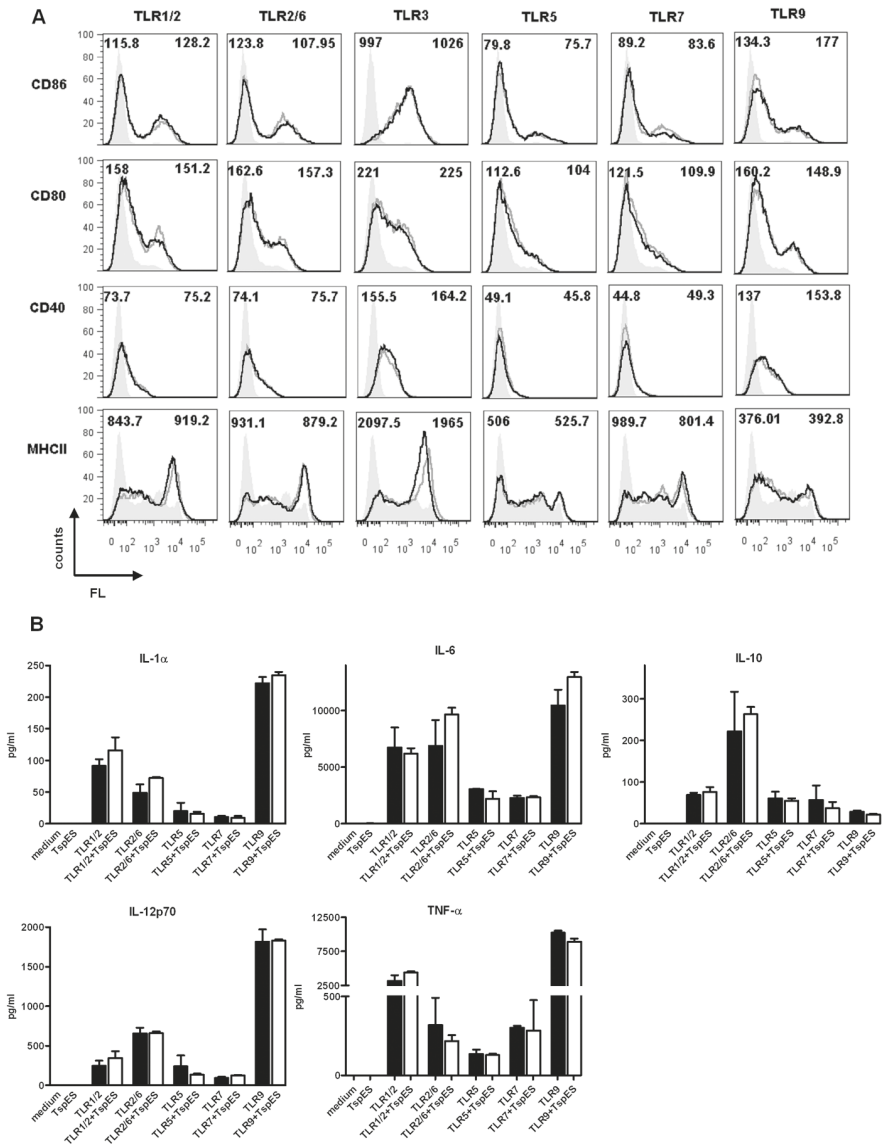

Figure 3. Effect of TspES on DC maturation induced by different TLR ligands. DC were incubated for 24 h with TspES (excretory/secretory products from *T. spiralis*) 5 µg/ml alone or in combination with 100 ng/ml of TLR ligand Pam3CSK4 (TLR1/2), 100 ng/ml FSL-1 (TLR2/6), 10 µg/ml Poly I:C (TLR3), 1 µg/ml, flagellin (TLR5), 5 µg/ml ssRNA 40 (TLR7) and 5 µM ODN 1826 (TLR9). (A) Expression of surface markers on DC incubated in medium only (filled histogram), with TLR ligand (grey line) or TLR ligand + TspES (black line). Numbers inside each histogram are the MFI of TLR ligand alone (left) and TLR ligand $^{+}$ TspES (right). (B). Production of cytokines by DC is represented as mean of triplicate values (pg/ml $^{+}$/- SEM). The data shown are representative of three independent experiments.

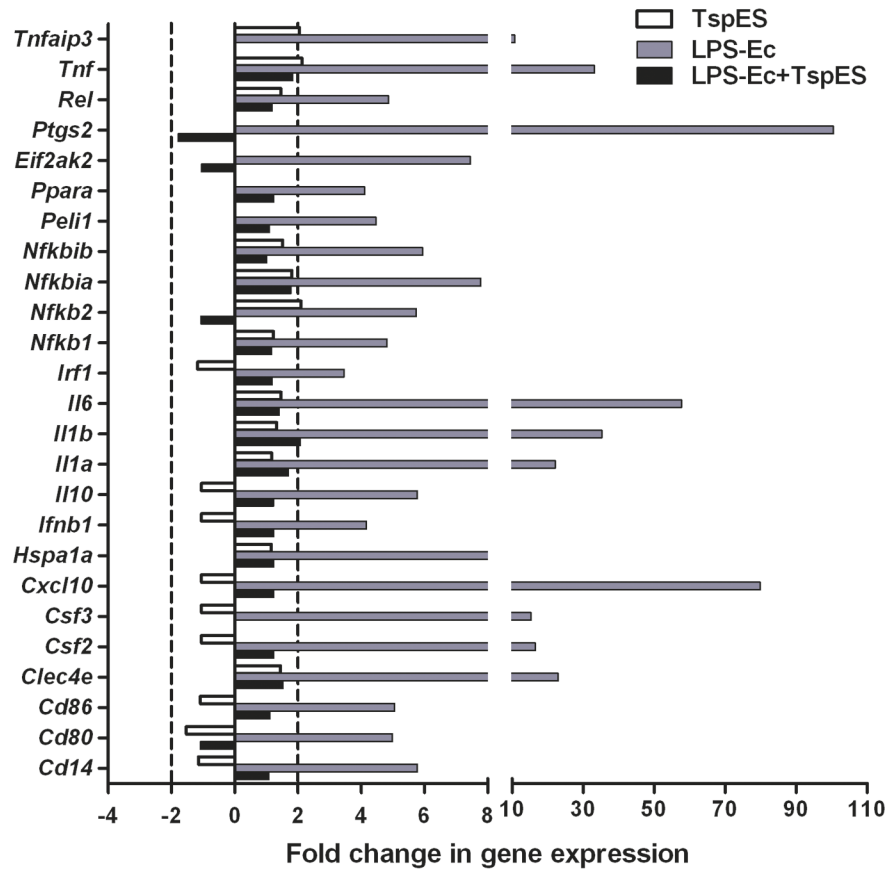

Figure 4. Effect of TspES on *E. coli* LPS S-form-induced expression of TLR signaling pathway genes. DC were incubated with 5 µg/ml TspES (excretory/secretory products from *T. spiralis*), 1ng/ml LPS-Ec or the combination of both for 6 h. The expression of 84 genes involved in the TLR signalling pathway (http://www.sabiosciences.com/rt_pcr_product/HTML/PAMM-018A.html) was assessed by real-time quantitative RT-PCR array and calculated relative to five housekeeping genes. Only the genes affected by TspES, LPS-Ec or LPS-Ec + TspES are shown in the graph. The data is shown as fold-change expression over unstimulated cells and are representative of three independent experiments. Up-regulation or down-regulation of gene expression was considered for values above or below 2 or -2 units of fold change of gene expression, respectively. LPS-Ec: *E. coli* LPS S-form

TspES induce expansion of Treg cells and increase TGF-β production

In addition to determining the effect of TspES on the functionality of DC, we were interested in investigating the effect of these helminth products on T cell activation. For this purpose DC previously incubated with TspES (TspES-DC) for 24 hours, were cultured with splenocytes from OVA-TCR tg mice in the presence or absence of the OVA protein. T-cell responses were measured after 4 days. Results show that in the $CD4^+CD25^+$ T cell population derived from splenocytes cultured with DC+OVA compared to splenocytes cultured with TspES- DC+OVA, the Foxp3 expression increased in average from 7% to 15.5% and the TGF-β expression from 1.5% to 8.6% (Fig. 5A-B). The intracellular expression of IL-10 by the $CD4^+CD25^+$ T cell population was lower when splenocytes were cultured with TspES-DC+OVA but the difference was not significant (Fig 5C). The cytokine production of splenocytes incubated with TspES-DC+OVA was different compared to cells incubated with DC+OVA. The levels of IL-2, IL-4, IL-5, IL-13 and IL-17 production were comparable while the production of IFN-γ, IL-10 and TGF-β varied (Fig. 6). The production of IFN-γ as well as IL-10 decreased in supernatants of splenocytes incubated with TspES-DC while the TGF-β production increased (Fig. 6). In order to determine whether the Treg population expanded by TspES-primed DC is due to the TGF-β produced, a blocking experiment was performed. Figure 7A shows that by adding an antibody against TGF-β, the expanded CD4+CD25+FOXP3+ T cell population was blocked. As a positive control rhTGF-β was added to the D011.10 splenocytes incubated with OVA and DC only (without TspES) which resulted in an increase from 9% to 13% of the CD4+CD25+FOXP3+ T cell population. This expansion was blocked by anti-TGF-β (data not shown). Cytokine analysis of the supernatants indicated that addition of anti-TGF-β significantly abrogates the decreased IFN-γ production due to TspES-primed DC. The decreased IL-10 production is also abrogated by anti- TGF-β but it is not significant (Fig 7B). The presence of anti-TGF-β did not affect the level of the other cytokines mentioned above (data not shown).

TspES induce functional Treg cells with suppressive activity

To determine whether the $CD4^+CD25^+$ T cell population induced by TspES-primed DC are functional Treg cells in terms of suppressive capacity, functional assays were performed. Figure 8 shows suppression of the effector cell proliferation induced by Treg cells isolated from spleen of OVA-TCR tg mice cultured with DC+OVA in the presence or absence of TspES. Results indicate that the percentage of effector cells decreased from 81% to 67.3 % when Treg isolated from the splenocytes incubated with OVA and

TspES-primed DC were used, indicating that this $CD4^+CD25^+$ T cell population has a suppressive activity *in vitro*.

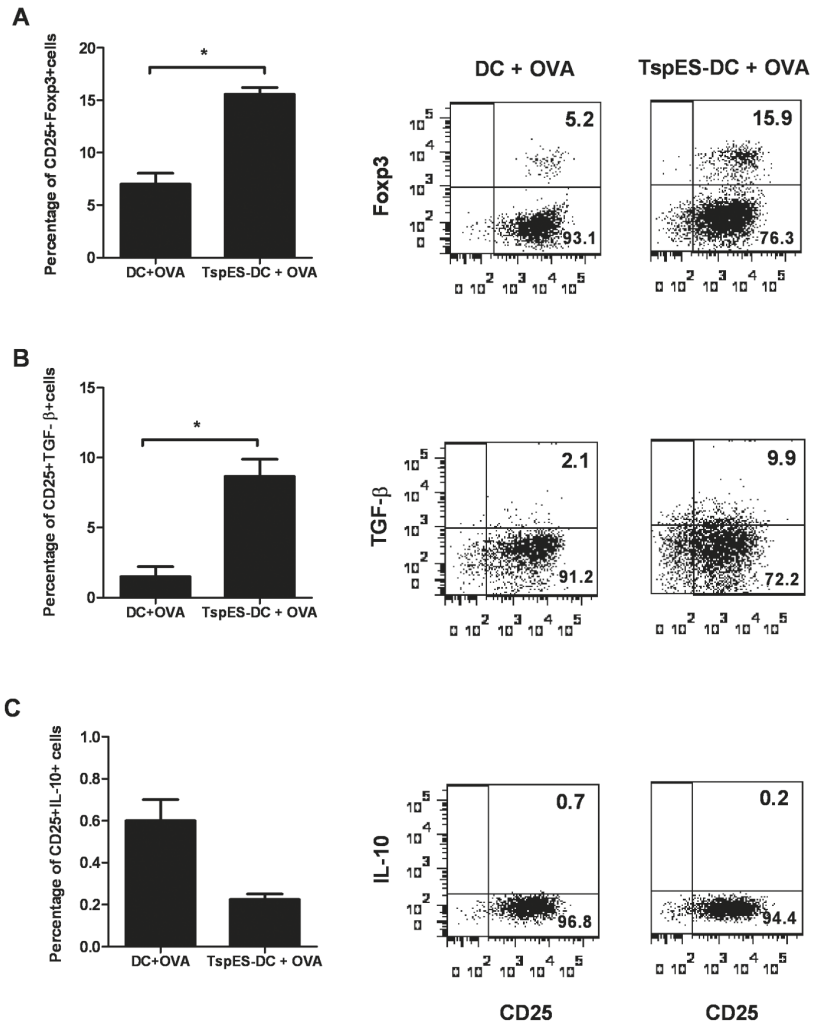

Figure 5. TspES-pulsed DC increase the expression of $Foxp3^+$ and TGF-β in the $CD4^+CD25^+$ splenocyte cell population. Splenocytes from D011.10 mice were cultured for 4 days in the presence of DC, DC+OVA or TspES-DC+OVA. (A) Percentage of $CD25^+$ cells expressing $Foxp3^+$ gated on $CD4^+$ T cells and representative plots (B) Percentage of $CD25^+$ cells expressing TGF-β gated on $CD4^+$ T cells and representative plots. (C) Percentage of $CD25^+$ cells expressing IL-10 gated on $CD4^+$ T cells and representative plots. Percentages are represented as mean values (% $^+$/- SEM). Numbers within the plots represent the percentage of cells in the corresponding quadrant. Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: *$p<0.05$. Data are representative of three independent experiments. TspES: excretory/secretory products from *T. spiralis*.

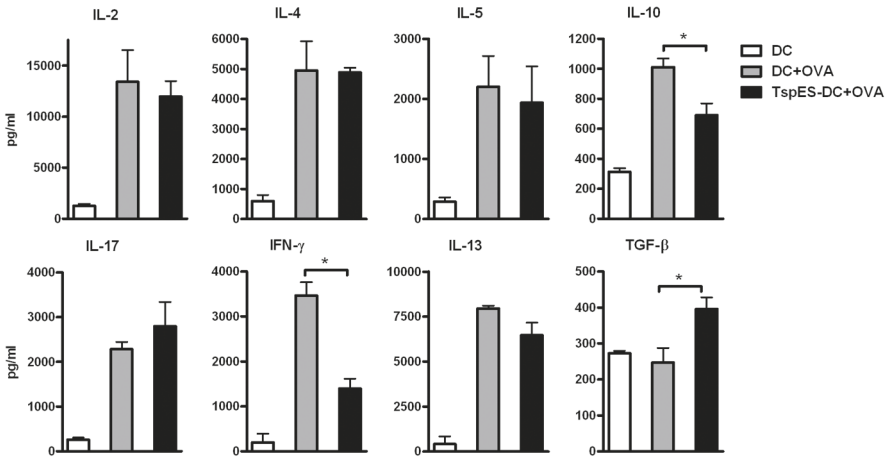

Figure 6. TspES-pulsed DC increase TGF-β production by splenocytes from D011.10 mice and decreases IFN-γ and IL-10 production. Splenocytes from D011.10 mice were cultured for 4 days in the presence of DC, DC+OVA or TspES-DC+OVA. Production of cytokines is represented as mean of triplicate values (pg/ml $^{+}$/- SEM). The data shown are representative of three independent experiments. Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: *p<0.05, **p<0.01. TspES: excretory/secretory products from *T. spiralis*.

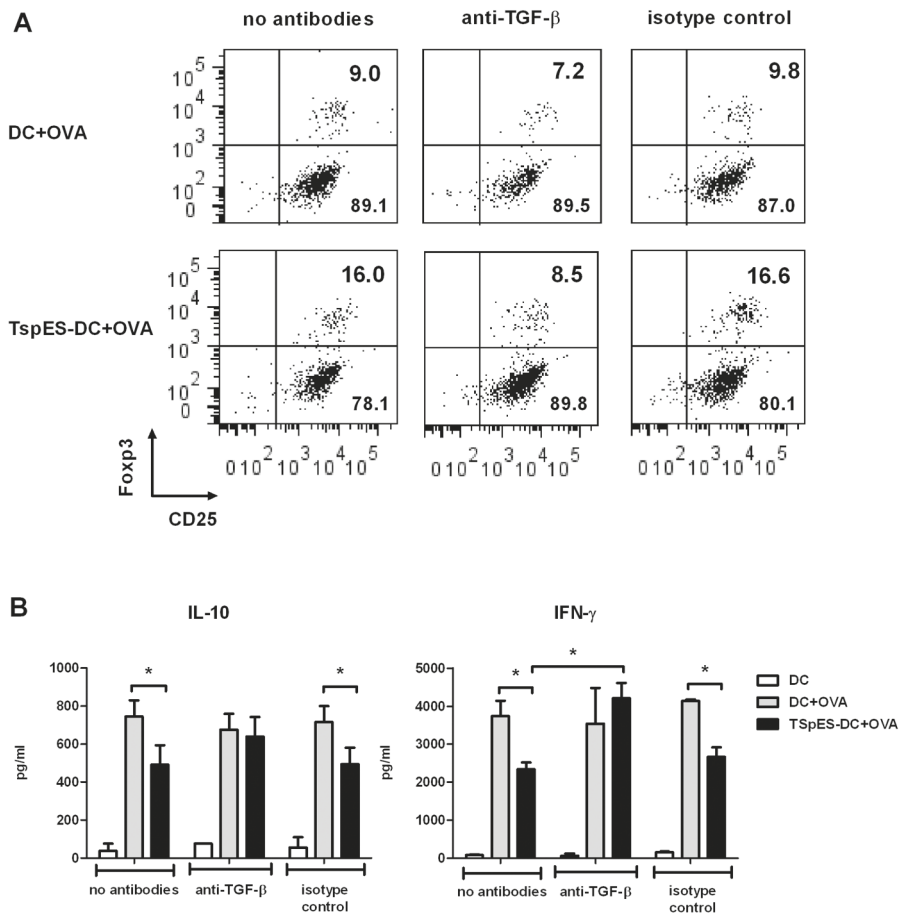

Figure 7. Anti- TGF-β abrogates the expansion of Foxp3$^+$ expression and the inhibition of IFN-γ and IL-10 production induced by TspES. Anti-TGF-β and isotype controls (IgG1) were added to splenocytes from D011.10 mice cultured for 4 days in the presence of DC, DC+OVA or TspES-DC+OVA. (A) Percentage of CD25$^+$ cells expressing Foxp3$^+$ gated on CD4$^+$T cells. Numbers within the plots represent the percentage of cells in the corresponding quadrant. (B) Production of cytokines is represented as mean values (pg/ml $^+$/- SEM). Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: *p<0.05. Data are representative of two independent experiments. TspES: excretory/secretory products from *T. spiralis*.

