Helminth infections, allergic disorders and immune responses: studies in Indonesia

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Chapter 8

Activity of the toll like receptor ligands in children living in areas endemic for intestinal helminth Infections

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Abstract

Children in developing countries, who are exposed to a great number of microbial infections, are expected to benefit most from introduction of novel/improved vaccines. Toll like receptor (TLR) ligands by stimulating the innate immune system can modulate adaptive immune responses and are therefore considered as important molecules that may be used as adjuvants. Helminth infections that are highly prevalent in the developing countries influence the immune system profoundly by magnifying Th2 responses and exerting anti-inflammatory effects. To characterize how TLR ligands stimulate the immune responses of children living in areas endemic for helminth infections, we stimulated blood from children with light, heavy and no helminth infections with ligand (L)s for TLR-2/1, -2/6, -3, -4, -5, -7, -9 and measured pro- and anti-inflammatory cytokines released. Upon 24 hour stimulation, the highest pro- versus anti-inflammatory responses were found to the R-848 (TLR-7L), Poly I:C (TLR-3L) and CpG (TLR-9L) in all children. In group with high intensity of infections, responses to TLR-L were characterized by strong TNF-α and IL-10 production in 24 hours culture supernatants compared to children with light/no infections. However, when supernatants were analyzed at 72 hours post stimulation, high production of IL-10 was maintained only in children with highly infected, whereas TNF-α production declined in all groups. Thus upon prolonged stimulation, a strong anti-inflammatory response profile is seen in children with intense helminth infections. The subtle differences in the dynamics of immune responses to TLR-L have to be considered when designing vaccines for use in areas endemic for helminth infections.

Introduction

Intestinal helminths are estimated to infect more than two billion people, mostly in developing countries. Of these at least 50 per cent are school aged children [336]. Intestinal helminth infections tend to be chronic in nature, which in time exert a formidable and continuous antigenic burden on the immune systems. Helminth infections cause T helper (Th)2 expansion [58;90] and some level of T cell hyporesponsiveness with increased production of suppressory cytokines such as IL-10 and TGF-β [66;435]. The helminth antigen specific immunological hyporesponsiveness appears to spill over to non-related antigen as reported by several studies in endemic areas. It was found that responses to vaccines such as bacillus Calmette-Guarin (BCG) [277-279;436] as well as toxoid tetanus (TT) [280-282] were in lower in helminth-infected subjects. Moreover, there is evidence that helminth co-infection can reduce the incidence of Th1-mediated diseases such as diabetes mellitus [437], *Bordatella pertussis* infections [438] and *Helicobacter pillory* infections [439] as well as Th2-mediated diseases like allergy [414]. With a view to the immune modulatory activity of helminth infections, these parasites need to be taken into account when designing or assessing novel/improved vaccines/adjuvants.

A prerequisite for a good immune response is a state of inflammation. In terms of vaccination this inflammation has to be induced to achieve specific immunity. Purified proteins lead to poor immune responses, thus combining pure antigens with adjuvants such as microbial products that promote the induction of specific effector responses is an important component of vaccine development. Adjuvants, non specifically activate the innate immune system through interaction with TLRs and other pattern recognition receptors (PRRs). These molecules are expressed on the cell surface or in intracellular compartments and upon binding to pathogen associated molecular patterns (PAMPs) initiate a signaling cascade that leads to activation of immune responses [284] that ends in differentiation of T cells into Th1, Th2 or T regulatory cells (Tregs) [440].

The capability of TLRs to polarize T cell responses has intensified research into the identification of effective and appropriate adjuvants for use in vaccination programs, not only to improve existing vaccines but also to use them for new vaccine initiatives. The question of how helminth infected children respond to a large array of TLR ligands has not been addressed and is of importance given that developing countries, where helminth infections are highly prevalent are most in need of vaccines that are being developed, for example the malaria vaccine. In this study we analyzed the ability of ligands for TLR-2/1, -2/6, -3, -4, -5, -7, and -9 to stimulate pro- and anti-inflammatory responses in children with varying burdens of helmith infections.

Material and methods

Study population

Two elementary schools in Makassar, Indonesia were screened for the presence of intestinal helminth infections. In one school (school A, SD Cambaya), 96% of children were found to be positive for at least one of the intestinal helminths and intensities of *Ascaris lumbricoides* as well as *Trichuris trichuria* infections were high (GM=8990 epg and 715 epg, respectively). In another school (school B, SD Mangkura), 7 km further, the prevalence of helminth infections was 23% with light intensities of infection (GM=2085 epg for *Ascaris* and 204 epg for *Trichuris*). From each school (school A and B), 27 children agreed to donate blood. As shown in table 1, the children from the two schools were sex and age matched. Informed consent was obtained from the parents of each child and the children. The study was approved by the ethics committee of the Faculty of Medicine, Hasanuddin University, Indonesia.

