Development of Immune Responses in Early Life: A Longitudinal Study in Indonesia

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To my dear parents and aunties

Contents

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).

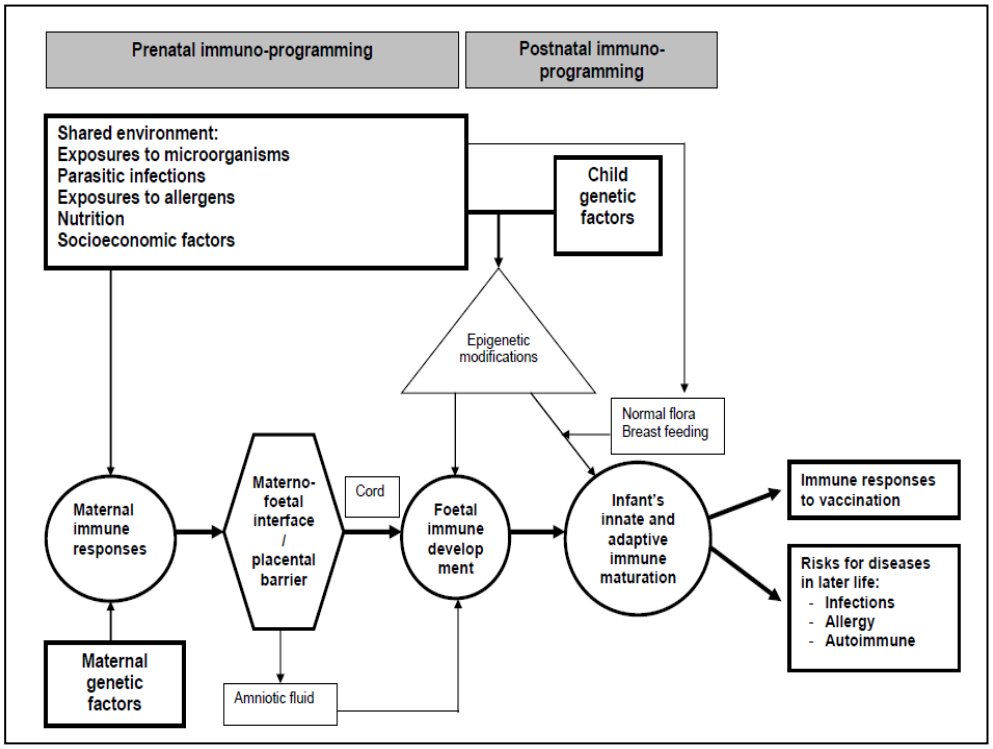

Figure 1. A proposed scheme for the impact of early exposure to environmental factors on the development of child's innate and adaptive immune responses, with consequences on immune responses to vaccination and on diseases in later life.

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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Chapter 2

The development of TH_2 responses from infancy to 4 years of age and atopic sensitization in areas endemic for helminth infections

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Abstract

Background: Helminth infections and allergies are associated with TH_2 responses. Whereas the development of TH_2 responses and allergic disorders in pediatric populations has been examined in affluent countries, no or little data exist from low income regions of the world.
The aim of this study is to examine factors influencing the development of TH_2 responses of children born in areas endemic for helminth infections and to relate these factors to atopic sensitization at 4 years of age.
Methods: Data were collected from pregnant mothers on helminth infections, education and socioeconomic status (SES). Total IgE, IL-5 in response to mitogen, and helminth antigens were measured in children at 2, 5, 12, 24 and 48 months of age. Skin prick testing (SPT) and allergen-specific IgE were determined at 4 years of age.
Results: Strong TH_2 responses were seen at 5 months of age and increased with time. Although maternal filarial infection was associated with helminth-antigen specific TH_2 responses, it was low maternal education or SES but not helminth infection, which was associated with the development of high total IgE and PHA-induced IL-5. At 4 years of age when allergen reactivity was assessed by SPT, the high general TH_2 responses did not translate into higher prevalence of SPT. The risk factor for SPT reactivity was low maternal education which decreased the risk of SPT positivity to allergens (adjusted OR, 0.32; 95% CI, 0.12 – 0.87) independently of maternal filarial infection which tended to reduce the child's risk for being SPT positive (adjusted OR, 0.35; 95% CI, 0.07 – 1.70).
Conclusions: In areas endemic for helminths, potent TH_2 responses were seen early in life, but did not translate into a higher SPT reactivity to allergens. Therefore, in many parts of the world TH_2 responses in general and IgE in particular cannot be used for diagnosis of allergic diseases.

Keywords: TH_2; IgE; IL-5; Helminth; Atopy; Skin prick test, Children

Background

Pregnancy and early childhood are critical periods in which the inherited immune system of a child is shaped by the environment both in utero and soon after birth. This in turn is thought to determine the disease outcome in older age. Previous studies have shown that the development of atopy can be linked to certain immunological patterns seen in cord blood or in peripheral blood during infancy. Several birth cohort studies such as in United Kingdom and Finland, revealed that higher cord total IgE levels were associated with atopic manifestations at 4, 10, or even at 20 years of age [1,2], while in another large birth cohort study in UK atopic children at 5 years of age had higher total IgE at 12 months [3]. In a small birth cohort study in Australia, children with atopy at 2 years of age already exhibited a decreased production of general adaptive T helper (Th)1 response at birth, followed by increasing allergen-specific-TH_2 from 6 till 18 months of age, indicating that immune responses can be already skewed early in childhood [4]. Another birth cohort study in United States showed that the levels of general adaptive TH_2 cytokines at 3 months and 1 year of age was positively associated with total IgE at 1, 2, 3 and 5 years of age [5]. All these studies demonstrated that any disturbance of immune responses which can translate into allergies in childhood seems to originate from very early life or in the first year of life. However, such important longitudinal studies have been conducted in affluent countries only. Given the alarming increase of allergic disorders in urban centers of low to middle income countries [6], it would be important to understand the relationship between TH_2 responses and allergic disorders in these countries where helminth infections can be highly prevalent.

Helminth parasites, like allergies, have been shown to be associated with TH_2-skewed immune responses characterized by the increased production of TH_2 cytokines (IL-4, IL-5, IL-13), polyclonal and specific IgE responses, and eosinophilia [7]. Interestingly, a number of studies show that the presence of helminth infections is inversely associated with atopy as defined by skin sensitization [8], while association with allergic diseases or asthma have been less convincing and if anything, helminths seem to be associated with increased IgE responses to environmental allergens [9]. However, in studies conducted in developing countries, the presence of IgE antibodies to allergens, has not always been a reliable predictor of skin prick test positivity or allergic diseases, especially in areas where helminth infections were prevalent [10]. Thus, many children with high total IgE or

positive allergen-specific IgE had negative skin test reactivity [9] or reported symptoms of allergy [11]. In this context, socioeconomic factors can play an important role as has been shown by the multicenter ISAAC study in affluent and less-affluent countries where the positive association between atopic sensitization and atopic wheeze was weaker in countries with low gross national income per capita [12]. Moreover it has been suggested that the result might apply to the association between IgE and skin test reactivity [13]. These studies were mainly cross-sectional and conducted among schoolchildren or adults. Little is known about the development of TH_2 responses at earlier time points in these less affluent countries, where helminth infections might be highly prevalent, and the relationship with allergic outcomes.