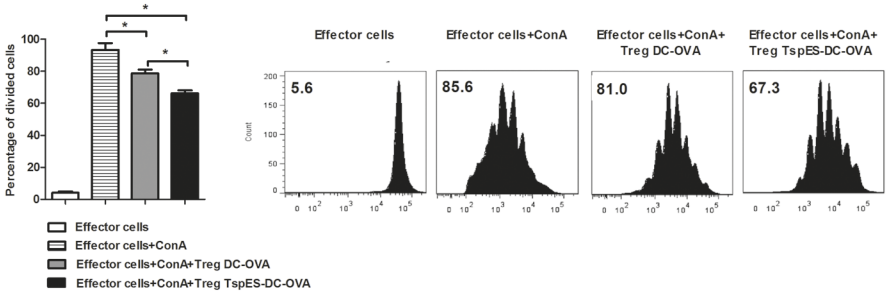

Figure 8. TspES-pulsed DC induce $CD4^+CD25^+$ T cells with suppressive activity. The proliferative response of effector $CD4^+CD25$- CFSE-labelled T cells cultured for 4 days with DC and ConA with or without $CD4^+CD25^+$ Treg cells isolated from splenocytes of D011.10 mice was measured by flow cytometry. Bars represent the percentages of the cells that have divided. These percentages are expressed as mean values (% $^+$/- SEM). Statistical analysis was performed by one-way ANOVA with the Bonferroni's test: *$p \leq 0.05$. The data shown are representative of three independent experiments. Treg DC-OVA: $CD4^+CD25^+$ T cells isolated from D011.10 spleen cells cultured with DC+OVA. Treg TspES-DC-OVA: $CD4^+CD25^+$ T cells isolated from D011.10 spleen cells cultured with TspES-DC+OVA. TspES: excretory/secretory products from *T. spiralis*.

Discussion

We have previously shown that TspES can suppress *in vitro* the maturation of DC induced by the S-form of *E. coli* LPS, but not by the R-form of *N. meningitidis* LPS [16]. These two different LPS forms have been shown to activate TLR4 differentially. In contrast to the S-form, the R-form of LPS does not depend on CD14 for TLR4 activation [18, 19]. This led us to investigate whether suppression by ES from *T. spiralis* was dependent on the form of LPS used, implying a role for CD14. Using now the R-form LPS from *E. coli* and the S-form LPS from *S. minnesota* we show that this suppression does not depend on the LPS form since TspES can suppress DC maturation induced by both LPS. Therefore the suppressive effect of TspES on LPS-induced DC maturation is CD14 independent. This suppressive effect depends most likely, on the bacterial source of LPS. The LPS structure of different enterobacteria such as *Escherichia coli* and *S. minnesota* are similar [25], whereas there is a clear difference between LPS from enterobacteria and *N. meningitidis* LPS. In the lipid A structure of *N. meningitidis* LPS, the localization of the secondary acyl chain is symmetrical while in enterobacteria LPS is asymmetrical [26]. Lipid A represents the conserved molecular pattern of LPS and is the main inducer of immunological responses to LPS. Its composition and number of acyl chains are important in the activation of TLR4/MD2. Different lipid A structures vary considerably in potency to activate DC and can differentially utilize signal transduction pathways leading to diverse patterns of inflammatory responses [18, 27, 28]. During migration from intestine to muscle, *T. spiralis* larvae may drag enterobacteria [29] which could lead to sepsis. Nevertheless, the number of patients with trichinellosis that develop sepsis is very low, 2.11% [30]. Perhaps, one of the strategies used by this helminth to prevent the induction of sepsis and ensuring host survival is to inhibit the immune response against enterobacteria LPS. Further studies should be carried out in order to determine whether *Trichinella* secreted products suppress DC maturation induced only by LPS derived from enterobacteria.

In this study, we also show that inhibition of LPS-induced DC maturation is restricted to the TLR4 signaling pathway since TspES does not suppress DC maturation induced by other TLR ligands. This finding is different to the one reported by Hamilton *et al*. (2009), who have shown that *Fasciola hepatica* tegumental products suppress cytokine production and expression of co-stimulatory markers on DC induced not only by TLR4 but also by a range of other TLRs [31]. In another study Kane *et al* showed that *Schistosoma mansoni* soluble egg products (SEA) suppress IL-12p40 production induced by engagement of TLR3, TLR4, or TLR9 [32]. These findings suggest that the effect of helminth

products on TLR induced DC maturation differs among helminths. TLR4 signaling involves two main intracellular pathways, namely the 'MyD88 (myeloid differentiation primary-response gene 88)-dependent' pathway which mediates the production of pro-inflammatory cytokines and 'MyD88-independent or TRIF' pathway which mediates the upregulation of co-stimulatory and MHCII molecules by DC. The MyD88 dependent pathway is shared by the other TLRs with the exception of TLR3, which signals through TRIF [33]. TspES inhibits the outcome of both, MyD88-dependent (cytokine production) and independent (expression of surface molecules) signaling pathway via TLR4 but not via other TLRs. It is therefore likely that the suppressive effect of these helminth products involves molecules of the TLR4 signaling pathway only and that it occurs at an initial stage of the cascade. Using PCR array, we examined the effect of TspES on the expression of genes involved in the TLR signaling pathways. Although, TspES on its own did not induce major changes, these helminth products impaired already after 6 hours, the expression of all genes downstream of the TLR pathway induced by LPS. Harnett *et al*. have recently shown that the glycoprotein ES-62 from the nematode Acanthocheilonema viteae suppresses TLR-mediated pro-inflammatory responses and that this process occurs as an early event [34]. These authors showed that ES-62 induces autophagy on antigen presenting cells which results in downregulation of TLR4 and MyD88 expression. Other mechanism by which this helminth could interfere with the TLR signaling pathways is through an indirect effect via interaction with other PRR (pattern-recognition receptors), such as C-type lectins [15]. For instance, the mycobacterial cell wall component ManLAM has been shown upon binding with DC-SIGN to interferes with TLR4-mediated signals down-regulating in this way DC-mediated immune responses [35]. Products secreted by helminths including those produced by *T. spiralis* are a complex mixture that may modulate the immune response in several ways. The possibility that enzymatic activity in the TspES is responsible for suppressing LPS-induced DC could be excluded since heat inactivation did not affect the suppressive activity of this helminth antigen (data not shown). Preservation of the suppressive activity after heat inactivation has been also reported for secretion products from *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* indicating that the products responsible for the suppressive effect are either of a non-protein nature or denatured proteins that are still functional [36]. Other components in the TspES such as lipids or glycans could also be responsible for the observed suppressive activity as has been reported for other helminths [14, 15]. Identification and characterization of the TspES components with immunomodulatory properties is currently being investigated.

Studies with *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis*, *Schistosoma mansoni*, *Litomosoides sigmodontis* among others have shown that these helminths can modulate the host immune response by inducing suppressive Treg cells that produce TGF-β and/or IL-10 [1, 37-42]. *Trichinella spiralis* has evolved to allow the larvae to complete their cycle in a single host, where the larvae migrate from the gut to the muscle, remaining alive for many years [6]. Immunoregulation by this helminth may, therefore, allow the larvae to invade and remain in the muscle, preserving the integrity of the occupied cells and thus keeping their host alive. Local inflammation against the muscle stage of this parasite has been shown to be limited by IL-10 during infection [43]. In vivo studies have shown that *T. spiralis* infection protects against experimental autoimmune encephalomyelitis and suppress airway hyperresponsiveness of OVA-challenged mice [10, 11]. These findings indicate that this helminth can modulate the host immune response. However, the role of Treg during infection with this helminth has not yet been well investigated. Here, we show that secreted products from *T. spiralis* muscle larvae expand suppressive Treg cells *in vitro*, as indicated by an increase in the $CD4^{+}CD25^{+}$ $Foxp3^{+}$ T cells population after splenocytes from DO11.10 mice were co-cultured with OVA and TspES-pulsed DC. Unlike our findings, Ilic *et al*. have recently reported that *T. spiralis* antigens do not induce de novo generation of Foxp3+ T cells *in vitro* [44]. Two major differences could explain the contrasting results. First of all, we used in our *in vitro* cultures OVA protein whereas these authors used an OVA peptide. *Trichinella spiralis* secreted products may affect DC antigen processing and presentation which has consequences for T cell activation. These effects of TspES cannot be evaluated when adding the peptide. In fact, in early studies we used an OVA peptide and indeed we did not observe expansion of Foxp3+ T cells *in vitro* (data not shown). Another important difference is the concentration of *Trichinella* antigen used to prime the DC. Ilic *et al* used 50 mg/ml which resulted in DC maturation whereas we used only 5 mg/ml. Although we did not observe increased expression of DC surface markers or cytokine production we did observe expansion of $CD4^{+}CD25^{+}$ $Foxp3^{+}$ T cells. Studies have shown that low antigen concentrations induce tolerogenic DC [45] and these DC can induce Treg cells [5]. Whether that is the case for low *Trichinella* antigen concentrations and whether higher concentrations result instead in the expansion of effector T cells remains to be investigated. Our study also shows that splenocytes derived from D011.10 mice produce TGF-β when co-cultured with OVA and TspES-pulsed DC. This cytokine has been shown to be required for both *in vitro* and in vivo generated Foxp3+ inducible Treg cells [46, 47]. By blocking TGF-β, we observed that the expansion of the CD4+CD25+ $Foxp3^{+}$ T cell population is abrogated and the production of IFN-g and IL-10 is restored.

Perhaps, the *in vitro* conditions used in this study may simulate natural conditions during chronic infection in which muscle larvae secreted products induce Treg cells in a TGF-β-dependent manner. This regulatory mechanism may favor parasite survival and could also benefit the host, as it has been shown for other helminths. For instance, infection with *H. polygyrus* has been reported to suppress the outcome of experimentally airway allergy induced by OVA and house dust mite. Here, the $CD4^{+}CD25^{+}$ $Foxp3^{+}$ T cells producing TGF-β and IL-10 were shown to down-regulate allergen induced lung pathology in vivo [1]. These findings demonstrate that helminth infections can elicit Treg cells able to modulate the response to bystander antigens. Currently, we are investigating whether in vivo *T. spiralis* induces Treg cells and what is the effect of infection with this helminth on other immunopathologies.

In this study, we show that *T. spiralis* products have immunomodulatory properties as indicated by suppression of DC maturation and expansion of functional Treg cells *in vitro*. While the exact mechanisms by which *T. spiralis* antigens mediate immune suppression remains to be elucidate, these findings are relevant since they could contribute to the development of new strategies to treat or prevent inflammatory diseases.

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Chapter 4

Protection against allergic airway inflammation during the chronic and acute phases of *Trichinella spiralis* infection

Carmen Aranzamendi, Alain de Bruin, Raoul Kuiper,
Claire J. P. Boog,Willem van Eden,
Victor Rutten, Elena Pinelli

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Abstract

Background

Modulation of the host immune response by helminths has been reported to be essential for parasite survival and also to benefit the host by suppressing inflammatory diseases such as allergies. We have previously shown that excretory-secretory products of *Trichinella spiralis* muscle larvae have immunomodulatory properties and induce *in vitro* the expansion of $CD4^+CD25^+$ $Foxp3^+$ Treg cells in a TGF-β-dependent manner.

Objective

We aimed at determining the effect of the acute (intestinal) and the chronic (muscle) phase of *T. spiralis* infection on experimental allergic airway inflammation (EAAI) to OVA (ovalbumin) and the involvement of Treg cells.

Methods

The chronic phase was established before OVA-sensitization/challenge and the acute phase at two-time points, before and after OVA-sensitization. Mice were infected with 400 *T. spiralis* larvae and after euthanasia different pathological features of EAAI were measured. Adoptive transfer of $CD4^+$ T cells from *Trichinella* infected mice to OVA sensitized/challenged recipients was also performed.

Results

We found that the chronic as well as the acute phase of *Trichinella* infection suppress EAAI as indicated by reduction in airway inflammation, OVA-specific IgE levels in sera, Th2-cytokine production and eosinophils in BAL. This protective effect was found to be stronger during the chronic phase and to be associated with increased numbers of splenic $CD4^+CD25^+Foxp3^+$ Treg cells with suppressive activity. Adoptive transfer of splenic $CD4^+$ T cells from chronically infected mice with elevated numbers of Treg cells resulted in partial protection against EAAI.

Conclusions & Clinical Relevance

These results demonstrate that the protective effect of *T. spiralis* on EAAI increases as infection progresses from the acute to the chronic phase. Here, Treg cells may play an essential role in the suppression of EAAI. Elucidating the mechanisms and molecular helminth structures responsible for this regulatory process is relevant in order to develop alternative tools for preventing or treating allergic asthma.

Introduction

Helminthic infections and allergies are characterised by a dominant Th2 type of immune response that mediates high levels of circulating IgE antibodies and eosinophilia. Even though in general a common type of immune responses is induced, an inverse correlation between certain helminth infections and allergy has been observed [1].

A series of epidemiological findings indicate that infection with helminths such as *Schistosoma spp*., *Necator americanus* and *Brugia malayi* is associated with reduction of allergic manifestations in humans [2-5]. Using murine models it has also been shown that certain helminths can suppress experimental allergic airway inflammation and that regulatory T (Treg) cells play an essential role in this process [6-8]. In addition, helminth-induced alternatively activated macrophages [9] and regulatory B cells [10, 11] have also been reported to down modulate immunopathologies. Creating an immunosuppressive network appears to be a strategy of helminths to survive in their host and its effect can be extended to third-party antigens such as allergens.

Different factors however, may influence whether helminth infections protect or exacerbate allergic manifestations. These factors include the helminth species and whether humans are definitive or accidental hosts [12]. We have previously shown that infection with *Toxocara canis*, a roundworm of dogs that can also infect humans exacerbates EAAI [13]. Since humans are accidental hosts for *Toxocara* infections, we were interested in determining the effect on allergic manifestations by a helminth that not only infects humans but also mice and completes its life cycle in a single host (definitive host). For this reason we chose *Trichinella spiralis* for the present study. After ingestion of *T. spiralis* infected meat the larvae are released in the stomach and migrate to the small intestine where they mature into adult worms. Female worms release within a week after infection, newborn larvae that rapidly disseminate throughout the host, and eventually enter skeletal muscles to remain there for many years [14]. Trichinellosis is characterized by two phases; the acute enteral (intestinal) phase that begins few days after ingestion of infected meat and the chronic (muscle) phase that occurs weeks after infection. We have shown that the excretory-secretory (ES) products of this helminth's muscle larvae have immunomodulatory properties as indicated by its suppressive effect on dendritic cell maturation *in vitro* [15]. In addition, using splenocytes derived from OVA-TCR transgenic D011.10 mice, we showed that *T. spiralis* ES products (TspES) induce *in vitro* the expansion of $CD4^+CD25^+$ $Foxp3^+$ Treg cells in a TGF-β-dependent manner [16] . Studies by others have shown that IL-10 and TGF-β control the level of inflammation induced by *T. spiralis* especially during the chronic phase of infection [17]. Modulation of

the immune response by *T. spiralis*, protecting the host against other immunopathologies such as experimental autoimmune encephalomyelitis and experimental colitis has been demonstrated [18, 19]. In this study we aimed at determining the effect of the acute versus the chronic phase of *T. spiralis* infection on experimental allergic airway inflammation (EAAI) to OVA and the involvement of Treg cells. Here, the acute phase was established at two-time points, before and after OVA-sensitization.

Our data show that the chronic as well as the acute phase of *Trichinella* infection protect against EAAI. This protective effect was found to be stronger as the infection progressed to the chronic phase and to be associated with increased numbers of $CD4^+CD25^+Foxp3^+$ T cells in the spleen. The Treg cells induced after *T. spiralis* infection were shown to have suppressive activity and adoptive transfer of $CD4^+$ T cells from the spleen of chronic infected mice containing elevated numbers of Treg cells resulted in partial protection against EAAI. All together these findings indicate that *T. spiralis* suppresses OVA-induced EAAI which increases as infection progresses. Furthermore, Treg cells may play an essential role in this suppression.

Materials and methods

Animals and parasites

Six- to 10-week-old BALB/c female mice (Harlan, Zeist, The Netherlands) were housed under specific pathogen-free conditions at the animal care facility of the National Institute for Public Health and the Environment (RIVM). All animal experiments were approved by the Committee on Animal Experimentation of the RIVM.

Infection with *Trichinella spiralis* and induction of experimental allergic airway inflammation

Mice were either infected with *T. spiralis* muscle larvae, treated (sensitized/ challenged) with OVA to induce EAAI or the combination of both. Infection was performed by oral administration of 400 *T. spiralis* larvae per mouse in 500 µl sterile phosphate-buffered saline (PBS) using a syringe fitted with a blunt needle. The experimental allergic airway inflammation was induced by sensitizing/challenging mice with OVA as previously described by Smits *et al* [10]. Briefly, mice were first sensitized on days 0 and 5 by intraperitoneal (i.p.) administration of 10 mg OVA (grade V, Sigma-Aldrich, Steinheim, Germany) adsorbed onto 2.25 mg alum (aluminium hydroxide, Pierce, Thermo Scientific, Rockford, USA) in 100 µL PBS (OVA/Alum). To induce allergic airway inflammation, OVA challenges were given on days 15, 16 and 17 days after sensitisation by exposing the mice for 30 minutes to OVA/PBS aerosol (1%v/v) that was generated by a nebulizer (Ultra-NebTM2000; DeVilbiss, Langen, Germany). Control uninfected mice received alum i.p. and only PBS during aerosol challenge. The effect of established chronic or acute phases of *T. spiralis* infection on EAAI was investigated by infecting mice 25 days (-25 d + OVA) or 5 days (-5 d + OVA) respectively, before OVA-treatment (sensitization/ challenge). In order to determine the effect of the acute phase of infection on the onset of EAAI, mice were infected 12 days (+12 d + OVA) after OVA-sensitization (Fig. 1) which was three days before the OVA challenge and 5 days before mice were sacrificed. Three other groups of mice were infected with *T. spiralis* (without OVA-treatment) at the same time points described above and are referred as -25 d and -5 d and +12 d. In total there were 8 groups of 8 mice each: One uninfected PBS/alum-treated control group, one OVA-treated group, three *T. spiralis*-infected (-25 d, -5 d and +12 d) groups and three groups that received both infection and OVA-treatment (-25 d + OVA, -5 d + OVA and +12 d + OVA). Mice from all groups were euthanized on day 18 as indicated in Fig 1. It is important to mention that since *T. spiralis* establishes persistent infection, the infected animals from group -25 d, -5 d, and +12 d treated or not with OVA were by the time they

were sacrificed, 43, 25 and 5 days infected. In order to verify that mice were infected with *T. spiralis*, adult parasites and larvae were counted in the intestines and carcasses of the infected mice. Carcasses were digested by overnight incubation with 4.3 % hydrochloric acid and 0.9% pepsin in PBS at 37 °C to obtain the larvae [20]. Intestines were incubated for 2-4 h with PBS containing 0.85% sodium chloride at 37 °C in order to release adult worms [21]. Adults and larvae where counted by light microscopy. After 43 and 25 days of *T. spiralis* infection larvae were only present in the carcass and no adults were present in the intestine of infected mice indicating the establishment of the muscle (chronic) phase of infection. After 5 days of infection only adult worms were observed in the intestine, while no muscle larvae were found in the carcass indicating the establishment of the intestinal (acute) phase of infection.

