Helminth infections induce immunomodulation : consequences and mechanisms

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About the cover:
The girl on the front represents the *consequences* of helminth infection. She just received two vaccinations, one in each arm, and she is holding a cotton against the sites of injection. Will her helminth status affect the efficacy of the vaccinations? The findings to this question are described in chapters 3 and 4. The pathway represented on the back is an artist's impression of the TLR2 pathway involved in intracellular signalling by helminth derived lipids (described in chapter 5) and represents the *mechanisms* involved in helminth infection. Photo: Linda Bombet, photographer: Bart Everts, cover design: Tim Jacobs & Elly van Riet.

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General introduction

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Helminths and the immune response

Worldwide, more than a billion people are infected with helminths. These worm infections generally do not lead to mortality, however, they are chronic in nature and can lead to considerable morbidity. Immunologically these infections are interesting; chronic helminth infections are characterized by skewing towards a T helper 2 type response as well as regulatory responses. The regulatory network is associated with chronic helminth infections and is thought to prevent strong immune responses against parasitic worms, allowing their long-term survival and restricting pathology. This regulatory network is thought to also temper responses to non-helminth antigens, like allergens or self antigens, possibly leading to lower prevalence of allergies and autoimmune diseases in subjects that are chronically infected with helminths. This raises the interesting idea that helminths may bear molecules that have potential therapeutic action against allergies and possibly other inflammatory diseases. However, on the other side of the coin, this would predict that helminth infected subjects might not respond strongly to third party antigens like vaccines. This is an important issue, since most vaccines that are being developed against diseases such as HIV, tuberculosis or malaria will be introduced in areas where helminth infections are highly prevalent. Moreover, these vaccines are proving difficult to develop and are often weak, thus any confounder that would affect their efficacy needs to be taken into consideration. Helminth derived molecules have been identified that induce T helper 2 and regulatory responses via modulation of dendritic cells and some appear to do so via TLR signalling. New insights into these pathways could be useful to antagonize suppression and hence boost vaccine efficacy or to optimize suppression induced by helminth derived molecules and control inflammatory diseases.

Scope
Helminth infections are characterized by a strong T helper 2 (Th2) response as well as an overall downregulated immune system. In this general introduction first the immune responses evoked by helminths will be described. Second the effect of helminth infections or helminth derived molecules on responses to unrelated antigens will be discussed and third an overview will be given of the mechanisms involved in helminth induced immune modulation.

Host immune responses to helminths

Helminths are multicellular organisms of which many species are parasitic. Those infecting humans are mainly found in two phyla; the phylum of Platyhelminths includes digenean flukes (trematodes) and tapeworms

(cestodes), and roundworms belong to the phylum of Nematoda (http://www.path.cam.ac.uk/~schisto/General_Parasitology/Hm.helminths.html). Worldwide millions of people are infected, causing mainly indirect effects on health, as they contribute to malnutrition, and hence a decrease in children's growth, and impairment of cognitive functions. Generally, a small population of infected individuals suffer from severe symptoms, but most people remain asymptomatic.

Helminths vary greatly in their biology. Their intermediate hosts range from snails for schistosomes to flies in case of filarial worms. Also the route of infection can differ from oral infection (e.g. *Ascaris lumbricoides*) to direct penetration of the human skin (schistosome species) or infectious bites of a fly (*Onchocerca volvulus*). In addition helminths can exist within the human host in different developmental stages, such as eggs, larvae or adult worms. Finally, different species affect different organs, including the colon, the small intestine, the lymphatics, the lungs and the liver. Despite this broad range of characteristics, most members of the helminth parasites evoke similar adaptive immune responses in their human host. Helminths are known to skew the immune response towards Th2, characterized by Th2 related cytokines, that typically include IL-4, IL-5 and IL-13 that induce B-lymphocytes to switch to IgE antibody production. The active molecules present in helminths that are inducing these responses include not only proteins but also lipids (described in Chapter 2 and Chapter 5). If the role of the Th2 response is to eradicate helminth infections, as shown in animal models [1], the question is why these organisms would carry Th2 inducing molecules? Presumably these molecules are essential for parasite biology.
Helminths are long-lived organisms (up to 10 years for filarial worms) and mostly they are not able to replicate within their human host. Therefore the use of antigenic variation to escape from the hosts' immune attack is not possible. In addition they are too large for sequestration in specialized niches away from the immune system. However, helminths are thought to have developed different strategies for survival in their human host. For example schistosomula can compromise complement function [2] and degrade host immunoglobulins [3], which may weaken a direct immune attack. Helminths may shield themselves by molecular mimicry. For example schistosomula can acquire surface molecules from the host, including blood group determinants and MHC molecules or they can produce cytokine mimics (reviewed by Maizels and coworkers [4]). Their ability to neutralize host derived immune molecules was shown recently when *Schistosoma mansoni* eggs were found to secrete a chemokine binding protein (smCKP) that blocks IL-8 induced neutrophil migration. In a purified recombinant form, smCKP was able to inhibit IL-8 induced pulmonary neutrophilic inflammation in a contact hypersensitivity model [5], indicating that this molecule may protect the parasite from an

inflammatory attack. Finally, helminths can interact with the hosts' adaptive immune response by downregulation of T and B-cell responses via the induction of regulatory T cells or the anti-inflammatory cytokines IL-10 and TGF-β in the chronic phase of infection, as reviewed before [6].

Immunomodulation is hypothesized to be beneficial to both the human host and the parasite, as it could protect helminths from being eradicated, and at the same time protect the host from excessive pro-inflammatory responses that may lead to organ damage. Importantly, immune hyporesponsiveness is evident mostly in case of chronic or high level infections; upon infection the immune system will be activated to try and eradicate the worm, however, as the burden or time after infection increases, the worms seem to modify and downregulate these responses in order to survive. The modulation of the immune response is not only directed to helminths, but also to non-related antigens. Below we will give an overview of how this affects co-infections, vaccinations, as well as allergic and autoimmune diseases.

Spill-over effects of helminth-induced immunomodulation

Helminths and co-infections

Helminths have been shown to induce immune hyporesponsiveness, which might affect the immune reaction to concomitant infections that occur with high frequencies in helminth-endemic areas. The most prevalent and threatening diseases in this respect are malaria, tuberculosis, and HIV, caused by protozoa, bacteria and viruses, respectively.

Helminths are prevalent in tropical areas where malaria parasites thrive and co-infections are common. The effect of helminth infections on the outcome of malaria is the subject of recent studies, however results are variable depending on the type of helminth, the intensity of infection and the age of the study population. An effective immune response against primary malaria infection requires a strong Th1 response. Therefore it would be expected that helminth infections would decrease the development of protective immunity by inducing a Th2 response, and this has been confirmed in several studies of helminth and malaria co-infections [7, 8]. However, the results on schistosome infections indicate that light schistosome infections might be protective in young children as it was observed that children between 4-8 years of age infected with schistosomes showed less malaria, increased time to the first clinical infection and lower parasitemia compared to non-infected controls [9]. Further, the influence of helminth infections on the symptoms of severe malaria could be different from the effects on parasitemia. Although in one study it was found that ascaris infections were associated with an increase

in prevalence of severe malaria [10], others found an association between ascaris infections and protection from cerebral malaria [11] or from renal failure [12]. As cerebral malaria has been associated with increased levels of pro-inflammatory cytokines, a concomitant helminth infection may be able to suppress these cytokines by production of IL-10 and/or TGF-β (upregulated by helminth infections [13]) and therefore decrease the chance of developing severe malarial disease.

Animal models of malaria and helminth co-infection showed that in C57BL/6 mice infected with both *Heligmosomoides polygyrus*, a gastrointestinal nematode, and *Plasmodium chabaudi* AS, a strain that cannot induce cerebral malaria, the proliferation of $CD4^+$ T cells and IFN-γ production was compromised and malaria parasites were not eliminated. Interestingly, treatment of the mice with an antihelminthic drug before malaria infection fully restored protective antimalarial immunity and TGF-β levels were downregulated [14]. However, in another study, infection with *P. chabaudi* in CBA mice resulted in lower parasitemia in the schistosome-infected mice, whereas in the same model schistosome infection did not have an effect on parasitemia caused by another parasite species, *Plasmodium yoelii* [15]. These studies support the notion that helminths could alter the course of malarial infection and disease as discussed in recent reviews [16, 17]. However, there is a need for more extensive studies with uniform methodology to understand the nature of interactions in different stages of malaria co-infections.

Protection from tuberculosis is characterized by strong mycobacterium-specific Th1 responses and it has been hypothesized that co-infections with helminths will prevent the necessary Th1 response by either disturbing the Th1-Th2 balance, or by driving the immune response towards a more anti-inflammatory status. Early observations showed that the incidence of lepromatous leprosy, caused by *Mycobacterium leprae*, was twice as high in areas where onchocerciasis was hyperendemic [18]. Others have shown that PBMC from onchocerciasis patients have decreased IL-4 responsiveness to mycobacterial antigens *ex vivo* [19]. Moreover, in a murine model, an established Th1 response to *Mycobacterium avium* was decreased by a subsequent co-infection with *S. mansoni*, affecting both Th1-cytokine production and IgG2a antibody responses [20].

Although there are not many epidemiological studies on the effects of helminth infections on tuberculosis, more is known on the modification of immune cells by helminths. For example, dendritic cells (DCs) and macrophages exposed to live microfilariae *in vitro* show reduced maturation after subsequent *Mycobacterium tuberculosis* infection, indicating a compromised activation of the immune system by mycobacteria upon interaction with helminths. In addition dendritic-cell-

specific ICAM-3 grabbing non-integrin (DC-SIGN) lectin receptor, used by *M. tuberculosis* to enter DCs, was shown to be downregulated on the surface of these filarial-infected DCs [21], thereby possibly reducing susceptibility of these DCs for infection by *M. tuberculosis*. Thus, *in vitro* studies indicate that helminths can suppress the immune response to mycobacterial infections. However, the *in vivo* relevance of these laboratory-based *in vitro* findings has to be more intensively examined in immuno-epidemiological studies in areas where these two infections are prevalent.

Recently the effects of helminth infections on the course of HIV infection have gained much attention. The question was raised whether it would be beneficial to treat HIV-infected people in helminth endemic areas with anti-helminthics in addition to HIV medication. The results on human studies so far are ambiguous as some studies find no association between treatment of intestinal helminth infections and reduction in viral load [22], whereas others find a decrease in viral load upon de-worming [23] or even find a transient increase [24]. Variation between the studies may be explained by difference in age groups studied, prevalence of helminth species, type and frequency of medication as well as length of time post treatment before determination of viral load. In this respect, infection with hookworm (an intestinal helminth) showed most consistent results. Hookworm infections were shown to be associated with reduced HIV disease progression, represented by higher $CD4^+$ T cell counts and lower mortality [25]. In another study hookworm was negatively associated with sensitivity to HIV infection, whereas in the same study *Wuchereria bancrofti*, a filarial nematode, was positively associated with infection [26]. Several studies have indicated that the characteristic depletion of $CD4^+$ memory T cells in HIV infected subjects does not occur gradually and systemically as originally thought, but at a high speed at the mucosal surfaces of the intestine (reviewed in [27]). Therefore, one could speculate that intestinal parasites like hookworm could play a role in influencing the anatomy of the mucosal surface, and thereby prevent T-cell death. However, both the epidemiological relations and the possible mechanisms involved in the interaction between helminths and HIV need to be further studied in more detail.

Helminth infections and vaccination efficacy

For successful vaccination against most bacterial and viral diseases an efficient Th1 response is required. When it was found that responsiveness to live (attenuated) oral vaccines like cholera, polio and rotavirus was impaired in developing countries it was hypothesized that the presence of helminths in the gastro-intestinal tract might have affected efficient uptake of oral vaccines, as these infections are also associated with reduced absorption of nutrients [28]. It was found that *A. lumbricoides* infections impair immune responses to oral cholera vaccine by decreasing

seroconversion as well as mean antibody titres to the vaccine. In addition, the magnitude of the Th1 cytokine response (IFN-γ and IL-2) to cholera toxin subunit B after oral cholera vaccination was greater in albendazole treated subjects compared to placebo-treated controls [28, 29]. Roughly, two mechanisms can contribute to this. First, helminths could decrease effective uptake of the vaccine by cells present at the mucosal surface of the small intestine by interfering with mucus production and intestinal motility. Second, the Th2 skewing or immunomodulation induced by helminth infections could decrease the strength of the Th1 response mounted against the vaccine. Moreover, these mechanisms might not only add up, but could also be interfering with each other as for example goblet cell hyperplasia and intestinal muscle contractility are considered to be under direct immunological control, and are associated with a Th2 response where CD4 T cells, STAT6, MHCII and CD40L play a role [30].

Considering non-oral vaccines, BCG (Bacille Calmette-Guérin) has received much attention as tuberculosis is still affecting many people worldwide, causing great mortality. In a study in Ethiopia, BCG vaccination improved cellular PPD-specific immune responses in de-wormed young adults, but not in placebo-treated subjects infected with intestinal helminths [31]. In a mouse model it was demonstrated that schistosomal infection reduced the effect of BCG vaccination [32]. In addition it was found that prenatal sensitization to filariasis and schistosomiasis biases T cell immunity away from protective INF-γ responses [33]. This could be of particular interest as currently BCG vaccination is mostly administered directly after birth, indicating that the effect of maternal helminth infections on newborns may be important.

Experimental data on effectiveness of other non-oral vaccines generally support the results seen for BCG. For example, after tetanus vaccination, the responsiveness to tetanus toxoid (TT) is decreased by filarial infections [34]. Infections with filarial worms reduced IFN-γ production and increased TT specific IL-10 production in an Indian population [34]. A study in areas where onchocerciasis was endemic indicated that following vaccination with a single dose of concentrated tetanus toxoid vaccine, 7.1% of onchocerciasis patients seroconverted versus 44.5% of the uninfected control group [35]. In another study of onchocerciasis, infected subjects showed lower antibody, proliferation and IFN-γ responses compared to non-infected subjects, whereas IL-10 levels were elevated in infected subjects [36]. Although light *O. volvulus* infections did not affect the anti-tetanus antibody response, heavier infections impaired the antitetanus IgG response [37]. For schistosomal infections similar results were found in Brazilian adults; TT-specific Th1-like responses, represented by IFN-γ production, were low in schistosome infected subjects in comparison to non-infected controls [38]. We found similar results in a study in Gabon

where rural children with concurrent schistosomiasis and intestinal helminth infections showed reduced IFN-γ responses to TT compared to semi-urban subjects, having less infections, after tetanus vaccination (described in Chapter 4). In addition these children received an influenza vaccine and similarly it was found that the IFN-γ response to influenza was higher in non- or low infected semi-urban children, whereas IL-5 and IL-13 production was increased in infected rural children (described in Chapter 3). Thus, in Chapters 3 and 4 we found differences between semi-urban and rural cohorts, and helminths are likely to play a role, but we find that other factors might be involved as well. Moreover, it needs to be investigated whether the differences can be attributed to helminth infections, and whether the effect is dependent on their actual presence at time of vaccination, (chronic) helminth infections in the past and / or the worm or egg load. For this, larger studies are needed, that for example also include groups of children that are treated with anti-helminthics before vaccination.

The influence of helminth infections on vaccination efficacies should be taken in consideration when designing new vaccines. It has for example proven difficult to design an effective vaccine against HIV. Notably, most trials on HIV vaccines are conducted in the western world and its effectiveness in developing countries could be even further reduced due to concurrent helminth infections. Interestingly, a study using schistosome-infected mice, with a pre-existent Th2 immune background, demonstrated that CpG immunostimulatory sequences co-administered with inactivated, gp120-depleted HIV-1 viral particles lead to potent Th1 anti-HIV-1 immune responses overcoming the Th2 bias. In contrast, schistosome-infected mice immunized with HIV-1 immunogen in incomplete Freund's adjuvant, induced Th2 anti-HIV-1 immune responses [39]. These findings suggest that an effective vaccine for those living in helminth endemic areas might need a modified adjuvant compared to a vaccine for use in the western world.

Helminths and allergic diseases

Infection patterns have been changing enormously in the westernised countries over the last few decades. Infections are declining but other diseases, like allergies and autoimmune diseases, are emerging. One of the questions arising is whether or not there is a correlation between these two phenomena, as together with an improved hygiene other environmental factors changed as well in the same time frame. The possibility of a direct correlation between allergies and helminth infections has been intensively studied, and results often show negative associations, suggesting that helminth infections could protect from allergic responses, although some studies find the opposite to be true. An overview of these studies is given in table 1.1A. The inconsistencies might be explained by different factors:

the age and genetic background of the study population, as well as the helminth species studied. However, the intensity of infection is thought to be the most important factor, as chronic, heavy helminth infections are increasingly associated with a regulatory response and protection from allergies, whereas acute, low level infections are thought to potentiate allergic reactions by increasing the Th2 response, without inducing regulation [40, 41].
Whether or not there is a causal relationship between helminths and allergy can only be investigated by assessing the effects of introducing or removing helminths. Some of these studies have been conducted, showing that treatment of schistosomiasis or intestinal helminths in humans indeed increases skin reactivity to house dust mite [42]. However, a recent, large scale study has shown that one year anti-helminth treatment had no effect on SPT reactivity in Ecuador [43] (table 1.1B). Differences between the outcomes of antihelminth treatments may be explained by differences in length and frequency of treatment given [44].

In murine models introduction of helminths can be studied. For example the gastrointestinal nematode *H. polygyrus* was shown to suppress inflammation in a model of allergic airway infection induced by either OVA or Derp1 [45]. In addition, in a model of chronic schistosome infection it was found that allergic reactions in response to OVA challenge were reduced, as measured by a decrease in airway responsiveness and eosinophilia in the lungs [46]. Other murine intervention models that have been studied are described in table 1.1B, all showing a protective effect of helminths on allergy.

The cellular mechanisms that mediate suppression of allergic immune responses by helminths was studied by Wilson and colleagues, who showed that adoptive transfer of T cells from mice infected with a nematode, *H. polygyrus*, could suppress allergic inflammation in recipient mice. $CD4^+CD25^+$ cells were shown to be the most potent cells in inducing suppression. While elevated levels of IL-10 and TGF-β and increased foxp3 expression were found, $CD4^+CD25^+$ cells from IL-10 deficient mice could also transfer suppression of allergic inflammation. These experiments indicate that regulatory T cells are important in helminth induced inhibition of allergic inflammation but that IL-10 was not required for the suppressive actions of regulatory T cells in this model [45]. Also other studies on helminth infections have shown the induction of regulatory T cells. These cells were heterogeneous with respect to production of regulatory cytokines and expression of regulation-associated markers such as IL-10, TGF-β, CTLA4 [47], GITR or foxp3 [48](reviewed in [49]).

Table 1.1. Studies that have examined the relationship between helminths and allergies. **A.** Correlations between helminth infections and allergies.

Study area	Population (age range)	Helminth	Outcome	Ref.
Allergy: Human association studies				
Reported negative associations				
Venezuela	children	Geohelminhths	Reduced skin reactivity to environmental and ascaris antigen	[51]
Brazil	175 subjects	*Schistosoma mansoni*	Reduced skin response to aeroallergens	[52]
Gabon	520 children (5-14)	*Schistosoma haematobium*	Reduced skin reactivity to mite	[53, 54]
Ethiopia	604 adults (>16)	Hookworm	Reduced risk of wheeze	[55]
Gambia	448 adults (>15)	Geohelminhths	Protection from skin reactivity	[56]
Ecuador	2865 children 5-19 years	Geohelminths	Reduced skin reactivity to allergens	[57]
Brazil	84 asthma patients (6-35)	*Schistosoma mansoni*	Reduced course of asthma	[58]
Ethiopia	563 children (1-4)	*Ascaris lumbricoides*, hookworm	Reduced wheezing	[59]
Uganda	62 infants	Maternal helminthiasis (filariasis and hookworm) at delivery	Protection against infantile eczema	[60]
Reported; no association				
Ecuador	4433 children (5-18)	Geohelminths	No association with allergic symptoms	[61]
Ethiopia	7649 (>5)	Geohelminths	No association with weeze/asthma	[62]
Reported positive associations				
The Netherlands	1379 children (4-12)	*Toxocara* spp.[1]	Increase in allergic manifestations	[63]
China	2164 children (8-18)	*Ascaris lumbricoides*	Increased sensitization to aeroallergens, increased risk of asthma	[64]
South Africa	359 children (6-14)	*Ascaris lumbricoides* (ascaris-specific IgE)	Increased SPT positivity to aeroallergens	[65]

[1] *Intestinal parasite of cats and dogs, human is a non-compatible host*

Table 1.1B. Intervention studies in human (treatment) and mouse (infection).

Study area Population (Age range) (*mouse*:strain)	*	Helminth	Intervention strategy	Outcome	Ref.
Allergy: Intervention					
Human studies					
Gabon 317 children (5-13)	+	Geohelminth	Treatment; Praziquantel Mebendazole every 3 months for 30 months	Increased atopic reactivity to mite	[42]
Ecuador 1632 school children	0	Geohelminths	Treatment; Albendazole; 2-monthly for one year	No effect on atopy or clinical allergy	[43]
Brazil 33 asthma patients (6-39)	+	Geohelminths *Schistosoma mansoni*	Treatment; Albendazole and oxamniquine once	Less production of Derp1 specific IL-10	[66]
Venezuela 89 asthma patients	-	*Trichuris trichiura, Ascaris lumbricoides*	Treatment; Albendazole; monthly for 1 year	Decreased numbers of asthmatic crises and need for asthma medication	[67]
Mouse studies					
BALB/c and C57BL/6	+	*Schistosoma mansoni*	Infection	Suppression of asthma symptoms	[46]
BALB/c and C57BL/6	+	*Heligmosomoides polygyrus*	Infection	Suppression of allergic airway inflammation	[45]
B10.A and C57BL/6	+	*Ascaris suum*	Treatment with worm extract	Suppression of lung inflammation	[68]
BALB/c	+	*Ascaris suum*	Treatment with PAS-1 protein	Suppression of allergic responses induced by an allergic protein of *A. suum* (APAS-3)	[69]
BALB/c and C57BL/6	+	*Schistosoma mansoni*	Infection	Inhibition of anaphylaxis	[50]
C3H/HeJ	+	*Heligmosomoides polygyrus*	Infection	Inhibition of production of allergen-specific IgE and anaphylaxis	[70]

**Effect of helminths is beneficial for the host with respect to the effect on allergy (+)*
Effect of helminths is detrimental for the host with respect to the effect on allergy (-)
No effect of helminths on allergy (0)

These molecules may contribute differentially to the regulatory response mediated by these cells in various models of allergic inflammation. In addition, cells other than regulatory T cells could play a role in the suppression of allergies by helminth infections. For example, adult *S. mansoni* worms could protect mice in an experimental model of fatal allergic anaphylaxis. In this model $CD4^+CD25^+$ T-cells and IL-10 produced by these cells did not seem to be involved in protection from anaphylaxis, but it was found that IL-10 and B cells were involved in this protection as transfer of IL-10 producing B-cells from infected IL-4 deficient mice ameliorated disease [50]. In summary, although epidemiological studies suggest that helminth infections can suppress allergic inflammation, solid evidence comes from animal models, where chronic nematode and trematode infections suppress allergic inflammation. Cell transfer experiments support the notion that several cell subsets are involved in the protective mechanisms.

Helminths and auto-immune diseases

The hygiene hypothesis proposes that the lack of serious childhood infections impairs development of an appropriately educated immune system. Although the rise of autoimmune diseases in the western world is correlated with improved hygiene, causal proofs are difficult to find, as most auto-immune diseases are influenced by multiple genetic and environmental factors [71] and, unlike allergic disorders, are less prevalent and therefore more difficult to study.

In murine models of autoimmune diseases, the presence of helminth infections have been shown to have a protective effect. Infection with *S. mansoni*, exposure to its eggs or to soluble extracts from either adult worms or eggs all inhibit development of type1 diabetes in NOD mice. The T cells of the protected mice could not transfer diabetes to NOD-SCID mice, whereas T cells from non-infected diabetic mice did [72, 73].

In addition, helminths were found to be protective in several models of inflammatory bowel disease (IBD), where the disease and the parasites are co-localized. A concurrent *S. mansoni* infection in a semi-permissive rat model attenuated the course of colitis [74] and the intestinal nematode *Trichinella spiralis* suppressed macroscopic and histological symptoms of colitis in mice [75]. Currently, *Trichuris suis* is used to treat patients with ulcerative colitis and Chrohn's disease and initial results look very promising [76, 77].

Furthermore, *S. mansoni* was shown to be protective in EAE, a murine model for multiple sclerosis [78]. Interestingly the reduction of EAE severity by *S. mansoni* infection was shown to be mediated by STAT6, indicating a role for this molecule in the induction of a Th2 environment.

Table 1.2. Studies that have examined the relationship between helminths and auto-immune diseases.

Model	*	Helminth	Antigen	Outcome	Ref.
Auto-immune diseases and helminths					
Human					
Human	+	*Trichuris suis*	Infection	Amelioration of Crohn's disease	[77]
Human	+	*Trichuris suis*	Infection	Reduced ulcerative colitis	[79]
Rat					
Wistar	+	*Schistosoma mansoni*	Infection	Reduced duration of colitis	[74]
Mouse					
NOD	+	*Schistosoma mansoni*	Infection of ova treatment	Inhibition of development of type I diabetes	[72]
NOD	+	*Schistosoma mansoni*	Egg treatment or egg or worm derived proteins	Inhibition of development of type I diabetes	[73]
C57BL/6J	+	*Schistosoma mansoni*	Infection	Suppression of symptoms of type I diabetes	[80]
NOD	+	*Dirofilaria immitis*	Recombinant antigen	Inhibition of development of type I diabetes	[81]
IL-10 deficient C57BL/6	+	*Heligmosomoides polygyrus*	Infection or transfer of MLN from infected mice	Suppression of established colitis	[82]
BALB/c	+	*Schistosoma mansoni*	Egg treatment	Inhibition of development of colitis	[83]
BALB/c	+	*Hymenolepis diminuta*	Infection	Suppression of symptoms of colitis	[84]
C57BL/6	+	*Trichinella spiralis*	Infection	Reduction of severity of colitis	[75]
C57BL/6J	+	*Schistosoma mansoni*	Infection (but not ova treatment)	Reduced induction and progression of EAE	[85]
SJL,C57BL/6	+	*Schistosoma mansoni*	Ova treatment	Reduction of severity of EAE	[85]
BALB/c	+	*Schistosoma mansoni*	Infection (but also αGalCer)	Graves' hyperthyroidism	[86]
DBA/1	+	*Acanthocheilonema viteae*	Secreted protein ES-62	Reduced initiation, progression and severity of collagen induced arthritis	[87]

** effect of helminths is beneficial for the host with respect to the effect on the auto-immune disease (+)*

In general the effects of helminths on autoimmune diseases are consistent (table 1.2), showing that these parasites can protect a host from developing autoimmune disease and/or can relieve symptoms of established autoimmune inflammation. In the modulation of autoimmune diseases by helminths both the generation of a Th2 environment as well as the immunomodulation might be beneficial.

Molecular mechanisms of immune modulation by helminths

Introduction

Effects of parasitic helminths on the host immune response have been studied extensively as described above. In several models, it has been shown that these infections lead to the generation of Th2 responses as well as anti-inflammatory/regulatory responses. However, the molecular immunological pathways that induce these Th2 or regulatory responses are still being characterized and in this section we will discuss what is known about cell characteristics and molecules involved in this particular immunological cross talk.

Instructions for the development of specific immune responses are largely mediated by dendritic cells, which are present in peripheral tissues as sentinel cells and upon activation migrate to draining lymph nodes to activate naïve T cells, not only by presenting antigen but also by providing signals that determine polarization of T cell development towards a Th1, Th2 or regulatory T cell phenotype [88]. In this way dendritic cells play a central role in providing information on the nature of the invading/residing pathogen by integrating signals received and conveying them to T cells via expressing a variety of factors that will affect T cell differentiation into polarized subsets. Although several lineages of DCs have been identified both in man and mouse [89] the paucity of them has restricted their extensive use. However, the generation of large numbers of DCs has become feasible through the *in vitro* culturing of monocyte derived DCs from peripheral blood mononuclear cells in man or bone marrow derived DCs in mice, which has enabled experimentation on these cells to understand how they sense the presence of viruses, bacteria or parasites in their microenvironment. Encountering various stimuli can change the maturation status of these cells, an important step in whether these cells participate in immune activation or not. The characteristics of dendritic cells in terms of expression of co-stimulatory molecules or released cytokines, are determined by the receptors that are engaged and the downstream signalling that follows. This is an intense area of research with some surprising findings regarding the activity of helminth derived molecules.

In the following sections, there will be a focus on what is known about the modulation of dendritic cell function by helminths and helminth derived molecules, which may be involved in triggering of Th2 and/or regulatory T cell responses by these cells. Helminths and their products can modify DCs in different ways ranging from influencing the DC maturation status to affecting the downstream signalling within the DCs.

Helminths and maturation status of dendritic cells

One of the differences found between DCs that induce a Th1 response (DC1), DCs inducing a Th2 response (DC2) and DCs inducing tolerance or regulatory T cells (DCreg) is their activation status. In DC1, there is often a high expression of the maturation marker, CD83, as well as increased expression of co-stimulatory molecules such as CD80 and CD86. When these DCs encounter naïve T cells, they induce strong Th1 responses. However, when stimuli that are associated with a Th2 response are considered, they are often less strong inducers of DC maturation. For example, soluble egg antigens of *S. mansoni* (SEA), strong inducers of Th2 responses *in vivo*, when incubated with murine DCs *in vitro*, do not lead to strong maturation of these cells, but do lead to the development of Th2 responses when they encounter T cells [90]. Partial upregulation of some markers associated with maturation, have been seen with excretory/secretory proteins of a parasitic nematode, *Nippostrongylus brasiliensis* (NES) an infection associated with a Th2 immune skewing. Here CD86, CD40 and OX40L were upregulated compared to medium, but no increase in CD80 or MHC class II molecules was found [91]. Similarly, products released by schistosome larvae did upregulate MHC class II, CD40 and CD86, but to a lower extent than a Th1 stimulus, and in addition CD80 and OX40L were not upregulated [92]. In human DC experiments, *in vitro* maturation of DCs in the presence of some Th2 inducing stimuli, such as an ascaris derived phosphatidylserine fraction (ascaris PS), results in a partial reduction of CD83 expression compared to DCs matured in the absence of ascaris PS, suggesting that this Th2 stimulus may inhibit DC maturation (Van Riet *et al.*, unpublished results).

Next to these antigen mixtures also little upregulation of DC maturation markers was seen in DCs stimulated with the glycoconjugate lacto-N-fucopentaose III (LNFPIII), expressed on molecules present in SEA [93]. When injected into mice, LNFPIII stimulates an immune response dominated by Th2. Another well characterized helminth derived molecule, the filarial PC-containing secreted glycoprotein ES-62 [94], when incubated with bone marrow derived DCs, resulted in the upregulation of some of the maturation markers but to a lower degree than what is seen with stimuli that lead to the development of DC1 and thereafter Th1 responses. The partially activated status was confirmed when SEA [95] or NES [91] stimulated murine DCs were analysed by microarrays and only a limited

set of genes was found to be upregulated in comparison to fully mature DC1.

Thus, DC2 usually do not reach a fully activated status, although some maturation markers are upregulated. However, the question arises whether the partial maturity of DCs is responsible for driving Th2 responses? When human DCs are exposed to helminth derived molecules during their maturation (induced by factors such as LPS or TNF-α plus IL-1), they mature and when co-cultured with T cells, they are still able to stimulate Th2 responses. This would suggest that fully mature DCs can also trigger Th2. An important point to consider is what exactly is a mature phenotype; in human DCs a high expression of CD83 only? Or is it important to also have CD80 or CD86 upregulated or both? For example, although human DCs exposed to SEA or schistosome PS, in the presence of maturation factors, can show high expression of CD83 [96], they might be defective in the expression of other maturation markers. For example, SEA was found to reduce CD86 expression induced by LPS in human monocyte derived DCs (Van Riet *et al.*, unpublished results).

With respect to regulatory responses, it is known that immature DCs can induce a tolerogenic immune response and may stimulate naïve T cells to become regulatory T cells [97]. Although LNFPIII as well as ES-62, under some experimental conditions, induce an anti-inflammatory response [98, 99], so far only lyso-phosphatidylserine from *S. mansoni* (lysoPS) when cultured with DCs during their maturation process has been shown to stimulate IL-10 producing T cells with regulatory activity. The dendritic cells that induced this regulatory response were fully mature, with respect to CD83 [96].