Parasitological examination

Stool samples were collected and analyzed for *A. lumbricoides, T. trichuria* and hookworm infections using the Kato Katz method [441].

	school A (n=27)	school B (n=27)	P-value
Age (yrs)			
mean	9	9	ns
(range)	(8-11)	(8-11)	
Sex			
girls/boys	14/13	14/13	ns
A.lumbricoides prev. (%)	96.3	25.9	<0.001
A.lumbricoides intensity(epg)*			
GM	10919	275	<0.001
IQR	(4704-31422)	(96-504)	
T.trichuria prev. (%)	92.6	33.3	<0.001
T.trichuria intensity (epg)*			
GM	2005	196	<0.001
95% CI	(744-5864)	(84-564)	
Mix infections prev. (%)	88.9	14.8	<0.001

Table 1. Description of study children from school A and B.
*Egg loads are given for infected children only
ns = not significant

Whole blood collection and stimulation

Approximately 2 ml of venous blood was collected from the participants into heparinated tubes and processed within 1 hour. Whole blood was diluted 5 times in RPMI-1640 medium (Invitrogen) and stimulations were performed in 96-wells round bottom tissue-culture plates (Nunc, VWR International, the Netherlands). To 100 µl of whole blood, 100 µl of medium containing a TLR-L was added to each well. RPMI-1640 was used as a negative control. Whole blood cultures were incubated with 5% CO2 at 37 °C for 24h and 72h. The stimuli were used in the following final concentrations: Pam3Cys-ser-(lys)4 (Pam3) 100 ng/ml, heat-killed *Listeria monocytogenes* (HkIm) 1 X 10^6 bacteria/ml, Poly I:C 50 µg/ml, lipopolysaccharide (LPS) 100 ng/ml, Flagellin 100ng/ml, resiquimod-848 (R-848) 1µg/ml and CpG2006 5µM. These were cultured for 24 hours. Stimulation with LPS was performed for 24 and 72 hours. At the end of the incubation period, the supernatants were harvested, and stored at -20°C. Supernatants were transported to the Netherlands on dry ice and stored at –80°C until analysis.

Cytokine measurement

Levels of IL-10 and TNF-α were determined in the supernatants by enzyme linked immunosorbent assay (ELISA) using PeliKine Compact™ Human Interleukin commercial kits (Sanquin/CLB, Amsterdam, The Netherlands) following manufacture's recommendation. Detection limit for IL-10 and TNF-α was 2.4 pg/ml and 2.8 pg/ml,

respectively. The level of each cytokine in response to TLR ligands was subtracted from the level of each cytokine in response to medium only.

Statistical analysis

The distribution of sex and helminth infections between the two schools was tested by using Fisher's exact test. Age, cytokines levels and infection intensities (calculated in infected children) between two schools were compared by using the Mann-Whitney test. Outcomes of statistical tests were considered significant when p-values were smaller than 0.05.

Result

Pro- and anti-inflammatory cytokine production in response to TLR ligands after 24 hours of stimulation

As shown in figure 1, the highest TNF-α production was in response to R-848 and the lowest was in response to CpG. The IL-10 production in both groups was highest in response to LPS in both groups and lowest in response to CpG. When ratios of TNF-α: IL-10 were calculated (fig 1c and f), R-848 lead to the strongest pro-inflammatory profile in both groups, followed by Poly I:C and CpG. When responses were compared between children with heavy and light/no infection, the level of TNF-α in response to HkIm (GM=53.57 pg/ml vs. 19.37 pg/ml) and LPS (GM=401.69 pg/ml vs. 98.45 pg/ml) as well as IL-10 in response to LPS (GM=267 pg/ml vs. 167 pg/ml) were significantly higher in children with high intensity helminth infections compared with those carrying light/no infections. When looking at the TNF-α:IL-10 ratios, stronger pro-inflammatory in response to R-848, Poly I:C and CpG was found in the group with high intensity of infections at 24 hours after stimulation.