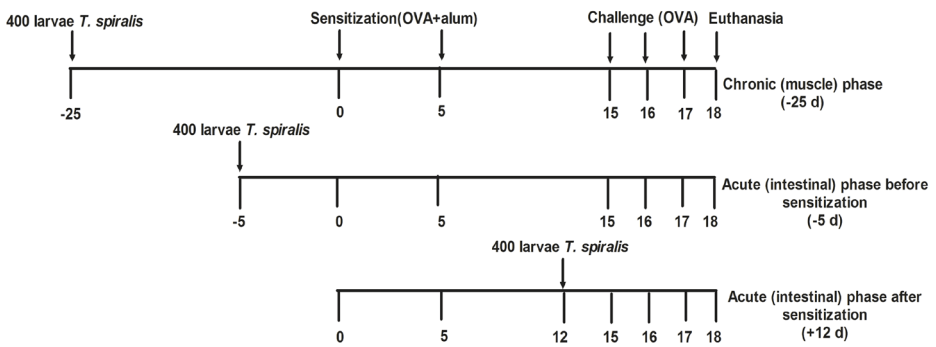

Figure 1. Schematic representation of the experimental design. The chronic phase of *Trichinella* infection was established 25 days (-25 d + OVA) before OVA-sensitization/challenge. The acute phase was established at two-time points, 5 days (-5 d + OVA) before and 12 days (+12 d + OVA) after OVA-sensitization. Mice from the control group were uninfected PBS/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. OVA-sensitization was done on days 0 and 5 and OVA-challenges on days 15, 16 and 17 followed by euthanasia on day 18.

Analysis of bronchoalveolar lavage cells

The bronchoalveolar lavage (BAL) fluid was harvested after mice were euthanized by injecting 1 ml PBS with a syringe through the tracheal tube into the airways and carefully recovering the fluid by aspiration with the same syringe. The BAL fluid was centrifuged at 400 ×g, at 4 °C, for 10 min. The supernatant was stored at -20 ºC for cytokine determination and the cell pellet was resuspended in 1 ml PBS. Total cell numbers were determined by mixing 0.5 ml of the cell suspension with 9.5 ml Isoton II (Beckman Coulter BV, Mijdrecht, The Netherlands) and counted using a cell counter (Beckman Coulter). Cytospins using the cell suspension were made, and differential cell counts were performed after staining with May-Grünwald-Giemsa. At least 400 cells per slide were counted by light microscopy.

Histology of the lungs

After harvesting the BAL fluid, the lungs were perfused via the right cardiac ventricle by injection of 10 ml PBS and thereafter removed. The right lung was fixed with 10% neutral-buffered formalin and embedded in paraffin (Kendall, Tyco Healthcare, Boston, MA, USA). Transverse sections of 3 μm were stained with haematoxylin–eosin (HE) and pathological changes (peribronchiolar inflammation, perivascular inflammation, hypertrophy and hyperplasia of goblet cells) were blindly scored by board certified veterinary pathologists using a semiquantitative scoring system from absent (0), minimal (1), slight (2), moderate (3) to marked (4). Sections were also stained with periodic acid schiff (PAS) reagent to identify goblet cells and determine mucus production.

Total IgE and OVA-specific IgE

Total serum IgE antibodies were measured by using the OptEIATM Set according to the manufacturer's recommendations (BD Pharmingen, San Diego, CA, USA). Determination of OVA-specific IgE levels was done using the OVA-IgE ELISA kit according to the manufacturer's recommendations (AbD serotec, Oxford, UK).

Flow cytometric analysis

Splenocytes were stained for 30 min at 4°C with LIVE/DEAD Fixable Violet Dead Cell Stain (Invitrogen, Carlsbad, CA, USA). Next, cells were pre-incubated with Fc receptor blocking antibodies (purified CD16/CD32) (BD Pharmingen) for 10 min. at 4°C. Anti-CD4-APCH7 and anti-CD25-PerCPCy5.5 (BD Pharmingen) were added to the cells and incubated for 30 min at 4°C. For intracellular staining, fixation and permeabilization of the cells were performed according to the manufacturer's recommendation (eBioscience, Frankfurt, Germany). Then, blocking antibodies were added for 10 min followed by addition of anti-Foxp3-APC (Bioscience, San Diego, CA, USA) for 30 min at 4°C. Cells ($5x10^5$ events) were acquired and analysed on a FACSCanto II (BD Biosciences, Erembodegem, Belgium). Data were analysed using the FlowJo software (version 7.6, Tree Star Inc, Ashland, OR.).

In vitro suppression assay

Suppression assays were carried out as previously described [16]. Briefly, 5 $x10^4$ $CD4+CD25^+$ T cells isolated from 4 pooled spleens per group were cultured in 96-well round-bottom plates (Corning) in complete RPMI-1640 medium (Invitrogen) with 5 $x10^4$ CFSE (carboxyfluorescein succinimidyl ester)-labelled effector T cells ($CD4^+CD25^-$) from naïve BALB/c mice. As antigen presenting cells, naïve bone marrow-derived dendritic cells (BM-DC) (1 $x10^5$) were added in the presence of 2.5 μg/ml Concanavalin-A (Con-A) (Sigma-Aldrich, St. Louis, MO, USA) during 4 days. The BM-DC were collected from euthanized naive mice by flushing femurs and tibiae with PBS (Invitrogen) as previously described [14]. These cells were resuspended at 2.5×10^5 cells/ml in complete RPMI-1640 medium containing 1% penicillin/ streptomycin, l% glutamine (Gibco-Invitrogen, Carlsbad, CA, USA), 50 μM β-mercaptoethanol (Invitrogen) and 10% fetal bovine serum (FBS) (Gibco, Invitrogen). Granulocyte–macrophage colony-stimulating factor (GM-CSF) (Cytocen, Utrecht, The Netherlands) at 20 ng/ml was added on days 0, 2 and 4. On day 7, cells were ready to be used. Isolation of $CD4^+CD25^+$ T cells from spleen cells was done by using the Regulatory T Cell Isolation Kit (Miltenyi Biotec GmBH, Bergisch Gladbach, Germany) according to the manufacturer instructions. Briefly, $CD4^+$ T cells were purified by depletion of non-$CD4^+$ cells with negative selection. From purified $CD4^+$ T Cells, $CD25^+$ T cells were isolated by positive selection of $CD4^+CD25^+$ regulatory T cells using $CD25^+$ magnetic microbeads. The purity of the $CD4^+CD25^+$ Treg cell and $CD4^+CD25^-$ T effector cell population was typically 90% and 85%, respectively. Effector T cells derived from the spleen of naïve BALB/c mice were also isolated by using the

Regulatory T Cell Isolation Kit (Miltenyi Biotec GmBH). The effector $CD4^+CD25^-$ cells were labelled at 10^6 cells/ ml in PBS containing CFSE at a concentration of 2 μM for 10 min at 37°C. Cells were washed and added to the suppression assays. Proliferation of effector cells was measured by flow cytometry using a FACSCanto II (BD Biosciences, Erembodegem, Belgium). The percentage of cell division was calculated using the FlowJo software (Tree Star Inc.).

Cytokine determination

The concentration of IL-2, IL-4, IL-5, IL-10, IL-17 and IFN-γ in BAL fluid and culture supernatants from lung-draining mediastinal lymph node cells were measured on the Luminex 100 (Luminex, Austin, TX, USA) using a Bio-Plex assay (Bio-Rad, Hercules, CA, USA) according to manufacturer's instructions.

Adoptive transfer of $CD4^+$ T cells

Spleens from mice chronically infected (43 days) with *T. spiralis* were aseptically collected and $CD4^+$ T cells were isolated using the $CD4^+$ T Cell Isolation Kit II (Miltenyi Biotec GmBH) according to manufacturer's instructions. $CD4^+$ T cells from uninfected mice were isolated using the same protocol. The purity of the isolated $CD4^+$ T cells was >80% based on flow cytometry. For adoptive transfer, isolated $CD4^+$ T cells were first washed in PBS, and 5×10^6 cells were injected into the tail vein of naive mice. Adoptive transfer for each recipient mouse was performed one day before each OVA challenge.

Culture of lymph node cells

Freshly removed lung-draining lymph nodes were pooled from 4 mice per group. These lymph nodes were dispersed by pressing them through a cell strainer using a plunger. Erythrocytes were removed by resuspending the lymph nodes cells in 4 ml RBC lysis buffer (Biolegend, San Diego, CA, USA) and incubating for 5 minutes on ice. The lymph nodes cells were washed and resuspended in RPMI-1640 containing 1% penicillin/ streptomycin, l% glutamine (Gibco-Invitrogen), 50 μM β-mercaptoethanol (Invitrogen), 10% fetal bovine serum (FBS) (Gibco, Invitrogen). Cells were cultured in triplicate in 96-well round-bottomed plates (Corning) at 5×10^5 cells/well in complete RPMI-1640 with or without OVA. After 5 days, supernatants were collected and stored at -20 C for cytokine analysis.

Statistical analyses

One-way analysis of variance (ANOVA) was performed by the Bonferroni's Multiple Comparison test to analyse differences in means between different groups. For analysis of the histological scores of the lungs, the Kruskal-Wallis-Dunn's Multiple Comparison test was used. Analyses were performed using GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA).

Results

Chronic phase of *T. spiralis* infection reduces OVA-IgE serum levels in EAAI

To determine the effect of *Trichinella* infection on EAAI, total IgE and OVA-specific IgE levels were measured in sera taken from all mice at the time they were sacrificed. Figure 2A shows that the levels of total IgE in OVA-treated mice were higher than those in control mice. *Trichinella* infection resulted for all three group of animals (-25 d, +5 d and +12 d) in increased levels of total IgE, which were higher than that of the OVA-treated animals but not significantly different from that of mice that were both infected and OVA-treated. Interestingly, the level of OVA-specific IgE was significantly lower in the group of mice that were chronically infected and OVA-treated (-25 d + OVA) compared to mice that were OVA-treated only. Acute *Trichinella* infection either 5 days (-5 d + OVA) before or 12 days (+12 d + OVA) after OVA-sensitization did not affect the levels of OVA-specific IgE.

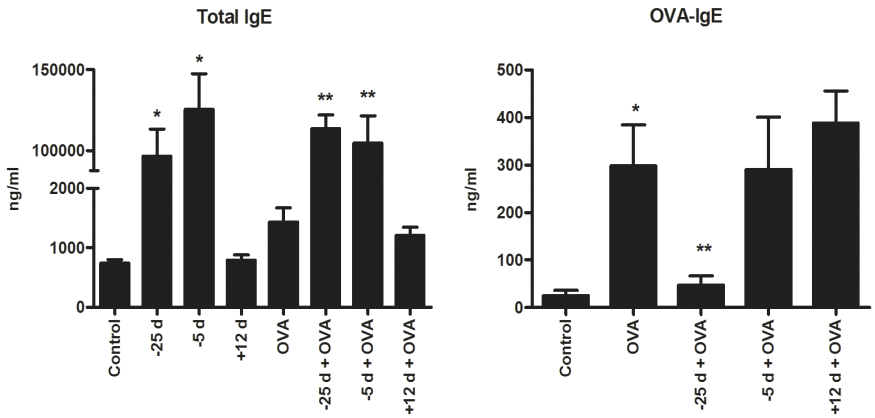

Figure 2. Effect of *T. spiralis* infection on OVA-IgE serum levels in EAAI. The chronic phase of *Trichinella* infection was established 25 days (-25 d + OVA) before OVA-sensitization/challenge. The acute phase was established at two-time points, 5 days (-5 d + OVA) before and 12 days (+12 d + OVA) after OVA-sensitization. Mice from the control group were uninfected PBS/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. On day 18, blood was collected and total IgE or OVA-specific IgE levels in serum were determined by means of ELISA. OVA-specific and total IgE levels for the different groups (8 mice/ group) are represented as mean (ng/ml ± SE). Data shown are pooled from two independent experiments.
* Significantly different *($p<0.05$)* from Control group. ** Significantly different *($p<0.05$)* from OVA group.

Acute and chronic phases of *T. spiralis* infection before OVA sensitization reduces BAL-Th2 cytokine in EAAI

To determine the effect of *T. spiralis* infection on EAAI, local cytokines in BAL fluid were measured at day 18 after OVA-treatment. The different cytokines measured in BAL of infected animals only were not significantly different from those of the control group. In contrast, BAL from the OVA-treated mice showed significantly higher levels of IL-2, IL-4, IL-5 and IL-10 (Fig. 3) compared to the control group. The concentrations of these cytokines were significantly lower in the mice infected either 25 (-25 d + OVA) or 5 (-5 d + OVA) days before OVA sensitization compared to the OVA-treated mice. No significant changes in the levels of these cytokines were observed in BAL of animals infected 12 (+12 d + OVA) days after OVA sensitization. The levels of the cytokines IL-13, IL-17, IFN-γ and TGF-β were not significantly different among the different groups (data not shown).

Acute and chronic phases of *T. spiralis* infection reduces airway eosinophilia and lung pathology in EAAI

Differential counting of cells in BAL revealed that infection with *T. spiralis* either 25 days or 5 days before as well as 12 days after OVA sensitization resulted in a significant decrease in the number of eosinophils in BAL compared with mice that were OVA-treated only (Fig. 4). Other cells (macrophages, neutrophils and lymphocytes) were also counted however; their numbers were not significantly different compared to the OVA-treated animals (data not shown). Figure 5A shows that no lung tissue inflammation (upper row) and production of mucus (lower row) were found in the *Trichinella* infected mice or in the uninfected PBS/alum-treated control animals. In contrast, mice treated with OVA showed multifocal cellular infiltrates around bronchiole and blood vessels. These peribronchiolar and perivascular cell infiltration were composed of many eosinophilic and neutrophilic granulocytes as well as lymphocytes (Fig. 5B, upper row). Furthermore, there were increased numbers of mucin-containing goblet cells present at the respiratory surface of bronchi and bronchiole, indicating an increase in mucus production (Fig. 5B, lower row). In mice infected 25 days (-25 d + OVA) before OVA-sensitization, tissue inflammation after OVA challenge was greatly reduced with significantly less peribronchiolar and perivascular cellular infiltration compared to OVA-treated mice (Fig. 5C). This effect was also observed in mice infected 5 days (-5 d + OVA) before OVA-sensitization however less compared to mice that were infected 25 days before OVA-sensitization. A decrease in

pulmonary inflammation for mice that were infected 12 days (+12d + OVA) after OVA-sensitization was also observed however, it was not significantly different compared to the group of uninfected OVA-treated mice (Fig 5).

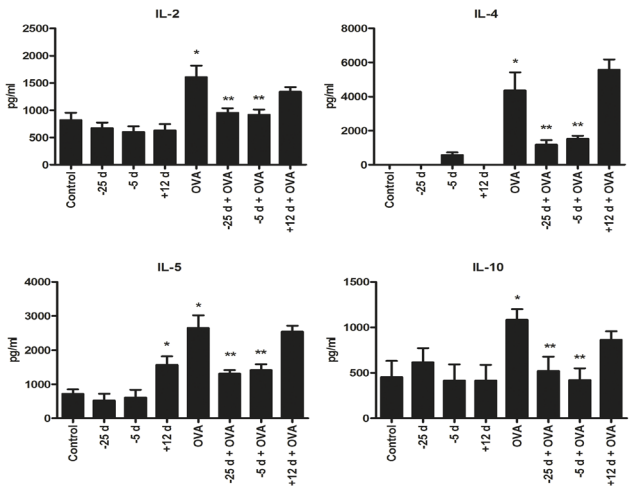

Figure 3. Effect of *T. spiralis* infection on BAL-cytokines in EAAI. The chronic phase of *Trichinella* infection was established 25 days (-25 d + OVA) before OVA-sensitization/challenge. The acute phase was established at two-time points, 5 days (-5 d + OVA) before and 12 days (+12 d + OVA) after OVA-sensitization. Mice from the control group were uninfected PBS/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. On day 18, BAL was collected and the cytokine levels measured using a multiplex bead-based assay. Cytokine levels for the different groups (8 mice/ group) are represented as mean (pg/ml ± SE). Data shown are pooled from two independent experiments. * Significantly different *(p<0.05)* from Control group. ** Significantly different *(p<0.05)* from OVA group.

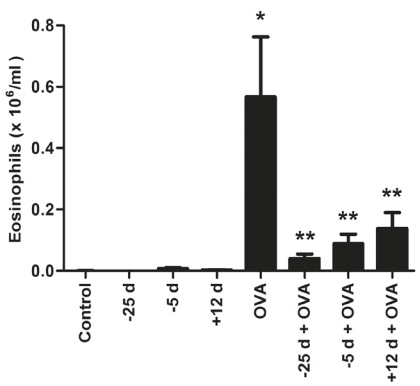

Figure 4. Effect of *T. spiralis* infection on BAL-eosinophils in EAAI. The chronic phase of *Trichinella* infection was established 25 days (-25 d + OVA) before OVA-sensitization/challenge. The acute phase was established at two-time points, 5 days (-5 d + OVA) before and 12 days (+12 d + OVA) after OVA-sensitization. Mice from the control group were uninfected PBS/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. BAL fluid was collected for eosinophil count. Data for the different groups (8 mice/ group) are presented as bars indicating mean values (cells/ml ± SE). Data shown are pooled from two independent experiments. * Significantly different *(p<0.05)* from group Control. ** Significantly different *(p<0.05)* from group OVA.

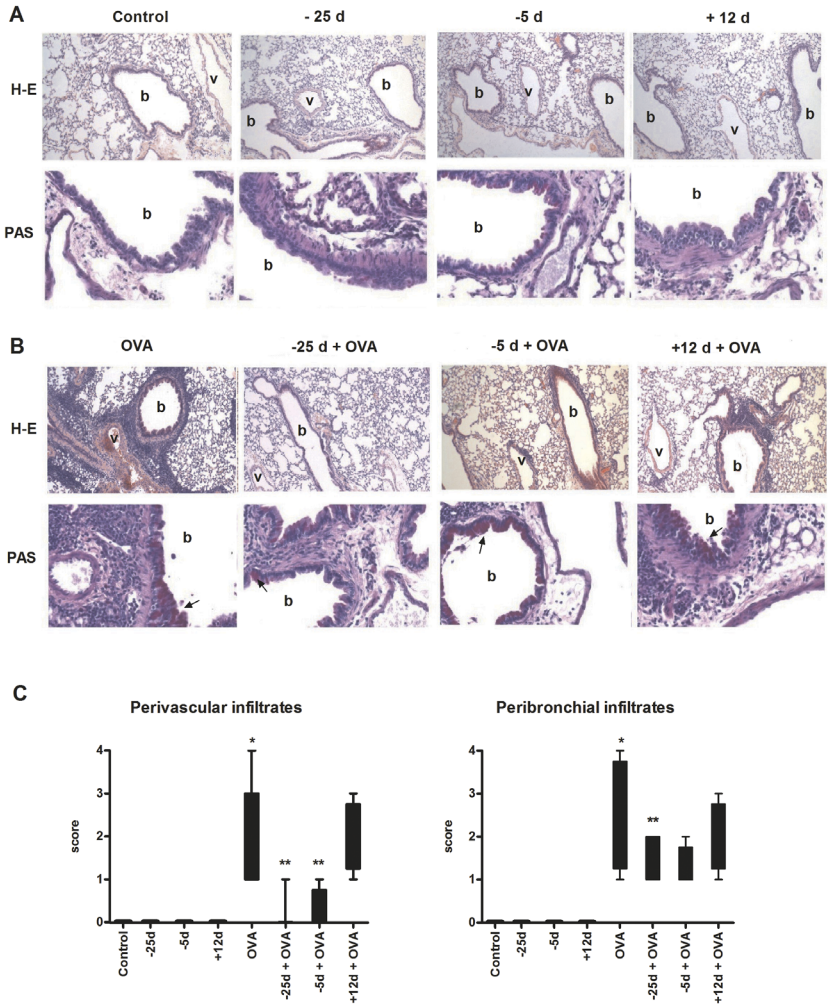

Figure 5. Effect of *T. spiralis* infection on pulmonary inflammation in EAAI. The chronic phase of *Trichinella* infection was established 25 days (-25 d + OVA) before OVA-sensitization/challenge. The acute phase was established at two-time points, 5 days (-5 d + OVA) before and 12 days (+12 d + OVA) after OVA-sensitization. Mice from the control group were uninfected PBS/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. Lungs were fixed with formaldehyde and sections were cut and stained with H-E (A) and PAS (B). Arrows indicate the mucin-filled cells (bright-fuchsia) and the bronchiole (b) and vessels (v) are shown. These figures represent the average of the histological scores. (C) The perivascular and peribronchial infiltrates in histological sections of the lung were blindly scored using a semiquantitative scale from absent (0), minimal (1), slight (2), moderate (3) to marked (4). Data for the different groups (8 mice/ group) are presented as box and whiskers indicating the minimum and maximum values. Data shown are pooled from two independent experiments. *Significantly different *($p<0.05$)* from group Control. **Significantly different *($p<0.05$)* from group OVA.