Activation of Toll-like receptors by helminth derived molecules

Microbial products have been shown to interact with specific Pattern Recognition Receptors (PRRs) on, among others, dendritic cells and initiate a cascade of responses that could lead to generation of Th1, Th2 or regulatory T cell responses. The main groups of PRRs are Toll like receptors (TLRs), scavenger receptors and C-type lectin receptors (CLRs)[100], which are located the surface of the cell or on the surface of endosomal or lysosomal membranes. In addition, cytoplasmic PPR, including the “Nod like receptors” (NLRs) and “RIG-like receptors” (RLRs), can recognize ligands that are present in the cytosol [101, 102]. In general, it is thought that TLRs expressed on the surface of the cell might be particularly suitable for interaction with extracellular pathogens, whereas those expressed internally, would be specialized to interact with intracellular pathogens [103]. Helminth derived molecules have been shown to be involved in TLR signalling via different TLRs, including TLR2, 3 and 4 (figure 1.1). Schistosomal and ascaris derived lipids were found to

signal via TLR2 [96] (described in Chapter 5), dsRNA from schistosomal eggs can activate TLR3 [104] and the glycoconjugate lacto-N-fucopentaose III (LNFPIII) as well as the filarial PC-containing secreted glycoprotein ES-62 were both shown to act in a TLR4 dependent fashion (figure 1.1). Both LNFP-III and ES-62, stimulate TLR4 and act on dendritic cells to induce a Th2 response [93, 105]. These results might be surprising in the light of the fact that TLR-signalling is mostly associated with a Th1 response. There are several possible mechanisms that could be responsible for the difference in responses between the most intensively studied TLR4 ligand, LPS, and the two helminth derived TLR4 activating antigens; LNFPIII and ES-62. For example, different co-receptors may be involved that activate different downstream signalling or interfere with normal TLR4 signalling. A profound effect of co-receptor usage on TLR mediated effects is seen with TLR2 ligands. The TLR2 ligand zymosan does not induce a Th1 response, and it engages both TLR2 and Dectin-1 to activate DCs such that they prime IL-10 producing T cells [106]. Also for schistosome lysoPS, which activates TLR2 on dendritic cells and mediates induction of regulatory T cells, it is possible that, like zymosan, it engages co-receptors in combination with TLR2 and therefore results in DCs with capacity to induce regulatory T cells.

Regarding TLR4 activation by helminth derived molecules that have the capacity to trigger Th2 skewing, it has to be noted that TLR4 can either activate the MyD88 dependent or the TRIF dependent pathways. For helminth derived TLR4 ligands it is not yet known which adaptor molecule is involved in downstream TLR signalling. In any case the use of HeJ mice that have a point mutation in the TIR domain of TLR4 has shown that there are differences in how the mutant receptor responds to helminth derived TLR4 ligands and LPS. The HeJ mice do not produce cytokines in response to LPS whereas no differences are seen in responses to ES-62 in HeJ mice compared to WT mice, indicating that ES-62 engages and triggers TLR4 differently than LPS and possibly signals via a co-receptor in HeJ mice.

One interesting consequence of TLR engagement has come to light recently in studies of intracellular compartmentalization and this might have implications for the development of Th1, Th2 or regulatory T cell responses. A role for the TLR4 ligand LPS was found in the selection of antigenic cargo for MHCII presentation in dendritic cells. MHCII presentation of OVA (ovalbumin) and HEL (hen egg lysozyme) antigens was compared when these antigens were either conjugated to LPS bearing microspheres or to LPS free microspheres [107]. When conjugated to LPS-microspheres, the antigens were preferentially processed and presented in the context of MHC class II on the surface of DCs leading to T cell activation. Related to this, one elegant study has investigated the compartmentalization in dendritic cells of soluble egg antigen preparation

(SEA) from *S. mansoni* which induces a Th2 response, compared to *Proprionebacterium acnes* (Pa), which triggers a strong Th1 response [90]. It was found that Pa was colocalised with transferrin within DCs, whereas SEA was not. In addition, Pa was processed in LAMP2 positive antigen processing compartments, and SEA was either localised in LAMP2 negative or –dull compartments when administered alone, or in LAMP2 dull compartments when DCs were cultured with both Pa and SEA. These results indicate that processing of SEA might be less extensive than that of Pa, leading to a reduced antigen presentation that might in turn affect T cell polarization. Therefore, it is important to consider the possible consequences of TLR engagement in compartmentalization of antigens within antigen presenting cells and the subsequent effects this may have on the type of immune responses induced.

Downstream effects of helminth derived molecules: MAP kinases

It has been noted that the activation of TLRs by helminth derived molecules leads to different downstream activation of kinases involved in intracellular signalling compared to TLR activation by Th1 stimuli. Although the TLR4 ligand LPS has been found to strongly activate the MAP-kinases p38, JNK and ERK, the molecule LNFPIII that, as already discussed above, acts via TLR4, phosphorylates only ERK. Similarly, ES-62 that like LNFPIII activates TLR4 but in contrast to LPS leads to Th2 responses, suppresses activation of p38 and JNK, but induces ERK dependent inhibition of IL-12 production (figure 1.1). These results support the notion that helminth derived molecules with Th2 inducing capacity can engage TLR4 but obviously in a manner that is distinct from LPS which has no Th2 inducing activity. With respect to TLR2, PAM3CSK, a TLR2 activating molecule that can induce Th2 responses, was shown to shift the balance of p38 and ERK in favor of ERK [108], leading to stabilization of c-Fos and subsequently to a suppression of IL-12 production. Interestingly we found that schistosome and ascaris derived phospholipids also decrease the balance of *p*-p38 / *p*-ERK, like PAM3CSK, but in contrast to PAM3CSK these lipids do not increase the duration and magnitude of ERK phosphorylation, but instead, they seem to inhibit LPS-induced phosphorylation of p38 (described in Chapter 5).

Taken together, the results from studies of intracellular signalling suggest that helminth derived molecules that act via TLRs and very likely also by engaging a co-receptor such as DC-SIGN or Dectin-1, interfere with TLR signalling, thereby changing T cell skewing. A recent study has shown that engagement of DC-SIGN by antibodies can lead to activation of ERK and phosphorylation of Akt without p38 activation [109]. So a more general characteristic of molecules that lead to Th2 skewing, might indeed be the specific induction of dominance of ERK over p38. The question of how Th2 inducing and regulatory T cell inducing molecules differ in their signalling is yet unanswered and a target for future studies.

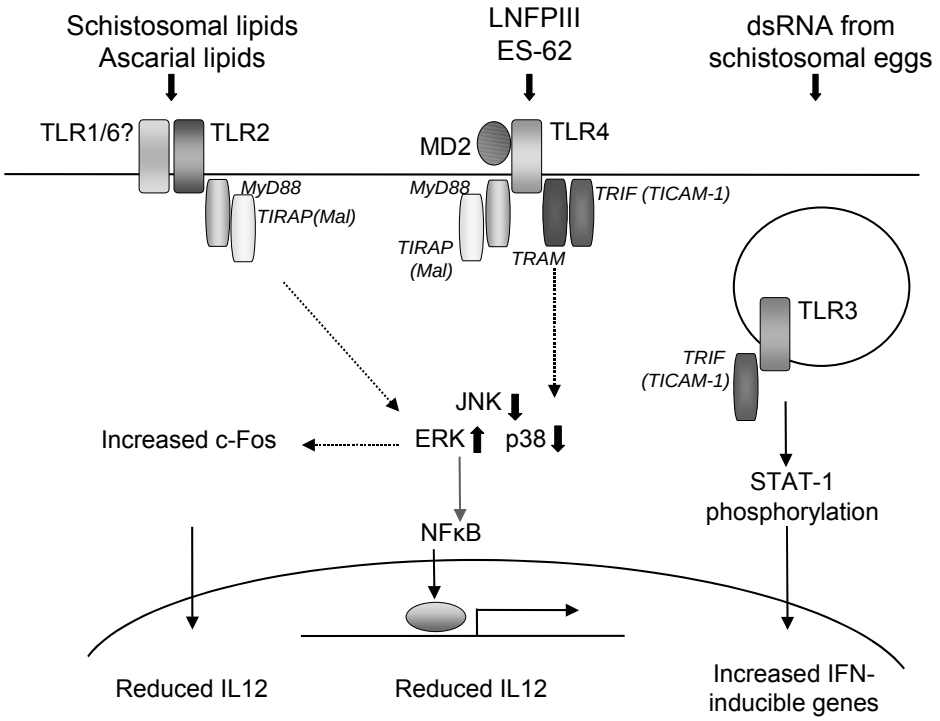

Figure 1.1. Toll-like receptor signalling in dendritic cells by helminth derived molecules. dsRNA from schistosomal eggs induces IFN-inducible genes via TLR3, whereas schistosomal and ascarial lipids as well as LNFPIII and ES-62 from schistosomes and filarial worms, induce a moderate Th2 response or immunomodulation via TLR2 or 4. For the TLR4 ligands LNFPIII and ES-62 this Th2 response was shown to be induced via increased phosphorylation of ERK. The lipids increased c-Fos expression in DCs, which has been shown to be stabilized by ERK, indicating that the Th2 inducing helminth antigens might signal via a similar pathway, although via different TLRs. Both lipids were shown to activate TLR2 and to be dependent on MyD88 and TIRAP, however, for the ascarial lipids it has not yet been shown that the reduced IL-12 and increased c-Fos is related directly to TLR2 signalling.

The remainder of this thesis "Helminth infections and immunomodulation; consequences and mechanisms" will describe the following:
In **Chapter 2** antibody profiles to glycolipids and (glyco)proteins of *Ascaris lumbricoides* were studied, as these different classes of compounds may have distinct roles in shaping of and interacting with humoral immune responses. In **Chapter 3** and **Chapter 4** examples of the consequences of helminth infections on vaccination efficacy were studied; the effect of influenza and tetanus vaccination on children living in a helminth endemic area of Gabon are described, respectively. Finally, to learn about the mechanisms involved in building immune responses upon helminth infections, human monocyte derived dendritic cells exposed to helminth derived molecules were studied at the molecular level and these results are portrayed in **Chapter 5**.

2

Antibody responses to *Ascaris*-derived proteins and glycolipids: the role of phosphorylcholine

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Summary

In addition to proteins, glycolipids can be targets of antibody responses and contribute to host pathogen interaction. Following the structural analysis of *Ascaris lumbricoides* derived glycolipids, the antibody responses of a group of children with no, light and heavy infections were analysed. The role of the phosphorylcholine moiety, present on *Ascaris* glycoproteins and glycolipids, in antibody reactivity of these infected individuals was determined.
Children carrying heavy infections showed highest IgG reactivity to glycolipids compared to lightly or non-infected children. Substantial IgG antibody reactivity to both (glyco)proteins and glycolipids was found to be directed to the phosphorylcholine moiety as determined by either removal of this group or a competition assay. This was most pronounced for glycolipids, where removal of the phosphorylcholine moieties by hydrofluoric acid treatment abrogated IgG antibody reactivity. Measurement of IgG4 and IgE isotypes showed no IgG4 reactivity to *Ascaris* glycolipids, but raised IgE responses were detected in subjects with light or no *Ascaris* infections, suggesting that IgE responses to glycolipids may play a role in controlling parasite burden. Differences found in antibody profiles to glycolipids and (glyco)proteins, indicates that these different classes of compounds may have distinct roles in shaping of and interacting with humoral immune responses.

Introduction

Ascaris infections affect one quarter of the population worldwide [110], causing low mortality, but considerable morbidity. Helminth infections, including ascariasis, are generally long lasting, occasionally causing severe pathology, however, major clinical symptoms are often absent. This is due to the dual effect of helminth infections on the immune system; helminths skew the host immune response towards a T helper 2 cytokine profile that induces B cells to switch to IgE antibody production but they are also associated with immune hyporesponsiveness, in part mediated by regulatory T cells, TGF-β and IL-10 [6]. It is known that IL-10 is involved in upregulation of the IgG4 isotype, thereby influencing the IgE/IgG4 balance, which has been shown to be important in immunity to helminth infections, including ascariasis [51, 111-113]. The current study was designed to compare antibody responses to both (glyco)proteins and glycolipids in well-characterized groups of children with different infection levels; a heavily infected group was compared to a lightly infected group and to a group of endemic controls who were free of infection.
Investigations of antibody profiles during infection have focused mainly on proteins and their carbohydrate conjugates [114, 115]. However antibody

reactivity to lipids and their conjugates has been reported, for example in schistosomiasis [116, 117], indicating that not only proteins but also lipids can be targets of humoral immune responses. Moreover, in schistosomes it has been shown that immunogenic carbohydrate epitopes can be shared between proteins and lipids [118, 119] and mediate antibody binding.

In this study we were particularly interested in phosphorylcholine (PC), a small hapten, often linked to carbohydrate epitopes. PC is a major antigenic determinant in a wide variety of organisms, varying from Gram positive and –negative bacteria, to protozoa and several intestinal nematodes [120-122]. It has been shown that PC is not only involved in physiological functions of micro-organisms (e.g. normal growth and cell division in *Streptococcus pneumoniae*), but also in modulation of the immune response of the host in favour of either the host or the pathogen. PC on *S. pneumoniae* can mediate the invasion of endothelial cells via interaction with platelet-activating factor, which can result in severe morbidity like pneumonia [123]. It has also been shown that antibodies against PC can help the host to eradicate *S. pneumoniae* [124]. Moreover, PC moieties on glycosphingolipids of *Ascaris suum* were shown to be able to modulate B cell, macrophage and T cell activation [125, 126]. PC-substituted molecules have been found to be present in large amounts in *Ascaris* worms with widespread anatomical location in the worm, including the epicuticle, indicating these components will be surface exposed and available to the host immune system [121]. Given the diverse functions of the PC moiety in host-parasite interaction [120], the development of antibodies to PC might play an important role in *Ascaris* infections.

Although glycolipids from *A. suum* have been characterized in detail, no information exists on the composition of glycolipids present in *A. lumbricoides*, the parasite of humans. In this study we have characterized these glycolipids and we have analysed IgG, IgG4 and IgE antibody responses to glycolipids, in a well-defined population residing in Indonesia where *A. lumbricoides* infections are prevalent. The antibody reactivity of children with both heavy and light infections to *Ascaris* derived glycolipids and (glyco)proteins was investigated and the contribution of PC moieties to these reactivities was determined.

Materials and methods

Study population

Banked plasma samples from Indonesian schoolchildren attending schools in different areas of the city of Makassar (Sulawesi) were studied. In one school (S.D. Cambaya) *A. lumbricoides* was highly endemic (76%) while in the second school (S.D. Mangkura) low prevalence of *A. lumbricoides*

infection was found (6.2%). The intensity of infection in S.D. Mangkura (geometric mean of 715 eggs per gram of faeces) was significantly lower than in S.D. Cambaya (geometric mean of 9,239 eggs per gram of faeces). Plasma samples were selected based on availability. For the non-infected group, children with coinfections with other intestinal parasites were excluded to prevent cross-reactivity. As indicated in table 2.1, we used plasma from 22 heavily infected children from S.D. Cambaya, 17 lightly infected children from S.D. Mangkura and 22 uninfected children from S.D. Mangkura. In addition, plasma from 14 European donors without a history of intestinal helminth infections was assayed for antibody responses. Informed consent was obtained from the parents of each child and blood withdrawal was approved by the ethics committee of the Faculty of Medicine, Hasanuddin University, Makassar, Indonesia.

Table 2.1. Characteristics of study population

	Ascaris infection intensity		
	High	Low	Not infected
Male / female	11/11	8/9	11/11
Median age, years	9	9	9.5
Range	8-11	8-11	8-11
Ascaris infection	+	+	-
Geometric mean (epg)	10084*	609	0
Range	288-87600	48-43704	-

**p<0.0001 compared to infected group with low level infections*
epg; number of eggs per gram of faeces.

Ascaris protein isolation

Proteins were extracted from *A. lumbricoides* worms that were collected in Indonesia from infected subjects. The worms were freeze-dried for two days and were then homogenized in phosphate-buffered saline (PBS) containing 1% (v/v) n-octyl β-D-glucopyranoside (Sigma) untill a homogeneous suspension was obtained, whilst keeping the mix on ice. The suspension was sonicated 6 times for 25 seconds at 20-second intervals. Samples were spun at 18,000 g and clear supernatants were collected. The supernatants were dialysed overnight against PBS to remove detergent and filtered (0.45 μm). Protein concentration was measured using a BCA-determination as described by Smith *et al* [127].

Ascaris lipid isolation

Glycolipids were extracted from *A. lumbricoides* (collected from Indonesian infected subjects) and *A. suum* (collected from pigs in a Dutch abattoir). Per extraction, lipids from 4 grams of homogenized *Ascaris* worms were extracted according to the method described by Bligh and Dyer [128]. The organic phase was dried by rotary evaporation, dissolved in 10 ml

chloroform and the total lipid extract was applied to a 20 ml column of the anion exchanger TEAE cellulose (Serva, Heidelberg, Germany) that was converted to the hydroxyl form. Lipids were eluted as described by Rouser *et al* [129]. The ceramide-containing fraction was eluted from the column as the third fraction (TEAE fraction 3) with a mixture of 67% chloroform and 33% methanol (v/v).

Hydrofluoric acid treatment for lipids
Lipids were transferred to a plastic vial and dried under nitrogen. Samples were dissolved in 50 μl of hydrofluoric acid (HF) and sonicated for 7 minutes. After incubation O/N at 4°C, HF was evaporated under a stream of nitrogen. Methanol (50 μl) was added and evaporated 3 times to remove traces of HF [130]. The lipids were transferred to glass vials and were stored at -20°C.

Mass spectrometry
Matrix assisted laser-desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS) of both *A. lumbricoides* and *A. suum* TEAE fraction 3 before and after HF treatment was performed in the reflector positive-ion mode on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) using 6-aza-2-thiothymine as a matrix (5 mg/ml in water; Sigma). Fragment-ion spectra were acquired with laser-induced fragmentation as outlined previously [131].

ELISA
For glycolipids, polysorp microtitration plates (Nunc, Denmark) were coated with 0.3% (IgG4 and IgE) or 0.05% (IgG) of the ceramide containing lipid fraction derived from 4 grams of worm, dissolved in methanol. Each well was coated either with 25 μl of lipid in methanol or with methanol only as a control, and the plates were air-dried overnight at room temperature.

For proteins, maxisorp microtitration plates (Nunc, Denmark) were coated overnight at room temperature with 100 μl 5 μg/ml (for IgG4 and IgE) and 0.83 μg/ml (for IgG) *Ascaris lumbricoides* proteins in PBS. As controls, wells were incubated with PBS only.

The following incubations were at 37°C while shaking, with a volume of 100 μl per well, unless stated otherwise. Between each incubation, plates were washed 4 times with 0.5 mM PBS / 0.01% (v/v) Tween-20. Plates were blocked by incubation with 200 μl of blocking solution 0.07% (w/v) bovine non-fat dry milk in PBS for an hour. Plasma samples as well as detection antibodies were diluted in blocking solution. Plates were incubated with plasma solutions (1/100, 60 minutes for IgG or 1/20, 90 minutes for IgG4 and IgE), followed by incubation with biotin-conjugated IgG (goat-anti-

human total IgG, 1/10,000, 60 min. Sanquin, Amsterdam, The Netherlands), biotin-conjugated IgE (goat-anti-human IgE, 1/1000, 90 min. Vector, Burlingame, CA) or HRP-conjugated IgG4 (goat-anti-human IgG4, 1/3000, 90 min. Vector, Burlingame, CA). The plates for determination of IgG and IgE antibody reactivity were in addition incubated for 1 hour with streptavidin-HRP (1/10,000 Sanquin, Amsterdam, The Netherlands). Assays were developed at room temperature with 3,3',5,5' tetramethylbenzidin (TMB, Sigma) as substrate. Reactions were stopped by adding 1.8 M H2SO4, and absorbance was read at 450 nm in an automated plate reader. For analysis, optical density (OD) values of each plasma samples from the control-coated wells (PBS or methanol) were subtracted from OD values of antigen-coated wells.

Competition with phoshorylcholine
To compete for antibody binding with the coated proteins or lipids, phosphorylcholine (Sigma) was diluted in blocking solution and added at a final concentration of 10 mM to the coated wells before adding plasma.

Statistical analysis
The Mann-Whitney test was used to compare data between the different patient groups. For comparison of the data with or without application of competition with PC or HF treatment, the Wilcoxon matched pairs test was used. The relationship between antibody levels and total IgE was examined using a Spearman correlation test.

Results

Structural characterization of glycolipids by mass spectrometry
Isolated lipids of *A. lumbricoides* were fractionated on TEAE cellulose according to Rouser [129]. TEAE fraction 3, containing the glycolipids, was analysed by MALDI-TOF-MS (figure 2.1A). The pattern of observed glycosphingolipid species was similar to that observed for *A. suum*, with conservation of oligosaccharide structures, phosphorylcholine substitution pattern, and ceramide structure [130, 132].

Based on the pronounced conservation of structural motifs indicated by the MALDI-TOF-MS data (figure 2.1A), the observed *A. lumbricoides* glycolipids, which also do occur in *A. suum*, were assigned as follows: the species at *m/z* 1000 corresponded to Man(β1-4)Glc(β1-1)ceramide, and the species at *m/z* 1203 to GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide [130]. For the latter glycolipid structure partial substitution with phosphorylcholine was found resulting in detection of PC-6GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide at *m/z* 1369 and *m/z* 1309 with the latter ion arising from specific fragmentation of the phosphorylcholine group, in accordance with

the described fragmentation behavior of PC-substituted biomolecules [132, 133]. The attachment of the PC moiety to the *N*-acetylhexosamine was shown by fragment ion analysis (figure 2.1C). This smallest PC-containing glycolipid of *A. lumbricoides* is also shared with *A. suum* [132]. In addition, a Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide was detected at *m/z* 1567, which may carry a phosphorylcholine substituent on the GlcNAc residue, as confirmed by fragment ion analysis of the species at *m/z* 1734 (figure 2.1B).

Larger structures were found to contain PC throughout and exhibited one or two additional hexose residues (*m/z* 1896 and *m/z* 2058, respectively) or one additional HexNAc unit (*m/z* 1937). The first two compositions are in accordance with the structures Gal(β1-3)Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide and Gal(β1-6)[Gal(β1-3)]Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide which both are also found in *A. suum* [132]. No mass spectrometric evidence was obtained for alternative biosynthetic pathways like in *C. elegans*, which expresses complex neutral glycosphingolipids with methylated fucose residues [134].

As for the ceramide structures, the major ceramide dihexoside signals observed for *A. lumbricoides* at *m/z* 998 (not labeled) and *m/z* 1000 (figure 2.1A) are in accordance with the major ceramide compositions observed for neutral and PC-substituted glycosphingolipids of other nematodes, namely C17 sphingosin or sphinganin, which may exhibit a branched hydrocarbon chain, and is amide-linked predominantly to hydroxylated tetracosanoic acid [121, 130, 132, 135, 136]. Based on the MALDI-TOF-MS data, the ceramide composition of the larger glycosphingolipids seems to be largely identical to the ceramide composition of ceramide dihexoside.

Taken together, we found that *A. lumbricoides* glycolipids exhibited typical nematode-type glycolipid structures, which were partially substituted with PC. The similarity with glycolipids of *A. suum* was very high, for which detailed structural information is available in literature [130, 132]. As the latter one is more readily available in larger amounts, *A. suum* glycolipids were used in this study. In addition, we found no differences in antibody responses when lipids from either *A. lumbricoides* or *A. suum* were used in a pilot study in 2 independent experiments with 4 plasma samples, which confirms that *A. lumbricoides* and *A. suum* are highly related [137]. *A. suum* glycolipids were prepared in the same way. Analysis of the *A. suum* preparation by MALDI-TOF-MS indicated nematode-specific glycolipids of 2 to 5 monosaccharide residues, with Gal(α1-3)GalNAc(β1-4)[PC-6]GlcNAc(β1-3)Man(β1-4)Glc(β1-1)ceramide as the major PC-containing species (data not shown). In order to be able to assign the role of PC in antibody-binding studies, the PC-moiety was selectively removed by HF-treatment, which

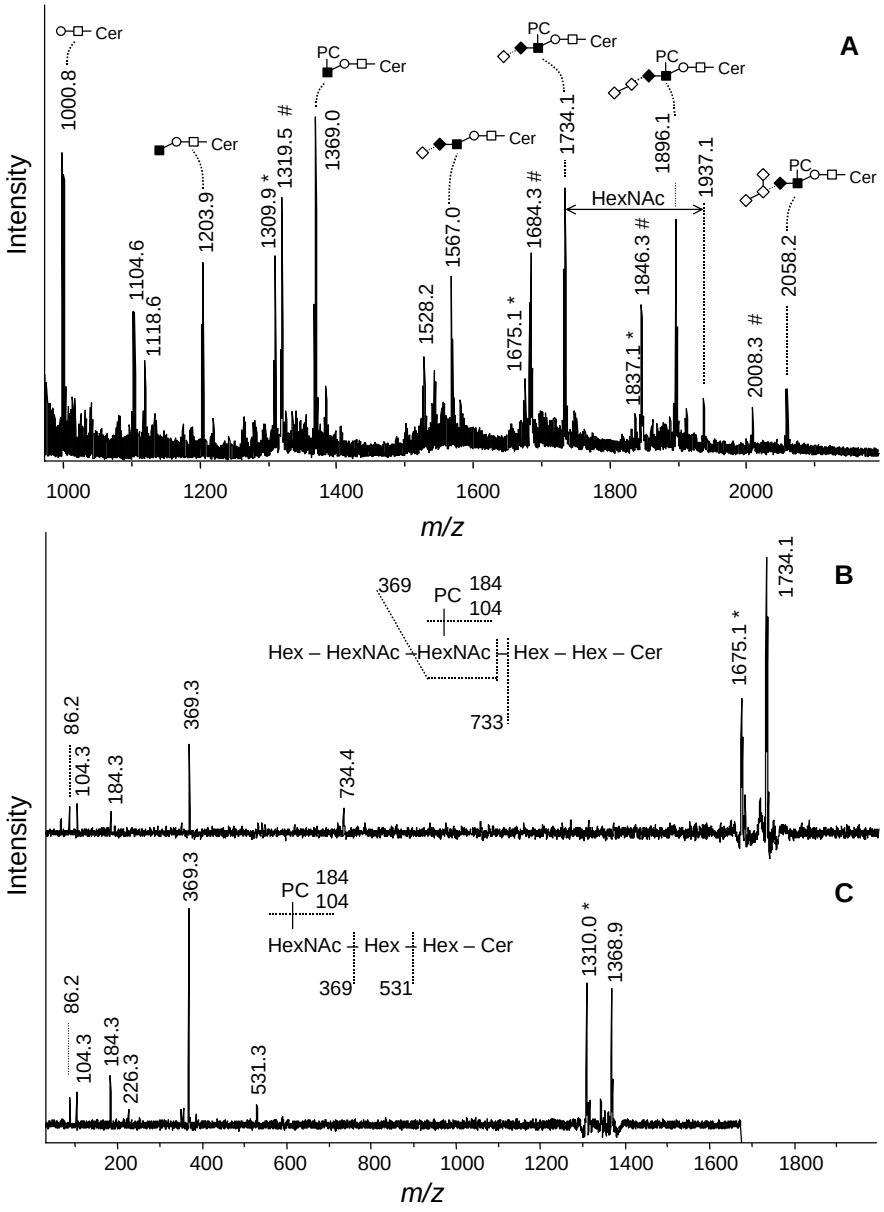

Figure 2.1. Mass spectrometric characterization of *A. lumbricoides* glycolipids.
((A) MALDI-TOF-MS of *A. lumbricoides* glycolipids in the positive-reflector mode. (B, C) MALDI-TOF/TOF-MS fragment ion spectra of two *A. lumbricoides* glycolipids. Fragment ions were registered for the two species at *m/z* 1734 (B) and *m/z* 1369 (C) observed in (A). The following symbols are used: open circle, mannose; open square, glucose; filled square, *N*-acetylglucosamine; open diamond, galactose; filled diamond, *N*-acetylgalactosamine. Lines between monosaccharide residues were placed as follows: continued line, β-linkage; dashed line, α-linkage; vertical line, 6-linkage; horizontal line, 4-linkage; oblique line, 3-linkage; Hex, hexose; HexNAc, *N*-acetylhexosamine; Cer, ceramide; PC, phosphorylcholine; #, metastable fragment arising from PC-containing glycolipids; *, loss of trimethylamine from the choline group (Δm=59 Da).

either removes the total PC molecule, or the choline part only. HF-treatment was monitored by MALDI-TOF-MS, which indicated the specific removal of the PC substituent, resulting in a preparation of unsubstituted nematode-type glycolipids of 2 to 5 monosaccharide residues (data not shown).

IgG antibody responses to *Ascaris* lipids

The IgG reactivity to *Ascaris* glycolipids was measured in plasma samples of 61 subjects (table 2.1) from Makassar, Indonesia, as well as in plasma samples obtained from 14 European controls. *Ascaris* glycolipids were recognized by IgG antibodies and IgG reactivity increased with increasing infection intensity (figure 2.2) with antibody levels in heavily infected children being significantly higher than in the European controls (p=0.017). In order to determine the role of phosphorylcholine (PC) as an epitope for antibody recognition of *Ascaris* glycolipids, the same experiments were performed in the presence of free soluble PC. In the presence of soluble PC the antibody reactivity decreased in all Indonesian groups, with the decrease reaching statistical significance in the group of heavily infected children (figure 2.2). In addition to the competition assay, the PC moieties were removed from *Ascaris*-derived lipids by hydrofluoric acid (HF) treatment. HF treatment abolished antibody reactivity in all groups (figure 2.2), showing PC plays a major role in recognition of *Ascaris* glycolipids by IgG.

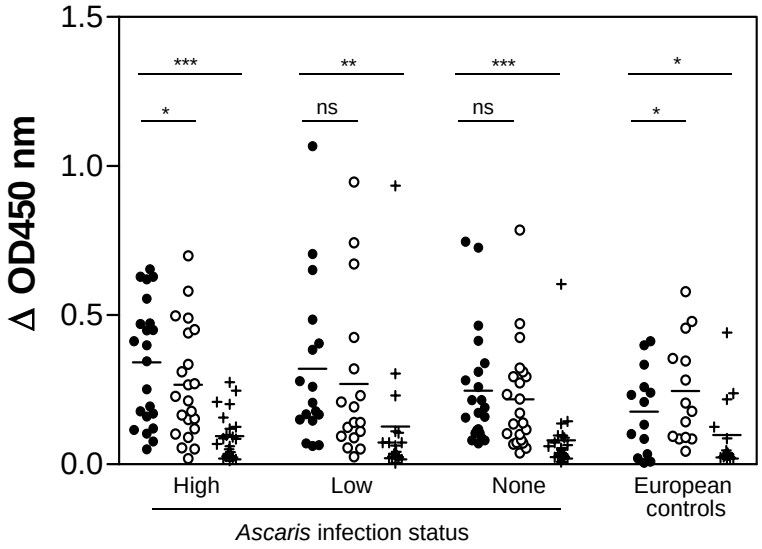

Figure 2.2. Reactivity of total IgG to *Ascaris* lipids. Antibody binding is plotted as ΔOD450 nm (OD coat with lipid – OD coat with methanol). Filled circles; no treatment, open circles; competition with PC, plus sign; HF treatment of the glycolipids. Within the groups antibody activity without treatment is compared to competition with PC (short line) or HF treatment (longer line) *: p<0.05, **: p <0.001, ***: p<0.0001, ns: not significant.

Total IgG responses to *Ascaris* proteins

In contrast to the IgG response to glycolipids, the highest IgG reactivity to *Ascaris* proteins was found in children with light infections; their antibody reactivity being significantly higher than endemic (p=0.016) or European (p=0.007) controls (figure 2.3). Although not statistically significant, children with high infection levels showed lower IgG reactivity to *Ascaris* proteins (p=0.11), suggesting that high intensity infections lead to diminished IgG responses to proteins.

Upon competition with soluble PC, IgG reactivity of the European controls was almost completely abolished (figure 2.3). The antibody responses of the infected groups and the endemic controls also showed a significant reduction in these competition experiments, indicating that PC plays a considerable role in antibody reactivity to *Ascaris* proteins. However, unlike European plasma samples, in Indonesian plasma samples substantial reactivity to *Ascaris* proteins remained after PC reactivity had been competed away. Again the highest IgG reactivity was seen in the lightly infected group, followed by those carrying intense infections (p=0.052). Both of the infected groups with light and heavy infections showed significantly higher remaining IgG reactivity than endemic (p=0.0003 and 0.03 respectively) and European (< 0.0001 and p=0.0005) controls. Proteins were not treated with HF in addition to the competition assay, as this treatment variably leads to precipitation of the proteins (Wuhrer, unpublished observation).