To start addressing this, we have examined the development of TH_2 responses and influencing factors in early life of children living in an area in Indonesia where helminth infections are prevalent. We hypothesize that early exposure to certain maternal environmental factors including helminth infection during pregnancy will affect the development of TH_2 responses. Furthermore, we have related these factors to atopic sensitizations at 4 years of age.

Methods

Study population and parasitological examination

The participants for this cohort study consisted of pregnant mothers and their subsequently born children living in two adjacent villages, which were endemic for filarial (*Wuchereria bancrofti*) and soil-transmitted helminth (STH) infections, in Bekasi District, Indonesia, as described previously [14]. Between 2002 and 2004, pregnant mothers who came to collaborating midwives for their prenatal care at the second or third trimester were enrolled. The children born full-term and healthy and whose mothers agreed to continue participation were followed up during house-to-house visits at 2 months (T0); 5 months (T5); 1 year (T12); 2 years (T24); and 4 years (T48) of age. A total of 84.3% (241/286) of children of enrolled mothers who continued to participate had immunological data available at least at 1 time point. The flow diagram of the study is shown in Figure 1. The study was approved by the Ethics Committee of Faculty of Medicine, University of Indonesia.

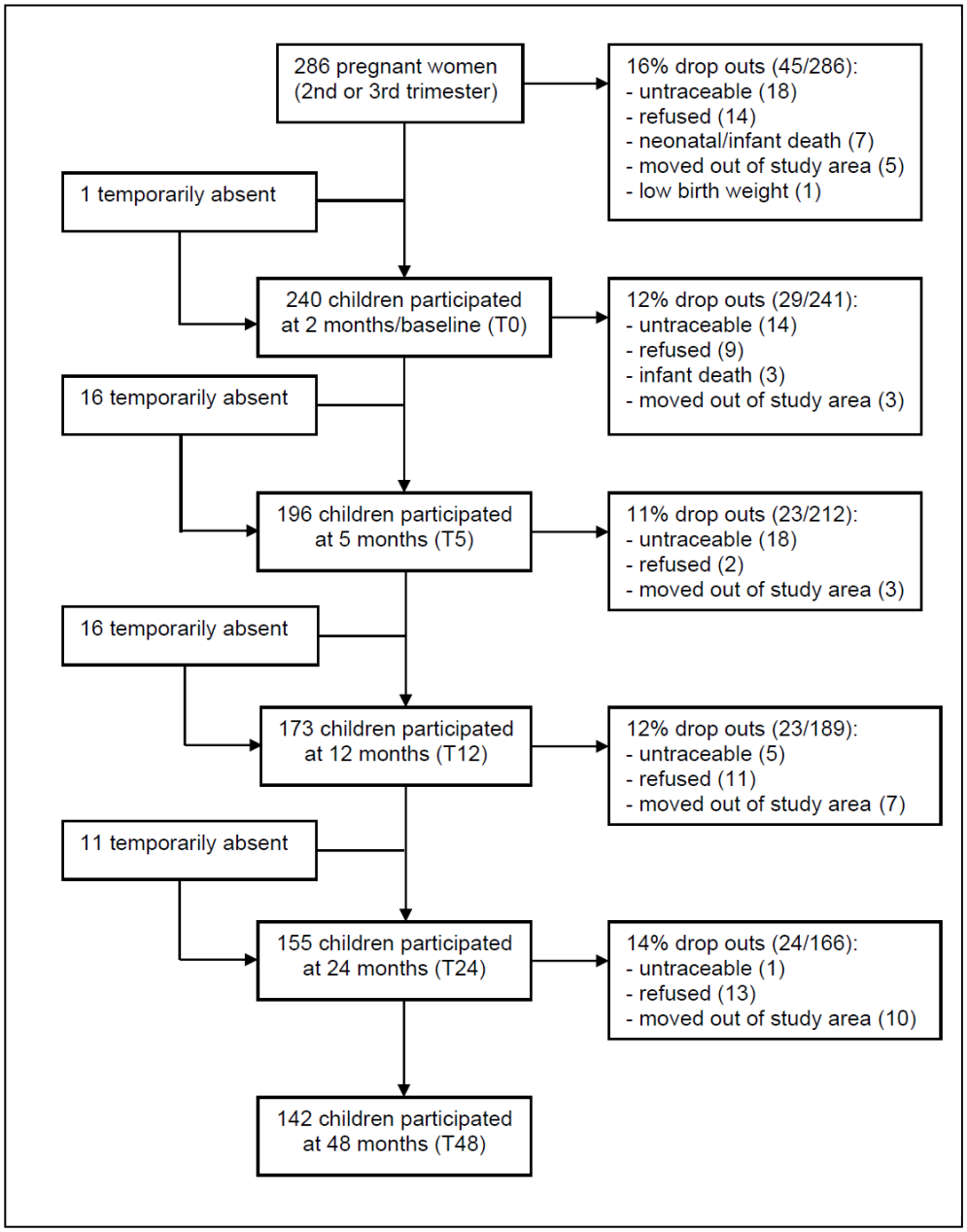

Figure 1. The flow diagram of the cohort study. Drop outs imply that the participants have no data from this time point onward. Temporarily absent implies that the participants have no data at this time point but have data available at other time point.

Maternal filarial infection was determined by detection of circulating antigens in blood using immuno-chromatographic test (ICT) as described by the manufacturer (Binax, Scarborough, ME, USA). Stool samples from mothers and children at 4 years of age were examined for the presence of STH eggs by lugol staining and microscopy, and positives were treated with single dose of albendazole 400 mg.

Data on socioeconomic status (SES) factors were collected via questionnaire during recruitment of pregnant women. Each factor was given a score: type of house wall (bamboo/wood/mixed with brick=0, brick=1), water supply (well=0, pump/pipe=1) and cooking fuel (wood=0, kerosene=1, gas=2). SES is low if the sum of scores ≤ 2, and high if ≥ 3. Educational level is scored 0 if no or primary schooling and 1 for higher education.

Preparation of adult worm antigens

Ascaris lumbricoides adult worms were obtained from infected individuals in Indonesia and *Brugia malayi* adult worms were recovered from infected jirds. The method for antigen preparation has been described in a previous study [15].

Whole blood culture and cytokine measurement

As previously described [16], whole blood was diluted 1:10 in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) before stimulation with phytohaemagglutinin (PHA; 2 µg/mL; Wellcome Diagnostics, Dartford, UK), *B. malayi* adult crude antigen (BmA; 12.5 µg/mL), *A. lumbricoides* adult crude antigen (20 µg/mL) or medium only. After six day culture at 37^{o}C and 5% CO_2, supernatants were collected and frozen (-20^{o}C) until cytokine measurement.

IL-5 in supernatant was measured by ELISA as described elsewhere [17] and with a detection limit of 2 pg/mL. All values below detection limit were given half of the detection limit. Between two and seven subjects within each time point were excluded from the entire IL-5 analysis due to very low response to mitogen (< 10 pg/mL).

Total IgE measurement

Total IgE levels in plasma of mothers and children at all time points were measured using ELISA, adapted from the previous study [18].

Specific IgE and skin prick testing (SPT)

Children at 4 years of age were skin tested against house dust mite (HDM, *Dermatophagoides pteronyssinus*), peanut, egg, shrimp, histamine dihydrochloride 10 mg/mL as positive control, allergen diluent as negative control (kindly provided by Paul van Rijn, HAL Allergy Laboratories, Leiden, The Netherlands) and german cockroach (*Blatella germanica*; Lofarma, Milan, Italy). The tests were performed using SPT lancets (Stallergens SA, Antony, France) and were assessed at 15 min. The result was considered positive if mean diameter of wheal (the sum of longest diameter to the perpendicular diameter /2), was at least 3 mm.