Acute and chronic phases of *T. spiralis* infection induce functional regulatory T cells in EAAI

To determine whether *Trichinella* infection induce functional Treg cells, we analysed the expression of CD4, CD25 and Foxp3 on splenocytes of infected mice. Flow cytometric analysis revealed that the proportion of $CD4^+25^+Foxp3^+$ T cells in $CD4^+$ gated splenocyte populations was significantly elevated in chronically infected (-25 d) mice compared to the control group. The number of these cells in the other two groups of infected PBS/alum-treated mice (-5 d and +12 d) were not significantly different from the control group (Fig 6A). Interestingly, the proportion of Treg cells in splenocytes of all infected OVA-treated mice was significantly higher compared to the OVA-treated mice only. The proportion of Treg cells in splenocytes of mice infected 25 days (-25 d + OVA) before OVA-sensitization was the highest (28%) compared to the other OVA-treated/infected groups: 18% (-5 d + OVA) and 16% (+12 d + OVA). To determine whether the $CD4^+CD25^+$ Treg cells induced during infection are functional Treg cells in terms of suppressive capacity, functional assays were performed. For this purpose $CD4^+CD25^+$ cells were purified from the spleens of control, OVA-treated and infected OVA-treated mice and incubated with CFSE-labeled effector cells (EC: $CD4^+CD25^-$) and DC in the presence or absence of ConA. Figure 6B shows a decrease in the effector cell proliferation from 89.9%, when these cells where incubated with Treg cells from control mice, to 67.5% when effector cells were incubated with Treg cells from mice infected 25 days before OVA-sensitization.

Adoptive transfer of $CD4^+$ T cells from *T. spiralis* infected mice reduces EAAI

To determine whether $CD4^+$ T cells from *T. spiralis* chronically infected mice containing high proportion of Treg cells, protect against EAAI, adoptive T cell transfer was performed. For this purpose $CD4^+$ T cells isolated from the spleens of chronically *T. spiralis* infected or uninfected mice were transferred to OVA-treated mice one day before each OVA-challenge. Figure 7(A-B) shows that the numbers of eosinophils and IL-5 levels in BAL were significantly reduced in OVA-treated mice that received $CD4^+$ T infected cells from chronically infected animals, compared to OVA-treated mice and OVA-treated mice that received $CD4^+$ T cells from uninfected animals. The levels of all other tested cytokines in BAL were not significantly different between these groups of animals (data not shown). Neither the levels of total IgE nor of OVA-IgE in sera of the OVA-treated recipient animals were affected by the $CD4^+$ T cell transfer (data not shown).

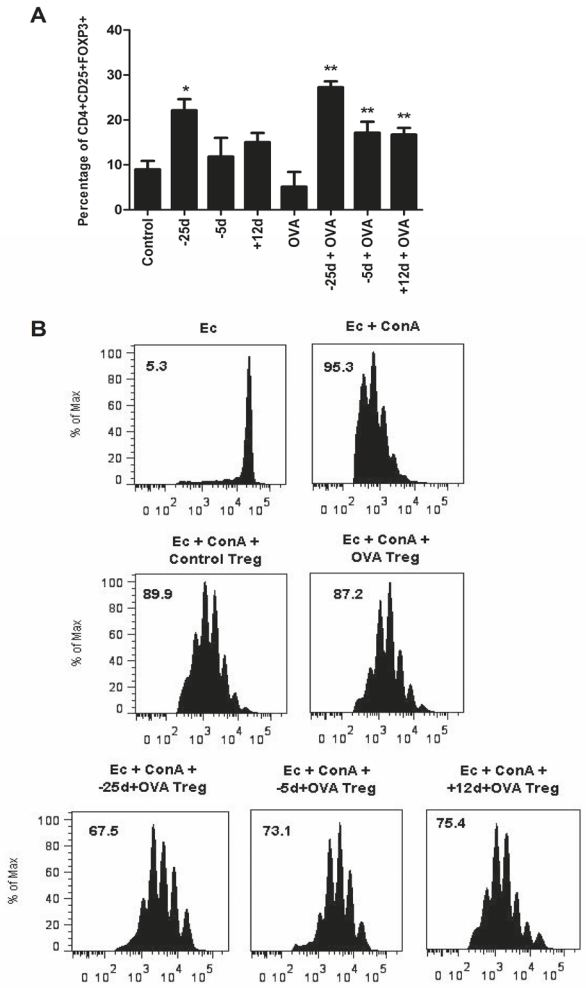

Figure 6. Effect of *T. spiralis* infection on the induction of functional Tregs in EAAI. The chronic phase of *Trichinella* infection was established 25 days (-25 d + OVA) before OVA-sensitization/challenge. The acute phase was established at two-time points, 5 days (-5 d + OVA) before and 12 days (+12 d + OVA) after OVA-sensitization. Mice from the control group were uninfected PBS/alum-treated. Mice from the OVA group were uninfected OVA- sensitized/challenged. Isolated splenocytes were stained and analysed by flow cytometry. (A) Percentage of $CD4^+$ $CD25^+$ cells expressing $Foxp3^+$ in $CD4^+$ gated cells. Data for the different groups (8 mice/ group) are represented as mean (% ± SE). Data shown are pooled from two independent experiments. *Significantly different *($p<0.05$)* from group Control. **Significantly different *($p<0.05$)* from group OVA. (B) Proliferative response of effector $CD4^+$ $CD25^-$ CFSE-labelled T cells (Ec) cultured for 4 days with DC and ConA with or without $CD4^+$ $CD25^+$ Treg cells isolated from splenocytes derived from the different groups. Histograms represent the percentages of the cells that have divided. Proliferation was measured by flow cytometry. Numbers inside the histograms indicate the percentage of dividing cells.

Histological examination of lungs (Fig. 7C, upper row) indicated that transfer of $CD4^+$T cells from infected mice to OVA-treated mice partially reduced cell infiltration around the bronchiole and vessels. The mucus production was also reduced in OVA-treated mice that received $CD4^+$T cells from infected mice (Fig. 7C, lower row). This result is summarized in the fig. 7D which shows that peribronchiolar cellular infiltration was reduced in OVA-treated mice that received $CD4^+$ T cells from infected mice. Perivascular cellular infiltration was also lower in mice that received $CD4^+$T cells from infected mice but it was not significantly different from mice that received $CD4^+$ T cells from uninfected mice.

Adoptive transfer of $CD4^+$ T cells from *T. spiralis* infected mice suppresses OVA-specific cytokine production in lung draining lymph nodes in EAAI

In order to determine the effect of the adoptive $CD4^+$T cell transfer from *Trichinella* infected to OVA-treated mice on cytokine production, lung-draining lymph nodes cells from the different groups were incubated with or without OVA for 5 days. Figure 8 shows that the levels of IL-4, IL-13 and IL-10, measured in the culture supernatants resulted as expected in increased levels of these cytokines in response to OVA. Interestingly, these cytokines were reduced to the background levels of the control mice when $CD4^+$T cell from spleens of *T. spiralis* chronically infected mice were given. No significant changes in cytokine levels were observed when mice received $CD4^+$ T cells from uninfected mice. It is noteworthy to mention that for the OVA-treated animals the levels of these cytokines in medium only were higher compared to the control uninfected PBS/alum treated mice. These increased background levels are probably due to the OVA sensitization and challenge treatment.

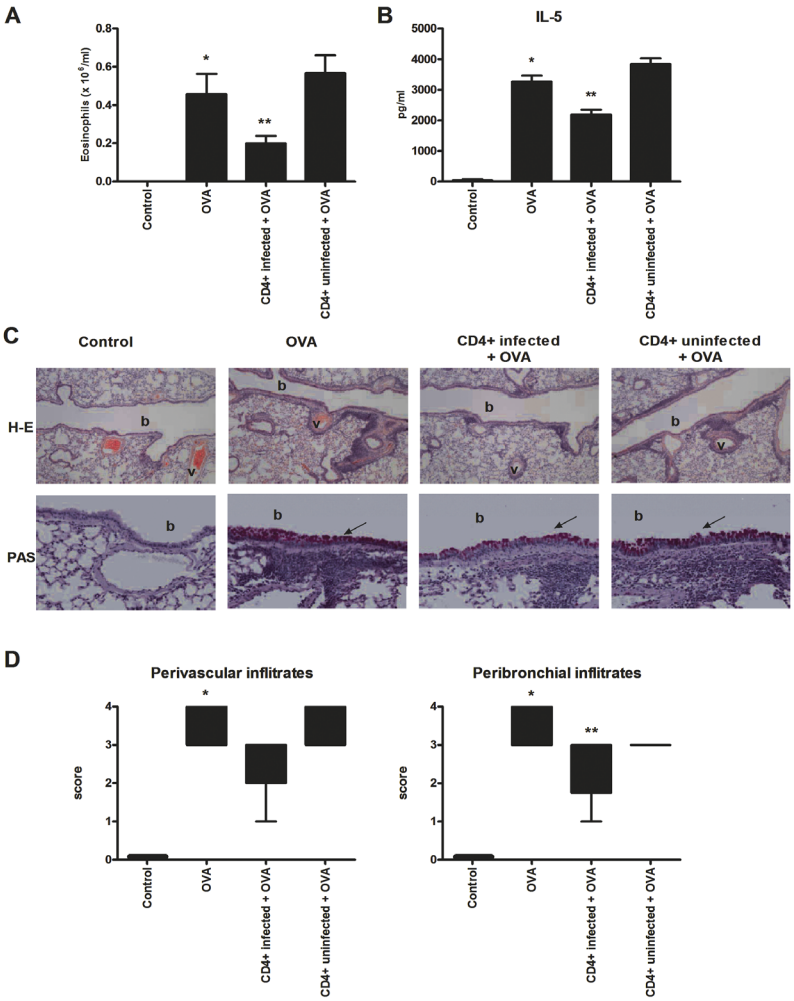

Figure 7. Effect of adoptive transfer of CD4⁺ T cells from *T. spiralis* infected mice on IL-5 levels in BAL and pulmonary inflammation in EAAI. Isolated CD4⁺T cells from either chronic *T. spiralis* infected (-25d) or uninfected mice were transferred to OVA sensitized mice one day before each challenge. (A) After euthanasia, BAL fluid was collected for eosinophil cell counting. (B) IL-5 levels in BAL were measured using a multiplex bead-based assay. (C) Lungs were fixed with formaldehyde and sections were cut and stained with H-E and PAS. Bronchiole (b) and vessels (v) are shown. Arrows indicate the mucin-filled cells (bright-fuchsia). The figures represent the average of the histological scores (D). The perivascular and peribronchial infiltrates in histological sections of the lung were blindly scored as mentioned in the legend of Fig 5. Data are presented as box and whiskers indicating the minimum and maximum values for the different groups (8 mice/ group). Data shown are pooled from two independent experiments. *Significantly different *($p<0.05$)* from group Control. **Significantly different *($p<0.05$)* from the OVA group. The CD4⁺ infected + OVA group refers to the OVA-treated mice that received CD4⁺ from infected mice. The CD4⁺ uninfected + OVA group are the OVA-treated mice that received CD4⁺ from uninfected mice.

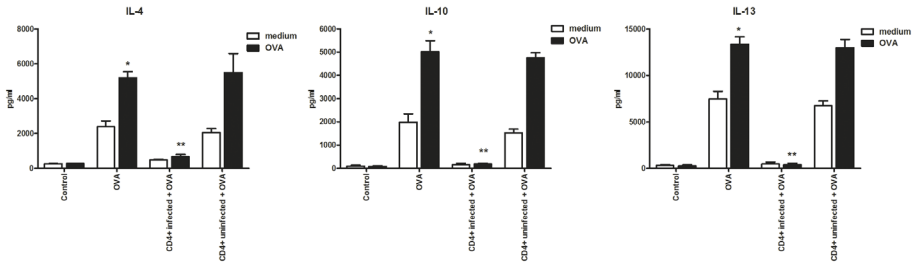

Figure 8. Effect of adoptive transfer of CD4$^+$ T cells from spleen of chronically *T. spiralis* infected mice on OVA-specific cytokine production. Isolated CD4$^+$T cells from either chronic *T. spiralis* infected or uninfected mice were transferred to OVA-sensitized mice one day before each OVA-challenge. Lung-draining lymph nodes cells from these mice were cultured with or without OVA for 5 days. Cytokine levels in the culture supernatants were measured using a multiplex bead-based assay. Results for the different groups (8 mice/group) are represented as mean values (pg/ml ± SE). Data shown are pooled from two independent experiments. *Significantly different *($p<0.05$)* from Control group. ** Significantly different *($p<0.05$)* from OVA group. The CD4$^+$ infected + OVA group refers to the OVA-treated mice that received CD4$^+$ from infected mice. The CD4$^+$ uninfected + OVA group are the OVA-treated mice that received CD4$^+$ from uninfected mice.

Discussion

Studies with certain helminths have shown that infection with these parasites can protect against allergic diseases. The inverse association between helminth infection and allergy often involves regulatory T (Treg) cells, which has given a new mechanistic basis to the *hygiene hypothesis* based on their role in dampening both Th1 and Th2 effectors responses. Several epidemiological studies support this inverse association between helminth infections and allergic disease in humans [2, 12, 22], and using murine models several groups have shown that certain helminths can reduce allergic responses [6, 8, 10, 23, 24].

In this study, mice infected with *T. spiralis* at three different time points were sensitized and challenged with OVA allergen in order to determine the effect of the different phases of infection on EAAI. We observed that events occurring during the muscle or chronic phase of *T. spiralis* infection protect against EAAI. Infected mice showed significantly reduced levels of OVA-specific IgE in serum, decreased levels of Th2 cytokines and low numbers of eosinophils in BAL. Histological analysis of the lungs indicated that mice in the chronic phase of infection had significantly lower OVA-induced peribronchiolitis and perivasculitis compared to mice that were OVA-treated only. Protection against experimental airway inflammation in mice chronically infected with other helminths has also been reported. For instance, *Schistosoma mansoni* infected BALB/c mice were protected against EAAI induced by OVA as indicated by reduction of eosinophils in BAL, Th2 cytokine production, OVA-specific IgE levels and reduction of the number of inflammatory cells in lungs. The authors also showed that the critical role of $CD4^+CD25^+Foxp3^+$ regulatory T cells was independent of IL-10 [7]. A study by Dittrich *et al*. investigated the effect of chronic infection with the filarial parasite *Litomosoides sigmodontis* on the mouse OVA-induced EAAI model. Infection with this filarial parasite suppressed all pathological features of the allergic model. Additionally, significantly increased numbers of Treg cells were observed in spleen and mediastinal lymph nodes in infected and sensitized mice compared with sensitized controls [8]. Similarly, Liu *et al*. showed that *Schistosoma japonicum* chronic infection suppressed airway eosinophilia, mucus production, OVA-specific IgE responses and production of IL-4 and IL-5 [25]. In a study that also used the OVA-induced EAAI model, Smit *et al*. (2007) showed that *S. mansoni* infection protects against EAAI only during the chronic but not the acute phase of infection [10].