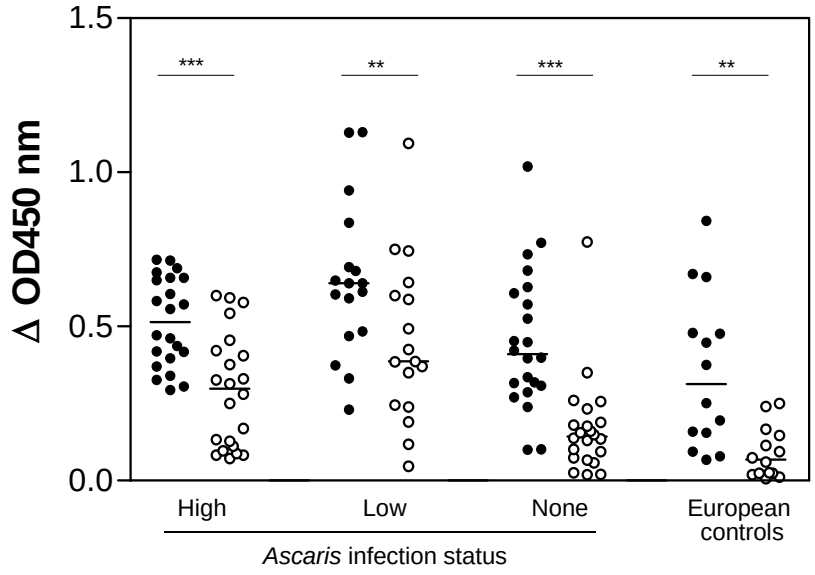

Figure 2.3. Reactivity of total IgG to *Ascaris* proteins. Antibody binding is plotted as ΔOD450 nm (OD coat with protein – OD coat with PBS). Filled circles; no treatment, open circles; competition with PC. Within the groups antibody activity without treatment is compared to competition with PC, indicated by a line. **: p <0.001, ***: p<0.0001.

IgE and IgG4 antibody responses

Since IgG4 and IgE are reported to be important antibody isotypes in helminth infections in the context of immunity, infection or reinfection, we analysed reactivity of these antibody isotypes against *Ascaris* glycolipids. With respect to IgG4 we found no responses in the European controls for either proteins or glycolipids. IgG4 antibody responses to proteins were most pronounced in the group of highly infected children (p=0.0003 and p<0.0001 compared to endemic or European controls respectively). There was a decreasing gradient of IgG4 antibodies to *Ascaris* proteins from the highly infected group to European controls (table 2.2). No IgG4 reactivity was found to *Ascaris* glycolipids in highly infected, non-infected and European control groups. Marginal levels of IgG4 to glycolipids were found in the lightly infected subjects. When examining IgE responses, highest levels of IgE to proteins were found in the group of heavily infected children (compared with endemic controls p=0.042), no significant differences were found between the other groups studied. IgE reactivity to glycolipids was weaker, but interestingly, individuals with light and low infections, showed higher IgE responses to *Ascaris* glycolipids compared with those with high intensity of *Ascaris* infections who had no detectable IgE reactivity to glycolipids. In addition, specific IgE reactivity to the proteins was positively correlated with total IgE (rho = 0.39, p = 0.0022) whereas no correlation was found between total IgE and IgE to *Ascaris* glycolipids.

Table 2.2. IgG4 and IgE antibody reactivity to *Ascaris* proteins and lipids (ΔOD values).

	Ascaris infection status			
	High	Low	None	European controls
Protein				
IgG4	0.137	0.073	0.028	0.010
IQR	0.049-0.397	0.024-0.377	0.010-0.047	0.010-0.010
IgE	0.247	0.226	0.195	0.213
IQR	0.183-0.314	0.162-0.286	0.160-0.218	0.167-0.275
Lipid				
IgG4	0.011	0.019	0.012	0.010
IQR	0.010-0.010	0.010-0.028	0.010-0.012	0.010-0.010
IgE	0.016	0.041	0.033	0.019
IQR	0.010-0.014	0.010-0.096	0.012-0.078	0.010-0.038

Values below a ΔOD of 0.01 were regarded as negative and were given the value 0.01. IQR: Interquartile range.

Discussion

Mass spectrometric characterization of the glycolipids of *A. lumbricoides*, obtained from infected Indonesian children, revealed for the first time that typical nematode-type structures are present in this species. In particular, the similarity to glycolipids of *A. suum* [130, 132] is very high, as indicated by the obtained MALDI-TOF-MS and MALDI-TOF/TOF-MS data. Structural features of glycolipids including oligosaccharide backbone, substitution with PC, and ceramide composition are to a large extent shared between all the parasitic nematode species for which glycolipids have been characterized so far, including *A. suum* [130, 132], *O. volvulus* [136], and *A. vitae* [138]. Notably, also the non-parasitic nematode *C. elegans* exhibits these PC-substituted glycosphingolipid structures [135] and it has just recently been shown that they have a role in development and fertility [139], next to the established immunomodulatory role of PC-substituted glycolipids and/or glycoproteins in parasitic settings [120]. The characterization of *A. lumbricoides* glycolipids here in combination with antibody profile analysis has indicated that antibody reactivity to *Ascaris* glycolipids develops during infection and that the PC moiety, which is expressed abundantly, plays a prominent role therein.

Antibodies to glycolipids are known to play a role in infections with mycobacteria, where these antibodies have been used for diagnostic purposes [140]. The presence of antibodies to *Ascaris* glycolipids indicates that this might also be common to humoral responses in helminth infections, as antibodies to glycolipids in humans with schistosomiasis were found to play a role as well [116, 117]. Although antibodies directed to glycolipids could arise from cross-reactivity with PC present on proteins, it is also possible that antibodies develop directly to glycolipids. The pathway via which these responses are generated is still unknown, however CD1d, a nonclassical MHC molecule presenting lipids, has recently been shown to play a role in the production of *Borrelia hermsii* specific IgM by marginal zone B cells, that have high expression levels of the CD1d [141].

Interestingly, the responses to *Ascaris* glycolipids could be partially inhibited by competition with free PC, but almost completely by HF treatment. This difference might be accounted for by the fact that some antibodies will recognize only the PC moiety and can bind free PC whereas other antibodies that bind to PC, do so in the context of structures it is attached to and therefore will not be competed away by free PC alone [142]. The possibility that HF treatment destroys glycolipids was ruled out not only by mass spectrometry, but also functionally by showing intact binding of monoclonal antibodies to LacdiNAc (GalNac(β1-4)GlcNAcβ1-) structures present on glycolipids of *Ascaris* both before and after treatment with HF (data not shown).

When we examined the role of PC in antibody responses to proteins we found that competition with PC resulted in inhibition of IgG binding to *Ascaris* proteins in all groups. The effect was much stronger than seen for lipids, implying that although carbohydrate epitopes can be shared between proteins and lipids [118, 119], the epitope nature might be different. The IgG reactivity to *Ascaris* proteins was totally abrogated in European controls indicating that antibody responses to *Ascaris* proteins in European controls result from cross-reactivity with PC-epitopes present on other organisms like bacteria or fungi, to which European controls would be exposed to. As antibody reactivity in plasma from infected Indonesian subjects was only partially reduced, epitopes on *Ascaris* proteins other than PC would be expected to also be recognised by IgG in infected individuals.

The IgG responses to *Ascaris* proteins tended to be highest in lightly infected and lower in heavily infected subjects, which would either argue that these antibodies are involved in reducing worm burden or alternatively, as seen in filarial infections, that with intense infections antibody responses are downregulated [143]. The pattern of IgG reactivity to glycolipids was distinct and correlated with intensity of infection. As PC moieties were the main target of IgG reactivity to glycolipids, this indicates that anti-PC antibodies develop as a function of *Ascaris* infection intensity.

The finding that protein specific IgG4 levels were higher in infected children and, although not significantly, they tended to be even higher in the heavily infected children, supports the notion that IgG4 plays a role in susceptibility to infection and/or is associated with chronic infections, which is in agreement with findings of Palmer and Hagan [112, 144]. In contrast there was minimal IgG4 reactivity to *Ascaris* glycolipids which is in agreement with what has been reported for antibody responses to schistosomal glycolipids [117] and to filarial PC containing antigens [145], indicating that these PC-bearing molecules might suppress IgG4 levels. The mechanism behind this is still unknown, however, PC containing molecules might enhance IgG4 to IgE switching, or these molecules might induce direct IgM to IgE switching by preventing the class switch from IgM to IgG4.

In our study we found no clear differences in IgE antibodies to *Ascaris* proteins in the different groups, although elevated IgE levels to proteins were more often found in the highly infected group. In contrast, IgE to glycolipids seemed present in individuals with light or no infections and absent in those heavily infected with *A. lumbricoides* raising the possibility that such reactivity may contribute to keeping infection levels low. Interestingly, in schistosomiasis, pretreatment levels of IgE to adult worm glycolipids were negatively associated with reinfection, indicating a

possible role of these antibodies in controlling also schistosome infections.

To conclude, this study reports for the first time the structural characterization of glycolipids from *A. lumbricoides* and antibody reactivity to this class of molecules. The phosphorylcholine moiety was a major component of antibody reactivity to both *Ascaris* (glyco)proteins and glycolipids. However, IgG, IgG4 and IgE antibody reactivity profiles to *Ascaris* glycolipids and (glyco)proteins were different. This indicates that these different classes of molecules participate in overlapping, yet distinct, immune responses and when analysing host parasite interactions, not only (glyco)proteins but also glycolipids should be studied.

Acknowledgements
We wish to thank all the Indonesian children and European controls who participated in this study for their cooperation. Further we thank the team in Indonesia and John Tuyp for the help with the field work and Jaap J. van Hellemond for critically reading the manuscript.

3

Cellular and humoral responses to influenza in Gabonese children living in rural and semi-urban areas

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Summary

With the current attention to the pandemic threat of avian influenza viruses, it is recognized that there is little information on influenza in Africa. In addition, the effect of influenza vaccination in African countries could be very different from the outcomes in regions with less exposure to micro-organisms and parasites. To monitor the presence of influenza viruses and investigate the immunological response to influenza vaccination, children in a semi-urban and a rural region of Gabon were studied. Influenza-specific antibody responses to the three strains represented in the vaccine were determined in the serum. Further, cytokine responses were measured after *in vitro* stimulation of whole blood with influenza antigens, before and after vaccination. Pre-vaccination antibody titres to H3N2 were high. Upon vaccination the titres to the three influenza strains increased significantly. Weaker anti-H1N1 and anti-B responses were found in rural versus semi-urban school children after vaccination. Influenza-specific cytokine responses were induced within a week, showing significantly lower IFN-γ and significantly higher IL-5 in the children from rural areas. Pre-vaccination antibody levels indicated influenza viruses circulate in Gabon. Altogether influenza vaccination induces weaker immune responses in a rural than in a semi-urban population of Gabonese schoolchildren.

Introduction

Little information is available on influenza in Africa. The influenza surveillance capacity is weak on this continent and only two countries, South Africa and Senegal, engage in active monitoring of the infection and disease [146]. Along with the global anxiety over the spread of H5N1 avian influenza, concerns have been raised on the lack of accurate data from the African continent [146-149]. From the few sporadic studies conducted in Africa, involving virological assessments, it appears that the problem of influenza virus is probably greatly underestimated. The virus was found to be present and circulate throughout the year or peak seasonally in the studied areas [147]. Even the reporting of influenza, based on clinical manifestations, is difficult in Africa, as symptoms are shared with many other infections that are prevalent in the region, making collection of data on morbidity and mortality particularly difficult. Such data are needed to trace the virus globally and also to identify people at the highest risk who would benefit from preventive vaccination.

Considering vaccination, even less is known about the effectiveness of influenza vaccines in Africa. With the current attention to the pandemic

threat of avian influenza viruses, its global spread and the preparation of preventive and curative vaccines, it is important to start asking the question of what the immunological consequences of influenza vaccination are in African populations. These vaccines are mostly developed and tested in Europe, in North America and in some parts of Asia. It is known that vaccines that have proven efficient in high income countries might perform less well in populations living in low income countries [150, 151].
In Africa, conditions in urban centers (those with high/middle wealth) might approximate those in western and industrialized countries, however, in rural areas lifestyle and exposures to infections are widely different. Many infectious diseases prevalent in Africa, particularly present in rural areas and chronic in nature, are known to be associated with profound alternations of the innate [152-154] and the adaptive [155-157] immune system, which may affect responses to third party antigens. For example, chronic helminth infections, highly prevalent in many rural areas in Africa, are known to be associated with skewing of immune responses towards Th2 [158]. Moreover, infections with some parasitic helminths and protozoa along with mycobacterial, malarial as well as viral hepatitis infections have been shown to induce regulatory immune responses that are characterized by production of high levels of suppressory cytokines such as TGF-β and IL-10 [6, 159-162]. These suppressory molecules might affect responses to vaccines as already documented for oral vaccines as well as tetanus and BCG vaccination [28, 32, 36].

To our knowledge influenza surveillance has never been conducted in Gabon and as symptoms of influenza infection resemble closely that of prevalent infections such as malaria, influenza is clinically not distinguishable. Therefore, antibody and cellular responses to influenza A and B strains were analysed in serum of schoolchildren residing in rural and semi-urban areas in Gabon. Subsequently the children received one dose of influenza vaccine and immune responses at various intervals after vaccination were monitored.

Materials and methods

Study population

The study was conducted in and around Lambaréné in Gabon, Africa. Children from two schools, one in a semi-urban area (Lambaréné) and another in a rural area (PK15), and their parents were informed of the study and written informed consent was received prior to inclusion. Exclusion criteria were: absence of informed consent, absence of stool, urine or blood samples for parasitological analysis or presence of any clinical symptoms. A total of 33 children from the semi-urban school and

22 children from the rural school agreed to participate in the study and met the inclusion criteria. The details of the children are shown in table 3.1. The children included in the study in the rural and semi-urban school, were similar with respect to age and sex ratio. The nutritional status was determined by measuring weight by age and sex and by comparing this with age and sex specific values provided by the Center for Disease Control and Prevention (CDC) [163]. For comparisons, we considered well-nourished children as those with a weight by age and sex >90% of the median corresponding weight by age and sex of CDC reference data.

In the cohorts investigated in this study, 33% of the children in the semi-urban school were infected with *Schistosoma haematobium* whereas in the rural school 100% of children were infected. These results were obtained after 3 independent urine samples were tested. The prevalence after testing one urine sample was 19% and 78% in semi-urban and rural school, respectively. The prevalence of intestinal helminth infections in the semi-urban school and rural school was also significantly different for both *Ascaris lumbricoides* (15% and 55%, respectively, $p= 0.002$) and *Trichuris trichiuria* (12% and 64%, respectively, $p< 0.001$). These values were obtained after two stool samples were examined using the Kato Katz method.

In total 12 out of 55 children were found to be positive for malaria during the study. 2 were infected with *P. malariae* and were treated with chloroquine 10 mg/kg/day for 2 days and 5 mg/kg/day the third day. The remaining 10 children were found to be positive for *P. falciparum* and were treated in case of clinical manifestations. Two children presented symptoms of malaria infection and were treated with sulfadoxin (25mg/kg, single dose) and pyrimethamin (1.25 mg/kg, single dose) (SP) as well as artesunate 4mg/kg/day for 3 days. An additional 4 children whose positivity was reported during the study were treated with SP due to physical complaints.

The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné.

Parasitological diagnostics

Infection with *S. haematobium* was determined by passing 10 ml of urine through a filter with 10 μm pore size and staining the eggs with a ninhydrin solution. From children tested negative two additional urine samples were analysed, to increase the accuracy of the diagnosis. Infection with malaria parasites was determined by staining of thick blood smears with Giemsa, as described elsewhere [164]. The presence of the intestinal helminths *A. lumbricoides* and *T. trichiura,* was determined by analysing stool samples using the Kato Katz method on at least one and when possible two stool samples [165]. In total 35 out of 55 children were

infected with one or more species of helminths examined. Subsequent to blood withdrawal at day 28 after vaccination, all children were treated with a single dose of albendazole (400mg) and those with eggs of *S. haematobium* detected in the urine samples, were additionally treated with a single dose of praziquantel 40 mg/kg.

Table 3.1. Study population

	Semi-urban	Rural	p-value[a]
Age in years; median age (range)	9 (7-12)	9 (7-11)	0.3
Sex M/F	14/19	10/12	0.8
Nutritional status; well-nourished children[b]	17/29 (59 %)	5/20 (25 %)	0.02
S. haematobium[c]	11/33 (33%)	22/22 (100%)	<0.0001
A. lumbricoides[d]	5/33 (15%)	12/22 (55%)	0.002
T. trichiuria[d]	4/33 (12%)	14/22 (64%)	<0.0001
Any helminth	13/33 (39%)	22/22 (100%)	<0.0001
Plasmodium infection[e]	4/33 (12%)	8/22 (36%)	0.033

[a]*Pearson Chi-Square test.*
[b]*Well nourished is defined as a weight by age and sex above 90% of CDC reference data*
[c]*Results of testing 3 independent urine samples.*
[d]*Results of testing 2 independent stool samples.*
[e]Plasmodium falciparum *or* Plasmodium malariae*, at day 0, 2, 4, 7 and/or 14.*

Vaccination and sample collection

On day 0, before vaccination, 3 ml of heparinated blood was used for immunological tests. Children were then vaccinated against influenza (Begrivac 2004/2005; Chiron Behring GmbH, Marburg, Germany) and tetanus (NIPHE, Bilthoven, The Netherlands). Subsequently, 3 ml of heparinated blood was used for immunological assays, drawn on day 2, 4, 7, 14 and 28 after vaccination. At all time points plasma was frozen and kept at -20 °C, and all days except at day 28, whole blood stimulations were performed to collect supernatants for cytokine analysis.

Antibody measurement

For the detection of serum antibodies to influenza virus the haemagglutination inhibition (HI) assay was used. The HI assay was performed in duplicate according to standard methods [166, 167], using turkey erythrocytes and four haemagglutinating units of the vaccine strains, which were propagated in 11-day old embryonated chicken eggs. Ferret sera raised against the test antigens were used as positive controls.

Serum samples were treated with cholera filtrate to remove non-specific anti-haemagglutinins. To ensure comparability, all serum samples collected at different time points, were tested at the same time. For sera with titres below the detection level, the value of 5 was assigned. The virus neutralization assay was performed according to standard procedures as described previously [168].

Whole blood culture and cytokine measurement

Cellular immunological analysis involved culturing of whole blood. 100 µl of blood was cultured with 100 µl of RPMI-1640 medium or influenza vaccine in medium (predetermined optimum of 1 µg HA/ml). Supernatant was collected after 72 hours of incubation and was kept at -20 °C until analysis.

Levels of IFN-γ, IL-5, IL-13, TNF-α and IL-10 were determined simultaneously in the supernatants by using the Luminex-100 cytometer (Luminex Corporation, Austin, TX, USA), equipped with StarStation software (Applied Cytometry Systems, Dinnington, UK). Buffer reagent kits and Luminex cytokine kits (BioSource, Camarillo, CA, USA) were used and cytokines were measured according to the protocol, with slight modifications. Briefly, assays were performed in 96-well round bottom plates (Nunc) at room temperature. A mix of beads was incubated with a standard, samples, or blank in a final volume of 50 µl for 2 hours under continuous shaking. Plates were washed twice and incubated with a cocktail of biotinylated antibodies (25 µl/well) for 1 hour. After removal of excess biotinylated antibodies by washing twice, streptavidin-RPE was added and incubated for 30 minutes. Subsequently plates were washed a final time and analysed using the Luminex-100 cytometer. The lower detection limit of the assays was 3 pg/ml for IL-5, 5 pg/ml for IL-10 and IFN-γ and 10 pg/ml for IL-13 and TNF-α. Samples with concentrations below the detection limit were assigned the value of this threshold. To determine influenza specific cytokine production, background cytokine production (cultured with medium only) was subtracted from the values obtained after stimulation of the blood cells with influenza vaccine.

Statistical analysis

The distribution of age, sex, nutritional status and prevalence of parasitic infections between the study groups were tested using Pearson's Chi square test. Cytokine and antibody levels were not normally distributed and the Mann-Whitney test was used to analyse these data. Logarithmic transformation of cytokine and antibody levels resulted in a normal distribution and these values were used for linear regression analysis. Results from statistical analyses, using SPSS or Graph Pad Prism, were considered significant when the p-value was below 0.05.

Results

Seroprevalence of antibodies to influenza viruses prior to vaccination
Results obtained with the haemagglutination inhibition (HI) assay showed that influenza virus specific antibodies were already present in the majority of sera before vaccination (figure 3.1), indicating that influenza A viruses of the H1N1 and H3N2 subtypes and influenza B virus have been circulating in Gabon prior to the study. Interestingly, a high seroprevalence was found of antibodies specific for the H3N2 strain A/Wyoming/3/2003 compared to that for the influenza H1N1 and B strains before vaccination. The presence of high titres of pre-existing antibody levels was confirmed using an alternative method for influenza serology. A virus neutralization assay was performed with the sera obtained from a subset of 19 children from both groups, showing similar titres; 1593 (range 160-20 480) and 1481 (range 80- 10240) for the HI and neutralization assay, respectively. Thus the pre-vaccination A/Wyoming/3/2003 X147 (H3N2) specific antibody levels indicate that an outbreak of infection caused by a strain related to A/Wyoming/3/2003 must have occurred recently.

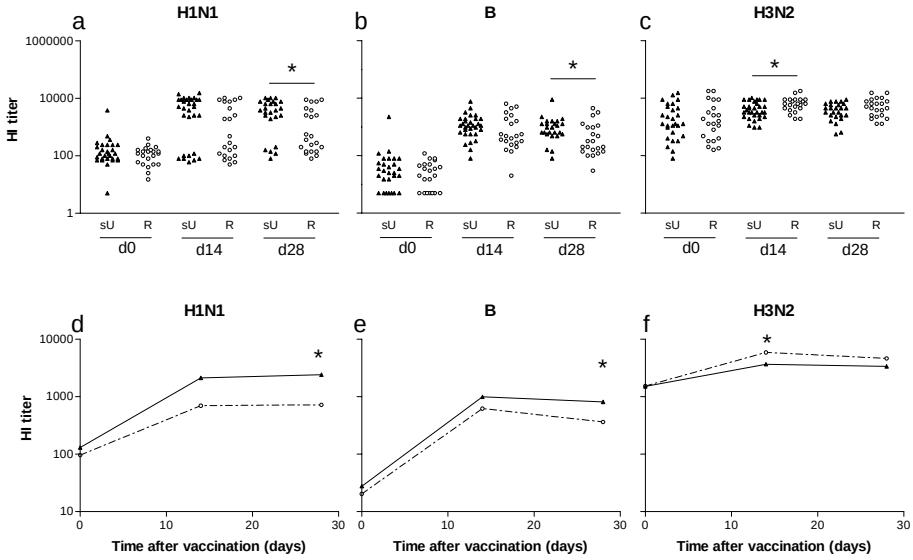

Figure 3.1. HI titres to the three influenza strains present in the vaccine before and 14 and 28 days after vaccination. Graphs a-c show the individual data; in d-f the kinetics of the responses are shown, geometric mean titres per group are given. a, d: A/New Caledonia/20/99, IVR-116(H1N1); b, e: B/Jiangsu/10/2003; c, f: A/Wyoming/3/2003 X147(H3N2). Closed triangles (and lines): semi-urban (sU) subjects; open circles (and dashed lines): rural (R) subjects, * $p < 0.05$ (Mann-Whitney test).

Efficacy of vaccination: antibody production

The antibody responses to the three different influenza virus strains present in the vaccine were determined not only before, but also 14 and 28 days after vaccination (figure 3.1). Upon vaccination, HI titres to the influenza A (H1N1) strain increased in both the rural and the semi-urban children, but reached significantly higher levels in the semi-urban than in the rural children on day 28 (figure 3.1a and d). Two groups of children could be identified, one group whose anti H1N1 titres remained at a level only slightly higher than the pre-vaccination values (low responders) and another group of children that showed a prominent increase in antibody responses (high responders) (figure 3.1a). The latter group was significantly larger in the semi-urban population (figure 3.2a), resulting in the overall higher titres in the semi-urban children.

Within the semi-urban children there was a clear difference in the H1N1 antibody response of those infected with helminths (*S. mansoni, A lumbricoides and/or T. trichiuris*) and those without helminth infection. Thus, when only the group of semi-urban children without helminth infections was compared to the rural cohort, the difference in levels of H1N1-specific antibody titres became highly significant ($p < 0.01$), at both day 14 and day 28 after vaccination. Indeed, also in terms of percentage high responders, it was clear that helminth infected children in the semi-urban area behaved more like rural children (figure 3.2a). In addition, infections with plasmodia affected anti-H1N1 titres in rural children but not semi-urban children. As shown in figure 3.2b only few rural children with plasmodia were high responders when anti H1N1 antibody titres were considered.

The antibody titres specific for the influenza B-strain increased upon vaccination, reaching a peak 14 days after vaccination, and slightly decreasing thereafter (figure 3.1 b and e). Also for this strain, responses were significantly higher in the semi-urban children on day 28.

The levels of antibodies to the influenza A (H3N2) strain, which were very high before vaccination compared to pre-vaccination antibody levels specific for the H1N1 or the B strain of the vaccine, still increased slightly upon vaccination. At day 14, antibody levels were significantly higher in rural children compared to the semi-urban group, but this difference was not significant anymore at day 28 (figure 3.1c and f).

For antibodies to influenza B or H3N2 strains, neither helminths nor malaria infection influenced the responses significantly.

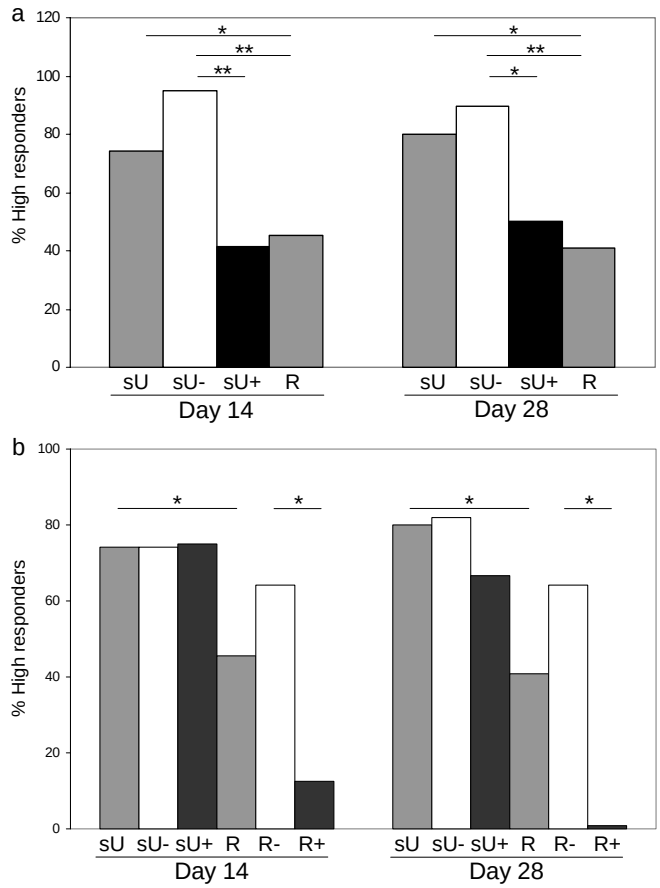

Figure 3.2 Influence of helminth (a) and malaria (b) infection on antibody responses to influenza A (H1N1). As shown in figure 3.1, antibody responses to the H1N1 strain were either low or high. In a, the percentage of high responders are shown for the total group of semi-urban (sU) children (both infected and uninfected; in grey), the semi-urban children without any helminth infection (sU-; white bars), the semi-urban children with a helminth (*S. mansoni, A lumbricoides* and/or *T. trichiuris*) infection (sU+; black bars) and the rural (R; grey) children (all infected). In b the percentage of high responders are shown for the total group of semi-urban (sU) and rural (R) children (both infected and uninfected; grey bars), the semi-urban and rural children without malaria infection (sU- and R-; white bars) and the semi-urban and rural children with malaria infection (sU+ and R+ black bars). * $p < 0.05$, ** $p \leq 0.005$; Chi Square Test.

Efficacy of vaccination: cytokine responses

The cytokine response to influenza was determined at different time points to follow the kinetics of cellular immune response development upon vaccination (figure 3.3). After day 2 following vaccination, cytokine responses started to rise. The IL-10 response was early and a tight peak was seen in both rural and semi-urban school children on day 4 (figure 3.3a).

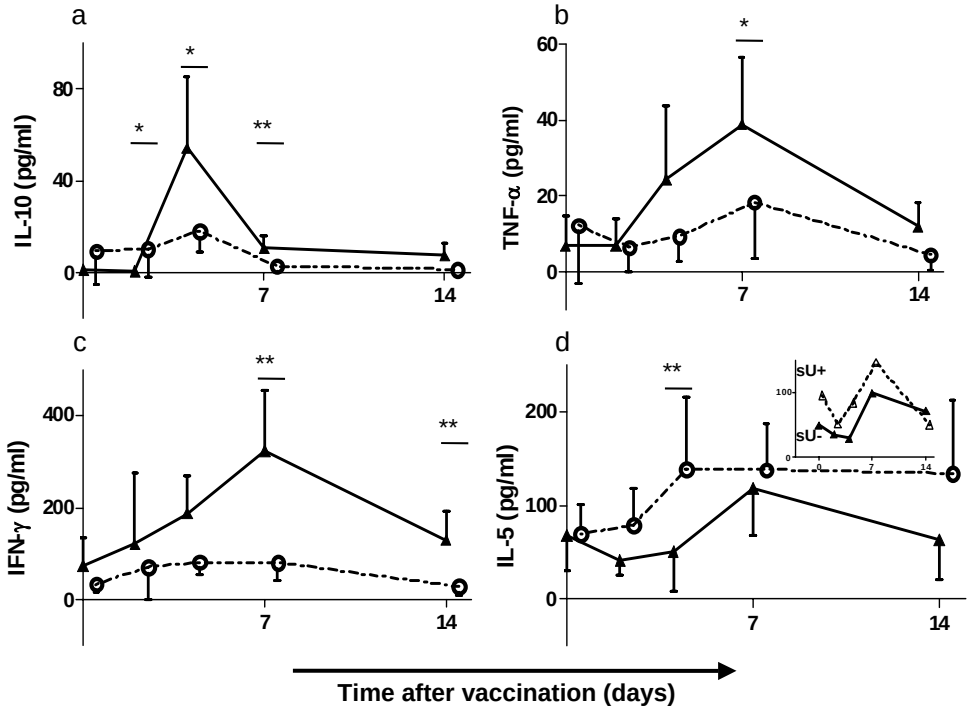

Figure 3.3. Kinetics of influenza-specific cytokine production determined by *ex vivo* stimulation of whole blood before and after influenza vaccination; mean values and the 95% confidence interval of a: IL-10; b: TNF-α; c: IFN-γ; d: IL-5 are shown. Closed triangles and lines: semi-urban cohort; open circles and dashed lines: rural cohort, * $p < 0.05$; ** $p \leq 0.005$ (Mann-Whitney test). In the insert in graph d, the IL-5 responses for the semi-urban children with helminth infection (sU+) and the semi-urban children without helminth (*S. mansoni, A lumbricoides* and/or *T. trichiuris*) infection (sU-) are shown.

The magnitude of the influenza specific IL-10 response was significantly higher in the semi-urban compared with rural school children. The TNF-α, IFN-γ and IL-5 responses peaked at day 7 (figure 3.3b-d). The TNF-α levels were significantly higher in semi-urban schoolchildren; the peak at day 7 was almost absent in the rural group (figure 3.3b). Starting from day 7 after vaccination IFN-γ responses were significantly higher in semi-urban children (figure 3.3c). In contrast, rural children showed a more Th2 skewed response, as they produced less IFN-γ, and higher levels of IL-5 compared to the semi-urban group (figure 3.3d). Interestingly, higher IL-5 responses to influenza were seen in the helminth infected semi-urban children compared to the non-infected semi-urban children (insert in figure 3.3d), indicating that the Th2 skewing that is more prominent in the rural group may, at least partly, be the result of the immune modulating effect of helminths. Malaria infection did not affect cytokine responses to influenza antigens.