Pro- and anti-inflammatory cytokine production in response to LPS after 72 hour of stimulation

In order to start examining the dynamics of IL-10 responses to TLR ligands, responses to LPS were examined not only after 24 hours but also after 72 hours post stimulation. Interestingly, differences were found in anti-inflammatory (IL-10) responses between children with high intensity infections and those with light/no helminth infections. After 72 hours, the levels of both TNF-α and IL-10 decreased significantly in cultures from children with light/no helminth infections and although TNF-α levels also decreases in cultures from children with heavy helminth loads, IL-10 production was maintained at high levels in this group (fig 2). This resulted in a strong anti-inflammatory response at 72 hours in children with high intensity helminth infections compared with those with light/no infections.

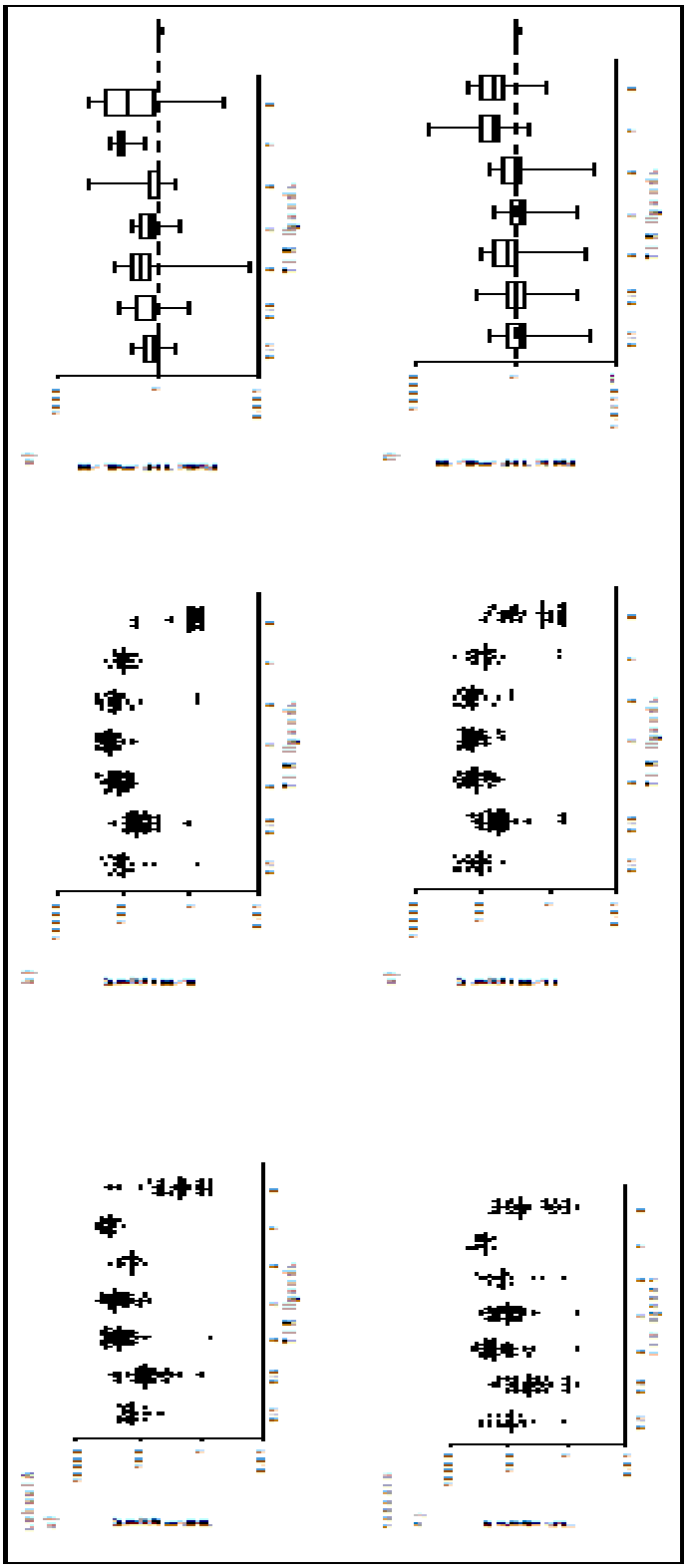

Figure 1. TNF-α (a and d), IL-10 (b and e) and ratio TNF-α /IL-10 (c and f) productions in response to TLR ligands in school A (top panel) and school B (bottom panel).