Our findings indicate that protection was most prominent during the chronic or muscle phase of *T. spiralis* infection which was already established before sensitization and

aerosol challenge with OVA. The acute or intestinal phase of infection established before OVA-sensitization/challenge had also a protective effect, although to a lesser extent. It is important to mention that by the time these animals received the OVA-challenge, mice were at a chronic phase of infection, which was perhaps the reason for the partial effect on EAAI. Since humans can be sensitized to aeroallergens early in life and before consumption of *Trichinella* infected meat, we included a group of mice that were *Trichinella* infected after OVA-sensitization and shortly before OVA-challenge. By the time the OVA-challenge was given these mice were in the intestinal or acute phase of infection. Here, partial protection against EAAI was also observed, which was restricted to significant decrease in the levels of eosinophils in BAL and partial reduction of pulmonary inflammation. Unfortunately it is not possible to study the effect of a chronic *Trichinella* infection after allergen sensitization since the period of time between OVA-sensitization and challenge in this EAAI model is too short. The observed difference between the effect of *T. spiralis* and *S. mansoni* acute infection on EAAI may be due to their different life cycles and immunopathology induced. Acute schistosomiasis is characterized by a systemic hypersensitivity reaction against the migrating schistosomula and eggs. Once in the blood capillaries, the schistosomula pass via the blood flow to the lungs and continues to the liver where they mature, pair and initiate egg deposition [26, 27]. These schistosomula can stay in the pulmonary capillaries from 3 to 16 days and there is evidence that during acute schistosomiasis individuals can suffer from lung eosinophilia, and pulmonary symptoms such as cough and shortness of breath [22]. Although *Trichinella* infection has also a migratory phase, where the newborn larvae pass through the lung microvascular system on its way to the skeletal muscle, this is a rapid process in which the larvae are usually not trapped in the lungs [28]. We did not observe any pulmonary inflammation in the mice that received *T. spiralis* infection only. Furthermore as suggested by Furze *et al* [29], *T. spiralis* larvae are particularly susceptible to immune attacks, and immunomodulatory mechanisms induced by adult worms in the early (acute) phase of *T. spiralis* infection might protect the future larvae development. The surface and excretory–secretory products from the adult parasite might act in multiple ways, provoking significant changes in the gut microenvironment which can have broad effects on the immune system, both within and beyond the gut as has been shown by other parasites [30]. One of these strategies might be the induction of Treg cells that have been found by us and others [29] to be enhanced also during the acute phase of infection. Protection of C57/BL mice against EAAI by chronic *T. spiralis* infection has been recently reported by Park *et al*.[31]. In the present study we show that also an acute phase of *T. spiralis* infection protects against EAAI and in addition we report on the

possible role of Treg cells in this process. Helminth induced Treg cells have been shown to contribute to immune suppression allowing the parasite to escape from host protective immune responses. Studies in humans have shown that individuals with chronic helminth infections develop prominent anti-inflammatory networks that reduce antigen-specific immune responses to both the helminth and unrelated antigens [32]. Using mouse models it has been demonstrated that $CD4^+CD25^+$ T cells induced by *Heligmosomoides polygyrus* suppressed allergic responses to OVA or Der P 1 in mice [6]. In our study, we observed increased number of $CD4^+CD25^+Foxp3^+$ cells in spleen of *T. spiralis* infected mice, particularly during the chronic phase of infection. This cell population was also found to be functional in suppression assays *in vitro*. Although the proportion of Treg cells in the spleen from the group infected 5 days before OVA-sensitization/challenge (-5 d + OVA) is comparable to that of the group infected 12 days after OVA-sensitization ($^+$12 d + OVA), the suppression of EAAI in the latter group was less. Here, timing may play an important role determining the overall effect of Treg cells on EAAI since mice from the group -5 d + OVA were sacrificed 25 days after infection while mice from the group $^+$12 d + OVA were sacrificed 5 days after infection. Whether at 5 days after infection the proportion of Treg cells that accumulate in lungs and draining lymph nodes is low and therefore they do not efficiently suppress EAAI remains to be investigated. Here we also show that transfer of $CD4^+$ T cells isolated from chronically infected mice containing higher proportions of suppressive $CD25^+Foxp3^+$ cells to OVA-treated mice conferred partial protection against EAAI. Our findings concur with those of Wilson *et al*, who showed suppression of airway eosinophilia in OVA-treated BALB/c mice after the transfer of $CD4^+$T cells isolated from mesenteric lymph nodes from infected mice with *H. polygyrus* [6]. Our results indicate that administration of $CD4^+$ T cells from chronically *Trichinella* infected mice containing high numbers of Treg cells just one day before each OVA-challenge, is sufficient for these cells to efficiently migrate into lymph nodes, and strongly suppress OVA-specific cytokine production. Studies by others have already shown that Treg cells can migrate into inflammatory sites in various inflammatory diseases and suppress peripheral effector T cell function where the various expression patterns of chemokine receptors and integrins contribute to Treg cell trafficking [33]. Our results show that Treg cells might be the cellular mediators responsible for the protective effect of *T. spiralis* infection, however other cell populations such as regulatory B cells, alternatively activated macrophages and even DC carrying parasite antigens might also be involved in immunosuppression [9-11, 16]. In conclusion, infection with *T. spiralis* confers protection against EAAI. This parasite induces a regulatory network that is probably orchestrated by secreted molecules as well as somatic antigens from the adult

and larval stages of the parasite. The magnitude of this protective effect increases as infection progresses from the intestinal or acute phase to the muscle or chronic phase of infection. During infection the immunological balance inclines toward a regulatory response where the Treg cells may play an important role. Future studies should focus on elucidating the mechanisms and molecular helminth structures responsible for inducing this regulatory process in order to develop alternative tools for preventing or treating allergic asthma.

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Chapter 5

Glycan microarray profiling of parasite infection sera identifies the LDNF glycan as a potential antigen for serodiagnosis of Trichinellosis

Carmen Aranzamendi, Boris Tefsen, Montse Jansen,
Lorena Chiumiento,Fabrizio Bruschi, Titia Kortbeek,
David F. Smith, Richard D. Cummings,
Elena Pinelli and Irma Van Die

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Abstract

Diagnostic methods for parasite infections still highly depend on the identification of the parasites by direct methods such as microscopic examination of blood, stool and tissue biopsies. Serodiagnosis is often carried out to complement the direct methods; however few synthetic antigens with sufficient sensitivity and specificity are available. Here we evaluated a glycan microarray approach to select for synthetic glycan antigens that could be used for serodiagnosis of parasitic infections. Using a glycan-array containing over 250 different glycan antigens, we identified GalNAcβ1-4(Fucα1-3)GlcNAc-R (LDNF) as a glycan antigen that is recognized by antibodies from *Trichinella*-infected individuals. We synthesized a neoglycoconjugate, consisting of five LDNF molecules covalently coupled to bovine serum albumin (BSA), and used this neoglycoconjugate as an antigen to develop a highly sensitive total-Ig ELISA for serological screening of trichinellosis. The results indicate that glycan microarrays constitute a promising technology for fast and specific identification of parasite glycan antigens to improve serodiagnosis of different parasitic infections, either using an ELISA format, or parasite-specific glycan-arrays.

Introduction

Infections with parasites cause worldwide suffering and death, particularly in developing countries. Diagnostic methods for some of these infections still highly depend on the identification of the parasites by direct methods such as microscopic examination of blood, stool and tissue biopsies [1]. Although these approaches can result in a specific diagnosis, the sensitivity is generally low. Serodiagnosis could be used to complement the direct methods. However, preparation of the parasite antigens is often complicated which can compromise reproducibility of the results. Improving serodiagnosis using new technology is required.

Here we evaluated the potential use of parasite glycan antigens for serodiagnosis of parasitic infections. Glycans, linked to proteins and lipids, are abundantly present on the surface and in the excretory/secretory products of parasites, and humoral immune responses to glycan antigens are a dominant feature of parasite infections, as illustrated by studies of helminth infections in mice, humans and primates [2-6]

To select for helminth glycan antigens that have serodiagnostic potential, we used an approach based on glycan microarray technology. A glycan microarray comprises a library of synthetic or natural glycan molecules that are covalently linked to a solid support such as glass slides [7-9]. The most common application of glycan-array technology is analyzing the binding specificity of lectins and antibodies. Such glycan microarrays have been already used to analyze the antibody response to pathogens, such as *Schistosoma* and *Salmonella* [10, 11]. The application of this technology could lead to novel glycan-based diagnostic tools for the serodiagnosis of parasitic infections.

A common parasitic infection which direct diagnosis is aided by serology is trichinellosis. This is a food borne zoonotic disease caused by nematodes of the genus *Trichinella*. Human infection occurs due to the ingestion of raw or inadequately cooked meat containing parasite larvae, mostly of *Trichinella spiralis* species [12]. The disease in humans can range from asymptomatic infection to a fatal disease, depending on the number of larvae ingested and the host immune status. According to reports from 55 countries worldwide, the yearly total number of trichinellosis cases is estimated to be 10,000, with a mortality rate of 0.2% [13].

The direct detection of muscle-stage larvae in muscle biopsies etiologically proves the diagnosis. The disadvantage of this method is that it requires surgical intervention and that the sensitivity of the diagnosis depends on the parasite load and the amount of muscle

sample tested [12]. In addition to the clinical history and results from the biopsy, serology by ELISA is used for the detection of specific anti-*Trichinella* antibodies in human sera. Most ELISA assays are based on the use of excretory/secretory (ES) products from the *T. spiralis* muscle larvae [12]. The use of the ES antigen, however, has serious disadvantages since the preparation of this antigen is laborious and requires the use of laboratory animals. Furthermore, micro-environmental factors during culture of the animal-derived larvae may affect antigen quality [14], resulting in standardization problems. Replacement of the ES antigen by synthetic antigens with sufficient sensitivity and specificity could solve these problems.

Our studies clearly show that specific parasite glycan antigens can be identified by glycan-array analysis of minute amounts of serum from infected individuals. In addition, we showed that an ELISA assay based on neoglycoconjugates carrying the GalNAcβ1-4(Fucα1-3)GlcNAc (LDNF) glycan antigen has a high sensitivity for serodiagnosis of trichinellosis, indicating the potential value of glycan microarray technology for diagnosis of parasite infections.

Material and methods

Human sera

A total of 29 *Trichinella* positive serum samples were tested. Seven of these sera were from the diagnostic laboratory at the RIVM, 12 were from an outbreak in Turkey (2004) that was confirmed to be caused by *Trichinella britovi* [15] and 10 were from an outbreak in Poland (1991) caused by *T. spiralis* [16]. The sera were tested both in a total Ig *Trichinella*-ELISA using ES antigen and confirmed with a *Trichinella*-immunoblot as previously described [16]. In addition, 90 serum samples from patients that were serologically positive to the following infections were used: ascariasis, leishmaniasis, toxocariasis, echinococcosis, cysticercosis, amebiasis, toxoplasmosis, borreliosis, and syphilis. Ten positive sera from patients with schistosomiasis and 10 from strongyloidiasis were kindly provided by Dr. J. Van Hellemond from the Rotterdam Harbour Hospital/ Erasmus MC, The Netherlands. Ten serum samples from healthy blood donors with no known history of parasitic infection were also used.

Preparation of *Trichinella spiralis* ES and crude antigen

Preparation of the ES antigen was performed as previously described by Gamble [17]. Briefly, 5-7 weeks old male Wistar rats obtained from Harlan-The Netherlands were orally infected with 3000 *T. spiralis* muscle larvae that were recovered by acid-pepsin digestion from chronically infected mice. After 42 days of infection, *T. spiralis* muscle larvae were recovered from infected rats by acid-pepsin digestion, washed and incubated at a concentration of 10^5 larvae per ml, for 19 h at 37 °C in 5% CO_2 in RPMI medium supplemented with 1% penicillin/streptomycin. After incubation, the medium was centrifuged and the supernatant containing the ES antigen was dialyzed and concentrated. The protein concentration was determined by the BCA protein assay (Pierce, Rockford, IL, USA). *T. spiralis* crude antigen was prepared from muscle larvae, in 100 mM Tris HCl (pH = 8), essentially as described by DeBose-Boyd *et al*.

Glycan-array

Glycan-array screening was performed at Core H of the Consortium for Functional Glycomics (CFG), Emory University School of Medicine, Atlanta, USA). The glycan-array is a microarray containing a library of natural and synthetic glycans with amino linkers printed onto NHS-derivatized glass slides to form a covalent amide linkage.

Printed array Version 2.1 containing glycan structures with the CFG numbers # 1-264 was used. The procedure for testing the glycan-array as well as all glycan structures used and their corresponding CFG numbers are available at the website of the CFG (http://www.functionalglycomics.org/fg/).

Glycan-array slides were incubated with human serum (1:100 dilution) derived from parasite-infected or healthy blood donors as indicated, and subsequently with Alexa labeled mouse anti-human IgG secondary antibodies in phosphate buffered saline (PBS) containing 0.5% Tween-20. The samples (100 µl) were applied directly onto the surface of a single slide, covered with a microscope cover slip and incubated in a humidified chamber for 60 min. Slides were subsequently washed by successive rinses in (*i*) PBS-0.05% Tween, (*ii*) PBS, and (*iii*) deionized water, and immediately subjected to imaging. Fluorescence intensities were detected by using a ScanArray 5000 (PerkinElmer) confocal scanner. Image analyses were carried out using IMAGENE image analysis software (BioDiscovery, El Segundo, CA). No background subtractions were performed. Data were plotted by using Microsoft EXCEL software.

Synthesis of neoglycoconjugates

Neoglycoconjugates presenting LDNF, GalNAcβ1-4GlcNAc (LDN) or N,N',N''-diacetylchitotriose (chitotriose, chi-3, Sigma) were synthesized essentially as described in Tefsen *et al*., 2009. In short, N,N'-diacetylchitobiose (chitobiose, chi-2, Sigma) or chitotriose was derivatized with 2,6-diaminopyridine (DAP, Sigma) in DMSO (Sigma), Acetic acid (Sigma) and $NaCNBH_3$ (Sigma) and purified on a preparative Zorbax NH_2 PrepHT column (250 x 21.2 mm, 7 µm, Agilent) using a gradient with acetonitrile (Sigma) and 50 mM ammonium formate, pH 4.4 (Sigma) with a column flow of 10 ml. Chi-2-DAP was converted to LDN-DAP using the recombinant enzyme β1,4-GalNAcT from *Caenorhabditis elegans* [18], and LDN-DAP subsequently was converted to LDNF-DAP using the fusion protein ProtA-FucT-VI as enzyme source [19]B. All DAP-derivatized glycans were purified by Sep-Pak C_{18} reverse-phase chromatography [20] and dried in a speedvac for subsequent conjugation to BSA. The DAP-derivatized glycans were activated with 3,4-diethoxy-3-cyclobutene-1,2-dione, (98%, di-ethylsquarate, Sigma Aldrich) in ethanol and coupled to BSA (Sigma) dissolved in conjugation buffer (boric acid (Gibco) and KCl (Fluka); the pH was adjusted with KOH (Merck). By varying the glycan to protein ratio, the amount of glycan antigens coupled to BSA was regulated, resulting in neoglycoconjugates carrying different amounts of LDNF antigen (LDNF2 and LDNF5). Samples were dialyzed in a Slide-A-Lyzer Dialysis Cassette with 10.000

MWCO (Thermo Scientific) against demi-H_2O and neoglycoconjugates were dried in a speedvac and stored at –20 °C. The molar ratio of glycans to carrier was analyzed in a 4800 MALDI-TOF/TOF Analyzer (Applied Biosystems) and was as follows: for chi-3-BSA 3.2:1, for LDN-BSA 4.5:1 and for LDNF-BSA 1.8:1 and 5.3:1, respectively.

ELISAs

Flat-bottomed, 96-well microtiter plates of medium binding (Nunc, Denmark) were coated with neoglycoconjugates at different concentrations in 0.1 M Na_2CO_3, pH 9.6 for 1 h at 37 °C. After washing, a blocking step was performed by adding BSA 1% for 1h at 37°C. The plates were then washed four times with (PBS), pH 7.2 containing 0.05% (v/v) Tween 20 (PBS/Tween). Serum samples were diluted at 1:10 in 1% BSA (Sigma, St. Louis, MO) in PBS/Tween and added to the wells. After incubation of 1 h at 37 °C, the plates were washed and incubated with horseradish peroxidase (HRP)-conjugated to anti-human total-Ig (Sigma) in 4% BSA/PBS/Tween for 1 h at 37 °C. The plates were washed again and the substrate containing H_2O_2 (0.05%) and 5-amino-2-hydroxybenzoic acid was added and incubated for 1 h at 22°C. Afterwards, the optical densities (OD) were measured at 450 nm. A cut-off value was calculated as the average OD value from sera (diluted 1:10) from 10 healthy blood donors plus threefold the standard deviation. The OD of the tested serum was divided by the OD of the cut-off value to obtain a ratio. Results from the ELISA were considered positive (+) when the ratio was higher or equal to 1, and it was considered negative (−) when it was less than 1. The procedures for the *Trichinella* ES-ELISA and the *Trichinella* ES-immunoblot have been described previously [16].

Results

Screening for glycan recognition by antibodies in sera from parasite-infected individuals

To identify specific glycan antigens with potential use for serodiagnosis of trichinellosis, the sera of five *Trichinella* positive individuals were screened for antibodies recognizing specific oligosaccharides within a large library of glycan antigens, using the glycan-array facility of the CFG (http://www.functionalglycomics.org) [7]. To enable selection of glycan antigens with specificity for serodiagnosis of trichinellosis, we additionally screened sera from five individuals serologically positive for echinococcosis and five for leishmaniasis as well as the sera from two healthy blood donors. Figure 1A shows the glycan-array profiles of two of these serum samples. IgG in sera of infected individuals and healthy blood donors recognized many different glycan antigens, but the types of glycans recognized differed between the infected versus uninfected individuals. To assess the specificity of glycan recognition in the sera of *Trichinella* positive individuals, we identified all glycan antigens that showed a high (> 10.000 RFU, relative fluorescence units) response in four out of five infection sera, and compared these values to the RFU's of all other sera to the same glycan antigens. This analysis showed that the sera of *Trichinella* positive individuals contain a relatively high level of serum IgG recognizing the glycan antigen LDNF (CFG #91), whereas sera from individuals positive for echinococcosis and leishmaniasis showed a low binding to this glycan antigen (Fig. 1B). Several of the mannose-containing glycan antigens also showed a differential recognition pattern with the sera used in this study (Fig. 1B). Multiple glycan antigens were inconsistently, or not recognized by any of the sera used here, such as the glycan antigen Galβ1-4(Fucα1-3) GlcNAc-R (Le^X, CFG #135) (Fig. 1B). In addition, we observed that several glycan antigens, for example CFG #156, strongly bound to antibodies in all sera, possibly representing a common glycan antigen.

Detection of LNDF in ES and crude larval antigen

The data from the glycan microarray showed that antibodies in sera from *Trichinella*-infected patients recognized glycans containing LDNF, but not other glycan antigens such as Le^X. To evaluate the presence of LDNF glycans on *T. spiralis* ES and/or muscle larvae crude antigen, an ELISA was performed using specific anti-glycan monoclonal antibodies. Anti-LDNF and anti-LDN antibodies recognized antigens in both *T. spiralis* ES and crude larval antigen (Figure 2), indicating the presence of these glycan antigens within *T. spiralis*. By contrast, the monoclonal antibody recognizing Le^X, a dominant

glycan antigen within schistosomes [21], did not recognize the *T. spiralis* antigens, whereas it recognized neoglycoconjugates carrying Le^X (not shown) suggesting the lack of this glycan in *T. spiralis*.

Establishment of a sensitive ELISA method using LDNF-neoglycoconjugates

To design a suitable LDNF-neoglycoconjugate for serodiagnosis of trichinellosis, we first determined whether the number of LDNF in the neoglyconconjugate would affect the sensitivity of the total Ig-ELISA by using sera from confirmed *Trichinella*-infected patients. LDNF glycan antigens were coupled to BSA at two different densities, resulting in BSA preparations carrying an average of two and five LDNF antigens per BSA molecule, LDNF2 and LDNF5, respectively. BSA and neoglycoconjugates (BSA-chitotriose (Chi), BSA-LDN (LDN) and BSA-LDNF (LDNF2 and LDNF5)) were used at concentrations of 5 and 10 µg/ml. The *Trichinella*-seropositive sera reacted strongly to LDNF5 coated at 5 and 10 µg/ml, whereas binding to LDNF2 was clearly lower (Figure 3). By contrast, binding of the sera to neoglycoconjugates carrying control glycans (LDN, the precursor glycan of LDNF lacking the fucose moiety, and chitotriose) were below the cut-off value. LDNF5 at a working concentration of 5 µg/ml was chosen to determine the sensitivity and specificity of this antigen for serodiagnosis of trichinellosis.

Sensitivity and specificity of the LDNF5 -ELISA

To determine the sensitivity of the LDNF5-ELISA, 29 serum samples from *Trichinella* positive patients were used. *Trichinella* infection was confirmed in these patients by positive biopsy in addition to conventional serodiagnosis using *Trichinella* ES-ELISA and *Trichinella* ES-Immunoblot analysis. Results indicate that from the 29 *Trichinella*-positive sera tested, 28 sera were positive in the LDNF5-ELISA (Figure 4) indicating that the sensitivity of the LDNF5-ELISA is 96%.

To determine the specificity of the LDNF5-ELISA, sera from individuals that were established seropositive for ascariasis, leishmaniasis, toxocariasis, echinococcosis, cysticercosis, borreliosis, amoebiasis, schistosomiasis, strongyloidiasis, toxoplasmosis and syphilis were used. We determined the number of samples that reacted positively and negatively in the LDNF5-ELISA (Table 1). From the 110 sera tested, 74 were found negative in the LDNF5-ELISA indicating a specificity of 67%. A positive reaction was mainly found in sera from individuals with cysticercosis and strongyloidiasis.

