Helminth infections induce immunomodulation : consequences and mechanisms

Riet, P.H. van

Citation

Riet, P. H. van. (2008, September 30). *Helminth infections induce immunomodulation : consequences and mechanisms*. Retrieved from https://hdl.handle.net/1887/13120

Version:	Corrected Publisher's Version
License:	Licence agreement concerning inclusion of doctoral thesis in the Institutional Repository of the University of Leiden
Downloaded from:	https://hdl.handle.net/1887/13120

Note: To cite this publication please use the final published version (if applicable).

4

Cellular and humoral responses to tetanus vaccination in Gabonese children

by E. van Riet, 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 & F. C. Hartgers

Vaccine. 2008, 26(29-30):3690-5

Reprinted with permission from Elsevier

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

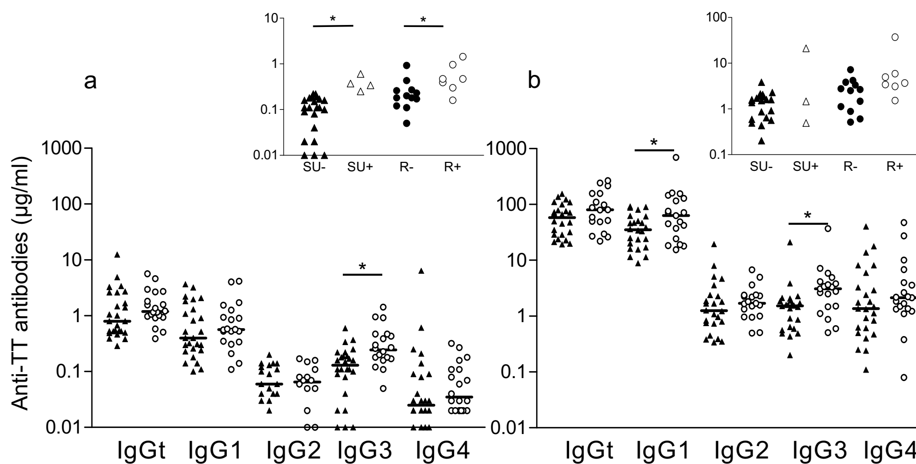

Figure 4.1. IgG subclass responses to tetanus toxoid before (a) and 28 days after vaccination (b). IgGt = total IgG anti-TT. Closed triangles: semi-urban subjects; open circles: rural subjects. Line indicates median value, * p<0.05. Inserts in both figure a and b: closed and open circles represent rural 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).

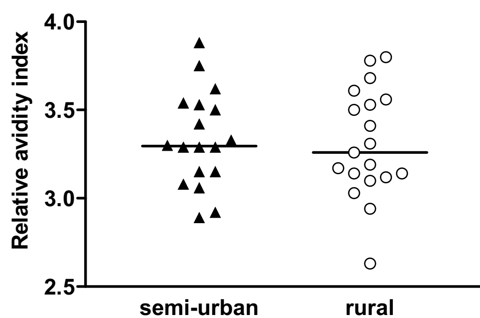

Figure 4.2. Avidity of the IgG1 anti-TT antibodies 28 days after vaccination. Closed triangles: semi-urban subjects; open circles: rural subjects, line indicates the median value.

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.

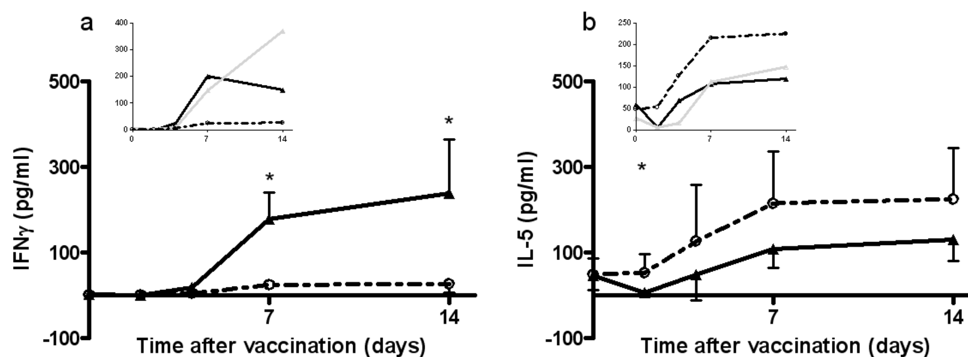

Figure 4.3. Tetanus toxoid-specific cytokine production after *ex vivo* stimulation of whole blood from children vaccinated with tetanus toxoid. Mean values and 95% confidence interval of IFNγ (a) and IL-5 (b) are presented. Closed triangles and solid lines: semi-urban subjects; open circles and dashed 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.