Specific IgE (sIgE) levels from pregnant women and children at 4 years of age were measured against two aeroallergens, HDM and german cockroach by ImmunoCAP® (Thermo Fisher Scientific, Uppsala, Sweden). The two allergens for sIgE measurement were chosen based on the most prevalent SPT positivity. Individuals were considered sensitized if the levels of sIgE were $\geq$ 0.35 kU/L.

Statistical analysis

Total IgE levels were normalized with log-transformation and comparisons between baseline and other time points were analyzed using Student t-tests. For child's IL-5 responses which could not be normalized with log-transformation, Mann-U Whitney tests were used. Bonferroni's correction was applied for multiple comparisons. Mean levels were expressed as geometric means with 95% confidence interval (CI), while median levels were presented with interquartile range (IQR).

To be able to analyze all children in a longitudinal fashion including those with missing data at a given time point, a linear mixed model for log total IgE was performed to include individuals as the random effect. Maternal helminth infections, village of residence, SES, education, time variable and child's baseline immunological data were treated as fixed effects. For the longitudinal analysis of binary outcomes of IL-5 (with median value as the cut-off), General Estimating Equation (GEE) analysis was performed to include time as the within subject variable and other factors as covariates. In addition to adjusting for the covariates in univariate models, all multivariate models were also adjusted for child's gender. An interaction term with time was tested only for factors that have effects on an outcome variable over time.

To explore the association between maternal factors and child's atopic sensitization (SPT or allergen-specific IgE), univariate and multivariate logistic regression analyses were performed. *P* values were two-sided and were considered significant if < 0.05. All statistical analyses were done in SPSS 17.0 (SPSS Inc., Chicago, IL, USA).

Results

Maternal and child's baseline characteristics

The baseline characteristics of mothers and children are shown in Table 1. Sixty four percent (153/240) of pregnant women had a low level of education and 43% (99/236) were classified as having low socioeconomic status (SES). Although low SES was more likely to be found in mothers with low education (49% in low-educated versus 29% in high-educated mothers), the overlap was only partial and therefore these two variables had to be considered separately.

Table 1. Baseline characteristics of mothers and children participating at the baseline (T0)

Participants	Characteristics	n/N (%)
Pregnant mothers	Village of residence	
	Jati Sampurna	109/241 (45)
	Jati Karya	132/241 (55)
	Filarial infections	45/241 (19)
	STH infections	68/217 (31)
	Ascaris lumbricoides	33/217 (15)
	Hookworms	29/217 (13)
	Trichuris trichiura	13/217 (6)
	Socioeconomic status	
	Low	99/236 (42)
	High	137/236 (58)
	Educational level	
	Low	153/240 (64)
	High	87/240 (36)
Children	Gender	
	Boys	114/230 (50)
	Girls	116/230 (50)
	STH infections at 4 yrs	10/126 (8)
	Ascaris lumbricoides	8/126 (6)
	Trichuris trichiura	5/126 (4)

STH: soil-transmitted helminth.

The percentage of pregnant women infected with filarial parasites was 19% (45/241) and with intestinal helminths was 31% (68/217). The loss to follow up at the end of the study was 41% (99/241). There were no significant differences in gender, maternal helminth infections, education and SES between those who remained in the study and the group that was lost to follow up, except for village of residence (Jati Karya village: 63% vs 43%, respectively, Chi-square test: p=0.002). At four years of age, 10 out of 126 children (8%) were infected with soil transmitted helminths (STH) and since the prevalence was low, it was not considered in data analysis.

The development of child's general and specific TH_2-type responses

The pattern of child's total IgE production was markedly increased between T0 and other time points (P < 0.001; Figure 2A). Geometric mean (GM; 95% CI) of total IgE was 2.4 (1.9 – 3.0) IU/mL at 2 months, 24.0 (19.3 – 29.8) IU/mL at 5 months, 59.3 (47.7 – 73.8) IU/mL at 1 year, 196.8 (156.1 – 248.1) IU/mL at 2 years, and 345.9 (280.6 – 426.4) IU/mL at 4 years of age. The increasing levels of general TH_2-type cytokine responses, represented by mitogen-stimulated IL-5 production, were seen in particular at T24 and T48 (Figure 2B).

In response to helminth antigens (BmA and *Ascaris*), IL-5 production increased significantly to reach a plateau at 4 years of age (Figure 2C and 2D). Spontaneous IL-5 production was low and did not increase significantly over the same time period (Figure 2E).

