Chapter 1

Introduction

Immunological footprint: the development of child's immune system in environments rich in microorganisms and parasites

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Introduction

The development of immune response is determined by the interaction between genetic factors and the environment. The influences from the environment are thought to start already *in utero* and continue after birth, with great and long-lasting impact on infants and young children whose immune system shows great plasticity and is amenable to modulation [1]. This foetal-neonatal programming such as shown in studies on low birth weight infants can have negative consequences on the number and function of thymus-derived T cells as well as the thymus size [2–4]. In this respect, nutritional deficiencies during pregnancy and infancy have been linked to diseases in adulthood, such as higher risk of cardiovascular diseases [5] and insulin resistance [6,7]. These epidemiological observations support the foetal origins hypothesis proposed by Barker [8]. One of the mechanisms which may explain the link between environmental exposures during foetal life and infancy to risk of diseases in adulthood is the alteration of epigenetic regulation [9]. Although this phenomenon is subject of a growing number of studies on the origins of metabolic diseases or cancer, the same phenomenon may possibly be applied to infectious and atopic diseases. In developing countries, an unborn child can be exposed to various pathogens or their components via the placenta, which may result in the engagement of innate as well as adaptive immune system and contribute to the shaping of child's immunity. The type of pathogen or compounds, the timing and intensity of exposure, the household environment as well as genetic and epigenetic factors are thought to determine the magnitude and direction of responses to specific and bystander antigens and altogether to the maturation of the immune network (Fig. 1).

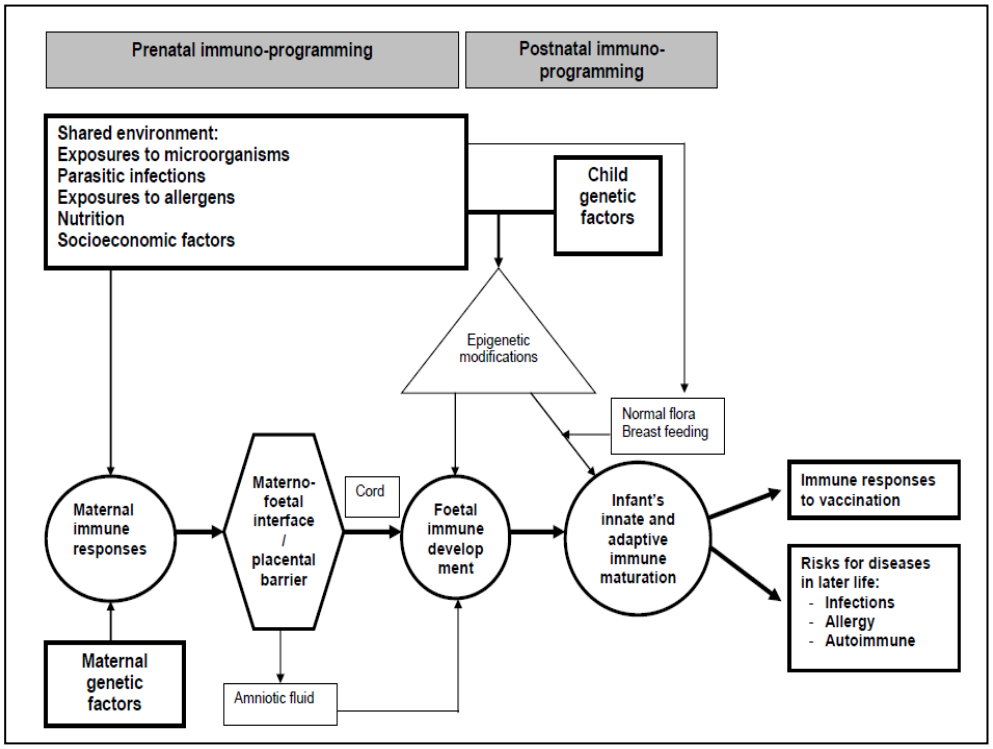

Figure 1. A proposed scheme for the impact of early exposure to environmental factors on the development of child's innate and adaptive immune responses, with consequences on immune responses to vaccination and on diseases in later life.