When children were categorized into those with heavy, light or no infection, it was clear that only in the group with heavy intensity of helminth infections, the IL-10 production was maintained at high levels leading to a profound anti-inflammatory profile at 72 hours post stimulation (table 2 and fig 3). Thus in children with intense helminth infections, the responses to TLR ligands might start with a pro-inflammatory profile, even higher than what is seen in children with light/no infections, but upon longer stimulation, the profile changes to a strong anti-inflammatory one

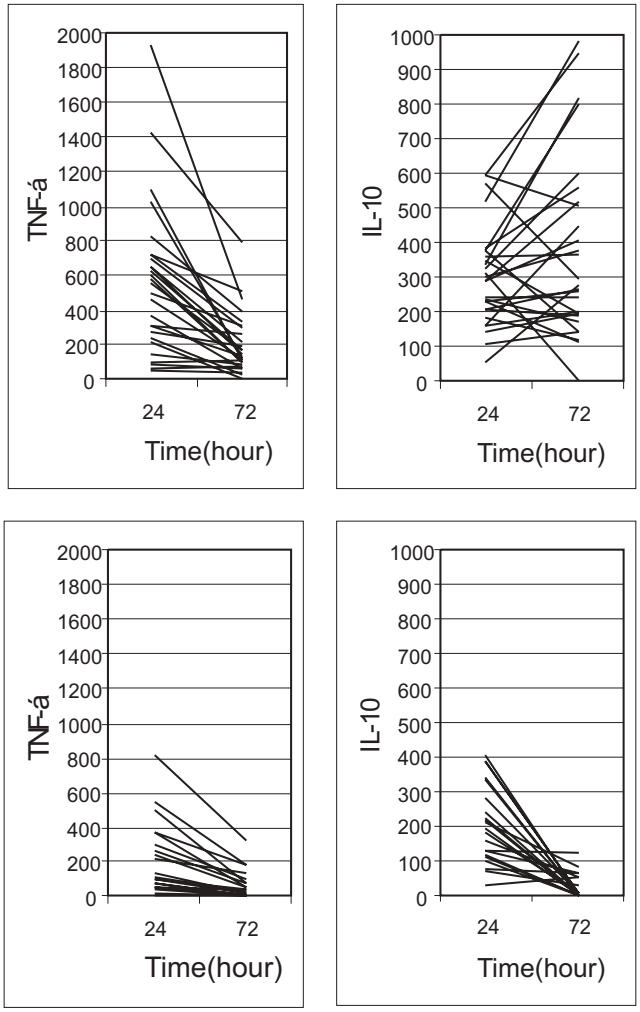

Figure 2. The production of TNF-α and IL-10 in response to LPS as a function of time in 24h and 72h culture supernatants in children from school A (top panel) and school B (bottom panel).

	TNF-α (LPS)				IL-10(LPS)			
school	24h		72h		24h		72h	
A (heavily-infected)								
GM	401.69	p<0.001*	123.30	P<0.009*	267.03	p<0.001*	268.44	p<0.001*
95% CI	(389.92 - 732.65)		(128.12 - 268.89)		(243.38-355.76)		(269.30-477.97)	
B (lightly-infected)								
GM	98.45		20.21		167.63		6.60	
95% CI	(104.95-258.07)		(22.98-81.55)		(157.13-241.70)		(-0.78-80.75)	
B1 (light-infected)								
GM	109.21	p=0.79#	24.69	p=0.68#	174.66	p=0.68#	10.60	p=0.32#
95% CI	(59.90 - 275.16)		(14.88 - 101.08)		(142.00 - 262.73)		(-33.74 -155.55)	
B2 (non-infected)								
GM	90.60		17.21		162.21		4.52	
95% CI	(73.01 - 312.37)		(3.01 - 92.38)		(131.23 - 262.80)		(1.01-45.47)	

Table 2. Mesurements as a function of age. Prevalence of microfilariae, filarial infection (determined by anti-filarial IgG4), skin test reactivity to mite (mite-SPT), IgE to mite (mite-IgE; low level sensitisation; >0.3 and high sensitization: >1.0 IU/ml), high level of total-IgE (>1400 IU/ml) and the levels of anti-filarial IgG4, mite-IgE and total-IgE are indicated for 466 subjects. P-values were measured using Pearson *chi-square* for comparing prevalences and one way ANOVA for comparing the levels of anti-filarial IgG4, specific- and total-IgE.