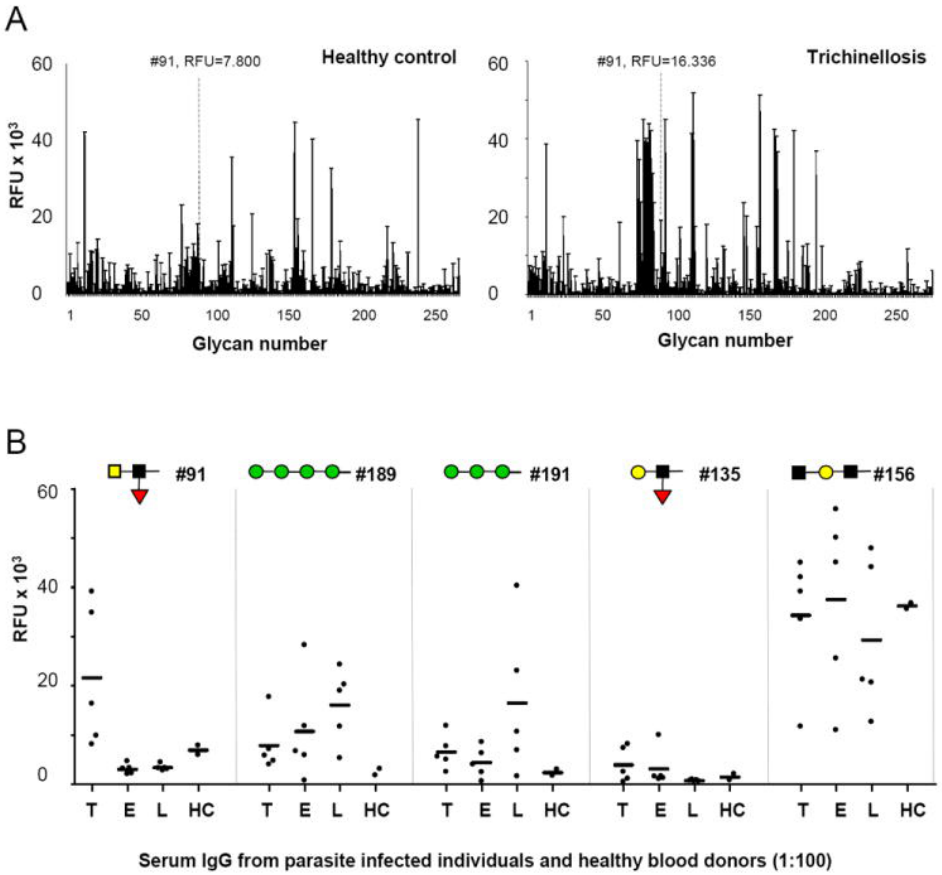

Figure 1. Glycan-array analysis of anti-glycan antibodies in the sera of parasite infected individuals. Sera from individuals seropositive for trichinellosis (T, n=5), leishmaniasis (L, n=5) or echinococcosus (E, n=5), as well as from healthy blood donors (HC, n=2) were 1:100 diluted and analyzed for the presence of anti-glycan IgG antibodies using printed array Version 2.1 of the Consortium for Functional Glycomics.

(A) Examples are shown of an array from an individual seropositive for trichinellosis and from a healthy blood donor. RFU=Relative Fluorescence Units. The RFU for the LDNF glycan (#91) is indicated in both arrays.

(B) Scatter plot of the RFU values for 5 selected glycan antigens, of all 17 sera analyzed. Three glycan antigens were selected that showed differential recognition by sera from parasite-infected individuals and healthy controls (#91, LDNF antigen, GalNAcβ1-4(Fucα1-3)GlcNAc-R; #189, Manα1-2Manα1-2Manα1-3Man-R; #191, Manα1-2Manα1-3Man-R). In addition, one glycan antigen was selected that showed a very low binding, and one that showed high binding, respectively (#135, Le^x antigen, Galβ1-4(Fucα1-3)GlcNAc-R; #156, GlcNAcα1-3Galβ1-4GlcNAc-R). CFG monosaccharide symbols used are: green circle: Man; black square, GlcNAc; yellow square, GalNAc; yellow circle, Gal; red triangle, Fuc.

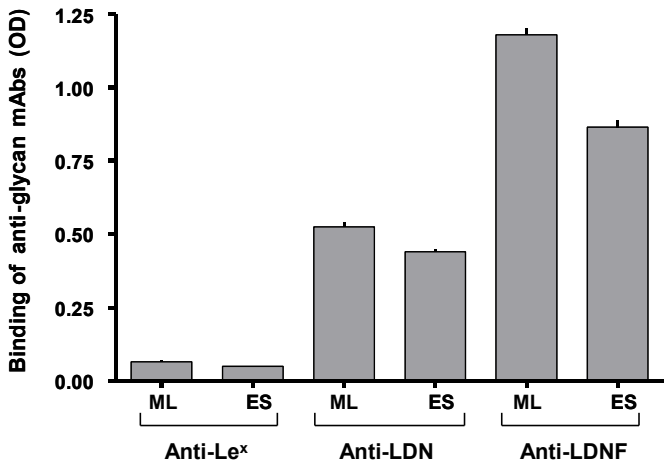

Figure 2. Recognition of glycan antigens in *Trichinella* by specific monoclonal antibodies in ELISA. Anti-Le^x (IgM, Calbiochem), anti-LDN (mAb SMLDN1.1) [35] and anti-LDNF (mAb SMLDNF) [30] antibodies were used for glycan recognition on crude antigen from *T. spiralis* muscle larvae (ML) and excretory/secretory (ES) antigen.

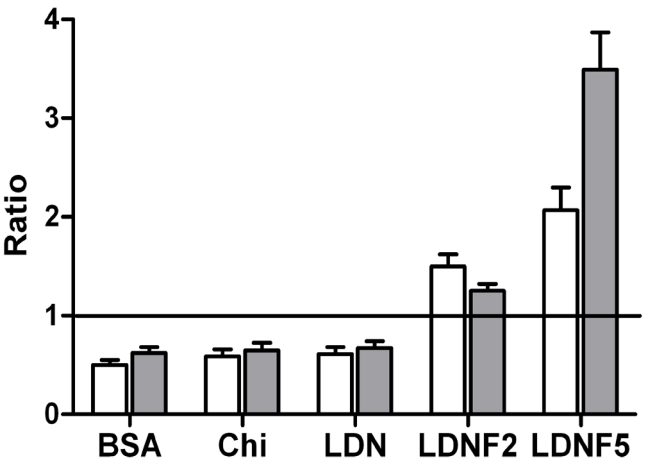

Figure 3. Analysis of the binding of anti-glycan Ig from serum of individuals seropositive for trichinellosis to different neoglycoconjugates in ELISA. BSA and neoglycoconjugates [BSA-chitotriose (Chi), BSA-LDN (LDN) and BSA-LDNF (LDNF2 and LDNF5)] were coated at concentrations of 5 (white bars) and 10µg/ml (grey bars). Bars represent the mean binding of five *Trichinella*-positive sera tested. Ratio values equal or higher than 1 (line) are considered positive.

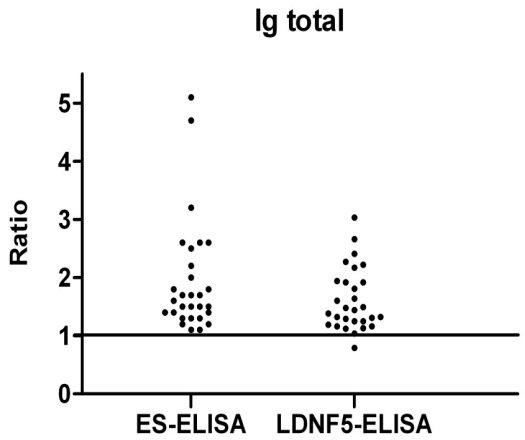

Figure 4. Plot of the ratio distribution of the *Trichinella*-positive serum samples tested by the ES-ELISA and LDNF5-ELISA for the detection of total Ig. Ratio values equal or higher than 1 (line) are considered positive.

Infections	Negative	Positive
Ascariasis	6	4
Leishmaniasis	6	4
Toxocariasis	7	3
Toxoplasmosis	6	4
Echinococcosis	8	2
Cysticercosis	3	7
Amoebiasis	9	1
Schistosomiasis	7	3
Strongyloidiasis	5	5
Borreliosis	7	3
Syfilis	10	0
Total	**74**	**36**

Table 1. Reactivity of serum samples from patients with different parasitic and bacterial infections in the LDNF5-ELISA. For each infection 10 serum samples (seropositive for the infections indicated) were tested. The numbers indicate the amount of serum samples that showed either a negative or positive result in the LDNF5-ELISA.

Discussion

The use of glycan antigens in serodiagnosis of helminth infections is an attractive approach and has been successfully applied for the detection of schistosomiasis, even in a field-applicable form as a dipstick [22, 23]. In addition to schistosomes, many other helminths express glycan antigens on proteins and lipids at the surface of larvae, adult worms or in the ES products, which in principle could be applied in serodiagnostic approaches. To evaluate the potential of a glycan microarray approach to select for glycan antigens that could be used for serodiagnosis of parasitic infections we focussed on serodiagnosis of *T. spiralis*. Structural analysis of *T. spiralis* glycans showed the presence of unique tri- and tetra-antennary N-glycans composed of LDNF (GalNAcβ1-4[Fucα1-3]GlcNAc-) antennae capped with β3-linked tyvelose moieties [24, 25]. An ELISA based on glycoconjugates containing terminal β-tyvelose-GalNAc-R for serodiagnosis of human trichinellosis showed a sensitivity ranging from 93% to 100% [26-28]. However, the use of the β-tyvelose is limited since it is not commercially available and its synthesis is complex. The present study aimed at identifying additional glycan antigens recognized by antibodies from *Trichinella*-infected individuals that could replace the use of ES antigen in the serodiagnosis of trichinellosis.

To select for potential antigenic glycan antigens that are recognized by *Trichinella* infection sera, but not by sera of individuals infected with other parasites, we initially screened a glycan-array containing over 250 different glycan antigens with a limited number of positive sera from trichinellosis, leishmaniasis and echinococcosis patients. We found that all *Trichinella*-positive sera recognized the LDNF glycan, in contrast to sera from individuals infected with other parasites or from healthy blood donors. To enable detection of anti-LDNF antibodies in sera of infected individuals we developed an LDNF-ELISA using a neoglycoconjugate, consisting of BSA containing LDNF antigen. The synthesis of this compound was recently described [29]. We showed that the number of LDNF molecules bound to the BSA is crucial for its recognition by antibodies from *Trichinella*-infected individuals. The coupling of an average of five LDNF moieties per BSA molecule resulted in a neoglycoconjugate which use in the ELISA resulted in a highly sensitive (96%) assay for the serodiagnosis of trichinellosis.

The specificity of the LDNF5 ELISA however was low (67%) which is not surprising since LDNF is present in different helminths. The presence of LDNF has been demonstrated in *Schistosoma mansoni* (not endemic in Europe), *Dirofilaria immitis* (heartworm of dogs), *Haemonchus contortus* (nematode of ruminants) and *Fasciola hepatica* (endemic in

sheep in The Netherlands) [25]. In the LDNF5-ELISA, several sera from individuals with cysticercosis and strongyloidiasis showed significant binding, suggesting the presence of LDNF moieties in these helminth species. Surprisingly, only 3 out of 10 sera from established schistosome infections were found positive in the LDNF5-ELISA. LDNF moieties are abundantly observed in different stages of schistosomes [5, 30] and the presence of anti-LDNF IgG and IgM has been demonstrated in schistosome infected humans, chimpanzees and mice [4, 5]. Whether factors such as the stage of *Schistosoma* infection, the parasite species and load may influence the results here reported, needs to be further investigated.

In this study, we show that the LDNF antigen is recognized by antibodies in sera from individuals infected with *T. spiralis*, and that LDNF5 is a suitable candidate to replace the ES antigen in a first screening for diagnosis of trichinellosis. Increasing the number of LDNF may lead to a further improvement of the sensitivity and/or specificity of the assay. The advantage of using LDNF neoglycoconjugates in a first screening is that enzymatic synthesis of this conjugate is a relatively easy and fast procedure that can be performed in every biochemical research facility.

Our data show that glycan microarrays constitute a promising technology for fast and specific analysis of anti-glycan antibodies in sera from individuals infected with different pathogens. It should be emphasized that the glycan microarray used in this study contains many glycan structures, but the microarray was not designed to present parasite-specific glycan structures. In serological assays which are based on the use of natural antigens, cross-reactivity due to the presence of common glycan antigens is often observed [31-33]. To improve the serodiagnosis of parasitic infections, the development of parasite-specific glycan microarrays would be an attractive approach. In such arrays the natural glycans of the parasites are isolated, fractionated and printed as glycan-arrays, thus facilitating the identification of glycan antigens specific for one, or a restricted group of parasites by a shotgun glycomics approach [34]. Such an approach is expected to result in the identification of glycan antigens which would allow both a sensitive and specific serodiagnosis of infections by a wide variety of different parasites.

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Chapter 6

Summarizing Discussion

The influence of helminth infections on allergic diseases is subject of both epidemiological and experimental animal studies. Many of these studies have shown how certain helminths suppress the host immune response which is essential for the parasite to survive and by doing so it also protect the host against immunopathologies such as allergies and autoimmune diseases. However, due to the diversity of helminth species and their interactions with the human host, the link between infection and reduction of allergy is not always evident.

Therefore, studies are necessary to uncover the complexity of the immune mechanisms during helminth infections to determine which helminths have a protective effect, whether the helminth infection and/or product can protect and whether it is safe to use them as a therapy. So far most evidence is based on epidemiological studies and experiments in animal models. The latter have shown that several helminth infections may protect against allergy. However, there is not yet conclusive evidence that helminth antigens including their excretory/secretory (ES) products can protect. Nowadays, clinical trials are being performed using infection with *Trichuris suis* as therapy for inflammatory bowel disease. Obviously infecting patients with a parasite is not the optimal situation. Therefore, it is relevant to identify the molecule or molecules involved in protection against allergic diseases and characterize the immune mechanisms involved.

In our search for helminth products that are able to modulate the immune response against allergens, we found *in vitro* that ES products from *Trichinella spiralis* can modulate DC maturation. To investigate this interesting finding in depth, we initiated a series of further *in vitro* and *in vivo* studies. This discussion will mainly focus on the modulation of the immune response by *T. spiralis* infection, or its ES products. In addition, the use of synthetic glycans to improve the diagnosis of human trichinellosis will be discussed.

Immunomodulation by excretory/secretory products of *T. spiralis*

After infection, helminths continuously modulate the host immune response against the parasite through the release of soluble mediators such as cystatins, serpins, glycans, mucins, lectins or cytokine homologs that could influence antigen processing, presentation and subsequent T-cell polarization [1]. *Trichinella spiralis* ES (TspES) products reduce the inflammation provoked by the invasion of muscle cells, modulate the immune response to protect both parasite and host and at the same time participate in orchestrating the biological process of host's cell remodelling to accommodate the larvae in the muscle [2].

Dendritic cells (DC) are part of the first line of defence against invading pathogens. They are essential for antigen presentation and interact with different populations of lymphocytes, to initiate the adaptive immune responses [3]. Therefore, a possible route by which helminths may exert their effects on the immune system is through the DC. In chapter 2 we showed that TspES inhibits DC maturation induced by LPS derived from certain bacteria. Interestingly, TspES on its own had no effect on DC maturation. However, in contrast to our results, studies by others using rat DC, showed that *Trichinella* antigens (crude muscle larvae antigen, ES from adult parasites and soluble extract of newborn larvae) on its own up-regulated the expression of costimulatory molecules [4]. In the same study ES from muscle larvae provoked moderate upregulation of CD 86 and ICAM 1, but no up- regulation of MHC II [5]. In other words, antigens derived from different stages of *T. spiralis* led to partial maturation of rat DC. Whether the difference between this study and ours relies on the different sources of DC; *Trichinella* antigens and the antigens concentrations used, still remains to be investigated.

Since we did not observe changes in DC maturation by stimulation with TspES alone, we used *E. coli* LPS which is a well-known, trigger of DC maturation. We found that TspES suppressed *E. coli* LPS-induced DC maturation. Our findings are similar to those found in previous studies using helminth products from *Heligmosomoides polygyrus*, *Fasciola hepatica*, Echinococcus multilocularis, Taenia crassiceps, Acanthocheilonema viteae *Schistosoma mansoni* among others [6-12]. For instance, Terrazas *et al* found that ES products from T. crassiceps did not induce maturation of human DC, but they were capable of reducing the LPS induced expression of maturation markers and the production of the inflammatory cytokines IL-1β, TNF, IL-12 and IL-6 induced [11]. Goodridge *et al*. found that the ES antigens from A. viteae (ES-62) [9] inhibit the IFN-γ *Salmonella* minnesota LPS-induced IL-12 and TNF-α production of murine DC and macrophages. Others have found that *Schistosoma mansoni* soluble egg antigens (SEA) suppress *E. coli* LPS-induced activation of murine DC as indicated by a decrease in

expression of MHCII and co-stimulatory molecules and production of IL-12, IL-6 and TNF-alpha [10, 12].These results indicate that these helminth antigens, just like TspES have the ability to induce the differentiation of DC into a tolerogenic-like phenotype inhibiting the effects of inflammatory stimuli such as LPS. There is evidence that immature DC can acquire a stable tolerogenic state that is preserved even in presence of concomitant maturation signals. These tolerogenic DC can induce or expand Treg cells and convert activated T cells into Treg cells [13]. Treg cells play an essential role in the immunosuppression induced by helminths as discussed below.

One of the novel and surprising findings from our studies is that although TspES suppressed DC maturation induced by *E. coli* LPS, it did not have any effect on DC maturation induced by *N. meningitidis* LPS. Using HEK (Human Embryonic Kidney) 293 cells transfected with mouse TLR4/MD2/CD14 we confirmed these results. Perhaps the difference relies on the types of bacterial LPS: while the *E. coli* LPS used in this study was a smooth form (S-form), the *N. meningitidis* LPS used was of the rough form (R-form). The LPS S-form requires the CD14 molecule as a co-receptor for TLR4 to activate the signalling pathway in DC [14, 15] whereas the LPS R-form does not [16]. Therefore, the CD14 molecule within the TLR4/MD2/CD14 complex [17, 18] was suspected to be involved in the suppressive effect of TspES.

In chapter 3, we show that CD14 was not involved in the suppressive effect of TspES. We carried out studies using, in addition to the E coli LPS S-form and *N. meningitidis* LPS (R-form) to mature the DC, also the *E. coli* LPS R-form and the *S. minnesota* LPS S-form. We found that suppression does not depend on the LPS form since TspES could suppress DC maturation induced by both smooth and rough LPS forms. Therefore the suppressive effect of TspES on LPS-induced DC maturation is CD14 independent. Most likely this suppressive effect depends on the bacterial source of LPS. Enterobacteria such as *Escherichia coli* and *S. minnesota* have a similar LPS structure [19], which differs from *N. meningitidis* LPS in the composition and number of acyl chains of the lipid A, the part which is recognized by TLR4/MD2. In the lipid A of *N. meningitidis* LPS, the localization of the secondary acyl chain is symmetrical while in enterobacterial LPS it is asymmetrical [20]. Since the biological activity of LPS is mainly determined by its Lipid A structure, these LPS variants vary considerably in their potency to activate DC. In addition, they can differentially utilize signal transduction pathways leading to diverse patterns of inflammatory responses [18, 21, 22]. This particular property of *T. spiralis* suppressing DC maturation induced by certain bacteria could be one of the strategies used by this helminth to survive by keeping the host alive. There is experimental evidence

indicating that infection with enteric helminths can lead to the movement of bacterial LPS into circulation [23, 24]. However, sepsis is not common during helminth infections. In fact, during migration from intestine to muscle, *T. spiralis* larvae drags along enterobacteria [25] however the number of patients with trichinellosis that develop sepsis is very low, 2.11% [26]. Perhaps, suppressing the immune response against enterobacterial LPS may prevent the induction of sepsis and ensure host survival.