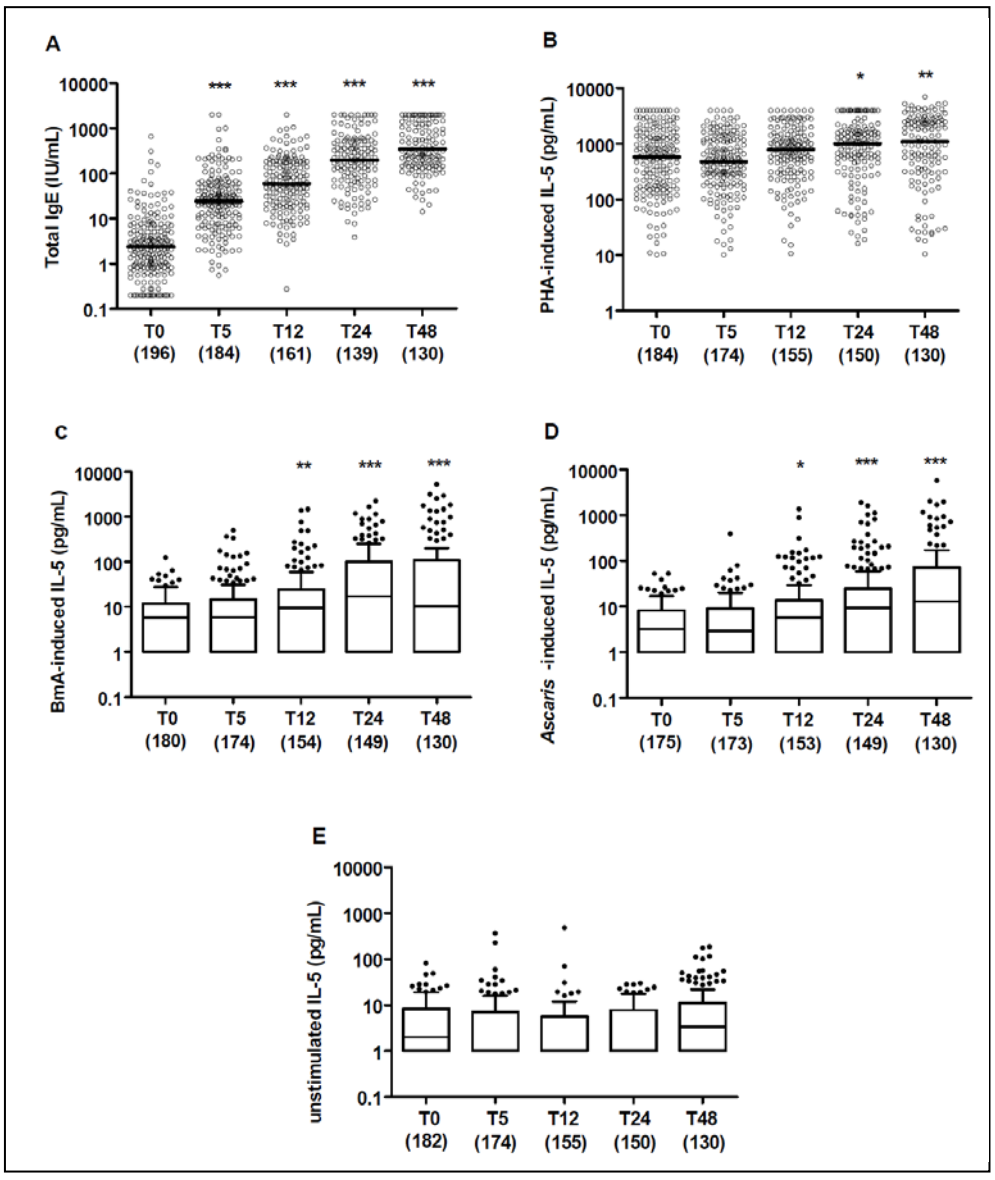

Figure 2. Longitudinal pattern of child's general and specific TH_2-type humoral and cellular immune responses. The levels of child's total IgE (**A**), PHA-induced IL-5 (**B**), BmA-induced IL-5 (**C**), *Ascaris*-induced IL-5 (**D**) and unstimulated IL-5 (**E**) of children at the age of 2 months (T0), 5 months (T5), 12 months (T12), 24 months (T24) and 48 months (T48). The horizontal lines in the bars represent the median, with the lower 25% and upper 75% percentiles and extended whiskers the 10% and 90% percentiles. Comparisons were made between the baseline (T0) and other time points: *p< 0.05; **0.001< p < 0.01, ***p< 0.001.

Association between maternal factors and development of child's TH_2-type responses

In the univariate linear model, village of residence, low maternal education and low SES were associated with higher total IgE levels (Table 2). In the multivariate model, maternal education was the strongest factor inversely associated with child's total IgE (adjusted estimate, 0.15; 95% CI, 0.05 – 0.25), followed by maternal SES (adjusted estimate, 0.12; 95% CI, 0.02 – 0.21) and village of residence (adjusted estimate, -0.21; 95% CI, -0.41 – (-0.01)). Figure 3 shows the difference in total IgE levels between children with low and high maternal educational level, where starting from 1 year of age, children with low-educated mothers had higher total IgE levels compared to those with high-educated mothers. This change with time is indicated by the significant interaction between maternal education and time (Table 2).

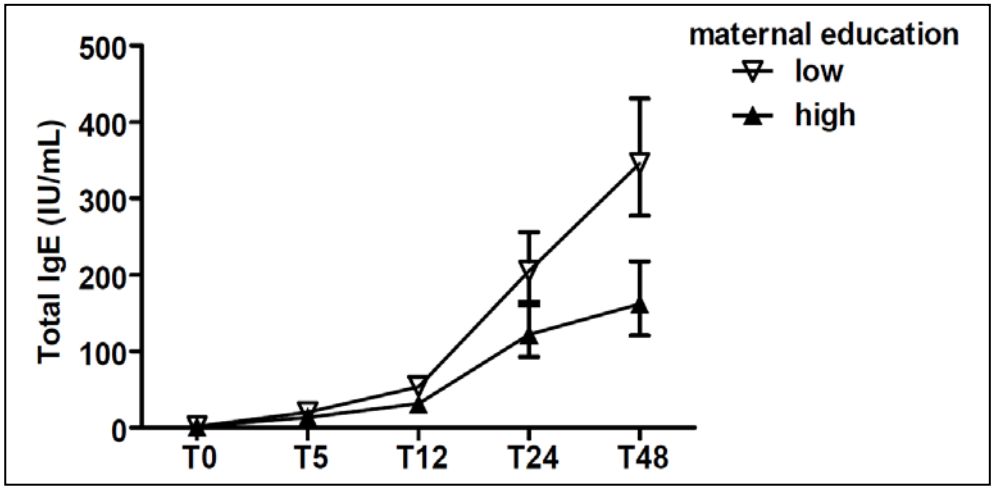

Figure 3. Total IgE of children as a function of maternal educational status. Open triangle are children born to low-educated mothers, closed triangle are children born to high-educated mothers. Error bars represent geometric means and 95% confidence intervals of child's total IgE, after adjustment for maternal helminth infection, village of residence, maternal education and socioeconomic status, child gender.

The child's general TH_2-type cytokine production (PHA-induced IL-5) up to 4 years of age was only affected by maternal SES (adjusted odds ratio [adj OR] 1.47; 95% CI, 1.11 – 1.95; Table 2). Regarding helminth specific TH_2-cytokine responses, there were positive associations between maternal

filarial infection and IL-5 induced by BmA or *Ascaris* antigen, that remained significant after adjusting for village of residence, maternal education and SES (adj OR, 1.64; 95% CI, 1.14 – 2.36 and adj OR, 1.69; 95% CI, 1.11 – 2.58, respectively).

Factors associated with child atopy

Of 132 children having SPT, there were 2 children with positive SPT for negative control and 3 children with no histamine wheal; these children were excluded from further analysis. Of 127 children with available SPT results, 24 children (19%) were positive for any allergen tested. There were 21 children (16%) positive for cockroach and 9 children (7%) positive for house dust mite (HDM), with a total of 22 children (17%) positive for either or both aeroallergens. Among the skin test reactivity for food allergens, three children (2%) were sensitized to shrimp, one child (0.8%) to peanut and none were sensitized to egg. From those children with available specific IgE (sIgE) measurements, 58% (61/105) of children were positive for HDM with median of 0.93 (interquartile range [IQR], 0.10 – 3.21) kU/L and 55% (58/106) were positive for cockroach with median of 0.60 (IQR, 0.09 – 1.88) kU/L. In total, 64% (68/107) of the children had positive sIgE to either HDM and/or cockroach.

To see whether SPT results correlated well with allergen-specific or total IgE measured at the same age, the IgE levels were compared between SPT-positive and SPT-negative children. No significant association was found between sIgE positivity and SPT. From a total of 93 children with available SPT and sIgE results, all 17 children with positive SPT, either to HDM or cockroach, had sIgE levels above 0.35 kU/L; while about half of children with negative SPT (41 out of 76) still had sIgE levels above 0.35 kU/L. We found no significant associations between total IgE and SPT reactivity, where SPT-positive children (n=19; GM, 396.4; 95% CI, 208.4 – 753.9 IU/mL) had similar levels of total IgE compared to SPT-negative children (n=95; GM, 330.3; 95% CI, 259.0 – 421.1 IU/mL).