Early life

The early immunological cross talk

Cord blood immune responses, reflecting the immature foetal immune system [10,11], are often used as a proxy for measure of effects that environmental stimuli exert through the foeto-maternal interface, the placenta. Neonatal immunoepidemiology is a relatively small area of research and has mostly been directed at studying allergic disorders. As allergy leads to serious pediatric diseases, much effort has gone into delineating *in utero* or early life events that might few years later lead to the development of allergic disorders. Robust epidemiological data linking early environmental exposures to the development of allergies have been obtained in studies of European children born to farming and non-farming families, which show that farmer's children develop less atopy or asthma [12–14]. The maternal exposure to stables and farm animals during

pregnancy, was strongly associated with upregulation of innate immune receptors and lower degree of allergic sensitization in a child born to a farmer mother [15]. In terms of cytokines, maternal exposure to microbial compounds and consumption of farm dairy products was associated with increased T helper 1 (TH_1)-type (IFN-γ) and pro-inflammatory (TNF-α) cytokines in cord blood [16]. These studies provide strong evidence for the early programming of the immune system in the developing fetus. Moreover, Schaub and coworkers were able to show that cord blood from mothers living on traditional farms in Germany responded to microbial Toll-like receptor (TLR) ligands by increasing number and function of T regulatory cells, characterized by expression of the forkhead/winged-helix family transcriptional repressor p3 (FOXP3), and decreasing TH_2-type cytokine (IL-5) [17]. Taken together these studies suggest that prenatal exposure to microbial compounds can modulate the foetal innate immune responses, which in turn can affect the development of adaptive immune responses during childhood. Furthermore when the children are still exposed to the same farming environment, the higher expression levels of TLR-2 and CD14 genes on peripheral blood mononuclear cells compared to non-farmer children is sustained [18].

It is important to note that immediately after birth, a newborn has to face tremendous exposure to various microorganisms such as normal flora which start to colonize body surfaces including the mucosa of the gastrointestinal tract. The introduction of normal flora to a newborn occurs first during delivery and it was shown that different modes of delivery could affect the composition of microbiota [19,20]. The microbial diversity and composition increases with age and is influenced by life events such as breast feeding, introduction of solid food and antibiotics administration [21,22], as well as different environmental exposures or lifestyles [23–25]. Furthermore De Filippo and coworkers demonstrated large differences in fecal microbial community between western European children and rural African children and for the first time linking this to the difference in the diet containing different proportion of carbohydrates and fibres in the daily food [26]. Despite growing studies exploring the diversity of human microbiota and its impact on health and disease, the studies on immunological impact exerted by the presence of gut microbiota in early childhood are still very scarce and only studied in the context of probiotics whose introduction has been shown to be associated with decreased prevalence of atopic disorders in childhood [27–29]. Taken together, it is thought that the

immune system of a newborn can benefit from the colonization of normal flora which helps the maturation of the immune system and contributes to the development of immuno-tolerant state in the gut [30,31]. Indeed certain species of early microbiota that colonize infant's gut are thought to be able to down-regulate pro-inflammatory responses [32].

Breast feeding is another way by which the neonatal immunity is affected, through the transfer of nutrients and bioactive factors present in breast milk, such as antibodies, soluble CD14, cytokines, immune cells and other immuno-active compounds [33]. Interestingly, the presence of immunological factors in breast milk can be influenced by maternal environment, such as shown in a study of Italian mothers living on farms or those not living on farms [34]. In this study the levels of TGF-β1, an anti-inflammatory cytokine, in breast milk from the farm-group was found to be higher and more sustained than the levels in breast milk from the non-farm group, regardless of maternal atopic status. Similar patterns of TGF-β1 in breast milk was observed in Swedish immigrant or Malian mothers compared to native Swedish mothers, and moreover Malian mothers had higher soluble CD14, a pro-inflammatory cytokine, than the other 2 groups [35]. The later study further performed the culture of breast milk with cord blood mononuclear cell (CBMC) and intestinal epithelial cell lines, and concluded that breast milk from immigrant mothers induces less cytokine or chemokine responses. Therefore the area of residence as well as the changing of environment (by migration) might affect the cytokine profiles in breast milk, with potential impact on child health. All these studies indicate that in addition to *in utero* exposures, the environmental exposures may act via alteration of the microbiota as well as changes in breast milk to further influence the development of the neonatal immune response.