Discussion

The current study indicates that influenza A and B viruses circulate in Gabon and the presence of high levels of A/Wyoming/3/2003 X147 (H3N2) specific antibodies before vaccination, as determined by a HI and a neutralization assay, provide evidence for a recent outbreak of a H3N2 virus. Sporadic monitoring of influenza in other African countries, has revealed outbreaks of H3N2 virus in South Africa in 2003 [169], in Madagascar in 2002 [147] and in the Democratic Republic of Congo in 2002 [147]. In addition, influenza B viruses have been reported to be circulating in South-Africa, Madagascar and Kenya [170, 171].

In some of these studies, a mortality of at least 3 percent of cases was reported, due to influenza A/Panama/2007/97-like (H3N2) infection, indicating that influenza can have a high impact also in African countries [147]. No data are available on influenza mortality or morbidity in Gabon and given our findings, it would not be surprising if considerable mortality in the study area may be attributed to influenza epidemics. This is particularly important for the area we studied, in fact for most of Africa, where malaria is endemic and high fever is often treated with anti-malarials. Thus, influenza infections in these areas can lead to considerable malaria over-medication on the one hand and over-estimation of malaria deaths, on the other.

Considerable differences were observed in vaccine induced antibody responses between rural and semi-urban school children. The responses to H1N1 and influenza B strains were higher in the children from the semi-urban area. As the pre-vaccination titres did not differ between semi-urban and rural children, it is unlikely that differences in exposure to influenza could explain these results. Why children in rural areas would respond differently than those in semi-urban areas has yet to be fully investigated. However, some vaccines like BCG or tetanus, that were shown to be effective in non-tropical countries, were found to induce a weak response in tropical countries and this has been associated with the presence of helminth infections [35, 150]. Moreover, although to our knowledge the effect of helminth infections on the efficacy of influenza vaccination has not been investigated previously, studies on the efficacy of cholera vaccine [28], BCG [31] and TT vaccine [34, 36] suggested that immune skewing in response to vaccines is affected by pre-existing helminth infections. These studies were performed in different areas covering intestinal helminth [31], filaria or schistosome infections [34, 38]. One of the major differences between the Gabonese cohorts of rural and semi-urban schoolchildren investigated in this study is the extent of exposure to parasitic infections as shown in table 3.1. Helminth infections were shown to affect H1N1-

specific antibody titres in semi-urban children as shown in figure 3.2a. In addition it was found that malaria-infected children in the rural cohort were more often low responders for H1N1 than children without malaria infection. This effect was not found for the semi-urban group, raising the possibility that malaria infection in the helminth infected group (all children in the rural area were infected with helminths) has strong suppressory effect. Alternatively, malaria treatment could have affected the outcome, although several studies did not find a negative effect of malaria treatment on outcome of immunization [172-174]; only long term treatment with chloroquine has been associated with impairment of vaccination efficacy [175]. However, malaria infection or treatment did not seem to affect the antibody titres to the influenza A (H3N2) and the influenza B strain nor the cytokine responses in this study.

Another difference noted between semi-urban and rural children was the nutritional status as shown in table 3.1. However, the differences between antibody titres could not be explained by differences in nutritional status.

The anti-A/Wyoming/3/2003 X147 (H3N2) antibody levels, which were very high before vaccination, increased further after vaccination. Interestingly, the post-vaccination antibody titres were higher in the rural children, but on day 14 after vaccination only. Thus, post vaccination antibody titres to a virus strain that had circulated recently, were not different or even higher in rural children. This is different to what is observed with post vaccination titres to strains that had not caused a recent epidemic. The mechanism behind this is not clear but may be due to differential requirement of central or effector memory T-cells to become activated [176].

In terms of cytokine responses, the semi-urban subjects show a stronger influenza-specific Th1 response as determined by increased IFN-γ and reduced IL-5 levels compared to children from a rural area. It is possible that next to the effect of helminth infections on antibody responses in rural areas, the characteristic Th2-like immune responses evoked by helminths, as well as immunological hyporesponsiveness, affect the efficacy of vaccines [35, 150], which are expected to induce a Th1 response, by skewing cytokine responses towards Th2.

Interestingly, there was a positive correlation (Pearson correlation coefficient = 0.17, $p = 0.034$) between levels of IFN-γ production and nutritional status (weight by age) in the urban children, but not in the rural cohort (data not shown). This correlation was not influenced by helminth infection. The influence of nutritional status on cytokine production has not been studied extensively but in a mouse model the cytokine production in response to helminth antigens was determined, finding decreased IFN-γ levels in response to zinc deficiency [177].

TNF-α responses to influenza vaccination were significantly lower in rural children. An increase in production of IL-10 and/or regulatory T cell responses could explain downregulation of Th1 as well as pro-inflammatory TNF-α responses. However, we observed an increased rather than a decreased IL-10 response to influenza vaccination in the semi-urban group. Although it is known that high levels of IL-10 are often associated with helminth infections [36, 53], little is known of the IL-10 response upon influenza vaccination. More studies on the source of IL-10 early after vaccination are needed to fully understand the detailed cellular responses.

Taken together, we report here that influenza vaccination seems to be more effective in a semi-urban than in a rural population of Gabonese schoolchildren for influenza virus strains that did not cause a recent outbreak. With respect to cytokine production upon *in vitro* stimulation of whole blood with the vaccine, lower IFN-γ responses are elicited in a rural population. Moreover, a better diagnosis of influenza and its dis-tangling from co-endemic diseases such as malaria can have important implications for over/mis-medication. Finally, epidemiological data on influenza as well as immune responses to vaccination will be critical for managing epidemic influenza properly in Africa.

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4

Cellular and humoral responses to tetanus vaccination in Gabonese children

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Summary

Protection to tetanus is often not optimal in developing countries due to incomplete vaccination schemes, or decreased efficacy of vaccination. In this study we investigated the immunological response to tetanus booster vaccination in school children living in a semi-urban or in a rural area of Gabon. Tetanus-specific total IgG as well as antibody subclasses of the IgG1, IgG2, IgG3 and IgG4 isotype and the avidity of the dominating IgG1 subclass were determined both before and one month after the booster vaccination. In addition, tetanus specific cytokine responses were determined. We found a polarization towards a T helper 1 (Th1) profile in the semi-urban children, whereas the cytokine responses of the rural children showed a T helper 2 (Th2) skewed response. Furthermore, tetanus specific antibodies of the different IgG subclasses were all increased upon a tetanus booster vaccination and levels of IgG1 and IgG3 were higher in the rural children. In conclusion, a tetanus booster vaccination induced a stronger Th2 over Th1 cytokine profile to tetanus toxoid (TT) in rural children who showed the highest levels of IgG1 and IgG3 anti-TT antibody responses.

Introduction

Tetanus toxoid (TT) is a T-cell dependent antigen and induces long-lasting immunity against tetanus. The immune response to TT vaccination has been extensively studied in populations in developed countries to show that protection is dependent on the amount of IgG antibodies, the subclass distribution, which is mainly dominated by IgG1, and the avidity of the produced antibodies [178]. Avidity is considered to be a parameter for the efficacy of the antibodies to neutralize the antigen as it reflects the collective functional affinities of the antibodies formed during a polyclonal humoral immune response [178]. Worldwide, TT vaccination has lead to a greatly reduced morbidity and mortality associated with tetanus infection. However, efficacy can still be improved as long-term protection is often not established [179]. Moreover, exposure is still high in certain areas and therefore protection is very important [180]. Previous studies examining tetanus vaccination in developing countries have mostly investigated the effect that parasite infections might have on vaccination efficacy. Studies in onchocerciasis patients have shown that helminth infected subjects responded less efficiently to tetanus vaccination as defined by reduced IFN-γ production by T cells and/or reduced levels of antibodies [35-37]. In schistosomiasis the cytokine balance towards the tetanus vaccine was also shown to be skewed towards a Th2 response compared to a Th1 or Th0 response in uninfected controls, but no antibodies were measured in this

study [38]. Moreover, earlier studies focused mainly on (young) adults.
In the current study the effect of booster vaccination with tetanus toxoid (TT) was investigated in children between 7 and 12 years of age in a rural and in a semi-urban area of Gabon. The antibody responses in all IgG subclasses were determined and, in addition, the avidity of the IgG1 antibodies was examined to study the potential efficacy of the antibodies in neutralizing the antigen. Furthermore, the kinetics of *in vitro* antigen-induced cytokine responses after vaccination were analysed.

Material and methods

Study cohort

The study was conducted in the vicinity of Lambaréné in Gabon, Central Africa. 131 children from Lambaréné, a semi-urban area and 120 children from the Nzilé rural area were examined for the presence of parasites. In the semi-urban area 19 percent of children were infected with *Schistosoma haematobium* versus 78 percent of the children in the rural area. In this study 33 children from the semi-urban area and 20 children of the rural area were vaccinated (table 4.1). However, two children from the rural area cohort were finally excluded, as plasma analysis revealed they had not been vaccinated with TT before, which could be deduced from the relatively low IgG1 anti-TT level measured after immunization, together with a significant level of IgG3 anti-TT. The latter IgG subclass is normally the only subclass being produced in relatively high amounts upon primary vaccination. Moreover, the avidity index (AI) of IgG1 anti-TT antibodies, which is rising after each subsequent vaccination, was much lower in these two subjects in comparison with all other subjects (1.25 and 1.27, whereas all other subjects, both semi-urban and rural, showed values within a range of 2.63 to 3.88).
The nutritional status was determined by measuring weight by age for gender and by comparing this with age and gender specific values provided by the Center for Disease Control and prevention (CDC) [163]. For comparisons, we considered well nourished children as those with a weight by age for gender values higher than 90% of the median corresponding weight by age for gender of CDC reference data.
The study was approved by the ethics committee of the International Foundation of the Albert Schweitzer Hospital in Lambaréné. Written informed consent was obtained from the parents or legal guardians of each child participating in the study.

Parasitological diagnostics and treatment

Infection with *S. haematobium* was determined by passing 10 ml of urine through a filter with 10 μm pore size and staining the eggs with a

ninhydrin solution. From children tested negative two additional urine samples were analysed, to increase the accuracy of the diagnosis.

The presence of the intestinal helminths *Ascaris lumbricoides* and *Trichuris trichiura,* was determined by analysing stool samples using the Kato Katz method [165]. After blood was drawn at day 28 after vaccination, all children were treated with a single dose of albendazole (400 mg) and those with eggs of *S. haematobium* detected in the urine samples were additionally treated with praziquantel (40 mg/kg). Infection with malaria parasites was determined on day 0, 2, 4, 7 and 14 by staining of thick blood smears with Giemsa, as described elsewhere [164]. In total 11 out of 53 children were found to be positive for malaria during the study. 2 were infected with *P. malariae* and were treated with chloroquine 10 mg/kg/day for 2 days and 5 mg/kg/day the third day. The remaining 9 children were found to be positive for *P. falciparum* and were treated in case of clinical manifestations. One child presented severe symptoms of malaria infection and was treated with sulfadoxin (25mg/kg, single dose) and pyrimethamin (1.25 mg/kg, single dose) (SP) as well as artesunate 4mg/kg/day for 3 days. An additional 4 children whose positivity was reported during the study were treated with SP due to mild symptoms.

Table 4.1. Study population

	Semi-urban n=33	Rural n=20	p-value[a]
Median age (range)	9.0 (7-12)	8.5 (7-11)	0.46
Gender (F/M)	19/14	12/8	0.86
Nutritional status; well nourished children[b]	17/29	3/18	0.005
Schistosoma haematobium[c]	11/33	20/20	<0.001
Intestinal helminths[d]	6/33	18/20	<0.001
Malaria[e]	4/33	7/20	0.052

[a]*Pearson Chi-square test*
[b]*Well nourished is defined as a weight by age for gender above 90% of CDC reference data*
[c]*Results of 3 independent urine samples*
[d]Ascaris lumbricoides *and/or* Trichuris trichiuria*; results of testing 2 independent stool samples*
[e]Plasmodium falciparum *or* Plasmodium malariae *at day 0, 2, 4, 7 and/or day 14*

Vaccination and sample collection

At day 0 children were vaccinated with tetanus toxoid (NIPHE, Bilthoven, The Netherlands) and influenza vaccine (Begrivac 2004/2005; Chiron Behring GmbH, Marburg, Germany). Before vaccination, as well as on day 2, 4, 7, 14 and day 28 after vaccination blood was drawn in heparin-coated tubes. At all time points plasma, for serology, was frozen and kept at -20 °C. At all days except day 28 a whole blood assay was performed.

Antibody measurement
Total IgG and IgG subclasses anti-tetanus toxoid (TT) antibodies were quantified in serum samples collected on day 0, day 28 and after 9 months by an antibody-capture enzyme-linked immunosorbent assay (ELISA) [181]. In short, the wells of a 96-well polystyrene microtiter plate were coated with tetanus toxoid, blocked with bovine serum albumin, and incubated with twofold serial dilutions of serum samples and standard sera. Total IgG anti-tetanus toxoid antibodies were measured by the addition of alkaline phosphatase-conjugated goat anti-human IgG (γ-chain specific) (Biosource). Antibodies in the IgG subclasses were measured by successive incubation with IgG subclass-specific monoclonal antibodies (anti-IgG1, MH 161-1, Sanquin, Amsterdam, The Netherlands; anti-IgG2, 35-1-27-2, TNO, Leiden, The Netherlands; anti-IgG3, NI 86, Nordic, Tilburg, The Netherlands; anti-IgG4, NI 315, Nordic, Tilburg, The Netherlands), followed by incubation with alkaline phosphatase-conjugated rabbit anti-mouse Ig (Dakopatts, Glostrup, Denmark). After incubation with substrate (p-nitrophenylphosphate), the reaction was stopped with 3 M NaOH, and the optical density at 405 nm was recorded with a Titertek Multiscan (Labsystems, Helsinki, Finland). Based on the standard sera containing known amounts of anti-tetanus toxoid antibodies, a reference curve was constructed. Use of this curve allowed the calculation of the amount of anti-TT antibodies of the respective classes or subclasses in the sera of the rural and the semi-urban children.
The avidity of IgG1 anti-TT was measured by a modified elution ELISA, in which well-chosen dilutions of serum samples were allowed to interact with TT coated on the wells of microtiter plates [182]. For each of the serum samples, dilutions containing 50 and 25% of the amount of the anti-tetanus toxoid antibodies which can maximally bind to the coated antigen were chosen. Thereafter, the wells were incubated with a variable molarity (range, 0.5 to 4.5 M) of the chaotropic agent sodium thiocyanate (NaSCN). IgG1 anti-TT antibody levels were then measured as described above. The relative avidity index is defined as the molarity of NaSCN at which 50% of the amount of IgG1 subclass antibodies that are bound to the coated TT in the absence of NaSCN, has been eluted from the antigen.

Cell culture and cytokine measurement
For stimulation of the cells 100 μl of blood was cultured with 100 μl of RPMI-1640 culture medium without (control) or with tetanus toxoid (1.5 lf/ml; SVM, Bilthoven, The Netherlands). Supernatant was collected after 72 hours of incubation and kept at -20 °C until further analysis. Levels of IFN-γ, IL-5, IL-13, TNF-α and IL-10 were determined simultaneously in the supernatants by using the Luminex-100 cytometer (Luminex Corporation, Austin, TX, USA), equipped with StarStation software (Applied Cytometry Systems, Dinnington, UK). Buffer reagent kits and Luminex cytokine kits (BioSource, Camarillo, CA, USA) were used and cytokines were measured

according to the instructions of the manufacturer, with slight modifications. Briefly, assays were performed in 96-well roundbottom plates (Nunc, Roskilde, Denmark) at room temperature. A mix of beads was incubated with a standard, samples, or blank in a final volume of 50 µl for 2 hours under continuous shaking. Plates were washed twice and incubated with a cocktail of biotinylated antibodies (25 µl/well) for 1 hour. After removal of excess biotinylated antibodies by washing twice, streptavidin-RPE was added and plates were incubated for 30 minutes. Subsequently, plates were washed a final time and analysed using the Luminex-100 cytometer. The lower detection limit of the assays was 3 pg/ml for IL-5, 5 pg/ml for IL-10 and IFN-γ and 10 pg/ml for IL-13 and TNF-α. Samples with concentrations below the detection limit were assigned the value of this threshold. For further analysis, background cytokine production (stimulated with medium only) was subtracted from the cytokine levels that were produced in response to TT. In order to calculate geometric means from the medium-corrected cytokine data, values below 0.1 pg/ml were arbitrarily set to 0.1.

Statistical analysis

The distribution of age, gender, nutritional status and infection status between the study groups was tested using Pearson's Chi square test. Analysis of cytokine and antibody responses was split into three parts. First, levels of antibodies and cytokines were compared between semi-urban and rural children at each time point. Since cytokine and antibody levels were not normally distributed, the Mann-Whitney test was used to analyse the difference between urban and rural children at each timepoint. For the following parametric analyses, we have used log-transformed antibody and cytokine responses, adjusting for the possible confounding variables age, sex and malaria infection (individuals considered positive if there was positive blood smear at any of the time points during the study). We have not adjusted for nutritional status, since this variable showed a strong negative correlation with the two groups (semi-urban and rural children). Second, we have performed multiple linear regression analysis of antibody responses at day 28 adjusting for baseline levels (antibody levels at day 0) in addition to adjusting for age, sex and malaria. Third, for cytokine data we have performed longitudinal analyses using the Proc Mixed procedure of the SAS statistical software release 9.01. To test changes in the cytokine measurements over time within and between groups, data were analysed by using repeated measurement analysis. Confounding variables were treated as fixed variables. Results from statistical analyses were considered significant when the p-value was below 0.05.

Results

Study population

All study subjects in the rural area were infected with *Schistosoma haematobium* and 18 from the group of 20 were also infected with the intestinal helminths *Ascaris lumbricoides* and/or *Trichuris trichiuria* (table 4.1). These infections were less prevalent in the children in the semi-urban area ($p < 0.001$), where 13 out of 33 children were infected with *Schistosoma haematobium* and/or intestinal helminths. Plasmodium infections were found in 7 out of 20 rural and 4 out of 33 semi-urban children. In addition, the nutritional status was better in the semi-urban compared to the rural cohort. Age and gender distributions were similar in the two study areas.

Antibody responses to tetanus vaccination

All children showed an increase in the levels of anti-TT antibodies in all IgG subclasses one month after vaccination compared to the levels before vaccination ($p < 0.001$ for all, in both the rural and the semi-urban group). As expected for individuals 7 to 12 years old and regularly vaccinated with TT during the first two years of life, both before and after the booster vaccination the total IgG anti-TT response consisted predominantly of IgG1

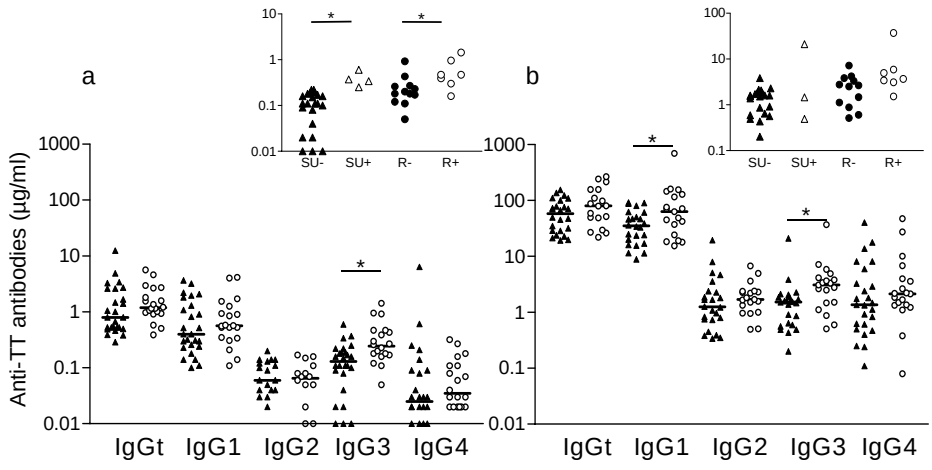

Figure 4.1. IgG subclass responses to tetanus toxoid before (a) and 28 days after vaccination (b). IgGt = total IgG anti-TT. Closed triangles: semi-urban subjects; open circles: rural subjects. Line indicates median value, * $p<0.05$. Inserts in both figure a and b: closed and open circles represent rural children without (R-) and with (R+) reported malaria infection during the study, respectively. Closed and open triangles represent rural children without (SU-) and with (SU+) reported malaria infection during the study, respectively.

(figure 4.1). Differences in IgG1 anti-TT responses were found between the semi-urban and rural subjects, being higher in the rural children one month after vaccination ($p < 0.05$, figure 4.1b).
The geometric mean values of the responses were 33 µg/ml for the semi-urban and 61 µg/ml for the rural children for IgG1 anti-TT. Regarding other IgG subclasses, no differences were found between the groups in levels of IgG2 and IgG4 anti-TT. However, the levels of IgG3 anti-TT were found to be higher in the rural children compared to semi-urban children both before and after vaccination ($p < 0.05$; figure 4.1a and 4.1b). The study children with plasmodium infection showed significantly higher levels of IgG3 anti-TT before vaccination compared to those without the infection, both in semi-urban and rural children ($p<0.05$; insets figure 4.1a). Multiple linear regression analysis indicated that there was still a significant difference between the urban and rural children at day 0 after adjusting for malaria infection ($p<0.01$). After vaccination the titres of IgG3 anti-TT increased in all children and there were no longer significant differences between malaria infected and uninfected children within either cohort (inset in figure 4.1b). Age and sex were very similar in urban and rural children (table 4.1), and adjusting for these variables did not change any of the differences observed between semi-urban and rural children. Baseline levels of antibodies at day 0 also did not change the significant differences found between urban and rural children. The avidity of IgG1 anti-TT 28 days after vaccination did not differ between the semi-urban and the rural group (figure 4.2), indicating that the quality of the antibody response was similar in the two cohorts. Moreover, malaria infection did not influence avidity (data not shown).

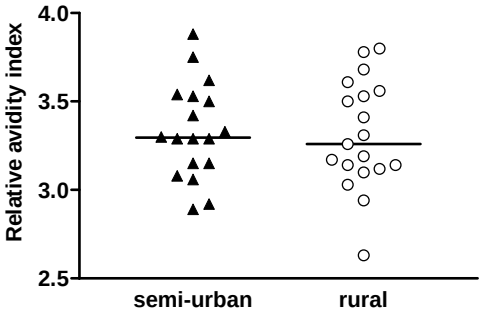

Figure 4.2. Avidity of the IgG1 anti-TT antibodies 28 days after vaccination. Closed triangles: semi-urban subjects; open circles: rural subjects, line indicates the median value.

Cytokine responses to tetanus vaccination
Considering the T cell responses after stimulation of whole blood with TT, the mean levels of IFN-γ were very low in both groups until 4 days after vaccination, thereafter levels increased in the semi-urban children, whereas levels in the rural cohort increased only slightly (figure 4.3a). Kinetics for IL-5 also showed little increase until 2 days after vaccination, but thereafter levels started to rise in both groups (figure 4.3b). The mean differences in

cytokine levels between semi-urban and rural children reached significance at day 2 after vaccination for IL-5 and at day 7 and 14 for IFN-γ (Mann-Whitney test, $p < 0.05$). When we included the possible confounding variables age, sex and malaria infection in the repeated measurement analysis, the overall IL-5 response during the 2 weeks after vaccination was significantly higher in the rural group ($p < 0.01$). Although levels of IL-5 were significantly higher in the rural children compared to semi-urban children when considering all time points after vaccination together, the increase in IL-5 levels over time was similar in both semi-urban and rural children. None of the confounding variables were significantly associated with the IL-5 response.

In contrast to IL-5, the levels of IFN-γ were not only higher over time, but in addition the levels of IFN-γ increased more rapidly in the semi-urban children than in the rural children, i.e. the slopes of the IFN-γ responses were significantly different between the semi-urban and the rural children ($p < 0.05$). The confounding variable age appeared to be significantly associated with the overall IFN-γ response over time. However, since age was similar in both semi-urban and rural children, this did not influence the difference in responses between the two groups of children.

Ratios of IL-5 to IFN-γ differed significantly between the rural and the semi-urban children at all days after, but not before, vaccination ($p < 0.05$ for day 2 and day 4 and $p \leq 0.001$ for day 7 and day 14; data not shown), showing that the Th2 (IL-5 producing T cell)/Th1 (IFN-γ producing T cell) balance is higher in the rural children. This was confirmed by the levels of another Th2 cytokine, IL-13, which correlated with the levels of IL-5 at all time points (data not shown). None of the cytokine levels were influenced by malaria infection.

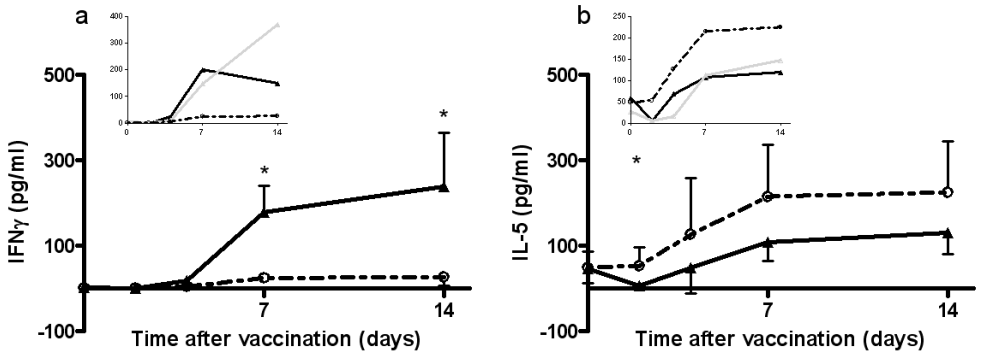

Figure 4.3. Tetanus toxoid-specific cytokine production after *ex vivo* stimulation of whole blood from children vaccinated with tetanus toxoid. Mean values and 95% confidence interval of IFNγ (a) and IL-5 (b) are presented. Closed triangles and solid lines: semi-urban subjects; open circles and dashed lines: rural subjects, * $p<0.05$. Insets in both figures a and b: black and grey triangles connected by solid lines represent semi-urban children without and with reported helminth infection at the time of vaccination, respectively. Open circles and dashed lines represent rural children that were all found positive for helminth infection. Mean values are given.

IL-10 and TNF-α production were also determined (data not shown). However, TT-induced IL-10 production was low with levels only slightly above the detection limit in both groups. TNF-α was produced in low amounts by cells from semi-urban children and hardly detectable in supernatants from cell cultures of rural children, but this difference did not reach statistical significance.

Discussion

In this study we found higher levels of IgG1 anti-TT antibodies after vaccination of the rural children compared to the semi-urban children, although IgG1 avidity was not different amongst the groups. Further, the observation that IgG3 anti-TT was elevated in the rural subjects was unexpected, as IgG3 is mainly induced upon primary vaccinations with protein antigens like TT [181]. High levels of IgG3 were present in children that were found to have plasmodia infections during the study, however, this could not account for the difference between the semi-urban and the rural cohorts.
Whether the observations in our study could be ascribed to helminth infections, could not be determined, as all rural children were infected with at least one helminth species. However, when comparing helminth infected children from the semi-urban area separately to non-infected semi-urban children or the rural cohort, these children showed antibody levels with a geometric mean more close to the semi-urban non-infected children than to the rural children. However, these differences did not reach significance, indicating that helminth infections might contribute to the difference, but are not the only factor. This is in accordance with other studies that have either found no effect of filarial infections on total IgG and IgG1 anti-TT antibodies following TT vaccination [34, 37], although in an other study a higher total IgG response in subjects free of onchocerciasis infections was found [36]. However, in these studies, antibody levels were analysed as fold increase above baseline pre-vaccination levels. When we transformed our data accordingly, no differences between the groups were found in any of the IgG subclasses. It remains to be discussed whether the actual levels or the percent increase represent the best indicator of the response upon immunization. We preferred to compare the actual levels, since higher levels before vaccination, which could be due to natural exposure or more effective prior vaccinations, would lead to reduced values in terms of fold increases, whereas the absolute levels of antibodies, which are thought to be an indication of protection, could be similar.

In general, other studies have investigated the difference between helminth infected and non-infected subjects. However, here we show that besides the helminth infections status, other factors are involved in the outcome of

vaccination. Factors that could influence this study as well as previous studies include the time after vaccination (in our study one month, in the other studies 2 months or longer), the helminth species and the number of vaccinations (in our study the subjects received one booster, compared to two or three in the other studies). Also, the age of the study subjects could make a difference as in our study schoolchildren (7-12 years old) were studied, whereas the other studies included adults or a combination of adults and children [34, 37]. Finally, as we show here, area-related conditions, other than helminth infection, could differ between the rural and the (semi-)urban populations. In all of the other studies, study subjects came from more than one area indicating that unknown area-related factors could have influenced the results. In this study for example, the nutritional status was significantly different between the semi-urban and the rural group. The effect of nutritional status on the outcome of tetanus vaccination has been studied before and, although one study reported a reduced anti-tetanus response in malnourished subjects [183], generally no association of nutritional status with protection to tetanus (defined as the induction of protective antibody levels) has been found, [179, 184]. In our study about half of the children showed protective antibody levels before vaccination. All children reached levels of antibodies that are considered protective one month after vaccination, and although the levels decreased over time, 9 months after vaccination levels were still above the threshold of 0.2 IU/ml (1 μg/ml) for all children. However, it has to be taken into consideration that for tetanus these levels that are considered protective (between 0.1 and 0.2 IU/ml as determined by ELISA) were never validated, and a number of cases of tetanus occurred in individuals with levels in this range, or even above [185].

Another factor found to be different between the areas in this study, was the incidence of malaria infection (table 4.1). This was only found to be influencing IgG3 anti-TT antibody titres, and the effect was significant only before vaccination (insets in figure 4.1). Moreover, it did not completely account for the difference observed between the rural and the semi-urban cohorts. It is known that IgG3 antibodies can be elevated during *P. falciparum* infection and some studies have indicated that this antibody might be associated with immunity [186]. It is possible that this affected the IgG3 antibodies to third party antigens as shown in a study where anti-schistosome IgG3 antibody reactivity can be higher in malaria co-infected subjects in Kenya [187].

It needs to be further investigated whether the observed differences in antibody responses between the semi-urban and the rural group are due to parasite (helminth or malaria) infections or to other factors that are different between rural and semi-urban areas. Moreover, the effect on long-term protection should be studied more intensively. Here we found no

differences between the antibody levels in the semi-urban and the rural cohorts 9 months after vaccination (data not shown), however, the most important feature of long-term protection would be the strength of the (memory) response after a challenge.

It has been hypothesized that a Th1 response is more beneficial than a Th2 response to ensure a strong anti-tetanus effect [38]. This could not be confirmed in the present study, since rural children, producing more IL-5 and less IFN-γ in response to TT, also had higher levels of serum IgG1 and IgG3 anti-TT. Thus, both Th1- and Th2-type cytokines seem to play a role in the process of tetanus-specific antibody production and they do not seem to counteract each other in the induction of humoral responses against tetanus. This positive effect of Th2 responses on the humoral response seems to be specific for tetanus and is not intrinsically related to the study population, as in the same area higher influenza-specific antibodies as well as a stronger Th1 response were found in semi-urban children [188]. Recently, more studies have concluded that a strong Th1 reactivity might not be essential for mounting antibody responses upon TT vaccination and new vaccines (Diphteria-Tetanus-Pertussis (DTP) combination) are being designed to induce a more Th2-like immune response to prevent averse Th1 reactions associated with the cellular DTP vaccine [189]. This study supports the idea that for booster responses, in contrast to earlier hypotheses, a strong Th1 response is not required to reach high anti-TT antibody responses.

In conclusion, this study revealed that the effect of tetanus vaccination differed between rural and semi-urban children in Gabon. Most clearly, the cellular immune response was more skewed towards a Th2 response in the rural children. In addition these children showed slightly higher levels of IgG1 and IgG3 anti-tetanus toxoid antibodies.

Acknowledgements
We thank the study participants in Gabon for volunteering in this study, Ludovic Mewono and Ghyslain Mombo Ngoma for collecting samples on day 28, Nestor Obiang, Brigitte Mingombet and Bart Everts for their technical assistance and all workers in the Albert Schweitzer Hospital for their hospitality and cooperation. Further we thank Revelino Vieira for performing the cytokine measurements and Anja Jansen-Hoogendijk for performing the tetanus serology.