Table 2. Association between maternal factors and child total IgE or IL-5 over time

Variables	Total IgE		IL5 PHA		IL5 BmA		IL5 Asc	
	crude β (95% CI)	adj β* (95% CI)	crude OR (95% CI)	adj OR* (95% CI)	crude OR (95% CI)	adj OR* (95% CI)	crude OR (95% CI)	adj OR* (95% CI)
Maternal filaria								
No	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
Yes	0.06	0.04	1.17	1.04	1.67	1.64	1.68	1.69
	(-0.04, 0.17)	(-0.08, 0.16)	(0.87, 1.57)	(0.76, 1.41)	(1.20, 2.33)	(1.14, 2.36)	(1.15, 2.46)	(1.11, 2.58)
P value	0.24	0.51	0.30	0.81	**0.003**	**0.008**	**0.007**	**0.01**
P-int**	NT	NT	NT	NT	**0.049**	**0.04**	0.52	0.50
Maternal STHs								
No	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
Yes	0.08	0.05	0.87	0.83	1.00	0.91	1.07	0.97
	(-0.02, 0.18)	(-0.05, 0.15)	(0.65, 1.18)	(0.61, 1.13)	(0.74, 1.36)	(0.67, 1.23)	(0.76, 1.50)	(0.69, 1.38)
P value	0.12	0.32	0.38	0.23	1.00	0.54	0.69	0.89
P-int**	NT	NT	NT	NT	NT	NT	NT	NT
Village of residence								
Jati Sampurna	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
JatiKarya	-0.13	-0.21	1.10	1.11	1.08	0.97	0.93	0.84
	(-0.93, 0.67)	(-0.41, -0.01)	(0.83, 1.45)	(0.83, 1.49)	(0.81, 1.44)	(0.70, 1.34)	(0.67, 1.28)	(0.60, 1.19)
P value	0.29	**0.04**	0.50	0.47	0.62	0.87	0.65	0.33
P-int**	NT	0.30	NT	NT	NT	NT	NT	NT
Socioeconomic status								
High	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
Low	0.13	0.12	1.44	1.47	1.20	1.16	1.39	1.20
	(0.04, 0.22)	(0.02, 0.21)	(1.10, 1.87)	(1.11, 1.95)	(0.89, 1.61)	(0.86, 1.58)	(1.02, 1.91)	(0.86, 1.68)
P value	**0.006**	**0.02**	**0.007**	**0.007**	0.23	0.33	**0.04**	0.28
P-int**	0.20	0.11	0.61	0.61	NT	NT	0.50	NT
Maternal education								
High	Ref	Ref	Ref	Ref	Ref	Ref	Ref	Ref
Low	0.16	0.15	1.08	0.93	1.02	0.87	1.43	1.22
	(0.07, 0.25)	(0.05, 0.25)	(0.81, 1.43)	(0.67, 1.27)	(0.75, 1.40)	(0.63, 1.21)	(1.03, 1.98)	(0.88, 1.70)
P value	**0.001**	**0.003**	0.60	0.63	0.89	0.42	**0.03**	0.23
P-int**	0.07	**0.03**	NT	NT	NT	NT	**0.03**	NT

*Adjusted for other factors including child gender. ** *P*-int: *P* value on the interaction between time and the corresponding variable. *P* value in bold if significant (< 0.05). Adj: adjusted, OR: odds ratio, Ref: reference, NT: Not tested, STH: soil-transmitted helminth, PHA: phytohemagglutinin, BmA: *Brugia malayi* adult worm antigen, Asc: *Ascaris* adult worm antigen.

Next we asked whether maternal factors would affect the child atopy at 4 years of age, a time point when SPT and sIgE to allergens were measured. Low maternal education was significantly associated with lower risk of child SPT positivity and remained significant in the multivariate analysis (adj OR, 0.32; 95% CI, 0.12 – 0.87; Table 3). Negative trends were seen for an independent association between maternal filarial infection during pregnancy and child SPT (OR, 0.27; 95% CI, 0.06 – 1.25); however when adjusted for other factors, the confidence interval widened (Table 3). In terms of child sIgE, no significant association was found with maternal education or filarial infection. In contrast to maternal education, socioeconomic status was not significantly associated with any of the atopic markers.

Table 3. Association between maternal factors and child's atopy at 4 years of age

Variables associated with child's skin prick test positivity	**n (%) N=126**	**crude OR (95%CI)**	***P* value**	**adj OR (95%CI)***	***P* value**
Maternal filaria	30 (24)	0.27 (0.06, 1.25)	0.09	0.35 (0.07, 1.70)	0.20
Village of residence (Jati Karya)	84 (67)	0.69 (0.27, 1.78)	0.44	0.84 (0.31, 2.28)	0.74
Socioeconomic status (low)	59 (47)	0.75 (0.29, 1.90)	0.54	1.02 (0.37, 2.82)	0.97
Maternal education (low)	87 (69)	0.29 (0.11, 0.74)	**0.01**	0.32 (0.12, 0.87)	**0.02**
Variables associated with child's allergen-specific IgE[§]	**n (%) N=105**	**crude OR (95%CI)**	***P* value**	**adj OR (95%CI)***	***P* value**
Maternal filaria	25 (24)	0.43 (0.17, 1.07)	0.07	0.43 (0.16, 1.15)	0.09
Village of residence (Jati Karya)	60 (57)	0.75 (0.33, 1.66)	0.47	0.83 (0.35, 1.97)	0.68
Socioeconomic status (low)	44 (42)	1.17 (0.52, 2.63)	0.70	1.41 (0.59, 3.32)	0.44
Maternal education (low)	69 (66)	0.68 (0.29, 1.59)	0.37	0.75 (0.30, 1.87)	0.54

*Adjusted for other factors including child's gender . [§]Allergen-specific IgE as the binary outcome using cut-off 0.35 kU/L.
P value in bold if significant (< 0.05). Adj: adjusted, OR: odds ratio.

Discussion

This follow-up study from birth to 4 years of age was conducted in an area endemic for helminth infections, and demonstrated development of strong TH_2 type responses. Here, the total IgE levels already present at 5 months of age were three times higher than those in the United States [19] and at 4 years of age the levels were at least ten times higher than those in the Netherlands [20]. These strong TH_2 responses early in life, did not translate into proportionally higher prevalence of SPT to allergens at 4 years of age which at 19% was similar or even lower than the prevalence in affluent countries: 23% at the age of 3 yrs in Denmark [21] and 19.6% at the age of 4 yrs in UK [22]. Surprisingly, the children from our study had two to four times higher prevalence of aeroallergen-sIgE compared to children of affluent countries at the same age [23,24]. To understand this we have assessed factors that affected the development of general TH_2 responses as well as atopic outcomes at 4 years of age.

While maternal filarial infection was strongly associated with child's helminth-specific TH_2 responses, it did not appear to play an important role in the development of general TH_2 responses. This indicates that maternal parasitic helminth status was not the strongest factor determining the TH_2 polarization in these young Indonesian children. Indeed further analysis revealed that maternal education and or socioeconomic status had a significant influence on the development of total IgE responses. Children born to mothers with low SES or low education showed stronger development of total IgE responses over time than children born to mothers with high SES or high education. Interestingly at 4 years of age, children born to low-educated mothers had less chance of skin test positivity while maternal filarial infection had a tendency, independently of education level, to reduce the chance of a child being SPT positive.

The increasing total IgE in children up to four years of age in our study was in line with a cross-sectional study in young Ethiopian children where it was shown that IgE levels were 16 to 20 times higher compared to the levels in healthy Swedish children [25]. Recent cohort studies which were mostly conducted in children from affluent countries have studied total IgE and related it to allergic outcomes. It was shown that increasing levels of IgE were associated with atopic sensitization (defined as positive SPT) [3] or allergy symptoms [26]. However, in our study the elevated total IgE did not seem to translate into high prevalence of SPT, the reason being that

the total IgE measured in our study reflects largely IgE whose specificity remains elusive and is possibly clinically irrelevant. The levels of total IgE, however, can be very different between countries and races, which is partly explained by genetic factors [27]; although other factors can play a role too. Often helminths have been shown to influence total IgE [25,28,29]. In our study population, higher maternal education or socioeconomic status led to lower total IgE in a child. We consider these maternal factors as proxies for specific factors that influence child's immune development. In previous epidemiological studies, higher maternal education and SES have been associated with better hygiene practices [30] which in turn could decrease exposure to microorganisms and parasites [31,32]. Although the prevalence of child's intestinal helminth infection at 4 years of age was low (8%), it is possible that past infection, exposure to helminth eggs without established infection or infection by other parasites induced TH_2-type responses. In addition, other orofecal pathogens which were not measured in this study have been associated with decreased atopy in infected individuals [33,34]. Besides hygiene, maternal education and SES can also be correlated with feeding practices (quality of nutrition and timing of first solid food introduction), maternal stress, antibiotics prescription, exposure to endotoxin and pollutants. All these factors can have long lasting impacts on a child's atopic outcomes [35].