During our studies, we also showed that inhibition of LPS-induced DC maturation is restricted to the TLR4 signalling pathway since TspES does not suppress DC maturation induced by other TLR ligands. In contrast, Segura *et al*. [6] reported on the inhibition of cytokine induction of DC with *Heligmosomoides polygyrus* ES for several TLR ligands. Similarly *Fasciola hepatica* tegumental products were shown to suppress cytokine production and expression of co-stimulatory markers on DC induced not only by TLR4 but also by a range of other TLR [27]. In another study it was shown that SEA suppresses IL-12p40 production induced by engagement of TLR3, TLR4, or TLR9 [28]. Finally, it has been demonstrated that TLR2-deficient but not TLR4-deficient bone marrow derived dendritic cells (BMDC) failed to produce IL-12p70 and IL-10 in response to schistosome antigens [29]. All these findings suggest that the effect of helminth products on TLR-induced DC maturation differs among helminths. In the case of TspES, it is clear that the suppressive effect is via the TLR4 complex or TLR4-mediated downstream signalling and/or mechanisms of activation. TLR4 signalling involves two main intracellular pathways, namely the 'MyD88 (myeloid differentiation primary-response gene 88)-dependent' pathway which mediates the production of pro-inflammatory cytokines and 'MyD88-independent or TRIF' pathway which mediates the upregulation of co-stimulatory and MHCII molecules. The MyD88 dependent pathway is shared by the other TLR with the exception of TLR3 which signals through TRIF [30]. Since TspES inhibits the outcome of both the MyD88-dependent and MyD88-independent pathway via TLR4 but not via other TLR, it is likely that the suppressive effect of these helminth products involves molecules specific for the TLR4 signalling pathway only. We observed that the expression of all genes downstream of the TLR pathway induced by LPS was impaired by TspES already after 6 hours which could indicate abrogation of TLR4 activation at a very early stage. This helminth could interfere with the TLR signalling pathways indirectly via interaction with other PRR, such as C-type lectins [31], class A scavenger receptor [32] , and possibly other receptors yet to be discovered. As an example , the mycobacterial cell wall component ManLAM has been shown to interfere with TLR4-mediated signals upon binding with DC-SIGN in this way down-regulating DC-mediated immune responses [33]. Furthermore, certain helminth products interfere with DC function at the

intracellular level, by blocking antigen processing [34] or degrading mRNAs within the host [32]. Given the complexity of the TspES, it is not surprising that various components can function synergistically or by cross-regulation to modulate the immune response. In our search to identify the possible molecules responsible for the TspES suppressive effect, TspES was heat-inactivated. We found that the TspES suppressive activity was still present, which suggest that the products responsible for the suppressive effect in the TspES are either of a non-protein nature or denatured proteins that are still functional. This result contradicts findings related to ES products from *Heligmosomoides polygyrus* and *Nippostrongylus brasiliensis* [35] showing that after heat-inactivation its suppressive activity was impaired. Finally, other components in the TspES such as lipids or glycans could also be responsible for the observed suppressive activity as has been reported for other helminths [31, 36].

Regulatory T cells, defined by the expression of CD4, CD25 and the transcription factor Foxp3, have a pivotal role in preventing or limiting autoimmune and allergic diseases. These cells can produce the inhibitory cytokines IL-10 and TGF-β which are mediators of Treg cell induced suppression [13]. In chapter 3, we also show that secreted products from *T. spiralis* muscle larvae induce expansion of suppressive Treg cells *in vitro*, as indicated by an increase in the $CD4^+CD25^+$ $Foxp3^+$ T cells population after co-culture of splenocytes from DO11.10 mice with OVA and TspES-pulsed DC. Unlike our findings, Ilic *et al*. have recently reported that *T. spiralis* antigens do not induce de novo generation of $Foxp3^+$ T cells *in vitro* [37]. Two major differences could explain these contrasting results. First of all, in our *in vitro* cultures OVA protein was used instead of OVA peptides used by Ilic *et al*. Most likely, antigen processing and presentation was affected by *T. spiralis* secreted products which has consequences for T cell activation and differs when using OVA protein compared to an OVA peptide. In fact, initially we used an OVA peptide (323-339) and indeed we did not observe expansion of $Foxp3^+$ T cells *in vitro* (data not shown). Another important difference is the concentration of *Trichinella* antigen used for DC stimulation. Ilic *et al* used 50 µg/ml which resulted in DC maturation whereas we used only 5 µg/ml. Although we did not observe increased expression of DC surface markers or cytokine production by DC, we did observe expansion of $CD4^+CD25^+$ $Foxp3^+$ T cells. Studies have shown that low antigen concentrations induce tolerogenic DC [38, 39] and these DC can induce Treg cells [13]. To initiate T cell responses, three concomitant signals are required: signal 1 is the antigenic stimulus provided by MHC molecules displaying a cognate peptide; signal 2 is provided by co-stimulatory molecules; and signal 3 is provided by cytokines produced by DCs or other microenvironmental sources [40]. Since many tolerogenic DC have an immature phenotype, evidence suggest that a major

mechanism for inducing Treg involves presentation of modest levels of an antigen in the absence of signals 2 and 3[13] which could be the case of TspES.

Our study also shows that splenocytes derived from D011.10 mice produce TGF-β when co-cultured with OVA and TspES-pulsed DC. By blocking TGF-β, we observed that the expansion of the $CD4^+CD25^+$ $Foxp3^+$ T cell population is abrogated and that the production of IFN-γ and IL-10 that was suppressed is restored. This cytokine has been shown to be required for both *in vitro* and in vivo induction of Treg cells [41] for other helminths such as *Heligmosomoides polygyrus*, *Nippostrongylus brasiliensis*, *Schistosoma mansoni*, *Litomosoides sigmodontis* among others [42-48]. Most likely, the TGF-β-induced Treg cells are part of the regulatory mechanism that allows the *T. spiralis* larvae to invade and remain in the muscle, preserving the integrity of the occupied cells.

Immunomodulation by *T. spiralis* infection

Several epidemiological studies support the inverse association between helminth infections and allergic disease in humans [49-51], and using murine models several groups have shown that certain helminths can reduce allergic responses by eliciting a prominent anti-inflammatory networks involving Treg cells [44, 45, 52-55]. In Chapter 4 we show that in the chronic as well as in the acute phase of *T. spiralis* infection mice are protected against experimental allergic airway inflammation (EAAI) and that Treg cells may have a role in this process.

In our study, mice were infected with *T. spiralis* at three different time points in the course of OVA–sensitization/challenge, in order to determine the effect of the different phases of *Trichinella* infection on EAAI. We observed that mice in the muscle or chronic phase of *T. spiralis* infection were protected against EAAI as indicated by significantly reduced levels of OVA-specific IgE in serum, decreased levels of Th2 cytokines and low numbers of eosinophils in BAL. Histological analysis of the lungs indicated that mice in the chronic phase of infection had significantly lower OVA-induced peribronchiolitis and perivasculitis compared to mice that were OVA-treated only. We also observed increased number of $CD4^+CD25^+Foxp3^+$ cells in spleen of mice with EAAI that were in the chronic phase of infection. This splenic Treg cell population was also found to be suppressive, as shown by suppression assays *in vitro*. Protection against experimental airway inflammation in mice chronically infected with other helminths has also been reported. For instance, *S. mansoni* infected BALB/c mice were protected against EAAI induced by OVA as indicated by reduction of eosinophils in BAL, Th2 cytokine production, OVA-specific IgE levels and reduction of the number of inflammatory cells

in lungs. Here, induction of $CD4^+CD25^+Foxp3^+$ regulatory T cells was independent of IL-10 [47]. Dittrich *et al*. found that chronic infection with the filarial parasite *Litomosoides sigmodontis* suppressed all pathological features of the OVA-induced EAAI model. Additionally, significantly increased numbers of Treg cells were observed in spleen and mediastinal lymph nodes in infected OVA-mice compared with OVA-mice controls [54]. Similarly, it was shown that *Schistosoma japonicum* chronic infection suppressed airway eosinophilia, mucus production, OVA-specific IgE responses and production of IL-4 and IL-5 [56]. In the case of *T. spiralis*, protection of C57/BL mice against EAAI by chronic infection has been recently reported by Park *et al*. [57]. However, the effect of the acute *T. spiralis* infection was not investigated.

In addition to confirming the suppressive effect of chronic *T spiralis* infection on EAAI, we showed that the acute or intestinal phase of infection established before OVA-sensitization/challenge also showed a protective effect against EAAI, although to a lesser extent. It is important to mention that by the time these animals received the OVA-challenge, all mice were at a chronic phase of infection, which was perhaps the reason for the partial effect on EAAI. Since humans can be sensitized to aeroallergens early in life and before consumption of *Trichinella* infected meat, we included a group of mice that were *Trichinella* infected after OVA-sensitization and short before OVA-challenge. By the time the OVA-challenge was given, these mice were in the intestinal or acute phase of infection. Here, partial protection against EAAI was also observed which was restricted to a significant decrease in the levels of eosinophils in BAL and partial reduction of pulmonary inflammation. In contrast to our findings, Smit *et al*. [52] using the OVA-induced EAAI model, showed that *S. mansoni* infection protects against EAAI only during chronic but not during the acute phase of infection. Between *T. spiralis* and *S. mansoni* there are clear differences in life cycles and immunopathology induced by infection. Acute schistosomiasis is characterized by a systemic hypersensitivity reaction against the migrating schistosomula and eggs. Once in the blood capillaries, the schistosomula pass via the blood flow to the lungs and continues to the liver where they mature, pair and initiate egg deposition [58, 59]. When these schistosomula stay in the pulmonary capillaries they can cause cough and shortness of breath [49]. Although *Trichinella* infection has also a migratory phase, the newborn larvae are usually not trapped in the lungs [60]. Therefore, it is not surprising that we did not observe any pulmonary inflammation in the mice that received *T. spiralis* infection only. It has been suggested by Furze *et al*. [61] that because *T. spiralis* larvae are particularly susceptible to immune attacks, the adult worm induce immunomodulatory mechanisms to protect the development of the newborn larvae. The surface and ES products from the adult parasite

might act in multiple ways, provoking significant changes in the gut microenvironment which can have broad effects on the immune system, both within and beyond the gut [62]. One of these immunomodulatory strategies might be the induction of Treg cells. During the acute phases of infection either before or after OVA sensitization, we observed in the spleen of *Trichinella* infected mice, increased number of $CD4^+CD25^+Foxp3^+$ cells with suppressive activity. The proportion of these Treg cells was however lower compared to the chronic phase of infection. Taylor *et al*. have shown that the number of Treg cells increased during acute *L. sigmodontis* infection in mice [63]. This rapid recruitment of Treg cells impaired the generation of $CD4^+$T effector cell responses and in this manner inhibited parasite killing. Indeed anti-CD25 treatment alone promoted protective immunity when given prior to infection to dampen the initial Treg cell responses. Perhaps, early involvement of Treg cells may reduce Th2-cell responses which can explain the observed protective effect against EAAI during the acute phase of *Trichinella* infection.

Using cell transfer studies, we showed that $CD4^+$ T cells isolated from chronically infected mice containing higher proportions of Treg cells that were transferred to OVA-treated mice conferred partial protection against pulmonary inflammation in EAAI. Similarly, Wilson, *et al*. [44] have previously shown suppression of airway eosinophilia in OVA-treated BALB/c mice after the transfer of $CD4^+$T cells isolated from mesenteric lymph nodes from mice infected with *H. polygyrus*. In our study the transferred $CD4^+$ T cells containing a high proportion of Treg cells strongly suppress OVA-specific cytokine production by lung-draining lymph nodes. It is possible that the transferred Treg cells can migrate into inflammatory sites and suppress peripheral effector T cell function as have been shown in studies in inflammatory diseases like experimental autoimmune encephalomyelitis (EAE), inflammatory bowel disease and type 1 diabetes, among others [64]. Our results show that Treg cells might be the cellular mediators responsible for the protective effect of *T. spiralis* infection, however other cell populations such as regulatory B cells and alternatively activated macrophages might also be involved [52, 65, 66].

We conclude that infection with *T. spiralis* may confer protection against EAAI by inducing a regulatory network where Treg cells may play an important role. The magnitude of this protective effect increases as infection progresses from the intestinal or acute phase to the muscle or chronic phase of infection.

Use of synthetic glycans to improve the diagnosis of human trichinellosis

Helminths express an broad array of glycan antigens on proteins and lipids at the surface of larvae the adult worms and are present in the ES products [36]. These glycans may modulate the host immune response through molecular mimicry, allowing parasites to escape antibody targeting [36]. In addition, some of these glycans are known to be antigenic [67] and in principle could be used for the serodiagnosis of *Trichinella* infections. Ingestion of heavily infected undercooked meat may lead to severe disease and if not diagnosed and treated on time it can be fatal. In chapter 5, we evaluated the potential use of a glycan microarray to identify glycan antigens that could be used for serodiagnosis of parasitic infections. *Trichinella* ES products are commonly used in ELISAs for the serodiagnosis of trichinellosis. However, these ES products have serious disadvantages. Its preparation is laborious and requires the use of laboratory animals. Furthermore, micro-environmental factors during culture of the animal-derived larvae may affect antigen quality [68], resulting in standardization problems. Therefore, replacing the ES products by synthetic antigens could solve these problems. An ELISA based on glycoconjugates containing terminal β-tyvelose-GalNAc-R has been previously used for the serodiagnosis of human trichinellosis [69-71]. However, the use of the β-tyvelose is limited since it is not commercially available and its synthesis is complex. Therefore, searching for other molecules, including other glycans is relevant for improving serodiagnosis of this disease. *Trichinella spiralis* contains unique tri- and tetra-antennary N-glycans composed of LDNF (GalNAcβ1-4[Fucα1-3]GlcNAc-) antennae capped with β3-linked tyvelose moieties [36, 72].

Using a glycan array containing over 250 different glycan antigens, we found that sera from *Trichinella* infected individuals contain a relatively high level of IgG recognizing the glycan antigen LDNF, whereas sera from individuals positive for echinococcosis and leishmaniasis showed a low binding to this glycan antigen. An ELISA was developed using a neoglycoconjugate that consisted of BSA containing LDNF as antigen. The synthesis of this compound was performed by a chemoenzymatic method to synthesize multivalent neoglycoconjugates carrying defined amounts of LDNF antigens [73]. We showed that the number of LDNF molecules bound to the BSA is crucial for its recognition by antibodies from *Trichinella*-infected individuals. The coupling of an average of 5 LDNF (LDNF5) moieties per BSA molecule resulted in a better antigen to use in the ELISA compared to BSA coupled to 2 LDNF molecules. Using sera from confirmed *Trichinella*-infected patients and LDNF5 as antigen an ELISA with 96% sensitivity was developed. Using 110 sera positive for other infections namely, ascariasis, leishmaniasis, toxocariasis,

echinococcosis, cysticercosis, borreliosis, amoebiasis, schistosomiasis, strongyloidiasis, toxoplasmosis and syphilis a specificity of 67% was determined. This low specificity was not surprising since the LDNF structure has been reported to be present in different helminths including *S. mansoni, Fasciola hepatica*, *Dirofilaria immitis*, and *H. contortus* [36, 74, 75]. In conclusion, the high sensitivity of the LDNF5 ELISA makes it a suitable assay to replace the *T. spiralis* ES-ELISA which could be used as a screening assay for the diagnosis of trichinellosis. Perhaps, increasing the number of LDNF may lead to a further improvement of the specificity of the assay. Furthermore, glycan microarrays with panels of different glycan structures could be used to identify a pattern of glycans recognized by one parasite or a group of parasites. Identifying specific or a pattern of glycan antigens for just one parasite or a group of parasites could improve the serodiagnosis of a wide variety of different parasitic infections.

Future perspectives

Helminth infections have been associated with protection against allergic and autoimmune diseases. This relationship has been demonstrated in epidemiological studies and in animal models. Present days challenge is to determine the mechanisms behind this protection for the development of future therapies against these diseases.

Therapy against different human immunopathologies with live helminths is under evaluation. So far, *Trichuris suis* has been tested in clinical trials with patients with multiple sclerosis (MS), inflammatory bowel disease (IBD), allergic rhinitis and food allergy and only mild-to-moderate gastrointestinal and mild eosinophilia have been observed as side effects [76]. Both the US Food and Drug Administration and the European Medicines Agency have formally approved the manufacturing process and allowed further testing of *T. suis* eggs as a drug [77]. Hookworms have also been tested in a few trials in healthy subjects, asthmatic patients, and patients with celiac disease with no clear clinical efficacy [78-80]. In addition to infection with *T. suis*, it has been shown that treatment with crude antigen from adult *T. suis* protects mice against induction of EAE, a model for MS [81]. Whether these *T. suis* extracts could confer protection against allergy has not been reported yet.

Other helminth products have only been tested in mouse models. Administration of ES-62 resulted in protection against collagen-induced arthritis, OVA-allergic airway inflammation and cutaneous immediate-type hypersensitivity [82, 83]. Products of *Ancylostoma caninum* [84], *Hymenolepis diminuta* [85], *S. mansoni* [86] can suppress pathology in mouse models of colitis while ES of *N. brasiliensis* [87] and *H. polygyrus*

[88] can inhibit allergic inflammation.

The use of *T. spiralis* as a therapeutic agent still remains to be investigated. Recently, crude muscle larvae antigen of *T. spiralis* was found to significantly reduce the severity of experimental colitis and EAE [81, 89]. We have shown that ES products modulate DC maturation and induce the expansion of Treg cells. Furthermore, infection with this helminth protects against EAAI. We suggest that TspES can convert immature DC into tolerogenic DC that induce and expand Treg cells. *Trichinella* products could also have an effect on Treg cells independently of DC, possibly through the production of molecules with regulatory properties. Examples of molecular mimicry such as TGF-β homologues have been identified in some parasitic nematodes including *Brugia malayi*, *S. mansoni* and *Ancylostoma caninum* [90-92]. For example, *B. malayi* was found to secrete TGH-2, a homologue of host TGF-β which can bind to the mammalian TGF-β receptor [92]. In this manner it may promote the generation of Treg cells as have been described for human TGF-β [93]. The precise role of the induced Treg cells and the mechanism involve in suppression of the host immune response against the parasite and against inflammatory diseases such as allergy and autoimmunity (Fig. 1) still remain to be elucidated. The more we learn about the immunoregulatory mechanisms during *T. spiralis* infection and the molecules involved, the more we will be able to develop therapies against inflammatory diseases.