The impact of in utero *priming by helminths on infant's immune responses to subsequent infections*

Many studies in humans have shown that neonate's immune responses can be sensitized by maternal parasite infections, particularly helminths, during pregnancy [36–41] but also by protozoan parasites of pregnant mother such as malaria [42–45], trypanosomes [46,47] and *Toxoplasma* [48]. The modulation of host immune responses by chronic helminth infections is characterized by increased TH_2-type cytokines and immune regulatory cytokines such as IL-10 and TGF-β causing immune hyporesponsiveness

which seems most prominent in the presence of tissue-dwelling helminths [49,50]. Although many population studies have examined the effect of helminth infections on the immune system, studies during pregnancy and in neonates are still relatively scarce, in particular those with a birth cohort design where the parasitic infection status of mothers during pregnancy is known. Pregnant women living in endemic areas seem to have either the same or higher risk of being infected with helminths compared to the rest of the population [51,52]. The impact of maternal helminth infections on their offspring has been studied in a number of papers suggesting that *in utero* exposure appears to be associated with increased susceptibility to filarial infection but less filarial pathology during childhood and adulthood [39,53,54]. Moreover, the offspring of *S. mansoni*-infected pregnant mice developed less liver granuloma and also had lower egg density in the liver or in the intestine after an experimental infection compared to offspring of uninfected pregnant mice [55–57]. It appears from these animal studies that prenatal exposure to maternal helminth infection leads to less worm burden and less pathology in the offspring. While in animal models the timing and duration of infection can be controlled, in human population many factors are not easily controlled and can lead to very diverse spectrum of infections and clinical presentations [58]: the diverse genetic background of the host, different levels of exposure to cercaria associated with behavioral patterns [59], as well as genetic diversity of the parasites within an individual or the community [60,61]. Up to date there is only one study in pregnant women with schistosomiasis where the treatment of infected women with praziquantel was shown to increase both the cellular and humoral responses to schistosome egg antigens [62,63]; however a follow-up study is needed to determine whether these immunological boosting effect that antihelminthics result in during pregnancy may have an impact on the child's immune responses in terms of susceptibility to the next infection or reduced pathology.

In human filariasis, Lammie and coworkers showed that maternal microfilaremic status, when the study was conducted (the infection status at pregnancy was not known), was associated with higher prevalence of microfilaremic children especially at the age of 10 years or younger [64]. Moreover, in a cross sectional study in Haiti the history of maternal filarial infection during pregnancy was found to be associated with cellular immune hyporesponsiveness to microfilarial antigen in non-infected young adults at 17-19 years of age, although the proportion of children with filarial specific

antibodies were not different between those born to infected and non-infected mothers [53]. Taken together, these findings suggest that cellular immune responses might be affected by exposure to maternal filarial infection during pregnancy and as such have long lasting effects. However no data was available on cellular immune hyporesponsiveness could lead to higher risk of getting filarial infection under a given exposure pressure in the community. In a birth cohort study up to seven years of age performed by Malhotra and coworkers, the study children were categorized not only by maternal filarial infection status but also by the presence or absence of cord cytokine responses to filarial antigen [39]. The study showed that children born to filarial-infected mothers but with no cord cytokine responses to filarial antigen, categorized as immuno-tolerant children, were more susceptible to filarial infection in childhood compared to the other groups.

The impact of in utero *priming by helminth on infant's immune responses to vaccination*

Immune hyporesponsiveness which is thought to result from immune modulation by helminth products may affect the immune responses to bystander antigens, such as to those present in vaccines. However depending on the nature of the vaccine, type and intensity of parasite infections, different outcomes have been seen. Bacille Calmette-Guérin (BCG) vaccination is known to induce a TH_1-type cytokine production in infants like in adults [65]. In a study carried out in Kenya, it was shown that neonates from mothers living in helminth endemic area were able to generate IFN-γ, IL-4 and IL-5 responses to mycobacterial antigens even before BCG vaccination [66]. In a later study the same authors raised the question of whether *in utero* exposure to helminth antigens can affect the immune responses to purified protein derivative (PPD) in infants aged 10-14 months, who were given BCG vaccination at birth. The peripheral blood mononuclear cells (PBMC) of infants born to helminth-infected mothers produced lower IFN-γ response to mycobacterium antigens but higher IL-5, compared to the infants born to helminth-free mothers [40]. Another birth cohort study with whole blood culture in Uganda showed that maternal hookworm infection was associated with reduced maternal IFN-γ responses to mycobacterial culture filtrate protein (CFP) but higher IFN-γ responses in their one-year old children [67]. Needless to say, in order to firmly establish whether helminth infections affect neonates' responses to vaccines, it