Our study showed that maternal filarial infection clearly led to helminth antigen specific TH_2 responses. Children can be sensitized to pathogen-derived antigens or allergens in utero [19,36–39]. In our study, maternal filarial infection affected the development of child's helminth-specific TH_2 responses indicating in utero sensitization of child's immune cells to filarial parasite antigens. Our results are in line with previous studies in areas endemic for filariasis showing that T cells and B cells of the newborns were capable of producing helminth-specific TH_2 cytokines and specific IgE antibodies [40,41].

While on the one hand maternal filarial infection clearly affected child's helminth-specific TH_2 responses, on the other hand, it showed a tendency to decrease child's skin reactivity and specific IgE production to allergens. Thus, early exposure to helminths might by stimulating helminth-antigen specific TH_2-type responses, attenuate and deviate from mounting a TH_2 response to allergens. Interestingly, exposure to filarial infection in earlier time points in these young children with a tendency for decreased skin reactivity does not appear different from what is seen in older subjects

where increasing prevalence of filarial infection was paralleled with decreasing skin reactivity to aeroallergens [42]. However, the difference seems to lie in the effect of helminth infection on allergen-specific IgE. In many studies so far, populations in areas where helminths are endemic bear high allergen-specific IgE [9,43,44]. Yet, in the current study we see that in early life, maternal filarial infection which is associated with helminth-antigen specific TH_2 responses, is associated with lower allergen-specific IgE. Whether the allergen-specific IgE induced during early childhood represents more of a "real" sensitization to aeroallergens compared to those at older age reflecting more of cross-reactivity between helminth- and allergen-specific IgE [45] needs to be investigated further. The possible effect of helminth infections during pregnancy on child's allergic disorders is supported by a randomized-controlled study in Uganda showing that praziquantel treatment of pregnant mother infected with *Schistosoma mansoni* increased the risk of eczema in their infants [46]. Moreover the group also found that eczema was positively associated with allergen skin test result in infants, although they did not look into the direct association between the effect of maternal treatment and child's SPT.

The percentage of children who were lost to follow up at the end of the study was considered high (41%). Since the participants were similar to the non-participants with only difference in the village of residence, we considered the possibility of bias minimal, as we have included village as a factor in the multivariate models. For child atopic outcomes, since the number of tested children decreased at the end of study and that the skin test reactivity prevalence was low, these factors could weaken the power of analysis. However as the negative trend of association between maternal filarial infection and child's skin test reactivity was also shown for allergen-specific IgE, this could indeed mean that exposure to maternal filarial infection affected child's atopy. For more confirmation on this finding, a similar cohort study with larger sample size would be needed by taking into account the prevalence of maternal helminth infection and child's skin test reactivity. With the limited number of potential confounders measured in our study and thus included in the analysis, it is possible that other uncontrolled confounders present during pregnancy and after birth could still affect the development of child's Th_2-type immune responses or atopy. Among these, family atopy, household crowding, duration of breastfeeding and pet ownership.

Conclusions

In summary, this longitudinal study shows that young children born in areas endemic for helminth infections develop strong TH_2 responses that increase with age at least up to 4 years of age, while the prevalence of SPT is not increased proportionally. Different maternal environmental factors determined by SES and education affect child's TH_2 responses. The challenge for the future is to delineate what precise factors mediate the effect of maternal SES and education on general TH_2 responses as well as SPT to allergens. In addition, further studies are needed to address whether the protection against atopy will translate into less asthma and allergic disorders in later life.

Consent

Written informed consent was obtained from the child's guardian/parent/ next in keen for publication of this report and any accompanying images.

Abbreviations

TH_2, T helper 2; sIg, specific Immunoglobulin; IL, Interleukin; PHA, phytohaemagglutinin; BmA, *Brugia malayi* antigen; SPT, skin prick test; HDM, house dust mite; SES, socioeconomic status; STH, soil-transmitted helminth; ICT, immuno-chromatographic test; ELISA, Enzyme-linked immunosorbent assay; ISAAC, International study of asthma and allergies in childhood; GEE, generalized estimated equation; OR, odds ratio; adj, adjusted; GM, geometric mean; CI, confidence interval; IQR, inter quartile range; IU, International Unit; kU/L, kilounits per liter; mg, milligram; µg, microgram; pg, pictogram; mL, milliliter; µL, microliter; °C, degrees Celsius.

Competing interests

All authors have declared that they have no competing interests.

Authors' contribution

MY conceived of the study and developed its design. YD carried out the field study, performed the statistical analyses and drafted the manuscript. TS participated in the study design and its coordination. HW participated in the study design and data collection. YCM participated in supervising the immunoassays and helped the interpretation of the results. SAV carried out the measurement of specific IgE. RvR supervised the measurement of specific IgE detection and helped to edit the manuscript. ES developed the immunoassays and helped to draft the manuscript. All authors read and approved the final manuscript.

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Chapter 3

A longitudinal study of BCG vaccination in early childhood: the development of innate and adaptive immune responses

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Abstract

BCG vaccine drives a strong T helper 1 cellular immunity which is essential for the protection against mycobacteria, however recent studies suggest that BCG vaccination can have non-specific beneficial effects unrelated to tuberculosis. In the present cohort study the development of cytokine profiles following BCG vaccination was investigated. Immune responses to PPD were assessed before vaccination and at ages of 5 months, 1 year, and 2 years, followed by BCG scar measurement at 4 years of age. BCG was shown to induce both TH_1 and TH_2 type responses against PPD at about 5 months of age after vaccination, and while TH_1 response was sustained, TH_2 responses declined over time. However, BCG scar size was strongly correlated with TH_2 responses to PPD at 5 months of age. Importantly, we observed no clear effects of BCG vaccination on innate immune responses in terms of early IL-10 or TNF-α production whereas some alterations in general adaptive immune responses to PHA were observed.

Introduction

Most immunological studies on the efficacy of Bacille Calmette-Guérin (BCG) vaccination focus on the production of IFN-γ as the main feature of TH_1 response, which leads to the activation of important cells such as macrophages to contain mycobacteria. Besides the partial protection against TB through IFN-γ production [1], BCG may decrease the mortality and morbidity in childhood and adulthood by its non-specific effects on the immune system [2,3]. The presence or absence of BCG scar has been used as one of the indicators for successful vaccination [4]; while not necessarily correlated with protection against tuberculosis [5], studies in Guinea Bissau have shown better survival and less respiratory infections in children with BCG scars [6,7]. These observations on the impact of BCG have been proposed to be caused by the enhancement of the maturation of the innate and adaptive immune responses [8]. However, very few studies have examined whether BCG vaccination in childhood alters not only responses to mycobacterial antigens, but also to mitogens or to stimuli of the innate immune system. Moreover, environmental factors can affect neonatal immune responses, influencing both specific and non-specific immune reactivities. Factors such as living on traditional farms or parasitic infections are known to affect the immune system in early life with possible consequences for disease outcome later in life [9,10] or for responses to vaccination at infancy [11]. Indeed, it is known that chronic helminth infection can modulate immune responses of the host to produce more TH_2 and regulatory cytokines against helminth and bystander antigens [12] . In developing countries, infants can be exposed to helminth antigens from early life, even in utero, which may affect the child's subsequent immune responses to Th-1 producing vaccines such as BCG [11,13]. There are so far very few studies that have examined the development and progression of cellular immune responses following BCG vaccination of neonates over time and the effect that environmental factors may have on this process.