5

TLR ligation in the context of bacterial or helminth extracts in human monocyte derived dendritic cells: molecular correlates for Th1/Th2 polarization

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Summary

Recognition of pathogens by dendritic cells (DCs) through interaction with pattern recognition receptors (PRRs), including Toll like receptors (TLRs), is crucial for the initiation of appropriate adaptive immune responses. Yet, the characteristics and differences in molecular profiles of DCs with different T cell polarizing capacities are still poorly defined. To address this issue, the molecular profile of human monocyte derived DCs was characterized after exposure to Th1 activating bacterial extracts from *Listeria monocytogenes* (HKLM) and *Escherichia coli* and the Th2 activating helminth derived phospholipids (PS) from *Schistosoma mansoni* and *Ascaris lumbricoides*, all with TLR2 activating capacity. We find that the ratio of activated MAP kinase *p*-ERK / *p*-p38 is lower in the DCs stimulated with the bacterial products compared to the DCs stimulated with the helminth products, which correlates with the Th1 and Th2 polarizing capacity of these compounds. Furthermore, the mRNA expression profiles induced by the bacterial and helminth derived products differ widely. Notch ligand delta-4 and transcription factor c-fos are differentially regulated and show a strong correlation with Th1 and Th2 polarization, respectively. The molecular profile induced by the two bacteria share a comparable molecular profile, while DCs exposed to the Th2 promoting lipid extracts have a profile that is similar to that induced by *S. mansoni* derived glycoproteins (SEA). These data show that TLR2 activating compounds embedded within different antigen sources can induce very distinct DC programming and suggest that the polarizing capacity of compounds can be predicted with the molecular signature they induce in DCs.

Introduction

Dendritic cells (DCs) are antigen presenting cells that play a pivotal role in the initiation of adaptive immune responses. These cells function as sentinels in the periphery where they are able to recognize and respond to stimuli from the environment they reside in, some of which could be products from invading micro-organisms or helminths. Upon such exposures DCs undergo phenotypic changes that allow them to effectively migrate to lymph nodes and prime appropriate T cell responses [88, 190].
The type of compounds encountered by DCs will determine to a large extent the nature of the T cell polarization promoted by these DCs. For this, DCs have to be able to distinguish these different classes of molecules. To this end, DCs express several classes of pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors, Nod-like receptors and RIG-I like receptors that are able to recognize specific pathogen derived components, the so-called pathogen associated

molecular patterns (PAMPs). Upon engagement of these receptors, signalling cascades are initiated that involve activation of the mitogen activated protein kinases (MAPKs) and NF-κB, and induction of expression of genes involved in DC maturation and the ability to prime and skew T cell responses. It is known that intracellular organisms are primarily capable of instructing DCs to induce T helper (Th) 1 responses [191], whereas extracts of parasitic helminths have been demonstrated to drive Th2 skewed responses [40, 96, 191].

Relatively much is known about the signalling pathways in DCs induced after triggering of PRRs [103, 192, 193], however, the molecular characteristics that are different for DCs that have been activated by Th1 or Th2 promoting PAMPs are much less understood [194, 195]. We set out to address this issue by characterizing human monocyte derived DCs after exposure to bacterial and helminth derived products. The characterization of the DCs comprised gene expression analysis of 25 genes that have been linked to activation and T cell polarizing properties of DCs. These molecular profiles of the DCs were correlated to their T cell polarizing capacity. In this study we used Gram-positive heat killed *Listeria monocytogenes* (HKLM) and Gram-negative *Escherichia coli*, both of which stimulate TLR2 activity and induce Th1 polarization. In addition, *Schistosoma mansoni* and *Ascaris lumbricoides* derived phosphatidylserine containing preparations (PS) were used, that also activate TLR2, but drive Th2 responses. We show that the signalling routes and the resulting mRNA expression profiles following stimulation by the bacterial and helminth derived products are very distinct. This indicates that not all extracts that contain TLR2 activating components lead to similar DC programming and suggests that there is a general molecular DC1 and DC2 signature that can be used to predict Th1 and Th2 skewing potential of DCs.

Materials and methods

Antigen preparation

Phosphatidylserine containing preparations (PS) were extracted from 4 gram of *A. lumbricoides* worms (expelled from infected humans) or from schistosomal worms, collected from golden hamsters 45-48 days after infection with *S. mansoni*, as described before [96]. Mass spectrometry was used to confirm the presence and composition of PS species in both lipid preparations, as described before [196]. Schistosomal egg antigen (SEA) was prepared from schistosomal eggs, collected from trypsin treated liver homogenate of the *S. mansoni* infected hamsters. *E. coli* (ATCC 11775) and *L. monocytogenes* (kind gift of J. van Dissel, LUMC, Leiden, The Netherlands) were grown at 37°C for 18 h in Brain Heart Infusion (BHI)

bouillon (Biomerieux). Cultures were washed with PBS, quantified, and frozen in aliquots. In addition *L. monocytogenes* was heat inactivated for 2 hours and 45 minutes at 80°C before storage.

Dendritic cell culture and naïve T cell polarization

Monocytes were isolated and immature DCs were cultured as described before [96]. At day 6 or 7 immature DCs were matured with LPS (ultrapure, *E. coli* 0111 B4 strain, invivogen) (100ng/ml) in the presence of IFN-γ (1000 U/ml), heat killed *L. monocytogenes* (HKLM; 10^8/ml), *E. coli* (10^7/ml), SEA (50 μg/ml), PS lipid extract derived from *Ascaris* worms (an equivalent of 120 mg of worm per ml) or PS lipid extract derived from schistosomal worms (an equivalent of 20 worm-pairs per ml). For RNA isolation, DCs were harvested 16 hours after stimulation, as pilot experiments in our lab indicated that the expression levels of most genes had changed at this time point. DCs were snap-frozen in liquid nitrogen and kept at -80 °C until RNA isolation. For measuring cytokine production by DCs and for co-culture with naïve T cells, DCs were matured for 48 hours after stimulation, after which produced cytokines were measured in the harvested supernatant. Levels of IL12p70 were determined by ELISA using monoclonal antibodies 20C2 and biotinylated mouse-anti-human IL-12 C8.6 (both Becton Dickinson) as coating and detection antibodies, respectively. Levels of IL-23 were determined by ELISA using monoclonal antibodies ebio473p19 and biotinylated mouse-anti-human IL-12 C8.6 (both Becton Dickinson) as coating and detection antibodies, respectively. To determine T cell polarization, 5 x 10^3 mature DCs were cocultured with 2 x 10^4 naïve T cells that were purified using a human CD4+/CD54RO- column kit (R&D, Minneapolis, MN) in the presence of SEB (100 pg/ml; Sigma) in 96-well flat-bottom plates (Costar). On day 5, rhuIL-2 (10 U/ml, Cetus Corp., Emeryville, CA) was added and the cultures were expanded for another 5-9 days. To measure the frequency of IL-4- and IFN-γ- producing T cells, Th cells were restimulated with PMA and ionomycin in the presence of brefeldinA (all Sigma) during 5 or 6 hours and stained with anti-hu-IL-4-PE and anti-hu-IFN-γ-FITC (both BD Biosciences).

RNA isolation, DNase treatment and cDNA synthesis

RNA isolation was performed using Trizol reagent (Invitrogen, Breda, The Netherlands) according the manufacturers' instructions, with a minor modification: 3 μl of glycogen (Invitrogen) was added to all samples after they were homogenized in Trizol for a few minutes at room temperature (RT). DNAse treatment and cDNA synthesis were performed following standard procedures.

Analysis of gene expression levels
Primers and Taqman probes were provided as a Taqman gene expression kit (Applied Biosystems, Foster City, California) or designed using Primer Express (Applied Biosystems) and synthesized by Biolegio (Malden, The Netherlands) and Eurogentec (Seraing, Belgium), respectively (sequences available upon request). Real time qPCR was performed using Eurogentec PCR reagents, in a volume of 25 μl on an ABI PRISM 7700 Sequence Detection System (SDS, Applied Biosystems), using the following program: 10 minutes at 95°C, 40 cycles of 15 seconds denaturation at 95°C and 60 seconds annealing and amplification at 60°C. Results were monitored and analysed with SDS software (Applied Biosystems).
Gene expression was normalized to the housekeeping gene TAF-1 and calculations were performed as described using the $2^{-\Delta\Delta CT}$ method [197]. Analysis of the expression of 6 different housekeeping genes in a subset of the samples indicated that TAF-1 was a stable housekeeping gene in our samples upon stimulation. Spotfire software (spotfire.tibco.com) was used to generate a heatmap and perform hierarchical clustering of the genes.

MAP Kinases
20 or 60 minutes after stimulation of immature DCs (day 6), cells were fixed for 10 minutes with 4% ultrapure formaldehyde (Polysciences) directly in the plate. Cells were harvested and washed twice in PBS/0.5% BSA. Subsequently, the DCs were permeabilized in 700 μl ice-cold 90% methanol in PBS in and left on ice for 30 minutes. Following two wash steps in PBS/0.5%BSA intracellular staining was performed for 2 hours at room temperature in the dark with anti-phospho-p44/42 MAPK AF-488 (T202/Y204), anti-phospho-p38 MAPK AF-647 (T180/Y182), anti-phospho-SAPK/JNK AF 647 (T183/Y185), anti-Phospho-Akt (Ser473) AF-488 (all Cell Signalling Technology), and anti-human c-fos-PE (BD). After one wash in PBS/0.5%BSA MAPK activation was determined by flow cytometry using a Becton Dickinson FACSCalibur flowcytometer (BD Biosciences) and analysed using FlowJo analysis software (Tree Star).

Statistical analysis
Data were analysed using SPSS (v14.0) and GraphPad Prism4. Differences amongst stimuli were analysed by a Mann-Whitney test. Differences relative to LPS stimulation were determined using a one sample t-test. Correlations between expression of genes and/or T-cell responses were calculated by a two-tailed Spearman's-rho test. Differences were considered significant when P-values were below 0.05.

Results

TLR2 activating components that induce Th1 or Th2 polarization via dendritic cells

To study the molecular characteristics of DCs exposed to compounds that engage TLRs, yet lead to differential skewing of immune responses in terms of Th1 and Th2 induction, different pathogen derived products from bacterial or helminth origin were chosen with a known Th1 and Th2 inducing capacity. For this study Gram-negative *E. coli* and Gram-positive heat killed *L. monocytogenes* (HKLM) were used as bacterial stimuli that induce Th1 responses. A schistosome (a trematode) derived phosphatidylserine containing lipid preparation (schPS) and a similar preparation from the nematode worm *A. lumbricoides* (ascPS), both containing mainly phosphatidylserine species with two attached acyl chains and some lysophosphatidylserine species (with only a single attached acyl chain) (figure 5.1A and B, respectively), were chosen as Th2 inducing compounds. Stimulation of HEK cells transfected with TLRs showed that all stimuli could activate TLR2, with additional potent TLR4 stimulation by *E. coli* (figure 5.1C). IFN-γ and schistosome derived soluble egg antigen (SEA), stimuli that do not show strong TLR activating capacity in our experiments (figure 5.1C), and induce Th1 and Th2 responses, respectively, were used as controls.

To assess the T cell polarizing capacity of DCs exposed to these compounds, stimulated human monocyte derived DCs were cocultured for two weeks with allogeneic naïve $CD4^+$ T cells and IL-4 as well as IFN-γ production was determined by intracellular staining upon T cell restimulation (figure 5.1D). DCs were stimulated with the different compounds in the presence of LPS, to ensure equal maturation and to rule out potential effects on polarization due to differences in maturation status of the DCs. We found that in all conditions expression of maturation markers was significantly higher than levels measured on immature DCs and overall similar to the levels induced by LPS alone (data not shown). As expected, *E. coli* induced a strong Th1 response comparable to DCs stimulated with IFN-γ, while HKLM induced a moderately polarized Th1 response. Conversely, the helminth derived compounds, as shown before for schPS [96], and SEA [96, 191], but also the *A. lumbricoides* derived phospholipids instructed DCs to drive Th2 skewed responses with the strongest polarization induced by SEA (figure 5.1D).

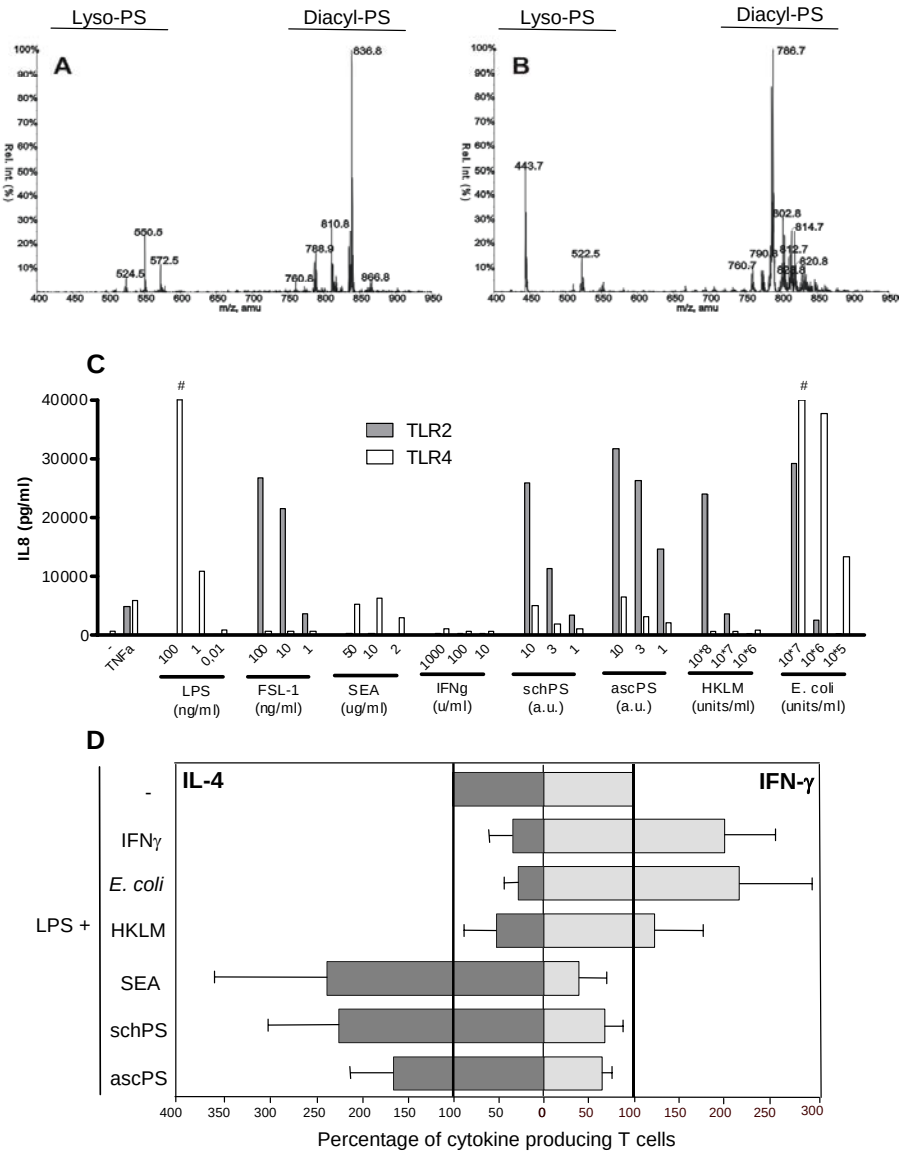

Figure 5.1. TLR activation and T-cell polarization by the different compounds. Mass spectrometry analysis of schPS (A) and ascPS (B). Samples were analysed by LC/MSMS in the negative mode. Neutral loss scans of 87 amu, corresponding to the loss of serine from the phospholipid were obtained. The relative intensity is shown of the detected phosphatidylserine species (indicated by their distinct m/z ratios). C. Activation of TLR2 and TLR4 transfected HEK293 cells. HEK cells were stimulated and IL-8 production in response to activation is shown. CD14 transfected HEK cells were used as negative controls (not shown). # out-positive, a.u. arbitrary units. D. T cell polarization was determined by measuring the percentages of cells with intracellular IFN-γ and IL-4 production by FACS analysis. T-cell polarization after LPS stimulation alone was set to 100% (indicated by the bold lines). Relative amounts of IFN-γ and IL-4 positive T cells induced by the stimuli in the presence of LPS are given. Dark gray (left); IL-4, Light grey (right); IFN-γ. Polarization profiles (IL-4/IFN-γ ratio; n ≥ 4) for all stimuli were significantly different from LPS stimulation alone; $p < 0.05$.

MAPK activation

To obtain a better understanding of the molecular processes in DCs that could underlie the observed differences in T cell polarizing capacity of these helminth- and bacteria-derived compounds, we set out to investigate in more detail the molecular characteristics of the DCs exposed to the different stimuli. To study the intracellular signalling routes activated upon exposure to the helminth and bacterial derived products, we analysed the activation of the MAPKs . ERK (ERK1/2) and p38 are two effector kinases of the MAPK family and are known to play an important role in shaping immune responses [198]. p38 has been shown to regulate DC maturation and proinflammatory responses, while activation of ERK has been related to anti-inflammatory and Th2 responses [199]. As has been described before [200], exposure of DCs to LPS alone led to preferential phosphorylation of p38 (figure 5.2A, B). The Th1 promoting stimuli IFN-γ and *E. coli* even further increased the activation of this MAPK resulting in a reduced *p*-ERK / *p*-p38 ratio, 20 minutes after stimulation (figure 5.2B,C), whereas for HKLM this ratio did not change. In contrast, the Th2 inducing compounds PS and SEA increased this ratio. Interestingly, the high *p*-ERK / *p*-p38 ratio induced by these Th2 polarizing stimuli was the result of different activation profiles for SEA versus the lipid preparations: SEA significantly induced phosphorylation of ERK, whereas the helminth derived lipids impaired p38 activation, but showed no effect on ERK activation (figure 5.2D and 2E). The *p*-ERK / *p*-p38 ratio showed a positive correlation with Th2, and negative correlation with Th1 polarization (R^2 = 0.36 and -0.47, respectively, figure 5.2F and G). In conclusion, for all components tested, the *p*-ERK/*p*-p38 ratio only 20 minutes after DC stimulation can be used to predict the outcome of the T cell response in terms of Th1 and Th2. This shows that very early events in DC activation already determine the fate of the DCs in terms of its T cell polarizing capacity.

Gene expression analysis

To further characterize the molecular profile of the differentially stimulated DCs we performed mRNA expression analysis, using real-time PCR, on a selected set of genes involved in TLR signalling and T cell polarization (table 5.1, figure 5.3A). Upon maturation with LPS, the expression of most genes was increased (data not shown). All data shown are relative to what is seen in mature DCs without any polarizing agents added, i.e. DCs stimulated with LPS. Stimulation of DCs from different individuals with the same stimulus showed very consistent profiles (data not shown). Clustering analysis revealed that the gene expression profiles of Th1 and Th2 polarizing agents clustered in separate groups (top of figure 5.3A). Within the Th1 stimuli, DCs exposed to bacterial products derived of *L. monocytogenes* and *E. coli* had a remarkably similar profile that was different from the profile induced by IFN-γ. For the Th2 stimuli,

both helminth derived lipid preparations showed a very comparable profile, which resembled the expression profile induced by SEA for most of the genes (figure 5.3A). However, expression levels in PS pulsed DCs were generally lower than in SEA stimulated DCs which is in accordance with the less pronounced effects on activation of the MAPKs by the PS preparations. Next, we related expression levels of individual genes to the T cell polarizing capacities of the DCs, to identify potential mechanisms through which different pathogen derived compounds induce differential T cell polarization. Members of the IL-12 cytokine family are well known for driving Th1 polarization [88]. Indeed the expression of both IL-12 p40 and p35, but also IL-23 p19 were shown to be upregulated in DCs stimulated with Th1 inducing stimuli and reduced in DCs stimulated with helminth derived compounds (figure 5.3A). This was confirmed at the protein level when IL-12 and IL-23 production by DCs were measured by ELISA (figure 5.3B,C).

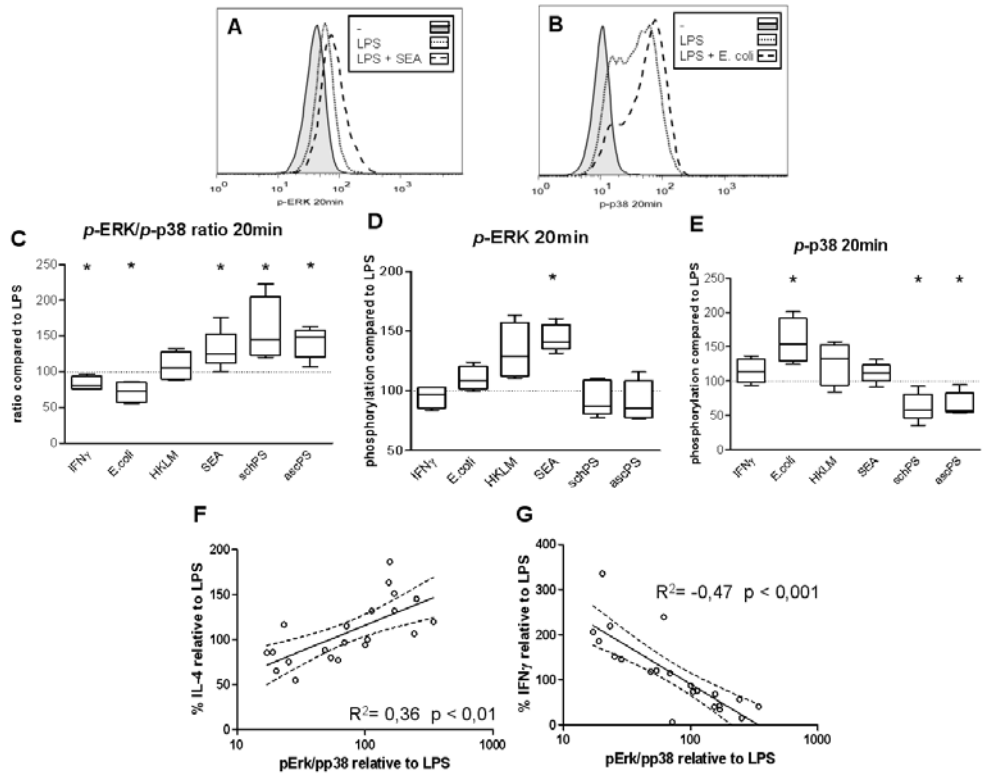

Figure 5.2. MAP kinase activation in dendritic cells. Representative histograms of (A) ERK and (B) p38 phosphorylation in DCs 20 minutes after stimulation. C-E. Ratios of p-ERK / p-p38 (C), phosphorylation of ERK (D) and p38 (E) 20 minutes after stimulation in the presence of LPS. Expression induced by LPS was set to 100% (dashed line), relative expression levels or ratios are shown. * P<0.05 compared to LPS stimulation. F and G. Correlation of p-ERK / p-p38 ratio and IL-4 (F) or IFN-γ (G) production by T-cells. All data are relative to stimulation with LPS only.

With respect to T cell polarization, other genes of interest are the notch ligand family members delta-1, delta-4 and jagged-2, since expression of delta and jagged on DCs has been associated with induction of Th1 and Th2 responses, respectively [230]. For jagged-2 and delta-1 no significant differences were found between the stimuli (figure 5.3A). However, delta-4 was upregulated by the bacterial Th1 inducing stimuli, and downregulated by the Th2 inducing lipids. Moreover, expression levels of delta-4 correlated with the IL-4/IFN-γ cytokine ratio produced by the T cells of the stimuli that activate TLR2 (R^2=-0.87, figure 5.3D). Yet, in SEA and IFN-γ stimulated DCs delta-4 expression was not altered. Therefore, Delta-4 seems to associate with T helper cell polarization only when TLR2 is also engaged.

Conversely, we found higher c-fos mRNA levels in PS and SEA pulsed DCs compared to HKLM and *E. coli* stimulated DCs. c-fos has been shown before to mediate SEA induced repression of IL-12 secretion by DCs [199]. Indeed, correlation analysis revealed that in DCs stimulated with bacterial products or helminth-derived lipids, mRNA levels of c-fos were positively correlated with Th2 induction (R^2 = 0.667, figure 5.3E).

Discussion

DCs express a range of PRRs that allow them to recognize different pathogens and initiate appropriate adaptive immune responses. Pivotal to this process is the proper integration of PRR derived signals into a molecular activation profile of DCs that leads to a particular T cell polarizing capacity. This study demonstrates that TLR activation in the context of different bacterial and helminth derived extracts can lead to very distinct molecular activation profiles of human DCs which correlate with their T cell polarizing capacity in terms of Th1 and Th2 skewing.

One of the major signalling cascades triggered upon engagement of TLRs is the MAPK pathway. Differential activation of MAPK p38 and ERK in DCs has been associated with specific T cell polarization; p38 is thought to be important in mediating DC maturation and pro-inflammatory / Th1 responses, whereas ERK activation has more often been associated with anti-inflammatory / Th2 responses [232]. At 20 minutes after stimulation, we observed decreased *p*-ERK/*p*-p38 ratios in the Th1 promoting DCs.

Of the two Th1 polarzing agents, *E. coli* induced stronger p38 activation, compared to HKLM. The fact that *E.coli*, unlike HKLM, strongly activates TLR4 (figure 5.1C), which is known to lead to preferential p38 activation and induction of pro-inflammatory responses, may explain this [200].

Table 5.1. Description of genes

		Gene	Function	Ref.
Cytokines & chemokines		IL-12 p35	Together with p40 forms the cytokine IL-12 involved in Th1 polarization	[191, 201]
		IL-12 p40	Together with p35 forms the cytokine IL-12 involved in Th1 polarization	[191, 201]
		IL-23 p19	Together with p40 forms cytokine IL-23 in DCs which augments Th1 and Th17 responses	[202-204]
		TNF-α	General pro-inflammatory cytokine mediating local inflammation. Its expression is dependent on NF-kB signalling	[205]
		CXCL-10 (IP-10)	Chemotactic factor for T cells. Its expression is dependent on the IFN-responsive gene pathway	[206, 207]
		CCL22	Chemokine involved in recruitment of Th2 effector cells	[208]
		TGF-β	Cytokine with anti-inflammatory properties, by inhibiting activity and function of both T cells and DCs	[209, 210]
		IL-10	Cytokine that potently suppresses immune responses and in particular DC and T cell responses, by downregulating pro-inflammatory effectors	[210, 211]
Toll like receptor signalling		TLR2	Receptor of innate immunity for recognition of mainly lipid containing compounds	[212, 213]
		TLR4	Receptor of innate immunity for recognition of LPS	[212, 213]
		MyD88	Proximal, most common adaptor of TLR signalling, shared by all TLRs except TLR3	[212, 214]
		NOD2	Intracellular peptidoglycan receptor implicated in activation of NF-kB but also in inhibition of TLR2 signalling	[215-217]
		TRIF (TICAM1)	TLR3 and TLR4 specific adaptor which mediates the MyD88-independent pathway preferentially leading to induction of IFN-responsive genes	[218, 219]
		Tollip	Inhibitor of IRAK activity and thereby TLR signalling	[220]
		SOCS-1	Inhibitor of LPS-TLR4 signalling pathway as well as TLR induced JAK/STAT signalling. Potential negative regulator of Th1 responses	[221, 222]
		SOCS-3	Inhibitor of JAK/STAT signalling but also positive regulator of APC function by suppression of STAT3, which normally inhibits TLR signalling.	[221, 222]
Modulators of T cell activation & polarization	Intracellular	DUSP2	Phosphatase modulating MAP kinase signalling balance	[223]
		IDO	Enzyme that catabolizes tryptophan to kynurenines, which are able to induce T cell apoptosis and inhibition of proliferation. Expression induced by IFN-γ	[224]
		c-Fos	Transcription factor activated by MAP kinases which induces IL-10 production and is involved in DC mediated Th2/anti-inflammatory responses	[108, 199]
	Membrane bound	PD-L1 (B7-H1)	Costimulatory molecule and ligand for PD-1 on T-cells. It has inhibitory function in T cell proliferation and cytokine production. Might be stimulatory for Th2 response	[225-227]
		PD-L2 (B7-DC)	Costimulatory molecule and ligand for PD-1 on T-cells. Reported to have synergic activity with other costimulatory molecules as well as inhibitory activity on T cell activation	[225-227]
		LIR-7 (ILT-1)	Receptor with unknown ligand(s) with possible immune suppressive properties, but also implicated in immune activation	[228, 229]
		Jagged-2	Ligand for notch-receptor on T cells; influences T cell skewing	[230]
		Delta-4	Ligand for notch-receptor on T cells; influences T cell skewing	[230, 231]
		Delta -1	Ligand for notch-receptor on T cells; influences T cell skewing	[230]

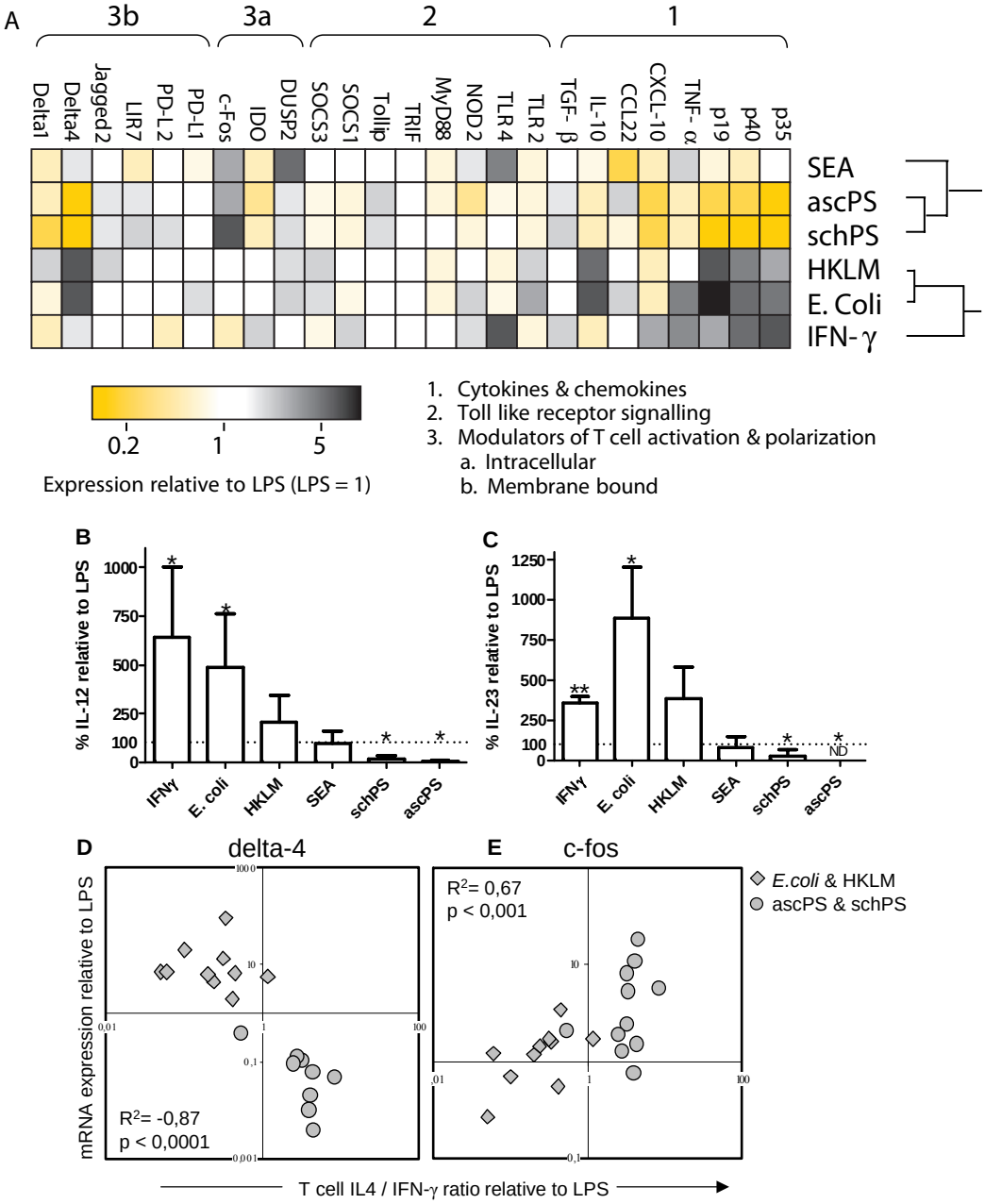

Figure 5.3. Distinct mRNA expression levels after stimulation of immature DCs with Th1 and Th2 inducing compounds. A. mRNA expression levels of the different genes compared to LPS (representing a value of 1). Black and yellow colors represent expression levels higher and lower, respectively, relative to LPS pulsed DCs. Expression was determined by real-time PCR with TAF-1 as housekeeping gene. Stimuli were clustered hierarchically according to expression profiles (top of figure). B and C. Amount of IL-12 and IL-23 present in the supernatant of DCs 48 hours after stimulation, relative to the amount produced in the presence of LPS only. ND: not detectable, * $p < 0.05$, ** $p < 0.01$ compared to LPS stimulation only. D and E. Association of delta-4 (D) and c-fos (E) mRNA expression levels with T cell polarization for TLR2 activating stimuli. Diamonds represent HKLM or *E. coli*, circles the helminth derived lipids. Relative expression levels to LPS control condition (set to 1) are shown.