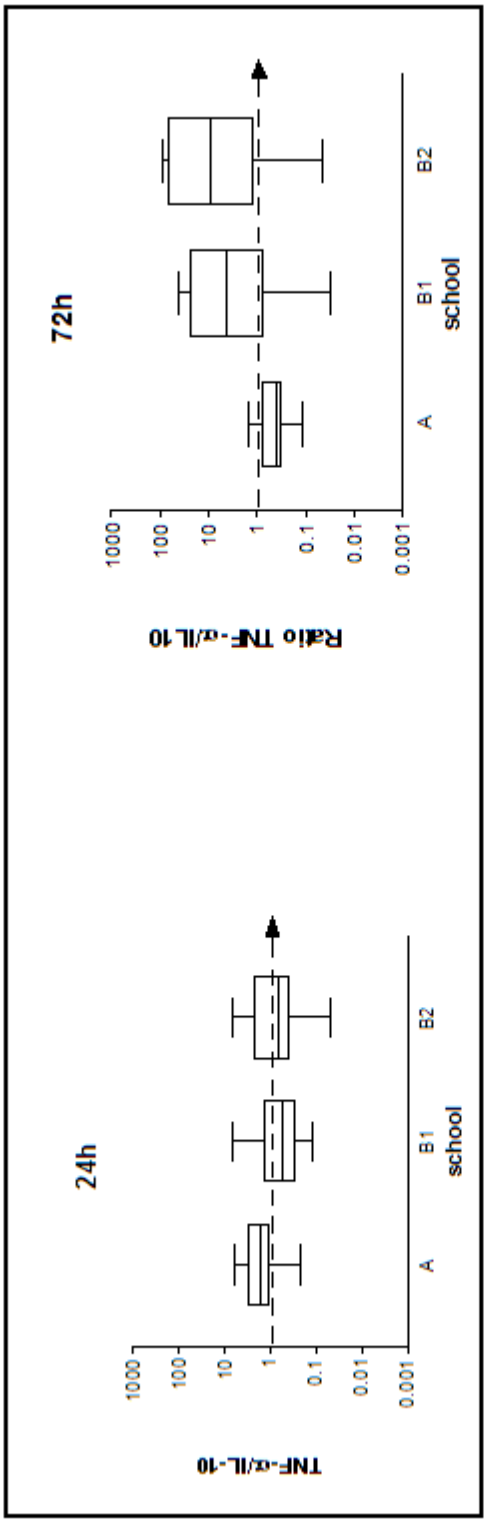

Figure 3. Ratio of TNF-á to IL-10 in school A (heavily-infected children), B1(light-infected children in school B) and B2 (non-infected children in school B) in 24h and 72h culture supernatants in response to LPS.

Discussion

Responses to vaccines can be influenced quantitatively and qualitatively by adjuvants used and TLR ligands, which stimulate innate immune responses. A number of studies have indicated that not only antigen specific adaptive immune responses but also innate immune responses might be modulated by chronic helminth infections [141;143;144]. This could have important implications for use of vaccines in populations living in areas where helminth infections are highly endemic. Considering that more than 1 billion people worldwide are infected with helminths, this can have important implications for developing new or improved vaccines. To start addressing this issue, the current work was undertaken to i) identify which TLR ligands induce strong pro-inflammatory responses and ii) determine whether responses to TLR ligands is different between helminth infected and uninfected children.

A varied range of responses was found to TLR ligands, which might reflect the differences in expression of TLRs and the strength of their signaling. It might also reflect the differences in solubility of the TLR ligands and thus access to receptors. Here we used the whole blood assay which has been regarded to be optimal for testing immunologic property of TLR ligands because it avoids inadvertent activation associated with cellular isolation techniques and maintains the influences of known and unknown soluble factors to affect experimental condition [442;443]. Using ratios of TNF-α to IL-10, it was possible to get an overall picture of the strength of the pro-inflammatory response induced by a TLR-L with the additional advantage that this parameter would be less affected by the differential solubility of the ligands.

R-848 induced the strongest TNF-α responses in heavy as well as lightly infected children. The receptor of this ligand (TLR-7) is located in the endosome and recognizes not only microbial components but also synthetic compound like Imidazoquiniline including Rasiquimod, Loxoribine and Bropirimine [444]. When the ratio of TNF-α to IL-10 was assessed as a measure of pro-inflammatory potential of TLR ligands, R-848, poly I:C and CpG were found to have the strongest pro-inflammatory property. It is interesting to note that these three TLRs are known to reside in the intracellular compartment of cells whereas other TLRs are expressed on cell surface and intracellular pathogens, in particular protozoa, are strong inducers of pro-inflammatory Th1 responses. Although the TNF-α: IL-10 ratio was high for CpG, the absolute amounts of TNF-α and IL-10 were low in response to this ligand. A poor TNF-α production in response to CpG has also been reported in a study of human peripheral blood mononuclear cells (PBMC) [445].