In the current longitudinal study following BCG vaccination of neonates in Indonesia, we have examined the production of TH_1 (IFN-γ) and TH_2 cytokines (IL-5, IL-13) in day 6 supernatants of whole blood in response to PPD and PHA to determine specific and non-specific adaptive immune responses. To assess the effect of BCG vaccination on the development of innate immune responses, IL-10 and TNF-α were measured

in one day culture supernatants of whole blood stimulated with LPS and PPD. We also analyzed the relationship between cytokine responses to mycobacterial antigens and scar formation at an age when the scar formation has stabilized. In order to assess how external factors might influence responses to BCG vaccination, we studied the effect of maternal parasitic infection status on the profile of cytokine production over time.

Methods

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by Ethics Committee of Faculty of Medicine, University of Indonesia. All mothers provided written informed consent for the collection of samples from their children and for subsequent analysis.

Blood collection, BCG vaccination and measurement of BCG scar

This study was performed as part of a birth cohort to examine the development of immune responses of children living in areas endemic for helminth infection. Maternal parasitological data such as filarial or intestinal parasite infection was obtained during the recruitment of pregnant women as described before [14]. Filarial antigenemia for *Wuchereria bancrofti* was determined by immunochromatographic test (ICT) as described by the manufacturer (Binax, Scarborough, ME, USA). The presence of intestinal helminth eggs and protozoa cysts was determined from direct stool examination by microscopy, using lugol staining.

Children were recruited from two adjacent villages in a peri-urban area, after their mothers gave informed consents for participation in this study. Blood was withdrawn by venipuncturist and was collected in a heparinized tube at several time points: T0: before BCG vaccination, T5: 5 months of age, T12: 1 year of age, T24: 2 years of age. In Indonesia, BCG vaccination is the first vaccine to be given according to the national vaccination program, followed by three Hepatitis B vaccination, three diphtheria-pertusis-tetanus (DPT) that are given together with three oral polio vaccination, and finally measles vaccination before the child reaches 1 year of age. BCG vaccination program requires every infant to be vaccinated soon after birth by health staff from local primary health care

center (PHC). BCG vaccine used here contains attenuated live *Mycobacterium bovis* strain Paris No. 1173-P2 (Biofarma, Bandung, Indonesia), and 0.05 ml is given by intradermal injection of the arm.

After BCG vaccination, the resulting BCG scars were measured at 4 years of age by the same person from the research team, and the mean diameter of scar size was calculated from diameters perpendicular to each other. The reason for measuring BCG scar at 4 years of age is because the scar formation is expected to have stabilized. A BCG scar was considered negative if the mean diameter was less than 2 mm.

Whole blood culture

Heparinized venous blood was processed for culture within 6 hours after venipuncture. As previously described [15], the whole blood was diluted 10 times in RPMI 1640 medium (Invitrogen, Breda, The Netherlands) and was stimulated with tuberculin purified protein derivative of *Mycobacterium tuberculosis* (PPD) batch RT 50 (10 μg/ml; Statens Serum Institut, Denmark, Copenhagen), phytohaemagglutinin (PHA; 2 μg/ml; Wellcome Diagnostics, Dartford, UK), lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich chemie, Zwijndrecht, The Netherlands) or medium only as a negative control. LPS stimulation was performed in 101 from 147 samples (69%). One hundred microlitres of stimuli were added to each well containing 100 μl diluted blood in a round-bottomed 96 well plates (Nunc, Roskilde, Denmark). The plates were incubated in the presence of 5% CO2, at 37^{o}C. Supernatants were collected on day 1 for interleukin (IL)-10 and TNF-α measurement to assess innate immune responses to LPS and PPD, and on day 6 for IL-5, IL-13, IFN-γ measurement to discern antigen specific (PPD) and non-specific polyclonal (PHA) adaptive immune responses. All supernatants were kept frozen in -20 ^{o}C until measurement. The cytokine measurements were performed by multiplex bead-based assay as described below.

Cytokine measurement in supernatants

IL-10, TNF-α, IL-13 and IFN-γ levels were measured in supernatants by in-house multiplex bead based assay using Luminex IS 100 (Luminexcorp, Austin, TX, USA) while IL-5 level was measured by ELISA as described elsewhere [14]. The detection limits for IL-10, TNF-α, IL-13, IFN-γ and IL-5 were 6.5 pg/ml, 1.7 pg/ml, 12.5 pg/ml, 3.6 pg/ml, 2 pg/ml, respectively. All

samples with values below detection limit were given half of these threshold values.

Statistical analyses

Cytokine levels were analyzed in two ways, either using raw cytokine levels when comparing cytokines between two groups of children or using net cytokine levels (substracted from the negative control) to obtain percentage of responders/ non-responders (above or below zero for substracted values). Mann-Whitney non- parametric test was used to compare cytokine levels which were not normally distributed. Since the sample size in each time point was not equal, Mann-Whitney test was used to compare cytokine responses between two time points of measurement. The correlations between cytokine responses to PPD and PHA and between BCG scar size and cytokine responses were analyzed using Spearman's rank correlation. In order to adjust cytokine levels for other variables (gender, birth weight, birth season, age receiving BCG, place of residence, maternal intestinal helminth or protozoan infection), multiple linear regression was performed with diameter of BCG scar as the outcome. Age receiving BCG, diameter of BCG scars and cytokine levels were previously log-transformed to get a normal distribution. The statistical analyses were performed in SPSS version 16. A two-tailed *p* value was considered significant if less than 0.05.

Results

Study subjects

A total of 147 children were recruited into the study and blood samples were obtained before BCG vaccination was given (T0). From these children, 120 children could be followed up at T5, 105 children at T12 and 98 children at T24. Apart from 66 children in whom blood samples were available at all time points, there were 81 children in whom blood samples were not available at one or two follow up time points due to the refusal of parents at the particular time point to allow blood sampling of their child (*n* = 14), child discomfort (crying) (*n* = 2), moving out of the study area (*n* = 5), death of the child (*n* = 1), not able to find the child and family (*n* = 37), and lack of sufficient blood for whole blood culture (*n* = 22). There were 6 infants who had received BCG with unclear dates but before any other vaccination; therefore they were included in the analysis. From 141 children

with known date of BCG vaccination, the average age at which BCG vaccination was given was at 5 weeks (IQR= 2.0 – 8.5). Of these, 4 infants (3%) received BCG at the age of less than 1 week, 63 infants (44%) at the age between 1 week and 4 weeks, 39 infants (28%) at the age between 4 weeks and 8 weeks, and 35 infants at the age more than 8 weeks (25%). The mean interval time between BCG vaccination and blood collection at 5 months of age was 20.1 weeks (SD= 5.8 weeks). As shown in Table 1, the child's characteristics were similar between those with complete and incomplete cytokine data. For the comparison of cytokine production before and after BCG vaccination, we used the data from all children (n=147), while for the relationship between cytokine data and BCG scar we analyzed data from children with complete cytokine results (n=66).