Comparison of the MAPK activation profile of IFN-γ with the Th1 inducing bacterial products revealed a similar reduction in the *p*-ERK/*p*-p38 ratio. This could be explained by the fact that IFN-γ induced STAT signalling is known to potentiate TLR4 mediated effects resulting in selective enhancement of p38 phosphorylation [233, 234]. These data are in agreement with the increased mRNA expression levels of pro-inflammatory mediators such as IL-12, IL-23 and TNF-α in all the Th1 promoting DCs. In contrast, all helminth derived stimuli increased the *p*-ERK/*p*-p38 ratio in the DCs. This is consistent with what others have found for SEA [108] and also for other helminth derived Th2 polarizing antigens such as LNFPIII [93] and ES-62 [235] in murine DC models. Taken together, an increased *p*-ERK/*p*-p38 ratio appears to be an important characteristic of antigen presenting cells exposed to helminth derived antigens that skew responses towards Th2.
Comparison of the MAPK activation profile induced by the helminth-derived lipids compared to SEA, revealed that SEA, like other helminth derived antigens such as LNFPIII and ES-62, induces a higher *p*-ERK/*p*-p38 ratio by increasing activation of ERK, whereas the lipids influenced the *p*-ERK/*p*-p38 ratio by specifically impairing p38 phosphorylation. Thus, although the lipids share the properties of other Th2 inducing helminth antigens described so far, they appear to achieve this differently which is exemplified by a different modulation of the MAP kinase signalling pathway.
This difference in MAPK activation between SEA and the lipid preparations is further substantiated by the finding that c-Fos protein levels were elevated and sustained in DCs stimulated with SEA, which is in line with a previous study showing that SEA stabilizes c-Fos through selective activation of ERK [108], whereas in PS pulsed DCs this increase was lower and more transient (Everts *et al*, unpublished data).

Comparison of the mRNA expression profiles of TLR activating bacterial and helminth derived compounds revealed that, unlike the Th2 inducing phospholipids, exposure of DCs to Th1 promoting stimuli preferentially led to the induction of the Th1 promoting IL-12 and IL-23, both at the mRNA and protein level. The level of expression of these cytokines reflected the degree of p38 activation that drives pro-inflammatory gene expression. The fact that these cytokines were expressed to a higher level in the *E. coli* and IFN-γ stimulated DCs, probably contributes to the stronger Th1 induction seen with these stimuli compared to HKLM pulsed DCs. Although the expression profile of the pro-inflammatory cytokines was shared by the IFN-γ stimulated DCs, expression of other genes like IL-10, delta-4 and TLR4 was clearly different from the bacteria conditioned DCs. These differences are likely to be caused by the fact that *E. coli* and HKLM

harbor PAMPs that activate DCs through multiple PRRs, whereas IFN-γ signals via PRR independent pathways that involve STAT signalling.
Far less is known about the mechanisms leading to induction of Th2 responses, compared to Th1 responses. Both lipid preparations induced a nearly identical molecular profile in DCs. This is in agreement with the similar molecular species composition present in the lipid preparations: both PS preparations contain predominantly diacyl phosphatidylserine species (molecules with m/z ratios above 700), and a few lysophosphatidylserine species (molecules with a single attached acyl chain and m/z ratios between 400 and 600) (figure 5.1A, B). Combining the mRNA expression levels and MAPK activation for the Th2 inducing stimuli we found that both lipid preparations as well as SEA induce Th2 polarization and a *p*-ERK / *p*-p38 ratio dominated by ERK. However, SEA induces higher levels of activated MAPKs and a stronger Th2 response. It appears therefore, that the ratio between *p*-ERK and *p*-p38 is important for the polarization of the immune response, whereas the strength of this response is dependent on the absolute amounts of the activated MAPKs.

Recent work highlighted a role for certain Notch ligands, including jagged-2, in skewing towards Th2 responses [230]. Our findings suggest that jagged-2 mRNA is not important in the Th2 skewing for the conditions tested in this study. However, high levels of delta-4 were found upon stimulation of human DCs with bacterial compounds and helminth derived compounds show a decreased delta-4 expression and induce a Th2 response, which is in agreement with the finding that the Notch ligand delta-4 is involved in Th1 skewing [230]. Moreover, recently it was found that delta-4 expressing mouse DCs not only drive Th1 by inducing IFN-γ production by T-cells, but also by actively inhibiting Th2 development via counteraction of IL-4 [236]. This is in agreement with our findings in human DCs that expression of delta-4 is not only positively associated with Th1 induction, but that delta-4 is also negatively associated with the development of a Th2 response. Moreover, we found that this up- and downregulation of delta-4 expression can both exist in the context of TLR2 signalling.

One of the genes that were found to be positively associated with Th2 inducing DCs was the transcription factor c-fos. c-Fos has been shown to mediate IL-12 suppression in SEA pulsed DCs, which is generally thought to be a prerequisite for Th2 induction [108, 199]. In addition, the observation that c-fos mRNA expression was strongly positively correlated with Th2 induction not only for SEA, but also for PS, further supports the notion that this transcription factor plays a role in the promotion of helminth antigen dependent Th2 skewing. However, as described above, the kinetics of c-fos translation seems different between the lipids and SEA. Therefore, it

remains to be established whether c-fos in PS pulsed DCs plays a similar role compared to the role in SEA induced Th2 polarization.

Several studies have shown that TLR2 activation may lead to many different outcomes; Th2 [108, 237], Treg [238] as well as Th1 [239]. It has been suggested that these different outcomes may be the result of heterodimerization of TLR2 with different receptors, such as TLR1 or TLR6 [240, 241], or liaisons with other receptors including Nod-like receptors and C-type lectins [194, 242]. In our study, the compounds used from helminths or bacteria are mixtures that would be expected to signal via additional receptors besides TLR2. *E. coli* has been shown to activate TLR4 and NOD1 [243, 244], whereas resistance to *Listeria* infection was related to the presence of functional NOD2 [245]. Relatively little is known about Th2 skewing by the helminth derived compounds, but in a previous study for schistosomal lipids it was shown that TLR2 activation was not needed for Th2, but rather for regulatory responses [96]. Therefore, these results suggest that the engagement of additional PRRs along with TLR2 and TLR4 are likely to play a role in the Th2 polarizing capacity of DCs [245, 246].

In conclusion, the study presented here indicates that TLR ligation on monocyte derived DCs in the context of bacterial or helminth derived extracts leads to profound differences in the activation or expression of various markers at the level of MAPK phosphorylation, mRNA expression levels and protein products thereof. Interestingly, several of these molecular markers are strongly correlated with the T cell polarizing capacity of these DCs. This not only gives new insights about the processes involved in Th1 and Th2 polarization but it also suggests that there is a common molecular Th1 and Th2 signature in human DCs that can be used to differentiate between Th1 and Th2 inducing DCs as well as to predict the strength of skewing in terms of Th1/Th2 balance. Thereby, this study provides new insights in molecular pathways involved in the capacity of human DCs to distinguish bacterial and helminth derived antigens via PRRs including TLRs and the proper integration of these signals leading to the induction of appropriate immune responses.

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6

General discussion

Introduction

Immune responses induced by helminth infection can best be characterized by a combination of Th2 and regulatory properties, a so called “modified Th2” response. Immune responses generated in response to antigens were initially divided into Th1 and Th2 responses. Th1 responses are usually associated with strong immune activation (e.g. IL-2, TNF-α), while Th2 is more associated with regulatory responses (e.g. IL-10). However, the regulatory component was later defined by a separate cell type, called regulatory T cells (consisting of natural Treg, Th3 or Tr1) that could suppress responses of both type 1 and 2 effector cells. More recently a third type of effector T cells was identified and named Th17 after the cytokine IL-17 that these T cells characteristically produce. This cell type is thought to be mainly involved in immune responses to extracellular bacteria as well as in tissue pathology and pathogenesis of autoimmune diseases [247]. It has also been proposed that Th17 cells, instead of a third type of effector T-cell, represent a more general inflammatory response opposite to regulatory T cells, thus forming a “regulation-activation” balance that crosses the “Th1-Th2” framework [248]. However, independent of the models proposed, the balance of the sum of all different types of polarizing responses will determine the final outcome of an immune reaction.
For helminth infections this could mean that there may be a Th17 component present, that could for example play a role in tissue damage, but in general the dominant immune response is a type 2 response with an overlapping regulatory component, the latter arises during infection characterized by high helminth loads and chronic inflammation [40]. This combination of a dominant type 2 and regulatory response is thought to be the basis of the long-lived partnership between the parasite and its host: the helminth is not expelled and the host does not suffer from severe tissue damage.

Consequences

Spill-over suppression

The regulatory component induced by helminth infections was found not to exert its effects solely on helminth antigens, but could also affect responses to non-related antigens. Depending on the type of antigen this can be either beneficial or detrimental for the host. For example, in case of allergic and autoimmune diseases this spill-over effect could alleviate the symptoms. How this works and how it can be used for clinical applications is widely explored and already some clinical trials have been performed by giving *Trichuris suis* eggs to patients with ulcerative colitis and Crohn’s

disease [76, 77]. In addition, several trials are underway for treatment of allergic disorders with helminths [249, 250].
However, helminth induced immunomodulation could also be detrimental when one considers coinfections with other pathogens (bacteria, protozoa or viruses) or in the case of vaccination. For the latter it would be important to know whether or not the presence of helminths is preventing efficient responses as in this case it would be relatively easy to provide antihelminthic treatment before vaccination to enhance responses. Further, knowledge about the influence of helminth infections on vaccination outcome is important for the current development of many vaccines (malaria, HIV, tuberculosis) that will be mostly needed in areas where helminths are also highly endemic. To optimize vaccines, possibly different adjuvants would be needed for populations in areas endemic for helminths than those used in vaccines for the western world.

Helminth infection and vaccine efficacy
In general it is found that the presence of helminth infections induces a Th2 bias in immune responses mounted to different types of vaccines, even when the exposure to the helminths is *in utero* [29, 33]. This Th2 bias is mostly defined as an increased antigen specific IL-5/IFN-γ ratio produced upon *ex vivo* stimulation of the immune cells after vaccination. Usually a Th1 biased immune response to vaccination is regarded effective, however, the exact influence of T helper cell polarization on the protective effect of the vaccines is not known in most cases. For example, when Gabonese children from a rural and a semi-urban area were vaccinated with tetanus toxoid (TT), rural children with a stronger TT specific Th2 bias, unexpectedly showed higher levels of anti-TT IgG1 antibodies compared to the urban children that induce a higher TT specific IFN-γ and a lower IL-5 response (chapter four).
In contrast the same children were also vaccinated with a trivalent influenza vaccine. Again, an antigen-specific Th2 skewed immune response was found in the rural children, who for influenza showed lower levels of anti-influenza haemagglutinin antibodies (chapter three). However, this was only true for two strains, antibody responses to the third strain, H3N2, were slightly, but significantly higher in the rural schoolchildren two weeks after vaccination. Interestingly, a recent outbreak with an H3N2 strain had probably occurred, as antibody titres specific to the H3N2 strain were very high already before vaccination. Would rural children mount a stronger antibody response to a strain that caused a recent epidemic? When considering the responses to TT vaccination, to which there is continuous exposure, also antibody responses were higher in the rural cohort. However, the mechanism behind this is not clear. It is possible that the difference in T cell polarization and associated cytokines affects the activation of central or effector memory T cells [176].

In the studies described in chapter three and four the aim was to compare vaccine efficacy in rural versus urban children. The presence of helminthes, determined just before vaccination, was the major difference between these populations. However, comparing antibody and cytokine production to current helminth infection status was difficult as all rural children were infected with at least one type of helminth. When comparing children with and without helminth infection within the urban cohort, helminth infection was found to significantly affect antibody production, but only for the influenza H1N1 strain. Different study designs are needed to specifically address the question of how helminth infections affect the responses to new vaccines.

It is often argued that not only current, but also early life exposure to infections may have a major influence on the immune system [251, 252]. Other characteristics of helminth infections can also influence the outcome, and these include the helminth species, the intensity of the infection, whether the infection is acute or chronic, and the history of infection. [40, 41, 253]. For example, the children in the rural area were all infected with at least one helminth species, and the chance that they were infected very early in life, or that they were even exposed *in utero* because their mothers were infected, is probably higher than for the semi-urban children. Also, the total time in life that they were infected is likely to be greater. In addition, schistosome infections might have a different influence on immune responses than intestinal helminths. These factors are difficult to study in a human population, as the populations studied need to be very large to distinguish amongst all these different characteristics, if possible at all. In animal models it has been shown that heavy and/or chronic infections can have a different effect on the immune system than when one considers acute or light infections [46]. In humans studies are needed to carefully examine these issues.

Read out of vaccine efficacy

The “ideal” responses to vaccines are mostly not known, even finding a good correlate of protection has proven to be difficult. What antibody levels are necessary? What kind of cellular responses are most effective? Which factors can improve the memory responses? Currently the correlates of protection that are used are mostly based on serum antibody titres. For example, vaccines to be developed for the yearly influenza vaccination are regarded to be effective when an haemagglutination inhibition (HI) titer of at least 40 can be induced, or alternatively a four times increase in HI titer compared to the prevaccination titer. However, when translating this to the titres found in Gabonese children, even the titres before vaccination would seem to be protective against influenza A viruses. It is possible that the viruses had been circulating very recently but it is also possible that in Gabonese population, exposed to many more pathogens than western

populations, the (persistence of) HI titres are influenced by unknown factors.

Also for TT, WHO describes protective levels as between 0.1 and 0.2 IU/ml, or 0.51-1.02 μg/ml found by ELISA (or 0.01 IU/ml in a neutralization assay). However, WHO emphasizes that often cases of tetanus occur in individuals with levels of antibodies in this range, or even above [185]. As for tetanus a fully standardized and readily used assay that correlates with toxin neutralization is not available, it is difficult to judge the situation. Consequently, antibody levels have not been validated against the relative risk of disease at a defined titer, something that has been done for e.g. pertussis, RSV, meningococci and pneumococci. Therefore, for tetanus, a "protective antibody concentration" may not be considered a guarantee of immunity under all circumstances.

Thus, the current correlates of protection are based on a single characteristic that mostly will not suffice to define efficacy of the complex immune responses induced by vaccines. Therefore, next to the humoral component, for many vaccines a T cell component should be included in the definition of protection. To this end, the quality of the antigen specific T cell populations should be defined and currently the role of the so-called poly- or multi-functional T cells is gaining more and more importance. The presence of T cells that produce more than one cytokine at the same time, have been found to be related to better protection in different infectious disease models (inclucing HIV, *Leishmania major* and *Mycobacterium tuberculosis*) than the presence of T cells producing only one cytokine as reviewed by Seder *et al* [254]. It was hypothesized that measurement of a relatively simple set of cytokines, IL-2, TNF and IFN-γ, in the same T cells can be used to define a vaccine-elicited response; TNF and IFN-γ are involved in clearance of the pathogen and IL-2 promotes expansion of $CD4^+$ as well as $CD8^+$ T cells, thereby having an indirect effect. The superior protective effects of multifunctional T cells was ascribed to increased efficacy at both the effector and the memory T cell level: multifunctional T cells produce more cytokines on a per cell basis and induce more efficient killing, but they were also found to serve as a reservoir of memory $CD4^+$ T cells with effector potential [254].
However, in order to be able to translate these multifunctional T cell responses to a correlate of protection for vaccination, a definition based on the magnitude as well as the quality of the multifunctional and memory T cells would be needed. Moreover, it could be necessary to include also cytokines produced by Th2, Th17 and Treg cells into the analysis, as the balance between the different T cells will define the final outcome of the immune response.

Mechanisms

Lipid molecules are immuno-active moieties

The helminth derived molecules studied in this thesis are predominantly of lipid nature. Traditionally, lipids were not thought to have an immunological role, as for lipid molecules, if involved, often the immunoactive group was found to be the headgroup and not the lipid moiety itself. However, the influence of these headgroups could differ between protein and lipid molecules, as for example shown in chapter two where antibody profiles to *A. lumbricoides* derived glycolipids and (glyco)proteins were described to be very different. The influence of the phosphorylcholine moiety was found to be stronger for lipids compared to proteins, which indicates that these different classes of compounds may have distinct roles in interacting with, and shaping of humoral immune responses.

Recently, also the immunoactivity of the lipids themselves is receiving more attention, especially since it was found that the lipid moieties present in products of pathogens can have an essential role in receptor recognition. In this respect, TLR2 is an interesting receptor that can be activated by several types of lipids, with or without forming heterodimers with TLR1 or 6 (for recognition of tri- and di-acylated lipids, respectively). Moreover, TLR2 signalling showed to be very diverse as it has been reported to be capable of inducing Th1 [239], Th2 [108, 237] as well as regulatory responses [96, 238, 255]. Understanding the pathways involved in downstream signalling upon TLR activation by different ligands is a topic of current research, as TLR ligands are thought to be interesting candidates for use as adjuvants in vaccines. In order to use them in clinical applications, it should be clear which parts of the pathways should or should not be targeted to cure for example an allergic disease but prevent adverse effects.

TLR related signalling

Starting close to the receptor itself, two main adaptor molecules can be involved in TLR signalling, MyD88 and TRIF. The latter is used by TLR3 and is optional for TLR4. TLR ligands activating both the MyD88 and the TRIF pathway were found to act synergistically [256]. This could also be true for other receptors, as still much is to be discovered about how signalling pathways of different TLRs interfere with pathways activated by other receptor families, including CLRs, NLRs, RLRs and scavenger receptors [194, 246].

All signals from activation of receptors or combinations thereof need to be integrated to mount a final immune polarization profile that should be

communicated to T cells. This signal integration is performed by antigen presenting cells, and of these the dendritic cells (DCs) are thought to be central to immune activation and silencing [88]. Phosphorylation of the MAP kinases is an early event in the signal integration after receptor ligation and differential activation of the MAP kinases p38 and ERK in DCs has been associated with specific T cell polarization. p38 is thought to be important in mediating DC maturation and pro-inflammatory and Th1 responses, whereas ERK activation has more often been associated with anti-inflammatory and Th2 responses. Thus, helminth derived antigens would induce an increased *p*-ERK/*p*-p38 ratio and this is described in chapter five as well as by others [93, 108, 235].

However, in chapter five we describe a difference between protein and lipid molecules derived from schistosomes; both SEA and the phospholipid preparation induced an increase in the *p*-ERK/*p*-p38 ratio, but SEA did so by increasing levels of activated ERK, whereas the phospholipid preparation reduced levels of activated p38. Other differences between the protein and lipid preparations derived from schistosomes include the strength of the T cell polarization, which was somewhat stronger for SEA and more moderate for the lipid preparation, and the levels of mRNA in the DCs 16 hours after stimulation. The mRNA profiles were very similar for the protein and the lipid preparation, thus the same type of mRNA molecules were usually up- (e.g. c-fos) or down-regulated (e.g. delta4), however, the expression levels were generally lower in the DCs pulsed with the lipid preparation. Together, the T cell polarization and levels of mRNA indicate that SEA has a stronger stimulatory component compared to the phospholipid preparations.
Relating this to the MAP kinase responses, the *p*-ERK/*p*-p38 ratio, which was similar for SEA and the lipid preparation, is indicative for the Th2/Th1 inducing potential which is in agreement with what others have found. But it is tempting to speculate that, in addition to the effect of the *p*-ERK/*p*-p38 ratio on the polarization, the absolute levels of phosphorylation of ERK and p38 influence the activation / regulation balance, with higher levels leading to more immune activation.

Involvement of additional TLR or non-TLR receptors
Concerning involvement of other receptors in addition to TLR2, the Th1 inducing bacteria described in chapter five, *E. coli* an *L. monocytogenes* were reported to involve additional receptors. *E. coli* has been shown to activate TLR4 and NOD1 [243, 244], whereas resistance to *Listeria* infection was related to the presence of functional NOD2 [245]. Relatively little is known about Th2 skewing by the helminth derived compounds, but in a previous study for schistosomal lipids it was shown that TLR2 activation was not needed for Th2, but rather for regulatory responses [96]. Our own preliminary results with stimulation of peritoneal macrophages from

MyD88, TIRAP, TLR1, TLR2 and TLR6 deficient mice, indicate that the phospholipid fractions activate MyD88 as well as TIRAP, of which the latter is specifically involved in TLR2 signalling. Further, when the preparation was separated into several subfractions, using HPLC, two of these fractions seemed to contain TLR2 activating molecules, of which one, undefined, subfraction involved TLR1, whereas another subfraction, containing lysophosphatidylserine molecules, as defined by mass spectrometry, was found to activate TLR2 in the presence of TLR6. However, further research is needed to confirm these results and to identify the molecules responsible for this activation.

Clinical implementations of helminth-derived molecules

Clinical implementations of helminth derived antigens could be manifold: many inflammatory diseases may be the possible targets of treatment with modulatory helminth based compounds. For example the filarial ES-62, at concentrations equivalent to those found in the bloodstream of filaria-infected humans, was recently shown to prevent degranulation of mast cells, thereby reducing allergic responses. Interestingly, also for this function TLR4 ligation of the ES-62 molecule was shown to be necessary [257].
Moreover, as helminth infections clearly influence the immune response to non-related antigens, also this more general effect of helminth derived antigens could be used in a clinical setting. In this respect, the treatment of patients with inflammatory bowel disease or asthma with live (porcine) helminth eggs or larvae respectively [77, 79, 249] is encouraging. However, to use this in a more controlled setting, more studies are needed to investigate the effects of single molecules, as these will be easier to administer, especially for use in larger populations. On the other hand, it is very likely that a mixture of at least a few molecules is needed to induce a controlled immune response. Moreover, research will be necessary to study long-term effects, as induction of a regulatory response might be interesting for curing allergies or autoimmune diseases, but when this response is not antigen specific, it might have adverse effects on protection from infectious diseases and cancer. Thus, influencing immune responses in a controlled and antigen specific manner is our future challenge.

R

References

References

[1] Finkelman FD, Shea-Donohue T, Morris SC, et al. Interleukin-4- and interleukin-13-mediated host protection against intestinal nematode parasites. Immunological Reviews 2004;201(1):139-55.

[2] Ouaissi MA, Auriault C, Santoro F, Capron A. Interaction between *Schistosoma mansoni* and the complement system: role of IgG Fc peptides in the activation of the classical pathway by schistosomula. J.Immunol. 1981;127(4):1556-9.

[3] Auriault C, Ouaissi MA, Torpier G, Eisen H, Capron A. Proteolytic cleavage of IgG bound to the Fc receptor of *Schistosoma mansoni* schistosomula. Parasite Immunol. 1981;3(1):33-44.

[4] Maizels RM, Bundy DA, Selkirk ME, Smith DF, Anderson RM. Immunological modulation and evasion by helminth parasites in human populations. Nature. 1993;365(6449):797-805.

[5] Smith P, Fallon RE, Mangan NE, et al. *Schistosoma mansoni* secretes a chemokine binding protein with antiinflammatory activity. J.Exp.Med. 2005;202(10):1319-25.

[6] Maizels RM, Yazdanbakhsh M. Immune regulation by helminth parasites: cellular and molecular mechanisms. Nat.Rev.Immunol. 2003;3(9):733-44.

[7] Spiegel A, Tall A, Raphenon G, Trape JF, Druilhe P. Increased frequency of malaria attacks in subjects co-infected by intestinal worms and *Plasmodium falciparum* malaria. Trans.R.Soc.Trop.Med.Hyg. 2003;97(2):198-9.

[8] Nacher M, Singhasivanon P, Yimsamran S, et al. Intestinal helminth infections are associated with increased incidence of *Plasmodium falciparum* malaria in Thailand. J.Parasitol. 2002;88(1):55-8.

[9] Lyke KE, Dicko A, Dabo A, et al. Association of *Schistosoma haematobium* infection with protection against acute *Plasmodium falciparum* malaria in Malian children. Am.J Trop.Med.Hyg. 2005;73(6):1124-30.

[10] Le Hesran JY, Akiana J, Ndiaye EHM, Dia M, Senghor P, Konate L. Severe malaria attack is associated with high prevalence of *Ascaris lumbricoides* infection among children in rural Senegal. Transactions of the Royal Society of Tropical Medicine and Hygiene 2004;98(7):397-9.

[11] Nacher M, Gay F, Singhasivanon P, et al. *Ascaris lumbricoides* infection is associated with protection from cerebral malaria. Parasite Immunology 2000;22(3):107-13.

[12] Nacher M, Singhasivanon P, Silachamroon U, et al. Helminth infections are associated with protection from malaria-related acute renal failure and jaundice in Thailand. Am J Trop Med Hyg 2001;65(6):834-6.

[13] Mahanty S, Mollis SN, Ravichandran M, et al. High levels of spontaneous and parasite antigen-driven interleukin-10 production are associated with antigen-specific hyporesponsiveness in human lymphatic filariasis. J.Infect.Dis. 1996;173(3):769-73.

[14] Su Z, Segura M, Morgan K, Loredo-Osti JC, Stevenson MM. Impairment of Protective Immunity to Blood-Stage Malaria by Concurrent Nematode Infection. Infect.Immun. 2005;73(6):3531-9.

[15] Lwin M, Last C, Targett GA, Doenhoff MJ. Infection of mice concurrently with *Schistosoma mansoni* and rodent malarias: contrasting effects of patent *S. mansoni* infections on *Plasmodium chabaudi*, *P. yoelii* and *P. berghei*. Ann.Trop.Med.Parasitol. 1982;76(3):265-73.

[16] Druilhe P, Tall A, Sokhna C. Worms can worsen malaria: towards a new means to roll back malaria? Trends in Parasitology 2005;21(8):359-62.

[17] Hartgers FC, Yazdanbakhsh M. Co-infection of helminths and malaria: modulation of the immune responses to malaria. Parasite Immunol. 2006;28(10):497-506.

[18] Prost A, Nebout M, Rougemont A. Lepromatous leprosy and onchocerciasis. Br.Med.J. 1979;1(6163):589-90.

[19] Stewart GR, Boussinesq M, Coulson T, Elson L, Nutman T, Bradley JE. Onchocerciasis modulates the immune response to mycobacterial antigens. Clin.Exp.Immunol. 1999;117(3):517-23.

[20] Sacco RE, Hagen M, Sandor M, Weinstock JV, Lynch RG. Established TH1 Granulomatous Responses Induced by Active *Mycobacterium avium* Infection Switch to TH2 Following Challenge with *Schistosoma mansoni*. Clinical Immunology 2002;104(3):274-81.

[21] Talaat KR, Bonawitz RE, Domenech P, Nutman TB. Preexposure to Live *Brugia malayi* Microfilariae Alters the Innate Response of Human Dendritic Cells to *Mycobacterium tuberculosis*. J Infect Dis 2006;193(2):196-204.

[22] Modjarrad K, Zulu I, Redden DT, et al. Treatment of Intestinal Helminths Does Not Reduce Plasma Concentrations of HIV-1 RNA in Coinfected Zambian Adults. J Infect Dis 2005;192(7):1277-83.

[23] Wolday D, Mayaan S, Mariam ZG, et al. Treatment of intestinal worms is associated with decreased HIV plasma viral load. J Acquir.Immune.Defic.Syndr. 2002;31(1):56-62.

[24] Brown M, Mawa PA, Joseph S, et al. Treatment of *Schistosoma mansoni* infection increases helminth-specific type 2 cytokine responses and HIV-1 loads in coinfected Ugandan adults. J.Infect.Dis. 2005;191(10):1648-57.

[25] Brown M, Kizza M, Watera C, et al. Helminth infection is not associated with faster progression of HIV disease in coinfected adults in Uganda. J Infect Dis. 2004;190(10):1869-79.

[26] Nielsen NO, Simonsen PE, Magnussen P, Magesa S, Friis H. Cross-sectional relationship between HIV, lymphatic filariasis and other parasitic infections in adults in coastal northeastern Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene 2006;100(6):543-50.

[27] Brenchley JM, Price DA, Douek DC. HIV disease: fallout from a mucosal catastrophe? Nat.Immunol. 2006;7(3):235-9.

[28] Cooper PJ, Chico M, Sandoval C, et al. Human Infection with *Ascaris lumbricoides* Is Associated with Suppression of the Interleukin-2 Response to Recombinant Cholera Toxin B Subunit following Vaccination with the Live Oral Cholera Vaccine CVD 103-HgR. Infect.Immun. 2001;69(3):1574-80.

[29] Cooper PJ, Chico ME, Losonsky G, et al. Albendazole treatment of children with ascariasis enhances the vibriocidal antibody response to the live attenuated oral cholera vaccine CVD 103-HgR. J Infect.Dis. 2000;182(4):1199-206.

[30] Khan WI, Collins SM. Immune-mediated alteration in gut physiology and its role in host defence in nematode infection. Parasite Immunology 2004;26(8-9):319-26.

[31] Elias D, Wolday D, Akuffo H, Petros B, Bronner U, Britton S. Effect of deworming on human T cell responses to mycobacterial antigens in helminth-exposed individuals before and after bacille Calmette-Guerin (BCG) vaccination. Clinical and Experimental Immunology 2001;123(2):219-25.

[32] Elias D, Akuffo H, Pawlowski A, Haile M, Schon T, Britton S. *Schistosoma mansoni* infection reduces the protective efficacy of BCG vaccination against virulent *Mycobacterium tuberculosis*. Vaccine. 2005;23(11):1326-34.

[33] Malhotra I, Mungai P, Wamachi A, et al. Helminth- and Bacillus Calmette-Guerin-Induced Immunity in Children Sensitized In Utero to Filariasis and Schistosomiasis. J Immunol 1999;162(11):6843-8.

[34] Nookala S, Srinivasan S, Kaliraj P, Narayanan RB, Nutman TB. Impairment of tetanus-specific cellular and humoral responses following tetanus vaccination in human lymphatic filariasis. Infect.Immun. 2004;72(5):2598-604.

[35] Prost A, Schlumberger M, Fayet MT. Response to tetanus immunization in onchocerciasis patients. Ann.Trop.Med.Parasitol. 1983;77(1):83-5.

[36] Cooper PJ, Espinel I, Paredes W, Guderian RH, Nutman TB. Impaired tetanus-specific cellular and humoral responses following tetanus vaccination in human onchocerciasis: a possible role for interleukin-10. J Infect Dis. 1998;178(4):1133-8.

[37] Cooper PJ, Espinel I, Wieseman M, et al. Human Onchocerciasis and Tetanus Vaccination: Impact on the Postvaccination Antitetanus Antibody Response. Infect.Immun. 1999;67(11):5951-7.

[38] Sabin EA, Araujo MI, Carvalho EM, Pearce EJ. Impairment of tetanus toxoid-specific Th1-like immune responses in humans infected with *Schistosoma mansoni*. J Infect Dis. 1996;173(1):269-72.

[39] Ayash-Rashkovsky M, Weisman Z, Diveley J, Moss RB, Bentwich Z, Borkow G. Generation of Th1 immune responses to inactivated, gp120-depleted HIV-1 in mice with a dominant Th2 biased immune profile via imunostimulatory oligonucleotides--relevance to AIDS vaccines in developing countries. Vaccine 2002;20(21-22):2684-92.

[40] Yazdanbakhsh M, van den BA, Maizels RM. Th2 responses without atopy: immunoregulation in chronic helminth infections and reduced allergic disease. Trends Immunol 2001;22(7):372-7.

[41] Cooper PJ. Intestinal worms and human allergy. Parasite Immunol. 2004;26(11-12):455-67.

[42] van den Biggelaar AH, Rodrigues LC, van Ree R, et al. Long-term treatment of intestinal helminths increases mite skin-test reactivity in Gabonese schoolchildren. J Infect Dis. 2004;189(5):892-900.

[43] Cooper PJ, Chico ME, Vaca MG, et al. Effect of albendazole treatments on the prevalence of atopy in children living in communities endemic for geohelminth parasites: a cluster-randomised trial. The Lancet 2006;367(9522):1598-603.