In terms of differences between children with intense helminth infections and those with light or no helminth infection, the data indicated that at 24 hours post stimulation, children with high intensity infections produced significantly higher levels of TNF-α in response to LPS and Hklm. With respect to IL-10, significantly higher levels were found when blood from children with intense infections was stimulated with LPS compared with those with low or no helminth infections. The higher responses to TLR ligands may seem to differ from previous findings that responses to TLR ligands are down-regulated in helminth infected subjects [143;144]. The differences may lie in the cells involved, types of helminth infections studied, as well as the time course. In studies by van der Kleij *et al*. and Babu *et al*.[143;144], PBMC were isolated prior to stimulation with TLR ligands. Here, whole blood cultures were used and although monocytes are a major source of TNF-α and IL-10 [446], other cell types such as granulocytes, NK cells [447;448] and eosinophils [449] are also capable of responding to TLR ligands to produce cytokines. Moreover, it is possible that these cells are manipulated during separation process and thus their responses are modified. The type of helminth infection might also influence the results. In the study by van der Kleij *et al*.[143], schistosome infection was the focus of the study where comparison was made between children with and without *Schistosoma haematobium* infections whereas intestinal helminth infections (*A. lumbricoides* and *T. trichuria*) were equivalent in the two groups. In the study by Babu *et al*., patients infected with filarial nematodes were studied [144]. Finally, with respect to time course, here we found that the dynamics of cytokine production can vary between helminth infected and uninfected subjects.

When ratios of TNF-α:IL-10 were considered at 24 hours post stimulation, children with high burden of intestinal helminths, showed a strong pro-inflammatory profile compared to those with light or no helminth infections. These results were surprising considering that helminth infections are associated with a more anti-inflammatory response [66]. However, we found that in response to LPS, the production of TNF-α and IL-10 was different at 24 and 72 hour cultures. The production of IL-10 was maintained at a high level in children with intense helminth infections, which resulted in a change from a pro- (at 24 hours) to an anti-inflammatory cytokine profile (at 72 hours). Two possibilities could account for this observation. First, in children highly infected with helminths, cells other than those involved in an early burst of response to TLR ligands, start to produce IL-10 at later time points during culture and such cells would not be present in children with light or no helminth infections. It is tempting to speculate that Tregs, such as Tr1, start to produce IL-10 at later time points. Indeed, Tregs are present during human onchocercal [434] and murine *Schistosome* [450] as well as intestinal helminth [451] infections. It is important to investigate this further in future studies by identifying cells producing IL-10 at early and later time

points after stimulation. Second, IL-10 consumption might be different between children with high or low intensity helminth infections; children with intense helminth infections might express low levels of IL-10 receptors compared with children with light/no infections. Again this needs to be investigated in future studies.

When schoolchildren were categorized into heavy, light and no helminth infections, the responses of children with light infections and those with no infections were indistinguishable and only children with high intensity of helminth infections showed sustained IL-10 production at 72 hours, and therefore an anti-inflammatory profile. This would indicate that light helminth infections do not have strong modulatory properties and a threshold level of infection is needed before systemic immune responses are affected. The question of how high intensities of parasitic helminths modulate innate immune responses, is not clear. Molecules expressed by nematodes (ES-62) [142] and trematodes (LNFP-III) [452] and Lysophosphatidylserine [453] with immunomodulatory properties and the ability to stimulate TLRs have been characterized. It is possible that abundant exposure of the immune system to such TLR activating molecules, possibly expressed by *A. lumbricoides*, hookworms or *T. trichuria* may lead to an altered expression of TLRs on/in cells as well as altered downstream signaling associated with the TLRs.

In conclusion, the ability of TLR-7 ligand to stimulate strong pro-inflammatory responses in children with or without intestinal helminth infections, along with the findings by others that newborns can also respond to TLR7 even though they do not respond well to TLR4 ligand [446] indicates that when strong inflammation is required with a vaccine, TLR-7 may be an appropriate target. However, we have shown that longer stimulation of TLRs in helminth infected subject results in an anti-inflammatory profile. The outstanding question is, whether the early or the later response to adjuvants will affect how adaptive response to vaccines develops. For this, time course studies of immune responses that develop to vaccines in children with or without helminth infections are needed.

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