would be important to conduct trials which administer anti helminthics during pregnancy. Such elegant design using double blind placebo-controlled trial was conducted by the group of Elliott who studied the immune responses of neonates after helminth infected pregnant women were treated with anti helminthics or placebo [67]. Here, maternal hookworm infection translated into increased IFN-γ response to CFP in one year old infants when the mothers were assigned to the placebo group but this increase was not as prominent when mothers were treated with albendazole during pregnancy. In a larger cohort study performed by the same group where one treatment was given during the second or third trimester of pregnancy, the effect of albendazole compared with placebo, was a 37% reduction in infant IFN-γ responses to CFP, but this fell short of statistical significance [68]. In this large study, there appeared to be a direct effect of albendazole; infant IFN-γ responses were higher when they were born to hookworm-uninfected mothers who were treated with albendazole [69], somehow complicating matters but highlighting the importance of caution when interpreting results from cross sectional or uncontrolled treatment studies [70]. This group, in earlier study, has found that maternal infection with *Mansonella perstans*, a filarial nematode, was associated with higher infant IL-10 responses to CFP and tetanus toxoid (TT) but with no significant effect on TH_1 and TH_2-type cytokines to the two vaccine antigens [68]. These studies all indicate that it is of outmost importance to have well powered and placebo controlled studies that will determine the impact of helminth infections on the immune responses to vaccine antigen [70]. Even larger studies will be needed to evaluate the effect of helminths not only on immune responses to vaccines but also on the efficacy of the vaccination.

The impact of in utero *priming by helminths on development of atopic disorders in early childhood*

A study in cord blood mononuclear cells (CBMC) in an area highly endemic for parasitic infections in Gabon, showed lower TLR2 expression on monocytes and myeloid dendritic cells but higher number of antigen presenting cells and antigen-experienced T cells along with lower expression of FOXP3 compared to Austrian CBMC [71]. The results were interpreted as increased activation and possible modulation of neonate's immune system in Gabon. Another study comparing neonate's innate immune responses between countries with different environmental settings,

showed that CBMC of Papua New Guinean (PNG) newborns expressed lower TLR4 but higher TLR2 and TLR9 compared to CBMC of Australian newborns [72]. The study also showed that stimulation with *Staphylococcus aureus* lipoteichoic acid (LTA) and lipopolysaccharides (LPS) resulted in lower IL-6, IL-10 and TNF-α responses in the PNG newborns. The downregulation of innate immune responses as shown in the studies in Africa and PNG appears to be different to the findings in the European farm studies described earlier where exposure to farms leads to increased TH_1 and TNF-α responses in cord blood [16]. It is expected that in European farms the exposure to helminths, if any, would be of low intensity and indeed that bacterial and fungal exposures are expected to be more prominent than helminths [73]. In fact, a birth cohort study in the Netherlands found that low transmission of *Ascaris suum*, measured by *Ascaris*-specific IgG antibodies in children of 4 years of age, was associated with higher prevalence of allergic disorders [74]. Thus different exposures, whether helminths or bacteria and the degree of exposures to micro-organisms (very high, high or low) may lead to different immune outcomes in terms of up or down regulation of Toll-Like Receptors but both indicating an early activation of the immune system which might later translate into less vigorous immune reaction to environmental insults such as those inflicted by allergens. In an elegant study of pregnant mice, it was shown that besides the type and origin of bacteria, maternal functioning of TLR signaling was needed in order to confer protection from experimental asthma in the offspring [75]. This study was interesting in that it showed that bacterial exposure of mothers, lead to a decreased expression of TLRs in the placenta; something that has not been studied up to date in humans.

Most studies have investigated the effect of helminths on atopic disorders during childhood or adulthood, showing either negative or positive associations [76]. So far studies investigating the impact of maternal helminths on the development of atopic disorders in their children are still scarce. A small birth cohort and interventional study in Uganda comparing mothers treated with albendazole or placebo showed that the presence of geohelminth infection, especially with hookworm, during pregnancy or at delivery was associated with decreased risk of infantile eczema up to 15 months of age while the treatment with antihelminthics reversed this association [77]. Placebo-controlled trials are needed in order to confirm this finding in areas with a different helminth species (gut or tissue-dwelling helminths) and levels of endemicity and if possible to compare findings

between areas with different degree of urbanization. While the benefit of antihelminthics administration during pregnancy on birth outcomes in hookworm endemic areas has not been confirmed [78,79], the possibility that maternal helminth infections may suppress atopic disorders in their children and therefore treatment would increase the risk of developing allergies, would even suggest that this treatment option is less favorable. It is interesting to note that total IgE levels found in amniotic fluid were correlated with the levels in maternal serum and although foetal levels of circulating IgE were very low, they expressed low-affinity IgE receptors in the lymphoid follicles of the gut, which was thought to educate foetal immune responses to deal with IgE-mediated antigens such as allergen and helminth antigens [80]. This finding together with the growing evidences for helminth-specific modulation of host immune responses [81], may open opportunities to use helminth-derived substances as vaccines for the mother to divert the child's immune responses into less atopic phenotype.