To date, we cannot tell which component(s) of the ES mixture is/are responsible for eliciting the observed polarization of the immune response that is beneficial for the host, protecting against EAAI. In our attempts to characterize these components, we have fractionated the ES antigens and findings so far indicate that separate fractions lose the immunosuppressive activity which is partially restored when the fractions are pooled. Although these experiments should be repeated, these preliminary finding indicate that purified molecules most probably do not have the effect that the live parasite or the complex mixture of secreted products have. Initial experiments to isolate lipid components were promising but need further investigation. So far only infection with *T. spiralis* seems to protect against diseases. However, introduction of infection as a therapy like with *T. suis* is not an option since *T. spiralis* infection could have adverse consequences for the infected patients. Therefore, isolation of the components in the *T. spiralis* products that modulate the host-immune response are mandatory and main focus of interest in the development of new therapeutic approaches for treatment of different autoimmune and allergic diseases. Finally, identification of other immunodominant *Trichinella* antigens that could be synthesized is also crucial to improve and standardize the diagnosis of

human trichinellosis worldwide.

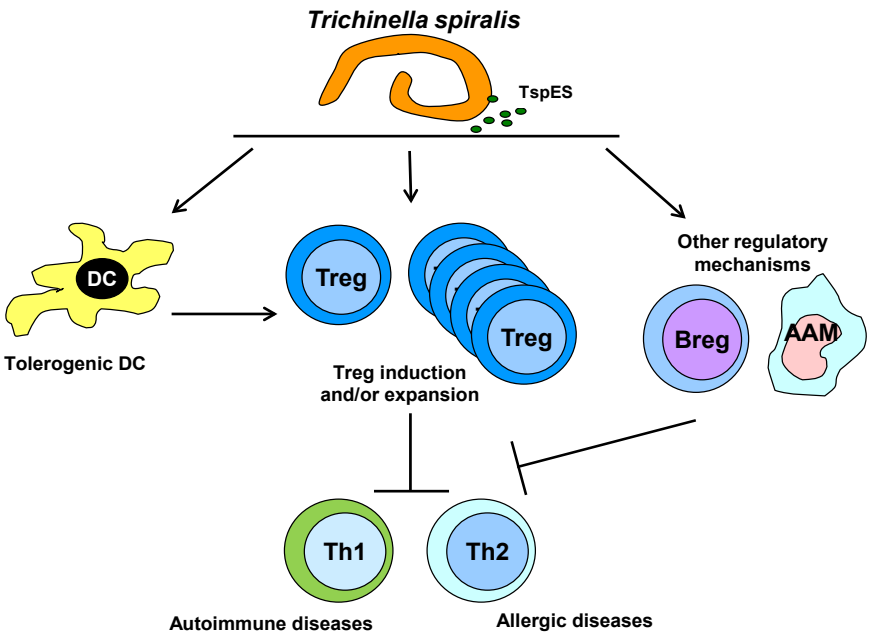

Figure 1. Mechanisms involve in *Trichinella*-induced immunosuppression. *Trichinella spiralis* excretory/secretory (TspES) products can suppress DC maturation induced by LPS. These helminth products do not induce DC maturation on its own but it can induce and expand $CD4^{+}CD25^{+}$ $Foxp3^{+}$ Treg cell population. It is therefore likely that TspES converts immature DC into tolerogenic DC. In addition, TspES could induce Treg cells directly independent of DC via host-like molecules like TGF-β homologues. Both strategies to induce Treg cells are potential mechanisms used by *Trichinella* to escape the host's immune responses which may also protect the host against inflammatory diseases such as allergy and autoimmunity. The induced immunosuppression benefits therefore both the parasite and the host. The role of B regulatory (Breg) cells and alternative activated macrophages (AAM) in suppression of the host immune response has been shown for other helminths but remains to be investigated for *T. spiralis*

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Appendices

Summary

Nederlandse Samenvatting

Acknowledgment

Curriculum Vitae

List of Publications

Summary

Improved sanitation, water treatment, and the use of vaccines and antibiotics have dramatically reduced bacterial, viral and parasitic infections in developed countries. However, growing evidence suggests that we have also eliminated organisms that kept our immune system in balance. For millions of years, our immune system has coevolved with countless bacteria, viruses, parasitic worms, and other infectious agents. Many of these produce substances that suppress our immune system and allow them to escape the host immune responses and maintain long-term, chronic infections. But the lack or reduced exposure to these organisms has led to alterations in the immune responses that favour the development of immunologic diseases, such as allergies.

In fact, several studies indicate that certain parasitic worms also called helminths suppress host immune responses. This suppression may benefit the parasite since it increases their chances of survival in their host. By doing so, the hosts may also benefit due to concomitant reduction of immune pathology associated with allergies. These immunomodulatory mechanisms differ nevertheless among helminths since each of these parasite species has evolved its own surviving strategy. Therefore, studying molecules and mechanisms involved in the modulation of the host immune responses by different helminths is necessary for a better understanding, particularly in the context of developing new strategies for treatment of allergies.

Here, we studied the helminth *Trichinella* spiralis. This parasite is sometimes referred to as the "pork worm" since pork products may contain the *Trichinella* infective cysts and ingestion of this undercooked meat can lead to the disease called trichinellosis. When the infected meat is ingested, the larvae are released from the cysts. These larvae become adults in the intestine (acute phase). After mating, female worms deposit large numbers of larvae that migrate through the intestinal wall into the blood stream. Few weeks after infection the adult worms are eliminated, but some of the larvae form cysts in the muscles and remain viable for many years (chronic phase).

The aim of the studies described in this thesis was to investigate the mechanisms involved in modulation of the host immune response by *T. spiralis*. We also investigated the effect of *Trichinella* infection on experimental allergic asthma. In addition, as a spin-off from this study we examined the potential use of parasite sugars also called glycans to develop new tools for the serodiagnosis of *T. spiralis* infection.

In chapter 2, we focused on the effect of *T. spiralis* antigens on dendritic cell (DC) maturation. DC are part of the innate immune system, which is the first line of defence

against pathogens. The main function of the DC is to process antigen and present it on their surface to other cells of the immune system. Upon interaction with antigens, DC undergo a transformation process known as maturation. In the studies here described, antigens from *T. spiralis* were added to DC either alone or in combination with lipopolysaccharide (LPS) derived from two different bacteria. LPS is the major component of the outer membrane of Gram-negative bacteria and it interacts with Toll-like receptor (TLR) 4, a protein expressed on DC. Interaction between LPS and TLR4 results in DC maturation. Results indicate that while *Trichinella* antigens alone did not induce DC maturation, it completely inhibited DC maturation induced by *Escherichia coli* LPS. In contrast, DC maturation induced by LPS from another bacterium, *Neisseria meningitidis,* was not affected by these *Trichinella* antigens. In conclusion, *T. spiralis* antigens lead to suppression of DC maturation but this effect depends on the bacterial source of LPS.

In chapter 3, we investigated this effect further by using LPS from other bacteria and also aimed at determining the effect of *Trichinella* antigens on T cell activation and the induction of T regulatory cells. T cells are important cells of the adaptive immune system. There are several types of T cells based on their specific function. Among these cells, there are the T regulatory cells. The main function of these cells is to maintain immune homeostasis, preventing autoimmune diseases and limiting chronic inflammatory diseases. To characterize further the effect of *Trichinella* antigens on DC maturation, we used other LPS forms derived from different bacteria. We found that *Trichinella* antigens suppress DC maturation induced by LPS from intestinal bacteria. During migration through the intestinal wall *Trichinella* larvae may drag bacteria on their surface. The presence of these larvae in the bloodstream can result in septic shock. Suppressing the effect of LPS from the dragged bacteria may be a strategy to avoid a septic shock and keep the host alive. In addition to the effect on DC maturation we also showed that *Trichinella* antigens induce the expansion of regulatory T cells with suppressive activity.

In chapter 4, we investigated whether infection with this helminth modulate the course of allergic asthma. For this, we combined a mouse model for experimental allergic asthma and the mouse model for trichinellosis. Our data showed that the chronic as well as the acute phase of *Trichinella* infection protects against experimental allergic asthma. This protective effect increases as infection progresses from the acute to the chronic phase. We also found that regulatory T cells may play an essential role in the suppression of experimental allergic asthma. Infection as a therapy is not an option for the treatment of allergy since *T. spiralis* infection could have adverse consequences for the infected patients. Therefore, identification of the *T. spiralis* molecules that modulate the host-

immune response and suppress allergic manifestations is relevant for the development of new therapy for the treatment of this disease.

Among the helminth molecules responsible for the immunomodulation of the host immune responses, certain glycans have been suggested to play an essential role. They have also been reported to be involved in the generation of host antibody responses during infection which make them excellent targets for diagnostic purposes. In chapter 5, we examined the potential use of parasite glycans for diagnosis of *T. spiralis* infection. We show that specific parasite glycans can be identified by using sera of infected patients and glycan microarrays. These microarrays are slides containing multiple glycans. In addition, we demonstrated that an ELISA (Enzyme-linked immunosorbent assay) using the identified glycans has a high sensitivity for diagnosis of trichinellosis, indicating the potential value of the glycan microarray technology for developing diagnostic assays of parasitic infections.

In conclusion, identification and isolation of the *T. spiralis* molecules responsible for the modulation of the host immune response are mandatory and main focus of interest in the development of new therapeutic approaches for treatment of allergic diseases. Finally, identification of other *Trichinella* antigens that could be synthesized is also crucial for improving and standardizing the diagnosis of human trichinellosis.

Nederlandse Samenvatting

Verbeterde hygiëne, schoon water, en het gebruik van vaccins en antibiotica hebben het aantal infecties met bacteriën, virussen, en parasieten drastisch verminderd in ontwikkelde landen. Er is echter groeiend bewijs dat we ons hiermee ook hebben ontdaan van de organismen die ons immuunsysteem in balans houden. Gedurende duizenden jaren is ons immuunsysteem geëvolueerd samen met ziekteverwekkers, zoals parasitaire wormen. Het immuunsysteem is als het ware gewend geraakt aan deze parasieten. De afwezigheid of verminderde bloostelling aan deze pathogenen brengt het immuunsysteem in de war, wat de kans vergroot op immunologische ziekten, zoals bijvoorbeeld allergieën.

Er zijn verscheidene onderzoeken die hebben aangetoond dat bepaalde parasitaire wormen het immuunsysteem kunnen afremmen. In zo'n geval zorgt de parasiet er voor dat hij niet wordt aangevallen door het immuunsysteem en daardoor kan overleven in de gastheer. Deze afremming van het immuunsysteem kan daarentegen ook voordelen hebben voor de gastheer omdat nadelige immuunresponsen, zoals bij allergieën, ook onderdrukt worden. De mechanismen waarmee parasitaire wormen het immuunsysteem manipuleren verschillen echter van worm tot worm, aangezien elk van deze parasieten zijn eigen unieke overlevingsstrategie ontwikkeld heeft. Daarom is het nodig om deze mechanismen en de betrokken moleculen te onderzoeken bij verschillende parasitaire wormen voor een beter begrip en vooral ook om nieuwe manieren te ontwikkelen om allergieën te bestrijden.

In dit onderzoek hebben we de parasitaire rondworm *Trichinella* spiralis bestudeerd. Deze parasiet wordt soms de "varkensworm" genoemd, aangezien rauw varkensvlees besmet kan zijn met *Trichinella*. Na het eten het vlees dat besmet is met de larven van de parasiet kan men een ziekte krijgen die luistert naar de naam trichinose. Eenmaal in de darmen komen de larven vrij en worden zij volwassen. Na de voortplanting laten de vrouwelijke wormen heel veel larven achter in de darm, die vervolgens via de darmwand in het bloed terechtkomen. Na enkele weken kunnen sommige van deze larven cysten vormen in de spieren en daar jarenlang overleven, wachtend totdat ze worden opgegeten om zo een nieuwe gastheer te besmetten.

Het doel van het onderzoek beschreven in dit proefschrift is om de mechanismen te onderzoeken waarmee *Trichinella* het immuunsysteem manipuleert. Ook hebben we onderzocht wat de invloed is van *Trichinella* infectie op experimentele allergische astma in muizen. Daarnaast hebben we de bruikbaarheid bekeken van bepaalde suikers die specifiek op deze parasiet voorkomen voor de diagnose van trichinose in het serum van

geïnfecteerde patiënten.

In hoofdstuk 2 hebben we de invloed van *Trichinella* antigenen onderzocht op de maturatie van dendritische cellen (DC). DC maken onderdeel uit van het aspecifieke (aangeboren) immuunsysteem, dat snel werkzaam is, maar minder specifiek voor de ziekteverwekker. De belangrijkste taak van DC is om antigenen van de ziekteverwekker te presenteren aan andere cellen van het adaptieve immuunsysteem om een meer gerichte respons op te wekken om zo de indringer uit te schakelen. Als DC in aanraking komen met deze antigenen worden ze geactiveerd wat maturatie wordt genoemd. In dit onderzoek werd *Trichinella* antigen toegevoegd aan DC, alleen of samen met lipopolysaccharide (LPS) van twee verschillende bacteriën. LPS is een belangrijke bouwsteen van Gram-negatieve bacteriën en wordt herkend door Toll-like receptor (TLR) 4, een eiwit dat voorkomt op de buitenkant van DC. Als DC in aanraking komen met LPS resulteert dat in maturatie van de DC. Wij laten zien dat *Trichinella* antigenen alleen geen invloed hebben op de DC, maar ze verhinderden maturatie geïnduceerd door LPS van *Escherichia coli*. Gek genoeg werd DC maturatie niet geremd als we LPS van een andere bacterie, *Neisseria meningitidis*, gebruikten. Dus we kunnen concluderen dat *Trichinella* antigenen DC maturatie kunnen remmen, maar dit effect is kennelijk afhankelijk van welke bacterie het gebruikte LPS afkomstig is.

In hoofdstuk 3 hebben we dit fenomeen verder onderzocht door nog meer verschillende soorten LPS te testen. We hebben gevonden dat *Trichinella* antigenen alleen DC maturatie remmen geïnduceerd door LPS van bacteriën die voorkomen in de darmen. Als de larven van de darmen naar het bloed reizen zouden ze ook wat van deze bacteriën kunnen meenemen. De aanwezigheid van deze bacteriën in het bloed is gevaarlijk, want hierdoor kan men erg ziek worden en zelfs dood gaan. Dit is niet in het belang van de parasiet, dus het onderdrukken van de immuunreactie tegen het LPS van de bacteriën kan een strategie zijn om de gastheer in leven te houden. Daarnaast hebben we in dit hoofdstuk het effect van *Trichinella* antigenen op T cellen onderzocht. T cellen maken onderdeel uit van het adaptieve immuunsysteem en kunnen worden onderverdeeld in verschillende subtypen. Een subtype T cel is de regulatoire T cel. De belangrijkste functie van dit subtype is het dempen van het immuunsysteem en daarmee het voorkomen van auto-immuunziekten en chronische inflammatoire ziekten. We laten zien dat *Trichinella* antigenen T cellen aanzetten om een regulatoire T cel te worden.

In hoofdstuk 4 hebben we bekeken of infectie van muizen met deze parasiet invloed heeft op het verloop van allergische astma. Hiervoor hebben we het muismodel voor experimentele allergische astma gecombineerd met het muismodel voor trichinose. In

het begin van de infectie bevinden de volwassen wormen zich in de darmen (acute fase), later zijn de volwassen wormen verdwenen en hebben hun larven zich genesteld in de spieren (chronische fase). Onze resultaten laten zien dat de chronische fase en de acute fase van *Trichinella* infectie de muizen beschermt tegen experimentele allergische astma, maar de bescherming is beter in de chronische fase. We hebben ook sterke aanwijzingen gevonden dat regulatoire T cellen een belangrijke rol spelen in de onderdrukking van experimentele allergische astma. Infectie van patiënten met *Trichinella* is geen optie voor de behandeling van allergieën vanwege de serieuze bijwerkingen die dat zou geven. Voor deze therapeutische toepassing zou het interessant zijn om uit te zoeken welke moleculen afkomstig van *Trichinella* verantwoordelijk zijn voor het manipuleren van het immuunsysteem.

Men heeft momenteel aanwijzingen dat bepaalde suikerstructuren van parasitaire wormen een centrale rol spelen in het manipuleren van het immuunsysteem. Er zijn ook onderzoeken die hebben laten zien dat deze suikerstructuren betrokken zijn bij het opwekken van antistoffen tijdens infectie. Daarom zouden de suikers uitermate geschikt kunnen zijn voor diagnostische doeleinden. In hoofdstuk 5 hebben we de bruikbaarheid van specifieke suikerstructuren getest voor de diagnose van *Trichinella* spiralis infectie. We laten zien dat suikerstructuren die uniek zijn voor de parasiet aangetoond kunnen worden met sera van geïnfecteerde patiënten en glycan microarrays. Deze microarrays zijn glaasjes met daarop allemaal verschillende suikerstructuren. We hebben ook gevonden dat de suikers geselecteerd met de microarray heel goed gebruikt kunnen worden voor de diagnose van trichinose met een andere veelgebruikte techniek (Enzyme-linked immunsorbent assay). Dus deze glycan microarray techniek is potentieel zeer waardevol voor de ontwikkeling van diagnostische testen voor parasitaire infecties.

We kunnen concluderen dat de identificatie en isolatie van de *Trichinella* moleculen die verantwoordelijk zijn voor de manipulatie van het immuunsysteem een belangrijke volgende stap is op weg naar de ontwikkeling van nieuwe strategieën om allergische ziekten te behandelen. Daarnaast zou het nuttig zijn om *Trichinella* antigenen te vinden die gesynthetiseerd kunnen worden om de diagnose van trichinose bij mensen te verbeteren.

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Curriculum vitae

Carmen Rosa Aranzamendi Esteban was born in Lima, Peru. She graduated at the Santa Rosa School in Barranco-Lima. She studied Medical Technology at the National University of San Marcos in Lima. In the last year of her study, she did an internship at the Dos de Mayo Hospital. Here, she was trained to perform chemical and biological analytical tests on body fluids and tissues, as well as interpreting the results. She worked for the US – Naval Medical Research Center Detachment in a malaria endemic area for around 5 months. Here the aim of the project was to assess the *Plasmodium falciparum* drug resistance to Chloroquine and Mefloquine. In her next job, she worked for the Pan American Center for Sanitary Engineering and Environmental Sciences in a project titled: Occurrence of Emerging Intestinal Parasites in Latin America. Here, she identified parasites in drinking water and wastewater with emphasis on *Giardia*, *Cryptosporidium* and *Cyclospora* by immunofluorescence microscopy. She also studied a master in Public Health at the National University of San Marcos. Her last job in Peru was for the General Office of Epidemiology of the Ministry of Health where she was part of the Surveillance Team for diarrhoea diseases.

In 2005, she moved to The Netherlands. She studied a master in Biomolecular Sciences at the VU University Amsterdam. From September 2007, she worked as a PhD student at the Centre for Infectious Disease Research, Diagnostics and Screening of the National Institute for Public Health and the Environment (RIVM) under the supervision of Dr. Elena Pinelli. This PhD project focused on the mechanisms by which the helminth *Trichinella spiralis* and its secreted products modulate the host immune response.

Carmen is married to Floris Fransen since 2011 and together they have a son, Sebastian (September 2012).

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