[44] Lau S, Matricardi PM. Worms, asthma, and the hygiene hypothesis. The Lancet 2006;367(9522):1556-8.

[45] Wilson MS, Taylor MD, Balic A, Finney CAM, Lamb JR, Maizels RM. Suppression of allergic airway inflammation by helminth-induced regulatory T cells. J.Exp.Med. 2005;202(9):1199-212.

[46] Smits HH, Hammad H, van Nimwegen M, et al. Protective effect of Schistosoma mansoni infection on allergic airway inflammation depends on the intensity and chronicity of infection. J Allergy Clin.Immunol. 2007;120(4):932-40.

[47] Steel C, Nutman TB. CTLA-4 in Filarial Infections: Implications for a Role in Diminished T Cell Reactivity. J Immunol 2003;170(4):1930-8.

[48] Taylor MD, LeGoff L, Harris A, Malone E, Allen JE, Maizels RM. Removal of Regulatory T Cell Activity Reverses Hyporesponsiveness and Leads to Filarial Parasite Clearance In Vivo. J Immunol 2005;174(8):4924-33.

[49] Maizels RM, Balic A, Gomez-Escobar N, Nair M, Taylor MD, Allen JE. Helminth parasites--masters of regulation. Immunol.Rev. 2004;201:89-116.

[50] Mangan NE, Fallon RE, Smith P, van Rooijen N, McKenzie AN, Fallon PG. Helminth Infection Protects Mice from Anaphylaxis via IL-10-Producing B Cells. J Immunol 2004;173(10):6346-56.

[51] Hagel I, Lynch NR, Di Prisco MC, Rojas E, Perez M, Alvarez N. *Ascaris* reinfection of slum children: relation with the IgE response. Clin.Exp.Immunol. 1993;94(1):80-3.

[52] Araujo MI, Lopes AA, Medeiros M, et al. Inverse association between skin response to aeroallergens and *Schistosoma mansoni* infection. Int.Arch.Allergy Immunol. 2000;123(2):145-8.

[53] van den Biggelaar AH, van Ree R, Rodrigues LC, et al. Decreased atopy in children infected with *Schistosoma haematobium*: a role for parasite-induced interleukin-10. Lancet 2000;356(9243):1723-7.

[54] van den Biggelaar AH, Lopuhaa C, van Ree R, et al. The prevalence of parasite infestation and house dust mite sensitization in Gabonese schoolchildren. Int.Arch.Allergy Immunol. 2001;126(3):231-8.

[55] Scrivener S, Yemaneberhan H, Zebenigus M, et al. Independent effects of intestinal parasite infection and domestic allergen exposure on risk of wheeze in Ethiopia: a nested case-control study. Lancet 2001;358(9292):1493-9.

[56] Nyan OA, Walraven GEL, Banya WAS, et al. Atopy, intestinal helminth infection and total serum IgE in rural and urban adult Gambian communities. Clinical & Experimental Allergy 2001;31(11):1672-8.

[57] Cooper PJ, Chico ME, Rodrigues LC, et al. Reduced risk of atopy among school-age children infected with geohelminth parasites in a rural area of the tropics. J.Allergy Clin.Immunol. 2003;111(5):995-1000.

[58] Medeiros J, Figueiredo JP, Almeida MC, et al. *Schistosoma mansoni* infection is associated with a reduced course of asthma. Journal of Allergy and Clinical Immunology 2003;111(5, Supplement 1):947-51.

[59] Dagoye D, Bekele Z, Woldemichael K, et al. Wheezing, Allergy, and Parasite Infection in Children in Urban and Rural Ethiopia. Am.J.Respir.Crit.Care Med. 2003;167(10):1369-73.

[60] Elliott AM, Mpairwe H, Quigley MA, et al. Helminth infection during pregnancy and development of infantile eczema. JAMA. 2005;294(16):2032-4.

[61] Cooper PJ, Chico ME, Bland M, Griffin GE, Nutman TB. Allergic Symptoms, Atopy, and Geohelminth Infections in a Rural Area of Ecuador. Am.J.Respir.Crit.Care Med. 2003;168(3):313-7.

[62] Davey G, Venn A, Belete H, Berhane Y, Britton J. Wheeze, allergic sensitization and geohelminth infection in Butajira, Ethiopia. Clinical & Experimental Allergy 2005;35(3):301-7.

[63] Buijs J, Borsboom G, Renting M, et al. Relationship between allergic manifestations and Toxocara seropositivity: a cross-sectional study among elementary school children. Eur Respir J 1997;10(7):1467-75.

[64] Palmer LJ, Celedon JC, Weiss ST, Wang B, Fang Z, Xu X. *Ascaris lumbricoides* Infection Is Associated with Increased Risk of Childhood Asthma and Atopy in Rural China. Am.J.Respir.Crit.Care Med. 2002;165(11):1489-93.

[65] Obihara CC, Beyers N, Gie RP, et al. Respiratory atopic disease, Ascaris-immunoglobulin E and tuberculin testing in urban South African children. Clinical & Experimental Allergy 2006;36(5):640-8.

[66] Araujo MI, Hoppe B, Medeiros M, Jr., et al. Impaired T helper 2 response to aeroallergen in helminth-infected patients with asthma. J.Infect.Dis. 2004;190(10):1797-803.

[67] Lynch NR, Palenque M, Hagel I, DiPrisco MC. Clinical improvement of asthma after anthelminthic treatment in a tropical situation. Am.J.Respir.Crit Care Med. 1997;156(1):50-4.

[68] Lima C, Perini A, Garcia ML, Martins MA, Teixeira MM, Macedo MS. Eosinophilic inflammation and airway hyper-responsiveness are profoundly inhibited by a helminth (*Ascaris suum*) extract in a murine model of asthma. Clin.Exp.Allergy 2002;32(11):1659-66.

[69] Itami DM, Oshiro TM, Araujo CA, et al. Modulation of murine experimental asthma by *Ascaris suum* components. Clin.Exp.Allergy. 2005;35(7):873-9.

[70] Bashir ME, Andersen P, Fuss IJ, Shi HN, Nagler-Anderson C. An enteric helminth infection protects against an allergic response to dietary antigen. J.Immunol. 2002;169(6):3284-92.

[71] Dunne DW, Cooke A. A worm's eye view of the immune system: consequences for evolution of human autoimmune disease. Nat.Rev.Immunol. 2005;5(5):420-6.

[72] Cooke A, Tonks P, Jones FM, et al. Infection with *Schistosoma mansoni* prevents insulin dependent diabetes mellitus in non-obese diabetic mice. Parasite Immunol. 1999;21(4):169-76.

[73] Zaccone P, Fehervari Z, Jones FM, et al. *Schistosoma mansoni* antigens modulate the activity of the innate immune response and prevent onset of type 1 diabetes. Eur.J.Immunol. 2003;33(5):1439-49.

[74] Moreels TG, Nieuwendijk RJ, De Man JG, et al. Concurrent infection with *Schistosoma mansoni* attenuates inflammation induced changes in colonic morphology, cytokine levels, and smooth muscle contractility of trinitrobenzene sulphonic acid induced colitis in rats. Gut 2004;53(1):99-107.

[75] Khan WI, Blennerhasset PA, Varghese AK, et al. Intestinal Nematode Infection Ameliorates Experimental Colitis in Mice. Infect.Immun. 2002;70(11):5931-7.

[76] Summers RW, Elliott DE, Qadir K, Urban JF, Thompson R, Weinstock JV. *Trichuris suis* seems to be safe and possibly effective in the treatment of inflammatory bowel disease. The American Journal of Gastroenterology 2003;98(9):2034-41.

[77] Summers RW, Elliott DE, Urban JF, Jr., Thompson R, Weinstock JV. *Trichuris suis* therapy in Crohn's disease. Gut 2005;54(1):87-90.

[78] Sewell D, Qing Z, Reinke E, et al. Immunomodulation of experimental autoimmune encephalomyelitis by helminth ova immunization. Int.Immunol. 2003;15(1):59-69.

[79] Summers RW, Elliott DE, Urban JF, Jr., Thompson RA, Weinstock JV. *Trichuris suis* therapy for active ulcerative colitis: a randomized controlled trial. Gastroenterology. 2005;128(4):825-32.

[80] El Wakil HS, Aboushousha TS, El Haddad O, Gamil NB, Mansour T, El Said H. Effect of *Schistosoma mansoni* egg deposition on multiple low doses streptozotocin induced insulin dependent diabetes. J.Egypt.Soc.Parasitol. 2002;32(3):987-1002.

[81] Imai S, Tezuka H, Fujita K. A Factor of Inducing IgE from a Filarial Parasite Prevents Insulin-Dependent Diabetes Mellitus in Nonobese Diabetic

Mice. Biochemical and Biophysical Research Communications 2001;286(5):1051-8.

[82] Elliott DE, Setiawan T, Metwali A, Blum A, Urban JF, Jr., Weinstock JV. *Heligmosomoides polygyrus* inhibits established colitis in IL-10-deficient mice. Eur.J Immunol. 2004;34(10):2690-8.

[83] Elliott DE, Li J, Blum A, et al. Exposure to schistosome eggs protects mice from TNBS-induced colitis. Am J Physiol Gastrointest Liver Physiol 2003;284(3):G385-G391

[84] Reardon C, Sanchez A, Hogaboam CM, McKay DM. Tapeworm Infection Reduces Epithelial Ion Transport Abnormalities in Murine Dextran Sulfate Sodium-Induced Colitis. Infect.Immun. 2001;69(7):4417-23.

[85] La Flamme AC, Ruddenklau K, Backstrom BT. Schistosomiasis Decreases Central Nervous System Inflammation and Alters the Progression of Experimental Autoimmune Encephalomyelitis. Infect.Immun. 2003;71(9):4996-5004.

[86] Nagayama Y, Watanabe K, Niwa M, McLachlan SM, Rapoport B. *Schistosoma mansoni* and {alpha}-Galactosylceramide: Prophylactic Effect of Th1 Immune Suppression in a Mouse Model of Graves' Hyperthyroidism. J Immunol 2004;173(3):2167-73.

[87] McInnes IB, Leung BP, Harnett M, Gracie JA, Liew FY, Harnett W. A Novel Therapeutic Approach Targeting Articular Inflammation Using the Filarial Nematode-Derived Phosphorylcholine-Containing Glycoprotein ES-62. J Immunol 2003;171(4):2127-33.

[88] Kapsenberg ML. Dendritic-cell control of pathogen-driven T-cell polarization. Nat.Rev.Immunol. 2003;3(12):984-93.

[89] Shortman K, Liu YJ. Mouse and human dendritic cell subtypes. Nat.Rev.Immunol 2002;2(3):151-61.

[90] Cervi L, MacDonald AS, Kane C, Dzierszinski F, Pearce EJ. Cutting Edge: Dendritic Cells Copulsed with Microbial and Helminth Antigens Undergo Modified Maturation, Segregate the Antigens to Distinct Intracellular Compartments, and Concurrently Induce Microbe-Specific Th1 and Helminth-Specific Th2 Responses. J Immunol 2004;172(4):2016-20.

[91] Balic A, Harcus Y, Holland MJ, Maizels RM. Selective maturation of dendritic cells by *Nippostrongylus brasiliensis*-secreted proteins drives Th2 immune responses. Eur.J.Immunol. 2004;34(11):3047-59.

[92] Jenkins SJ, Mountford AP. Dendritic Cells Activated with Products Released by Schistosome Larvae Drive Th2-Type Immune Responses, Which

Can Be Inhibited by Manipulation of CD40 Costimulation. Infect.Immun. 2005;73(1):395-402.

[93] Thomas PG, Carter MR, Atochina O, et al. Maturation of dendritic cell 2 phenotype by a helminth glycan uses a Toll-like receptor 4-dependent mechanism. J.Immunol. 2003;171(11):5837-41.

[94] Whelan M, Harnett MM, Houston KM, Patel V, Harnett W, Rigley KP. A Filarial Nematode-Secreted Product Signals Dendritic Cells to Acquire a Phenotype That Drives Development of Th2 Cells. J Immunol 2000;164(12):6453-60.

[95] Kane CM, Cervi L, Sun J, et al. Helminth Antigens Modulate TLR-Initiated Dendritic Cell Activation. J Immunol 2004;173(12):7454-61.

[96] van der Kleij D, Latz E, Brouwers JF, et al. A novel host-parasite lipid cross-talk. Schistosomal lyso-phosphatidylserine acivates Toll-like receptor 2 and affects immune polarization. J Biol.Chem. 2002;277(50):48122-9.

[97] Mahnke K, Qian Y, Knop J, Enk AH. Induction of CD4+/CD25+ regulatory T cells by targeting of antigens to immature dendritic cells. Blood 2003;101(12):4862-9.

[98] Atochina O, Daly-Engel T, Piskorska D, McGuire E, Harn DA. A Schistosome-Expressed Immunomodulatory Glycoconjugate Expands Peritoneal Gr1+ Macrophages That Suppress Naive CD4+ T Cell Proliferation Via an IFN-{gamma} and Nitric Oxide-Dependent Mechanism. J Immunol 2001;167(8):4293-302.

[99] Goodridge HS, Marshall FA, Wilson EH, et al. *In vivo* exposure of murine dendritic cell and macrophage bone marrow progenitors to the phosphorylcholine-containing filarial nematode glycoprotein ES-62 polarizes their differentiation to an anti-inflammatory phenotype. Immunology 2004;113(4):491-8.

[100] Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat.Immunol. 2001;2(8):675-80.

[101] Martinon F, Tschopp J. NLRs join TLRs as innate sensors of pathogens. Trends in Immunology 2005;26(8):447-54.

[102] Yoneyama M, Kikuchi M, Natsukawa T, et al. The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat.Immunol. 2004;5(7):730-7.

[103] Akira S, Uematsu S, Takeuchi O. Pathogen Recognition and Innate Immunity. Cell 2006;124(4):783-801.

[104] Aksoy E, Zouain CS, Vanhoutte F, et al. Double-stranded RNAs from the helminth parasite Schistosoma activate TLR3 in dendritic cells. J.Biol.Chem. 2005;280(1):277-83.

[105] Goodridge HS, Marshall FA, Else KJ, et al. Immunomodulation via Novel Use of TLR4 by the Filarial Nematode Phosphorylcholine-Containing Secreted Product, ES-62. J Immunol 2005;174(1):284-93.

[106] Dillon S, Agrawal S, Banerjee K, et al. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. J.Clin.Invest. 2006;116(4):916-28.

[107] Blander JM, Medzhitov R. Toll-dependent selection of microbial antigens for presentation by dendritic cells. Nature. 2006;440(7085):808-12.

[108] Agrawal S, Agrawal A, Doughty B, et al. Cutting edge: different Toll-like receptor agonists instruct dendritic cells to induce distinct Th responses via differential modulation of extracellular signal-regulated kinase-mitogen-activated protein kinase and c-Fos. J.Immunol. 2003;171(10):4984-9.

[109] Caparros E, Munoz P, Sierra-Filardi E, et al. DC-SIGN ligation on dendritic cells results in ERK and PI3K activation and modulates cytokine production. Blood 2006;107(10):3950-8.

[110] de Silva NR, Chan MS, Bundy DA. Morbidity and mortality due to ascariasis: re-estimation and sensitivity analysis of global numbers at risk. Trop.Med.Int.Health 1997;2(6):519-28.

[111] McSharry C, Xia Y, Holland CV, Kennedy MW. Natural immunity to *Ascaris lumbricoides* associated with immunoglobulin E antibody to ABA-1 allergen and inflammation indicators in children. Infect.Immun. 1999;67(2):484-9.

[112] Palmer DR, Hall A, Haque R, Anwar KS. Antibody isotype responses to antigens of *Ascaris lumbricoides* in a case-control study of persistently heavily infected Bangladeshi children. Parasitology 1995;111(Pt 3):385-93.

[113] Turner JD, Faulkner H, Kamgno J, et al. Allergen-specific IgE and IgG4 are markers of resistance and susceptibility in a human intestinal nematode infection. Microbes.Infect. 2005;7(7-8):990-6.

[114] Geiger SM, Massara CL, Bethony J, Soboslay PT, Carvalho OS, Correa-Oliveira R. Cellular responses and cytokine profiles in *Ascaris lumbricoides* and *Trichuris trichiura* infected patients. Parasite Immunol. 2002;24(11-12):499-509.

[115] Thomas PG, Harn DA, Jr. Immune biasing by helminth glycans. Cell Microbiol. 2004;6(1):13-22.

[116] Dennis RD, Baumeister S, Lauer G, Richter R, Geyer E. Neutral glycolipids of Schistosoma mansoni as feasible antigens in the detection of schistosomiasis. Parasitology 1996;112(Pt 3):295-307.

[117] van der Kleij D, Tielens AGM, Yazdanbakhsh M. Recognition of schistosome glycolipids by immunoglobulin E: possible role in immunity. Infect.Immun. 1999;67(11):5946-50.

[118] Robijn ML, Wuhrer M, Kornelis D, Deelder AM, Geyer R, Hokke CH. Mapping fucosylated epitopes on glycoproteins and glycolipids of *Schistosoma mansoni* cercariae, adult worms and eggs. Parasitology 2005;130(Pt 1):67-77.

[119] Weiss JB, Magnani JL, Strand M. Identification of *Schistosoma mansoni* glycolipids that share immunogenic carbohydrate epitopes with glycoproteins. J.Immunol. 1986;136(11):4275-82.

[120] Harnett W, Harnett MM. Phosphorylcholine: friend or foe of the immune system? Immunol.Today 1999;20(3):125-9.

[121] Lochnit G, Dennis RD, Geyer R. Phosphorylcholine substituents in nematodes: structures, occurrence and biological implications. Biol.Chem. 2000;381(9-10):839-47.

[122] Sloan T, Dooge D, Joyce P. Identification of phosphorylcholine containing antigens of *Fasciola hepatica*--successful tolerization against this epitope in experimental animals. Parasite Immunol. 1991;13(4):447-55.

[123] Cundell DR, Gerard NP, Gerard C, Idanpaan-Heikkila I, Tuomanen EI. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. Nature 1995;377(6548):435-8.

[124] Briles DE, Claflin JL, Schroer K, Forman C. Mouse IgG3 antibodies are highly protective against infection with *Streptococcus pneumoniae*. Nature 1981;294(5836):88-90.

[125] Deehan MR, Goodridge HS, Blair D, et al. Immunomodulatory properties of *Ascaris suum* glycosphingolipids - phosphorylcholine and non-phosphorylcholine-dependent effects. Parasite Immunol. 2002;24(9-10):463-9.

[126] Harnett MM, Deehan MR, Williams DM, Harnett W. Induction of signalling anergy via the T-cell receptor in cultured Jurkat T cells by pre-exposure to a filarial nematode secreted product. Parasite Immunol. 1998;20(11):551-63.

[127] Smith PK, Krohn RI, Hermanson GT, et al. Measurement of protein using bicinchoninic acid. Anal.Biochem. 1985;150(1):76-85.

[128] Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can.J.Biochem.Physiol. 1959;37 :911-7.

[129] Rouser G, Kritchevsky G, Yamamoto A, Simon G, Galli C, Bauman AJ. Diethylaminoethyl and triethylaminoethyl cellulose column chromatographic procedures for phospholipids, glycolipids, and pigments. Methods Enzymol. 1969;14:272-317.

[130] Lochnit G, Dennis RD, Ulmer AJ, Geyer R. Structural elucidation and monokine-inducing activity of two biologically active zwitterionic glycosphingolipids derived from the porcine parasitic nematode *Ascaris suum*. J.Biol.Chem. 1998;273(1):466-74.

[131] Wuhrer M, Hokke CH, Deelder AM. Glycopeptide analysis by matrix-assisted laser desorption/ionization tandem time-of-flight mass spectrometry reveals novel features of horseradish peroxidase glycosylation. Rapid Commun.Mass Spectrom. 2004;18(15):1741-8.

[132] Friedl CH, Lochnit G, Z„hringer U, Bahr U, Geyer R. Structural elucidation of zwitterionic carbohydrates derived from glycosphingolipids of the porcine parasitic nematode *Ascaris suum*. Biochem.J. 2003;369(Pt 1):89-102.

[133] Grabitzki J, Sauerland V, Geyer R, Lochnit G. Identification of phosphorylcholine substituted peptides by their characteristic mass spectrometric fragmentation. Eur.J.Mass Spectrom.(Chichester, Eng) 2005;11(3):335-44.

[134] Griffitts JS, Haslam SM, Yang T, et al. Glycolipids as receptors for *Bacillus thuringiensis* crystal toxin. Science 2005;307(5711):922-5.

[135] Gerdt S, Dennis RD, Borgonie G, Schnabel R, Geyer R. Isolation, characterization and immunolocalization of phosphorylcholine-substituted glycolipids in developmental stages of *Caenorhabditis elegans*. Eur.J.Biochem. 1999;266(3):952-63.

[136] Wuhrer M, Rickhoff S, Dennis RD, et al. Phosphocholine-containing, zwitterionic glycosphingolipids of adult *Onchocerca volvulus* as highly conserved antigenic structures of parasitic nematodes. Biochem.J. 2000;348:417-23.

[137] Schmidt GD, Roberts LS. Roberts LS, Janovy JJ, editors.Foundations of parasitology. fifth edition ed. Dubuque: Times Mirror Higher Education Group, Inc.; 1996; 26, Nematodes: Ascaridida, intestinal large roundworms. p. 419-31.

[138] Houston KM, Lochnit G, Geyer R, Harnett W. Investigation of the nature of potential phosphorylcholine donors for filarial nematode glycoconjugates. Mol.Biochem.Parasitol. 2002;123(1):55-66.

[139] Lochnit G, Bongaarts R, Geyer R. Searching new targets for anthelminthic strategies: Interference with glycosphingolipid biosynthesis and phosphorylcholine metabolism affects development of Caenorhabditis elegans. Int.J.Parasitol. 2005;35(8):911-23.

[140] Julian E, Matas L, Alcaide J, Luquin M. Comparison of antibody responses to a potential combination of specific glycolipids and proteins for test sensitivity improvement in tuberculosis serodiagnosis. Clin.Diagn.Lab Immunol. 2004;11(1):70-6.

[141] Belperron AA, Dailey CM, Bockenstedt LK. Infection-induced marginal zone B cell production of Borrelia hermsii-specific antibody is impaired in the absence of CD1d. J.Immunol. 2005;174(9):5681-6.

[142] Romaris F, Escalante M, Valinas B, et al. Characterization of two monoclonal antibodies raised in Btk(xid) mice that recognize phosphorylcholine-bearing antigens from *Trichinella* and other helminths. Parasite Immunol. 2001;23(6):313-22.

[143] Simonsen PE, Lemnge MM, Msangeni HA, Jakobsen PH, Bygbjerg IC. Bancroftian filariasis: the patterns of filarial-specific immunoglobulin G1 (IgG1), IgG4, and circulating antigens in an endemic community of northeastern Tanzania. Am.J.Trop.Med.Hyg. 1996;55(1):69-75.

[144] Hagan P, Blumenthal UJ, Dunn D, Simpson AJ, Wilkins HA. Human IgE, IgG4 and resistance to reinfection with *Schistosoma haematobium*. Nature 1991;349(6306):243-5.

[145] Lal RB, Dhawan RR, Tarrand JJ, Ayoub EM, Ottesen EA. Lack of IgG4 antibody response to carbohydrate antigens in patients with lymphatic filariasis. Immunology 1991;74(2):333-7.

[146] Schoub BD, McAnerney JM, Besselaar TG. Regional perspectives on influenza surveillance in Africa. Vaccine 2002;20(Supplement 2):S45-S46

[147] Nicholson KG, Wood JM, Zambon M. Influenza. The Lancet 2003;362(9397):1733-45.

[148] Normile D, Enserink M. Avian influenza. With change in the seasons, bird flu returns. Science. 2007;315(5811):448

[149] Moszynski P. Africa is ill equipped to fight a bird flu epidemic. BMJ. 2006;332(7538):380

[150] Baily GV. Tuberculosis prevention Trial, Madras. Indian J.Med.Res. 1980;72 Suppl:1-74.:1-74.

[151] Rook GAW, Dheda K, Zumla A. Immune systems in developed and developing countries; implications for the design of vaccines that will work where BCG does not. Tuberculosis 2006;86(3-4):152-62.

[152] van der Kleij D, van den Biggelaar AH, Kruize YC, et al. Responses to Toll-like receptor ligands in children living in areas where schistosome infections are endemic. J.Infect.Dis. 2004;189(6):1044-51.

[153] Fell AH, Smith NC. Immunity to Asexual Blood Stages of Plasmodium: Is Resistance to Acute Malaria Adaptive or Innate? Parasitology Today 1998;14(9):364-9.

[154] Babu S, Blauvelt CP, Kumaraswami V, Nutman TB. Chemokine receptors of T cells and of B cells in lymphatic filarial infection: a role for CCR9 in pathogenesis. J.Infect.Dis. 2005;191(6):1018-26.

[155] Mahanty S, King CL, Kumaraswami V, et al. IL-4- and IL-5-secreting lymphocyte populations are preferentially stimulated by parasite-derived antigens in human tissue invasive nematode infections. J.Immunol. 1993;151(7):3704-11.

[156] King CL, Medhat A, Malhotra I, et al. Cytokine control of parasite-specific anergy in human urinary schistosomiasis. IL-10 modulates lymphocyte reactivity. J.Immunol. 1996;156(12):4715-21.

[157] Cooper PJ, Chico ME, Sandoval C, et al. Human infection with Ascaris lumbricoides is associated with a polarized cytokine response. J.Infect.Dis. 2000;182(4):1207-13.

[158] Jankovic D, Steinfelder S, Kullberg MC, Sher A. Mechanisms underlying helminth- induced Th2 polarization: default, negative or positive pathways? Chem.Immunol.Allergy. 2006;90:65-81.:65-81.

[159] Redpath S, Ghazal P, Gascoigne NRJ. Hijacking and exploitation of IL-10 by intracellular pathogens. Trends in Microbiology 2001;9(2):86-92.

[160] Keller CC, Yamo O, Ouma C, et al. Acquisition of hemozoin by monocytes down-regulates interleukin-12 p40 (IL-12p40) transcripts and circulating IL-12p70 through an IL-10-dependent mechanism: in vivo and in vitro findings in severe malarial anemia. Infect.Immun. 2006;74(9):5249-60.

[161] Guyot-Revol V, Innes JA, Hackforth S, Hinks T, Lalvani A. Regulatory T cells are expanded in blood and disease sites in patients with tuberculosis. Am.J.Respir.Crit Care Med. 2006;173(7):803-10.

[162] Boettler T, Spangenberg HC, Neumann-Haefelin C, et al. T cells with a CD4+CD25+ regulatory phenotype suppress in vitro proliferation of virus-specific CD8+ T cells during chronic hepatitis C virus infection. J.Virol. 2005;79(12):7860-7.

[163] Ogden CL, Kuczmarski RJ, Flegal KM, et al. Centers for Disease Control and Prevention 2000 growth charts for the United States: improvements to the 1977 National Center for Health Statistics version. Pediatrics. 2002;109(1):45-60.

[164] Planche T, Krishna S, Kombila M, et al. Comparison of methods for the rapid laboratory assessment of children with malaria. Am.J.Trop.Med.Hyg. 2001;65(5):599-602.

[165] Katz N, Chaves A, Pellegrino J. A simple device for quantitative stool thick-smear technique in Schistosomiasis mansoni. Rev.Inst.Med.Trop.Sao Paulo. 1972;14(6):397-400.

[166] Palmer DF, Dowdle WR, Coleman MT, and Schild GC. Haemagglutination inhibition test. In: Anonymous, Advanced laboratory techniques for influenza diagnosis.Procedural guide. 25-62. Atlanta, US Dept. Hlth. ED. Welfare.

[167] Masurel N, Ophof P, de Jong P. Antibody response to immunization with influenza A/USSR/77 (H1N1) virus in young individuals primed or unprimed for A/New Jersey/76 (H1N1) virus. J.Hyg.(Lond). 1981;87(2):201-9.

[168] Rimmelzwaan GF, Baars M, van Beek R, et al. Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. J.Gen.Virol. 1997;78(Pt 4):757-65.

[169] Besselaar TG, Botha L, McAnerney JM, Schoub BD. Antigenic and molecular analysis of influenza A (H3N2) virus strains isolated from a localised influenza outbreak in South Africa in 2003. J.Med.Virol. 2004;73(1):71-8.

[170] WHO. Recommended composition of influenza virus vaccines for use in the 2005 influenza season. Wkly.Epidemiol.Rec. 2004;79(41):369-73.

[171] Gachara G, Ngeranwa J, Magana JM, et al. Influenza virus strains in Nairobi, Kenya. Journal of Clinical Virology 2006;35(1):117-8.

[172] Barry M, Patterson JE, Tirrell S, Cullen MR, Shope RE. The effect of chloroquine prophylaxis on yellow fever vaccine antibody response: comparison of plaque reduction neutralization test and enzyme-linked immunosorbent assay. Am.J.Trop.Med.Hyg. 1991;44(1):79-82.

[173] Faucher JF, Binder R, Missinou MA, et al. Efficacy of atovaquone/proguanil for malaria prophylaxis in children and its effect on the immunogenicity of live oral typhoid and cholera vaccines. Clin.Infect.Dis. 2002;35(10):1147-54.

[174] Fryauff DJ, Cryz SJ, Widjaja H, et al. Humoral immune response to tetanus-diphtheria vaccine given during extended use of chloroquine or primaquine malaria chemoprophylaxis. J.Infect.Dis. 1998;177(6):1762-5.

[175] Fryauff DJ, Church LW, Richards AL, et al. Lymphocyte response to tetanus toxoid among Indonesian men immunized with tetanus-diphtheria during extended chloroquine or primaquine prophylaxis. J.Infect.Dis. 1997;176(6):1644-8.

[176] Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu.Rev.Immunol. 2004;22:745-63.:745-63.

[177] Shi HN, Scott ME, Stevenson MM, Koski KG. Energy restriction and zinc deficiency impair the functions of murine T cells and antigen-presenting cells during gastrointestinal nematode infection. J.Nutr. 1998;128(1):20-7.

[178] Kroon FP, van Tol MJ, Jol-van der Zijde CM, van Furth R, van Dissel JT. Immunoglobulin G (IgG) subclass distribution and IgG1 avidity of antibodies in human immunodeficiency virus-infected individuals after revaccination with tetanus toxoid. Clin.Diagn.Lab Immunol. 1999;6(3):352-5.

[179] Dietz V, Galazka A, van Loon F, Cochi S. Factors affecting the immunogenicity and potency of tetanus toxoid: implications for the elimination of neonatal and non-neonatal tetanus as public health problems. Bull.World Health Organ 1997;75(1):81-93.

[180] Brabin L, Fazio-Tirrozzo G, Shahid S, et al. Tetanus antibody levels among adolescent girls in developing countries. Trans.R.Soc.Trop.Med.Hyg. 2000;94(4):455-9.

[181] Jol-van der Zijde C M., van der Kaaden M, Rümke HC, Gerritsen EJA, Vossen JM, and van Tol MJD. The antibody response against tetanus toxoid: a longitudinal study in healthy infants and adults. Chapel H M, Levinsky R J, Webster A D B. , editors. Progress in immune deficiency III., 238-240. 1991. London, United Kingdom, Royal Society of Medicine Services Ltd.

[182] Pullen GR, Fitzgerald MG, Hosking CS. Antibody avidity determination by ELISA using thiocyanate elution. J.Immunol.Methods 1986;86(1):83-7.

[183] Siekmann JH, Allen LH, Watnik MR, et al. Titers of antibody to common pathogens: relation to food-based interventions in rural Kenyan schoolchildren. Am.J.Clin.Nutr. 2003;77(1):242-9.

[184] Monjour L, Bourdillon F, Korinek AM, et al. [Humoral immunity, 5 years after anti-tetanus vaccination, in a group of malaria-infected and malnourished African children]. Pathol.Biol.(Paris) 1988;36(3):235-9.

[185] Borrow R, Balmer P, and Roper MH. Immunization, Vaccines and Biologicals. The immunological basis for immunization series. Module 3: Tetanus Update 2006. Online available: http://www.who.int/vaccines-documents/DocsPDF07/869.pdf. 2006.

[186] Eisenhut M. Immunity to blood stages of Plasmodium falciparum is dependent on a specific pattern of immunoglobulin subclass responses to multiple blood stage antigens. Med Hypotheses 2007;69(4):804-8.