As already alluded to earlier, the genetic make-up can govern the development of the immune system and disease outcome. The genetic material of living organisms or so called deoxyribonucleotide acid (DNA) is made of 4 nucleobases (A, adenine; C, cytosine; G, guanine; T, thymine) bound together by backbones of sugars and phosphate groups. Single nucleotide polymorphism or SNP is the variation of single base pair which occurs in at least 1% of the human population (minor allele frequency/ MAF $\geq$ 1%). Most SNPs are found outside exons, the protein-coding region of the gene. Some SNPs do not change the expression of genes between individuals, while others may cause functional differences or predispose to certain diseases.

Human Genome Project

The first large-scale genome project, International Hapmap, was founded in 2002 to develop a database of haplotype map of the human genome (www.hapmap.org). Haplotype map, comprising of 3.5 million SNPs, is an open-access source for researchers to find common pattern of human genetic variation. However it only covers those variations occurring in more than 5-10% of the populations. To overcome this in 2008 the 1000 genomes project was launched with the aim to build a catalog of human genetic variations from about 2500 people representing 27 population groups in the

world (www.1000genomes.org). This project covers five major populations in the world: Europe, East Asia, South Asia, West Africa and America, and can detect rare variants with MAF $\leq$ 1%.

More recent work on whole genome mapping of a South-east Asian population has been started by Singapore Genome Variation Project (SGVP), which covers three major populations in Singapore: Chinese, Malays and Indian. These ethnic groups are also reflected in the populations in neighboring countries such as Indonesia. So far SGVP has genotyped more than 2 million SNP polymorphisms, of which the online data was available for the public [82]. Historically Indonesian population in the western part was considered similar to Singaporean's Malays both linguistically and genetically [83]; therefore we use SGVP as the main source for finding SNP polymorphisms.

This thesis

The general objective of this thesis was to study the development of innate and adaptive immune responses of young children living in a helminth-endemic area in Indonesia. For this purpose we set up a birth cohort study, following up pregnant mothers and their children up to 4 years of age.

Study population

Our study site was located in two adjacent villages in Bekasi Distric, West Java Province, Indonesia: Jati Sampurna (JS) and Jati Karya (JK). The area is situated about 30 km east from Jakarta, the Indonesian capital. These two villages, together with three other villages are covered by Puskesmas (primary health centre) Jati Sampurna. JS village has about 21,000 residents and JK village about 8,000 residents, with area coverage of approximately 80 km^2. According to the census data in 2000 by Badan Pusat Statistik (BPS) several ethnic groups can be found in West Java such as Sundanese (79%), Javanese (11%), Betawi (5%) and Cirebonese (5%). (source: Suryadinata L, Arifin EN, *Indonesia's Population: Ethnicity and Religion in a Changing Political Landscape*. Singapore: Institute of Southeast Asian Studies; 2003). Data on ethnicity in the study area was not available; however we assumed that the distribution of ethnicity in the study area was similar to the general population of West Java.

Jati Sampurna has more direct access to the main busy road, and many of its inhabitants are industrial or construction workers, as well as

traders or government employee. While many people in Jati Sampurna are transmigrants, Jati Karya has more life-long residents. Some of Jati Karya residents work as farm labourers. People in both villages live close to each other, and sometimes one house can consist of the nuclear family plus the extended family such as grandparents or cousins. Some families live in brick-walled houses, while others in bamboo or wooden houses especially in Jati Karya. Characteristics of traditional houses or called “rumah panggung” which can still be found in the study area, has the floor uplifted with wooden pillars about 50 cm above the ground, and walls are made of bamboo. Some houses have pools of water sewage in their backyards, which can be good breeding places for mosquitos which are the vector of the lymphatic filarial worm (*Wuchereria bancrofti*).