[187] Naus CWA, Jones FM, Satti MZ, et al. Serological responses among individuals in areas where both schistosomiasis and malaria are endemic: Cross-reactivity between Schistosoma mansoni and Plasmodium falciparum. Journal of Infectious Diseases 2003;187(8):1272-82.

[188] van Riet E, Adegnika AA, Retra K, et al. Cellular and humoral responses to influenza in gabonese children living in rural and semi-urban areas. J Infect.Dis. 2007;196(11):1671-8.

[189] Rowe J, Yerkovich ST, Richmond P, et al. Th2-associated local reactions to the acellular diphtheria-tetanus-pertussis vaccine in 4- to 6-year-old children. Infect.Immun. 2005;73(12):8130-5.

[190] Mellman I, Steinman RM. Dendritic cells: specialized and regulated antigen processing machines. Cell 2001;106(3):255-8.

[191] de Jong EC, Vieira PL, Kalinski P, et al. Microbial compounds selectively induce Th1 cell-promoting or Th2 cell-promoting dendritic cells in vitro with diverse th cell-polarizing signals. J Immunol 2002;168(4):1704-9.

[192] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat.Immunol. 2004;5(10):987-95.

[193] Reis e Sousa. Toll-like receptors and dendritic cells: for whom the bug tolls. Semin.Immunol. 2004;16(1):27-34.

[194] Underhill DM. Collaboration between the innate immune receptors dectin-1, TLRs, and Nods. Immunol.Rev. 2007;219:75-87.

[195] MacDonald AS, Maizels RM. Alarming dendritic cells for Th2 induction. J Exp.Med 2008;

[196] Retra K, van Riet E, Adegnika AA, et al. Immunologic activity of schistosomal and bacterial TLR2 ligands in Gabonese children. Parasite Immunol. 2008;30(1):39-46.

[197] The Perkin-Elmer Corporation. User bulletin #2. ABI Prism 7700 Sequence Detection system. P/N 4303859 Rev.A, Stock No 777802-001 . 2005.

[198] Banerjee A, Gerondakis S. Coordinating TLR-activated signaling pathways in cells of the immune system. Immunol.Cell Biol. 2007;85(6):420-4.

[199] Dillon S, Agrawal A, Van Dyke T, et al. expDC-RNAA Toll-like receptor 2 ligand stimulates Th2 responses *in vivo*, via induction of extracellular signal-regulated kinase mitogen-activated protein kinase and c-Fos in dendritic cells. J.Immunol. 2004;172(8):4733-43.

[200] Han J, Lee JD, Bibbs L, Ulevitch RJ. A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells. Science 1994;265(5173):808-11.

[201] Balkhy HH, Heinzel FP. Endotoxin fails to induce IFN-gamma in endotoxin-tolerant mice: deficiencies in both IL-12 heterodimer production and IL-12 responsiveness. J.Immunol. 1999;162(6):3633-8.

[202] Langrish CL, McKenzie BS, Wilson NJ, de Waal MR, Kastelein RA, Cua DJ. IL-12 and IL-23: master regulators of innate and adaptive immunity. Immunol.Rev. 2004;202:96-105.

[203] Ivanov II, Zhou L, Littman DR. Transcriptional regulation of Th17 cell differentiation. Semin.Immunol. 2007;19(6):409-17.

[204] Chen Z, Laurence A, O'Shea JJ. Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation. Semin.Immunol. 2007;19(6):400-8.

[205] Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat.Rev.Immunol. 2003;3(9):745-56.

[206] Kawai T, Takeuchi O, Fujita T, et al. Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes. J.Immunol. 2001;167(10):5887-94.

[207] Weighardt H, Jusek G, Mages J, et al. Identification of a. Eur.J.Immunol. 2004;34(2):558-64.

[208] Delgado M, Gonzalez-Rey E, Ganea D. VIP/PACAP preferentially attract Th2 effectors through differential regulation of chemokine production by dendritic cells. FASEB J. 2004;18(12):1453-5.

[209] Strobl H, Knapp W. TGF-beta1 regulation of dendritic cells. Microbes.Infect. 1999;1(15):1283-90.

[210] Kabelitz D, Wesch D, Oberg HH. Regulation of regulatory T cells: role of dendritic cells and toll-like receptors. Crit Rev.Immunol. 2006;26(4):291-306.

[211] Mahnke K, Johnson TS, Ring S, Enk AH. Tolerogenic dendritic cells and regulatory T cells: a two-way relationship. J.Dermatol.Sci. 2007;46(3):159-67.

[212] Takeda K, Akira S. TLR signaling pathways. Semin.Immunol. 2004;16(1):3-9.

[213] Akira S, Takeda K, Kaisho T. Toll-like receptors: critical proteins linking innate and acquired immunity. Nat.Immunol. 2001;2(8):675-80.

[214] Burns K, Martinon F, Esslinger C, et al. MyD88, an adapter protein involved in interleukin-1 signaling. J.Biol.Chem. 1998;273(20):12203-9.

[215] Inohara N, Nunez G. NODs: intracellular proteins involved in inflammation and apoptosis. Nat.Rev.Immunol. 2003;3(5):371-82.

[216] Pauleau AL, Murray PJ. Role of nod2 in the response of macrophages to toll-like receptor agonists. Mol.Cell Biol. 2003;23(21):7531-9.

[217] Watanabe T, Kitani A, Murray PJ, Strober W. NOD2 is a negative regulator of Toll-like receptor 2-mediated T helper type 1 responses. Nat.Immunol. 2004;5(8):800-8.

[218] Yamamoto M, Sato S, Hemmi H, et al. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. Science 2003;301(5633):640-3.

[219] Hardy MP, McGGettrick AF, O'Neill LA. Transcriptional regulation of the human TRIF (TIR domain-containing adaptor protein inducing interferon beta) gene. Biochem.J. 2004;380(Pt 1):83-93.

[220] Zhang G, Ghosh S. Negative regulation of toll-like receptor-mediated signaling by Tollip. J.Biol.Chem. 2002;277(9):7059-65.

[221] Alexander WS, Starr R, Metcalf D, et al. Suppressors of cytokine signaling (SOCS): negative regulators of signal transduction. J.Leukoc.Biol. 1999;66(4):588-92.

[222] Yoshimura A, Naka T, Kubo M. SOCS proteins, cytokine signalling and immune regulation. Nat.Rev.Immunol. 2007;7(6):454-65.

[223] Jeffrey KL, Brummer T, Rolph MS, et al. Positive regulation of immune cell function and inflammatory responses by phosphatase PAC-1. Nat.Immunol. 2006;7(3):274-83.

[224] Grohmann U, Fallarino F, Puccetti P. Tolerance, DCs and tryptophan: much ado about IDO. Trends Immunol. 2003;24(5):242-8.

[225] Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed death-1 ligand 1 interacts specifically with the B7-1 costimulatory molecule to inhibit T cell responses. Immunity. 2007;27(1):111-22.

[226] Sharpe AH, Wherry EJ, Ahmed R, Freeman GJ. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. Nat.Immunol. 2007;8(3):239-45.

[227] Habicht A, Kewalaramani R, Vu MD, et al. Striking dichotomy of PD-L1 and PD-L2 pathways in regulating alloreactive CD4(+) and CD8(+) T cells in vivo. Am.J.Transplant. 2007;7(12):2683-92.

[228] Bleharski JR, Li H, Meinken C, et al. Use of genetic profiling in leprosy to discriminate clinical forms of the disease. Science 2003;301(5639):1527-30.

[229] Sloane DE, Tedla N, Awoniyi M, et al. Leukocyte immunoglobulin-like receptors: novel innate receptors for human basophil activation and inhibition. Blood 2004;104(9):2832-9.

[230] Amsen D, Blander JM, Lee GR, Tanigaki K, Honjo T, Flavell RA. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell 2004;117(4):515-26.

[231] Schaller MA, Neupane R, Rudd BD, et al. Notch ligand Delta-like 4 regulates disease pathogenesis during respiratory viral infections by modulating Th2 cytokines. J.Exp.Med. 2007;204(12):2925-34.

[232] Nakahara T, Moroi Y, Uchi H, Furue M. Differential role of MAPK signaling in human dendritic cell maturation and Th1/Th2 engagement. J Dermatol.Sci. 2006;42(1):1-11.

[233] Jurkovich GJ, Mileski WJ, Maier RV, Winn RK, Rice CL. Interferon gamma increases sensitivity to endotoxin. J Surg.Res. 1991;51(3):197-203.

[234] Schroder K, Hertzog PJ, Ravasi T, Hume DA. Interferon-gamma: an overview of signals, mechanisms and functions. J Leukoc.Biol. 2004;75(2):163-89.

[235] Goodridge HS, Harnett W, Liew FY, Harnett MM. Differential regulation of interleukin-12 p40 and p35 induction via Erk mitogen-activated protein kinase-dependent and -independent mechanisms and the implications for bioactive IL-12 and IL-23 responses. Immunology 2003;109(3):415-25.

[236] Sun J, Krawczyk CJ, Pearce EJ. Suppression of th2 cell development by notch ligands delta1 and delta4. J Immunol. 2008;180(3):1655-61.

[237] Yang D, Chen Q, Su SB, et al. Eosinophil-derived neurotoxin acts as an alarmin to activate the TLR2-MyD88 signal pathway in dendritic cells and enhances Th2 immune responses. J Exp.Med 2008;205(1):79-90.

[238] Netea MG, Sutmuller R, Hermann C, et al. Toll-like receptor 2 suppresses immunity against *Candida albicans* through induction of IL-10 and regulatory T cells. J.Immunol. 2004;172(6):3712-8.

[239] Sieling PA, Chung W, Duong BT, Godowski PJ, Modlin RL. Toll-Like Receptor 2 Ligands as Adjuvants for Human Th1 Responses. J Immunol 2003;170(1):194-200.

[240] Takeuchi O, Kawai T, Muhlradt PF, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int.Immunol. 2001;13(7):933-40.

[241] Takeuchi O, Sato S, Horiuchi T, et al. Cutting edge: role of Toll-like receptor 1 in mediating immune response to microbial lipoproteins. J.Immunol. 2002;169(1):10-4.

[242] Gringhuis SI, den Dunnen J, Litjens M, van het Hof B, van Kooyk Y, Geijtenbeek TBH. C-Type Lectin DC-SIGN Modulates Toll-like Receptor Signaling via Raf-1 Kinase-Dependent Acetylation of Transcription Factor NF-[kappa]B. Immunity 2007;26(5):605-16.

[243] Takeuchi O, Hoshino K, Kawai T, et al. Differential roles of TLR2 and TLR4 in recognition of gram-negative and gram-positive bacterial cell wall components. Immunity 1999;11(4):443-51.

[244] Kim JG, Lee SJ, Kagnoff MF. Nod1 is an essential signal transducer in intestinal epithelial cells infected with bacteria that avoid recognition by toll-like receptors. Infect.Immun. 2004;72(3):1487-95.

[245] Kobayashi KS, Chamaillard M, Ogura Y, et al. Nod2-dependent regulation of innate and adaptive immunity in the intestinal tract. Science 2005;307(5710):731-4.

[246] Lee MS, Kim YJ. Signaling pathways downstream of pattern-recognition receptors and their cross talk. Annu.Rev.Biochem. 2007;76:447-80.

[247] Ouyang W, Kolls JK, Zheng Y. The biological functions of T helper 17 cell effector cytokines in inflammation. Immunity 2008;28(4):454-67.

[248] Diaz A, Allen JE. Mapping immune response profiles: the emerging scenario from helminth immunology. Eur.J Immunol. 2007;37(12):3319-26.

[249] Mortimer K, Brown A, Feary J, et al. Dose-ranging study for trials of therapeutic infection with Necator americanus in humans. Am J Trop Med Hyg 2006;75(5):914-20.

[250] Falcone FH, Pritchard DI. Parasite role reversal: worms on trial. Trends Parasitol. 2005;21(4):157-60.

[251] Cooper PJ. The potential impact of early exposures to geohelminth infections on the development of atopy. Clin.Rev.Allergy Immunol. 2004;26(1):5-14.

[252] Ege MJ, Bieli C, Frei R, et al. Prenatal farm exposure is related to the expression of receptors of the innate immunity and to atopic sensitization in school-age children. J Allergy Clin.Immunol. 2006;117(4):817-23.

[253] Yazdanbakhsh M, Kremsner PG, van Ree R. Allergy, parasites, and the hygiene hypothesis. Science 2002;%19;296(5567):490-4.

[254] Seder RA, Darrah PA, Roederer M. T-cell quality in memory and protection: implications for vaccine design. Nat.Rev.Immunol. 2008;8(4):247-58.

[255] Layland LE, Rad R, Wagner H, da Costa CU. Immunopathology in schistosomiasis is controlled by antigen-specific regulatory T cells primed in the presence of TLR2. Eur.J Immunol. 2007;37(8):2174-84.

[256] Napolitani G, Rinaldi A, Bertoni F, Sallusto F, Lanzavecchia A. Selected Toll-like receptor agonist combinations synergistically trigger a T helper type 1-polarizing program in dendritic cells. Nat.Immunol. 2005;6(8):769-76.

[257] Melendez AJ, Harnett MM, Pushparaj PN, et al. Inhibition of Fc epsilon RI-mediated mast cell responses by ES-62, a product of parasitic filarial nematodes. Nat.Med 2007;13(11):1375-81.

Summary

Chapter 1

Chapter 1 provides a general introduction to immune responses evoked by helminths. It describes how the helminth-induced Th2 and immunomodulatory responses affect immune responses to other infections (bacterial, viral, parastic), to vaccines and to allergens as well as possible self antigens. In addition, the molecular mechanisms involved in immune modulation are reviewed, with a particular focus on the modulation of dendritic cell (DC) function. The effect of DC maturation as well as the role of Toll-like receptors and downstream MAP kinase activation in shaping the dendritic cell function have been described.

Chapter 2

In chapter 2 the presence of specific antibodies against *Ascaris lumbricoides* derived glycolipids in patients infected with this helminth were studied. The highest IgG reactivity to glycolipids was found in children carrying heavy infections, compared to lightly-infected or uninfected children. Substantial IgG antibody reactivity to (glyco)proteins was found to be directed to the phosphorylcholine moiety. For glycolipids this was even more pronounced, as removal of the phosphorylcholine moieties by hydrofluoric acid treatment abrogated IgG antibody reactivity. For helminth infections, IgG4 has been associated with susceptibility to reinfection after treatment, whereas IgE is thought to be protective against reinfection. Measurement of IgG4 and IgE isotypes showed no IgG4 reactivity to *Ascaris* glycolipids, but indicated increased IgE responses in subjects with light or no *Ascaris* infections, suggesting that IgE responses to glycolipids may play a role in controlling parasite burden.

Chapter 3

In chapter 3 the difference in efficacy of vaccination with a trivalent influenza vaccine in children living in a rural area, with a high prevalence of helminth infections, compared to a semi-urban area, with a lower prevalence of helminth infections, was compared. First it is described that, unexpectedly, anti-influenza antibody levels before vaccination were present in all children, indicating that influenza viruses had been circulating previously. Cytokine responses were induced within a week after vaccination and showed a Th2 bias in rural children; more IL-5 and less IFN-γ. Antibody levels were significantly increased in both rural and semi-urban children after vaccination and reached significantly higher

levels for the semi-urban children compared to the rural children one month after vaccination for 2 of the strains. However, post vaccination responses to the third strain (H3N2) were higher in the rural cohort compared to semi-urban group. The extremely high pre-vaccination levels specific for this strain meant that it had caused a very recent epidemic, and although purely speculation, this might indicate that recently memory is boosted more strongly in rural areas. The exact role of parasite infections in the differential response between rural and semi-urban children needs to be investigated in an appropriately powered study. However, for the H1N1 strain helminth infection in semi-urban children reduced the anti-H1N1 antibody levels compared to non-infected semi-urban children. Malaria infection in rural children suppressed antibody responses to the H1N1 strain. Overall, influenza vaccination induced weaker responses in rural compared to a semi-urban population of Gabonese schoolchildren.

Chapter 4

In chapter 4 the immune responses induced upon vaccination with tetanus vaccine in children from rural and semi-urban areas of Gabon is described. The same population as described in chapter 3 was studied and similarly, also in response to tetanus toxoid, more IL-5 and less IFN-γ, indicating a Th2 biased immune response, was found in the rural children. However, to this vaccine higher antibody levels were found also in the rural group. Total IgG as well as antigen specific subclasses of the IgG1, IgG2, IgG3 and IgG4 isotype and the avidity of the dominating IgG1 subclass were determined. For these, differences between rural and semi-urban children were found one month after vaccination for tetanus-specific IgG1 and IgG3; both were higher in the rural children. This might be in line with the finding in Chapter 3, that vaccine induced responses to the recently circulating influenza H3N2, were also higher in rural children. Subjects with plasmodium infections showed higher levels of IgG3, but multivariate linear regression analysis showed that this could not account for the difference in anti-TT IgG3 between the rural and the semi-urban children. Furthermore, current helminth infections could also not explain the difference between rural and semi-urban responses. This indicates that other environmental influences and/or the history of helminth infection might be important factors that could explain the Th2 and stronger antibody responses upon a tetanus booster vaccination in rural compared to semi-urban children.

Chapter 5

To link immune responses to lipids derived from helminths and *in vivo* vaccination responses in areas where helminth infections are highly

prevalent, dendritic cells, which are central to the immune system, were studied. In chapter 5 the pathways within dendritic cells that are activated by helminth derived lipids are described. The lipid fractions of both *Schistosoma mansoni* and *Ascaris lumbricoides* activate Toll Like Receptor 2. Activation of this receptor is an important step in the initiation of an appropriate adaptive immune response. However, several ligands activate TLR2 and these can have different effects on immune polarization. Dendritic cell activation by both helminth derived (Th2 inducing) and bacterial (Th1 inducing) TLR2 ligands was studied and the molecular profile of these DCs was determined. It was found that the MAP kinase activation correlated with the T cell polarizing effects. Thus, the bacterial ligands showed a low *p*-ERK/*p*-p38 ratio, whereas this was high for the helminth derived lipids. However, unlike a schistosomal egg extract (SEA) that increases this ratio by increasing the amount of activated ERK, the lipids specifically reduced the phosphorylation of p38. In addition, mRNA expression profiles were very different. Most clearly, notch ligand delta-4 was associated with a Th1 polarization and transcription factor c-fos showed a strong correlation with Th2 responses. The overall profile of the two Th1 inducing bacterial TLR2 ligands was very similar, whereas the Th2 promoting lipid extracts showed a profile closer to that of SEA. Thus, the activation of TLRs within different antigenic mixtures can lead to very different polarization of the immune system, which can be explained by involvement of additional receptors. More importantly, the molecular signature of the DCs upon activation by antigenic mixtures can be used to predict the polarizing capacity of those compounds.

Samenvatting

Hoofdstuk 1

Hoofdstuk 1 geeft een algemene introductie tot de immuunresponsen die worden opgewekt door helminthen; wormen, waarvan de meeste soorten parasitair zijn. Twee typen zijn te onderscheiden: T helper 2 (Th2) responsen en immuunsysteem regulerende responsen. Beide typen responsen zijn niet alleen gericht tegen de helminth maar ook tegen ongerelateerde immuunactiverende organismen of moleculen. Er wordt beschreven hoe de helminthen de immuunresponsen beïnvloeden die gericht zijn tegen a) andere infecties (bacterieel, viraal of parasitair), b) vaccins, c) allergenen of d) mogelijke zelf-antigenen. Verder worden de moleculaire mechanismen beschreven die een rol spelen in deze regulering van het immuunsysteem, waarbij de nadruk ligt op de modulering van de functie van dendritische cellen (DCs). Tevens wordt beschreven wat het effect is van helminth infectie op de maturatie van DCs, met aandacht voor de rol van Toll-like receptoren en de daarop volgende activering van MAP kinases, in het vormen van de immuun-regulerende eigenschappen van de DCs.

Hoofdstuk 2

In hoofdstuk 2 is de aanwezigheid van specifieke antilichamen tegen glycolipiden van *Ascaris lumbricoides* bestudeerd in patiënten die geïnfecteerd waren met deze helminth. De hoogste IgG reactiviteit tegen glycolipiden werd gevonden in kinderen met zware infecties, in vergelijking met kinderen met een lichte of geen infectie. Een aanzienlijk deel van de reactiviteit van de IgG antilichamen tegen de (glyco)proteïnen was gericht tegen phosphorylcholine eenheden. Voor glycolipiden was de rol van phosphorylcholine nog groter; na verwijdering van de phosphorylcholine eenheden was de reactiviteit van de IgG antilichamen zo goed als verdwenen. In infecties met helminthen wordt de aanwezigheid van de subklasse IgG4 geassocieerd met gevoeligheid voor de terugkeer van infecties na behandeling, terwijl IgE in verband gebracht wordt met resistentie tegen terugkerende infecties. Bepaling van de IgG4 en IgE isotypen toonde aan dat er geen reactiviteit van IgG4 tegen glycolipiden van *Ascaris* was, maar dat er wel verhoogde IgE responsen waren in kinderen met lichte of geen *Ascaris* infecties. Dit zou kunnen betekenen dat IgE responsen tegen glycolipiden een rol spelen in het inperken van deze parasitaire infectie.

Hoofdstuk 3

In hoofdstuk 3 is het verschil in effectiviteit van vaccinatie met een trivalent influenza vaccin bestudeerd in kinderen afkomstig uit twee verschillende gebieden in Gabon; een ruraal gebied, met een hoge prevalentie van helminth infecties, en een verstedelijkt gebied, waar helminth infecties minder vaak voorkomen. Tegen de verwachting in werd geconstateerd dat in alle kinderen al voor de vaccinatie antilichamen gericht tegen influenza aanwezig waren. Dit duidt er op dat influenza virussen recent gecirculeerd hebben in deze gebieden. Na vaccinatie waren de hoeveelheden antilichaam significant toegenomen in zowel de kinderen uit het rurale als het verstedelijkte gebied. Voor twee van de drie influenza stammen waren de niveaus van de antilichamen een maand na de vaccinatie significant hoger in de kinderen uit het verstedelijkte gebied dan in de kinderen uit het rurale gebied. Echter, de antilichaamresponsen specifiek voor de derde stam (H3N2) waren juist hoger in de kinderen uit het rurale gebied. De extreem hoge titers tegen deze stam die al voor de vaccinatie aanwezig waren duidden op een recente epidemie en, hoewel dit speculatief is, dit zou kunnen betekenen dat het immunologisch geheugen recent sterker gestimuleerd is in de kinderen in het rurale gebied. Verder zijn na de vaccinatie de influenza specifieke cytokine responsen gemeten en deze vertoonden een Th2 type profiel in kinderen uit het rurale gebied: meer IL-5 en minder IFN-γ in vergelijking met de kinderen uit het verstedelijkte gebied. In theorie zou voor een goede respons tegen influenza juist een Th1 respons gewenst zijn. De exacte rol van parasitaire infecties in het verschil in responsen tussen kinderen uit het rurale en het verstedelijkte gebied dienen in een nieuwe studie met grotere aantallen kinderen onderzocht te worden. Wel waren er in deze studie al aanwijzingen dat de kinderen uit het verstedelijkte gebied met een helminth infectie minder anti-H1N1 antilichamen aanmaakten in vergelijking met kinderen uit hetzelfde gebied zonder een helminth infectie. Bovendien onderdrukte een infectie met malaria, geconstateerd voor of binnen 2 weken na de vaccinatie, de antilichaam responsen tegen de H1N1 stam in de kinderen uit het rurale gebied. Samenvattend wekte het influenza vaccin zwakkere responsen op in schoolkinderen uit een ruraal gebied dan in kinderen uit een verstedelijkt gebied in Gabon.

Hoofdstuk 4

In hoofdstuk 4 is gekeken naar immuunresponsen in kinderen uit een ruraal en een verstedelijkt gebied in Gabon na vaccinatie met een tetanus vaccin. De groep kinderen was dezelfde als die beschreven is in hoofdstuk 3. Net als voor influenza, werd er in de kinderen uit het rurale gebied ook in reactie op tetanus toxoid meer IL-5 en minder IFN-γ aangemaakt. Echter, tegen tetanus toxoid werden sterkere antilichamen gevonden in de groep

rurale kinderen. Antigen specifiek totaal IgG, subklassen van het IgG1, IgG2, IgG3 en IgG4 isotype en de aviditeit van de dominerende IgG1 subklasse werden bepaald. Er werden verschillen gevonden in tetanus specifiek IgG1 en IgG3 tussen kinderen uit het rurale en het verstedelijkte gebied één maand na de vaccinatie; beide antilichamen waren in hogere mate aanwezig in de kinderen uit het rurale gebied. Aangezien kinderen uit de rurale gebieden vaker infecties oplopen, en dit ook voor tetanus het geval zou kunnen zijn, zou de verhoogde antilichaamrespons in de rurale kinderen in overeenstemming kunnen zijn met de bevindingen in hoofdstuk 3, waar de responsen tegen de recent aanwezige influenza H3N2 stam, geïnduceerd door vaccinatie, ook hoger waren in de kinderen uit het rurale gebied. Kinderen met malaria hadden grotere hoeveelheden IgG3, maar lineaire regressie analyse toonde aan dat malaria infecties niet het verschil in tetanus specifiek IgG3 tussen de kinderen uit het rurale en het verstedelijkte gebied kon verklaren. Ook konden aanwezige helminth infecties niet de verschillen tussen de twee groepen verklaren. Dit wijst erop dat andere omgevingsfactoren en/of de voorgeschiedenis van helminth infecties belangrijke factoren zouden kunnen zijn die de Th2 en sterkere antilichaam responsen in kinderen uit een ruraal gebied na een booster vaccinatie met tenanus vaccin zouden kunnen verklaren.

Hoofdstuk 5

Om de immuunresponsen tegen lipiden geïsoleerd uit helminthen (hoofdstuk 2) en de *in vivo* responsen tegen vaccinatie in gebieden waar helminth infecties heersen (hoofdstuk 3 en 4) aan elkaar te kunnen liëren, werden dendritische cellen bestudeerd. Dendritische cellen (DCs) spelen een centrale rol in het immuunsysteem. Wanneer ze in aanraking komen met bijvoorbeeld ziekteverwekkers, zullen naïve DCs hun aanwezigheid opmerken en matureren. Deze “volwassen” DCs zullen vervolgens de witte bloedcellen (T en B cellen) instrueren hoe ze moeten handelen. In hoofdstuk 5 worden de routes in de DCs beschreven die geactiveerd worden door lipiden afkomstig van helminthen. De lipiden fracties van twee verschillende parasitaire wormen, *Schistosoma mansoni* en *Ascaris lumbricoides*, activeren een receptor op het oppervlak van de DCs, genaamd Toll Like Receptor 2 (TLR2). Activering van deze receptor is een belangrijke stap in de initiatie van een passende specifieke immuunrespons. Echter, er zijn verschillende liganden die TLR2 kunnen activeren en deze kunnen uiteenlopende effecten hebben op de sturing van de immuunrespons. Activering van DCs door zowel Th2 inducerende, van helminthen afkomstige liganden, als Th1 inducerende, van bacteriën afkomstige TLR2 liganden, werden gebruikt om DCs te activeren en het moleculaire profiel van deze DCs werd geanalyseerd. Activering van TLR2 leidt tot activering van andere moleculen die op hun beurt weer de volgende signaalmoleculen

in de route kunnen activeren. Een groep van enzymen die ook deel uitmaken van deze cascade zijn de “mitogen-activated protein kinases“ (MAP kinases). Activering van de MAP kinases in de DCs was in overeenstemming met de effecten die deze DCs hadden op T cel polarisatie. Ofwel, the bacteriële liganden toonden een lage ratio van twee verschillende MAP kinases (*p*-ERK / *p*-p38), terwijl deze ratio hoog was voor zowel lipiden afkomstig van de helminthen, als voor SEA, een veel bestudeerd extract uit eieren van schistosomen. SEA verhoogt de *p*-ERK / *p*-p38 ratio door het verhogen van de hoeveelheid geactiveerd ERK, terwijl de lipiden juist de hoeveelheid geactiveerd p38 verlagen. Naast de verschillen in activering van eiwitmoleculen waren ook de expressieprofielen van messenger RNA (mRNA) erg verschillend tussen de Th1 en Th2 inducerende TLR2 liganden. Het meest in het oog springende resultaat was dat delta-4, een ligand van de notch receptor, geassocieerd was met Th1 polarisatie en dat de transcriptiefactor c-fos sterk geassocieerd was met een Th2 respons. Het mRNA profiel van de DCs geactiveerd met de twee Th1 inducerende bacteriële TLR2 liganden was erg vergelijkbaar, terwijl de lipiden een profiel in de DCs opwekten dat meer overeen kwam met dat geïnduceerd door SEA. Concluderend kan worden gezegd dat de activering van hetzelfde type TLR in de aanwezigheid van verschillende mixen van antigenen kan leiden tot een sterk verschillende polarisatie van het immuunsysteem. Bovendien kan het moleculaire profiel van de DCs gebruikt worden om de polariserende eigenschappen van deze stoffen te voorspellen.

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Curriculum Vitae

Petronella Helena (Elly) van Riet was born on march 25th 1977 in Hoogeloon c.a., The Netherlands. In 1995 she passed her secondary school exam at the Rythovius College in Eersel (Noord-Brabant) and in the same year started studying bioprocess technology at Wageningen University and Research Center. During her studies she performed research projects at the department of toxicology of Wageningen University (Prof. Rietjens), at Numico Research in Wageningen (Dr. van Tol / Prof. van Muiswinkel) and at the division of molecular biology, at the Dutch Cancer Institute in Amsterdam (Prof. te Riele / Dr. de Boer).
In September 2001 she graduated in bioprocess technology. In 2002 she continued as a PhD student at the department of Parasitology of the Leiden University Medical Center under supervision of Prof. M. Yazdanbakhsh and performed the research described in this thesis. In May 2007 she continued as a postdoc at the Leiden Amsterdam Center for Drug Research in Leiden, at the division of Drug Delivery Technology in the lab of Prof. J. Bouwstra and Prof. W. Jiskoot.

List of publications

van Riet, E., M. Wuhrer, S. Wahyuni, K. Retra, A.M. Deelder, A.G.M. Tielens, D. van der Kleij, and M. Yazdanbakhsh. 2006. Antibody responses to *Ascaris*-derived proteins and glycolipids: the role of phosphorylcholine. *Parasite Immunol.* 28(8):363-371.

van Riet, E., F.C. Hartgers, and M. Yazdanbakhsh. 2007. Chronic helminth infections induce immunomodulation: consequences and mechanisms. *Immunobiology* 212(6):475-490. *Review.*

van Riet, E., A.A. Adegnika, K. Retra, R. Vieira, A.G.M. Tielens, B. Lell, S. Issifou, F.C. Hartgers, G.F. Rimmelzwaan, P.G. Kremsner, and M. Yazdanbakhsh. 2007. Cellular and humoral responses to influenza in Gabonese children living in rural and semi-urban areas. *J Infect.Dis.* 196(11):1671-8.

Retra, K., *E. van Riet*, A.A. Adegnika, B. Everts, S. van Geest, P.G. Kremsner, J.J. van Hellemond, D. van der Kleij, A.G.M. Tielens, and M. Yazdanbakhsh. 2008. Immunologic activity of schistosomal and bacterial TLR2 ligands in Gabonese children. *Parasite Immunol.* 30(1):39-46.

van Riet, E., K. Retra, A.A. Adegnika, C.M. Jol-van der Zijde, H.-W. Uh, B. Lell, S. Issifou, P.G. Kremsner, M. Yazdanbakhsh, M.J.D. van Tol and F.C. Hartgers. 2008. Cellular and humoral responses to tetanus vaccination in Gabonese children. *Vaccine,* 26(29-30):3690-5.

Verheul, R.J., M. Amidi, S. van der Wal, *E. van Riet*, W. Jiskoot and W.E. Hennink. 2008. Synthesis, characterization and *in vitro* biological properties of O-methyl free N,N,N-trimethylated chitosan. *Biomaterials,* 29(27):3642-9.

van Riet, E. 2008. Protection from skin test reactivity by helminth infections: *Trichuris trichiura* induces protection in the long term. *Clin. Exp. Allergy, In press, Editorial.*

van Riet, E., B. Everts, K. Retra, M. Phylipsen, J.J. van Hellemond, A.G.M. Tielens, D. van der Kleij, F.C. Hartgers and M. Yazdanbakhsh. TLR ligation in the context of bacterial or helminth extracts in human monocyte derived dendritic cells: correlates for Th1/Th2 polarization. *Submitted.*