Public health care services are mainly provided by a primary health care (“Puskesmas”), while smaller public health units (“posyandu”) are formed among the community to give more direct access for pre-school child care. “Posyandu” takes place in a house, where local health staff and cadres help the administration of child vaccinations, vitamin A supplementation and growth monitoring. National policy for vaccination (EPI/ Expanded program on immunization) consists of BCG, DTP and oral Polio, Hepatitis B, Measles are available for free for children under 5 years of age. Prenatal care is provided by Puskesmas, hospital or midwifes who have private practice, and majority of pregnant women give birth with the help of midwives or traditional birth attendants who have been trained by Puskesmas.

In 2001 our preliminary survey with finger prick blood was performed in 14 subvillages or Rukun Warga (RW) in JS and JK, showing that the prevalence of filariasis in the population was ranging between 0 – 14%. Since then more blood screenings were done by the government in the surrounding areas till in 2004 it was declared that Bekasi district was endemic for filariasis and the campaign for filarial mass treatment started. In 2008, the year when our study ended, the mass treatment program reached JS and JK villages. Besides filarial infection, soil-transmitted helminth infections are still prevalent in the area.

Study design

The study project started between 2002 and 2004 with the recruitment of pregnant women. The research team from Department of Parasitology University of Indonesia, with the help of two collaborating midwives, invited

pregnant women in the second and third trimester to participate in the study. Demographic and socio-economic data were gathered and entered into a database. Filarial infection of mother was determined by the positive result of antigen detection in peripheral blood, while soil-transmitted helminth infection was detected in stool samples by microscopy. The rest of blood was used for whole blood culture and cytokine detection, while separated plasma was used for antibody measurements.

The children from the participating mothers were followed up five times, starting from the age before any vaccination was given (which was on average at 2 months of age), 5 months, 1, 2 and 4 years of age. Blood collections were performed during these indicated time points for the same immunological measurements as those of mothers once during pregnancy. Necessary information on child's vaccination dates were obtained from mothers and puskesmas Information on child's health and common illnesses were gathered by questionnaire during house-to-house visit, between time point of 2 and 4 years of age. At 4 years of age, the children were skin prick tested against common aeroallergens.

Scope of the thesis

Here in the Introduction, **Chapter 1**, we review the current understanding of how immune responses are shaped in early life and how this might affect disease outcome with particular focus on developing countries.

Chapter 2. Helminth infection and allergy are both associated with increased TH_2-type immune responses. While studies of allergic responses in children at very young age in developed countries are abundant, similar studies in developing countries are still scarce. In this chapter we looked at the development of child's TH_2-type cytokine responses to general and helminth-specific stimuli as well as total IgE production up to 4 years of age, and asked whether environmental factors during pregnancy could affect this development. In addition we correlated these factors with child's atopic sensitization at 4 years of age.

Chapter 3. BCG is one of the earliest vaccines given to infants, which is also a strong inducer of TH_1 responses. It is known that BCG vaccination can protect infants against severe form of tuberculosis (TB) such as meningitis and miliary TB. Besides that BCG can have non-specific beneficial effects unrelated to TB. In this chapter we studied the effect of

BCG vaccination on the development of TH_1- and TH_2-type immune responses to purified protein derivatives (PPD), as well as to mitogen as a general/polyclonal stimulus, in young children up to 2 years of age living in a helminth endemic area. We also correlated the BCG scar size at 4 years of age with the cytokine responses to PPD at earlier ages.

Chapter 4. Cytokines are mediators produced by immune cells and play an important role in innate and adaptive immune responses. Pregnant mothers are exposed to environmental factors which can induce maternal immune responses including cytokines, and in turn can influence the child's immune responses in the womb. To investigate the immunological relationships between mother and child, we measured the innate (LPS-induced IL-10 and TNF-α after 24 hours of incubation) and adaptive cytokine responses (mitogen-induced IFN-γ, IL-5 and IL-13 after 6 days of incubation) in pregnant mothers and their children at the age of 2 months, before any vaccination was given. Maternal characteristics including socioeconomic parameters and parasitic infections were taken into account in this maternal-child cytokine relationship.

Chapter 5. Early childhood is a critical period when a child is exposed to various environmental factors shared with his/her mother, and together with genetic factors can affect the development of child immune responses. In order to understand the extent of mother-child cytokine relationships beyond 2 months of age, we measured the child cytokine responses at 5 months, 1, 2, and 4 years of age and correlated this with maternal cytokine production. We also analyzed the single nucleotide polymorphisms of cytokine genes between mother and their children, and asked whether this could be related to cytokine production in vitro.

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