Adaptive immune responses before and after BCG vaccination: TH_1 and TH_2 cytokine production in response to PPD and PHA

In comparison with pre vaccination time point (T0), IFN-γ, IL-5 and IL-13 responses to PPD increased after BCG vaccination at 5 months of age ($p<0.001$ for all cytokines). In contrast to IFN-γ which remained high until the children reached 2 years of age, IL-5 and IL-13 responses showed a gradual decrease (Figure 1A). Before BCG vaccination (baseline time point), there were already a proportion of IFN-γ (69%), IL-5 (55%) and IL-13 (36%) responders to PPD (Figure 1C).

Table 1. Comparison of child's characteristics with BCG scar measurement and cytokine data at all time points (complete data), children with incomplete cytokine data and all children recruited into the study.

Child characteristics	All children (n = 147)	Children with complete cytokine data (n = 66)	Children with incomplete cytokine data (n = 81)
Place of residence			
Jati Sampurna	69 (47%)	34 (52%)	35 (43%)
Jati Karya	78 (53%)	32 (48%)	46 (57%)
Mean birth weight (kg) (SD)	3.2 (0.5)	3.2 (0.4)	3.2 (0.5)
< 3 kg	23/99 (23%)	13/50 (26%)	10/49 (20%)
≥3 kg	76/99 (77%)	37/50 (74%)	39/49 (80%)
Birth season			
dry season	74 (50%)	39 (59%)	35 (43%)
rainy season	73 (50%)	27 (41%)	46 (57%)
Gender			
male	70/141 (50%)	36/66 (55%)	34/75 (45%)
female	71/141 (50%)	30/66 (45%)	41/75 (55%)
Median age at BCG vaccination (week)(IQR)	5 (2.0 – 8.5) (n=141)	4 (1.7 – 8.2) (n=66)	5 (2.0 – 9.0) (n=75)
Mean interval BCG vaccination and T5 (week) (SD)	20.1 (5.8) (n=119)	20.7 (5.7) (n=66)	19.4 (5.9) (n=53)
Median BCG scar at 48 months (mm) (IQR)	3.3 (2.0 – 4.5) (n=96)	3.3 (1.5 – 4.1) (n=58)	3.5 (2.4 – 4.8) (n=38)
Maternal parasitic infections			
Filaria	35/147 (24%)	15/66 (23%)	20/81 (25%)
Intestinal helminth infection	51/139 (37%)	18/65 (28%)	33/74 (45%)
Hookworm	25/139 (18%)	8/65 (12%)	17/74 (23%)
Ascaris lumbricoides	21/139 (15%)	9/65 (14%)	12/74 (16%)
Trichuris trichiura	11/139 (8%)	4/65 (6%)	7/74 (9%)
Intestinal protozoan infection	39/139 (28%)	13/65 (20%)	26/74 (35%)
Blastocystis hominis	31/139 (22%)	10/65 (15%)	21/74 (28%)
*Others**	15/139 (11%)	3/65 (5%)	12/74 (16%)
Co-infection of intestinal helminth and protozoan	20/139 (14%)	6/65 (9%)	14/74 (19%)

* other intestinal protozoa species: *Entamoeba histolytica/ dispar, E. coli, Iodamoeba bütschlii, E. nana, E. hartmani*

To determine whether BCG vaccination influenced the polyclonal non-specific responses as well, cytokine production to PHA was measured. As shown in figure 1B and 1C, the pattern for IFN-γ responses to PHA was increased significantly from pre vaccination to 2 years of age (p<0.001).

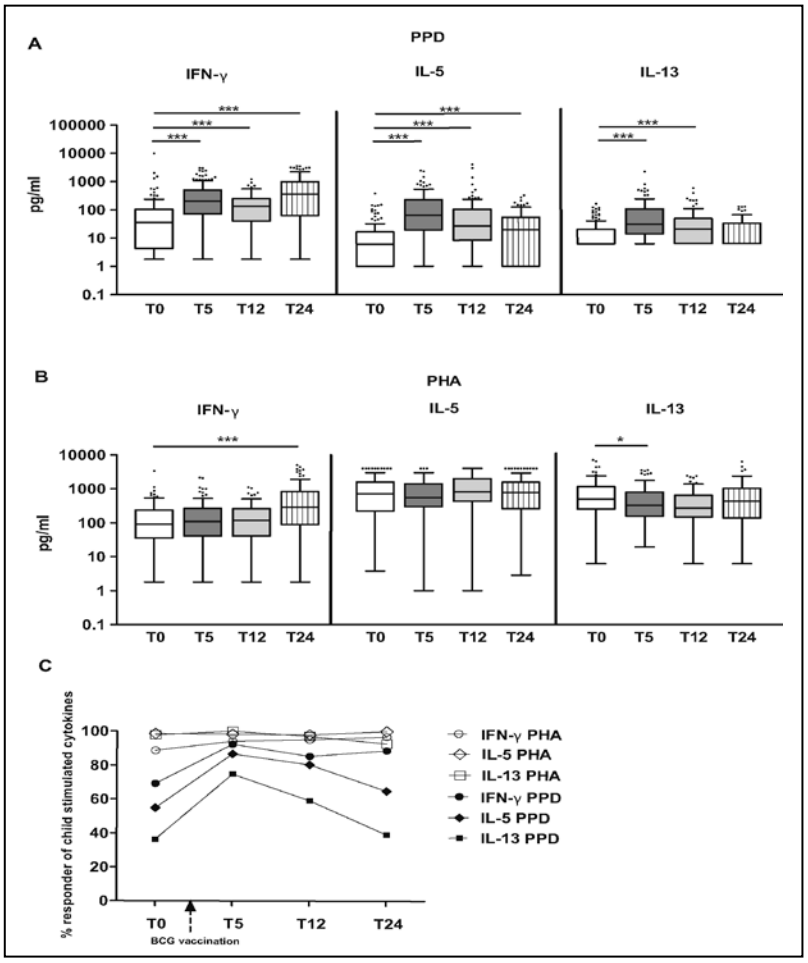

Figure 1. Adaptive cytokine responses to PPD and PHA before and after vaccination.

IFN-γ, IL-5, IL-13 responses to PPD **(A)** and PHA **(B)**. Levels of cytokines at T0 (before vaccination, n=143); T5 (5 months of age, n=119), T12 (1 year of age, n=103) and T24 (2 years of age, n=97).The horizontal lines in the bars represent the median, with the lower 25% and upper 75% percentiles and extended whiskers the 10% and 90% percentiles. Mann-Whitney test: *0.05 > $p \geq 0.01$; **0.01 > $p \geq 0.001$; ***0.001 > p. **C**: Percentage of PPD and PHA responders. T0: before BCG vaccination, T5: at 5 months of age, T12: at 1 year of age, and T24: at 2 years of age. A responder in terms of cytokine production in response to a stimulus is defined as producing cytokine after stimulation above the background production of cytokine when no stimulus is added (level in a stimulated culture above zero after substraction of background cytokine level).

The relations between IFN-γ production in response to PPD and to PHA were analyzed next (Figure 2A). A correlation was seen before vaccination but this was much stronger at 5 months of age which was the first time point after vaccination whereas at two years of age, the IFN-γ production in response to PPD was no longer correlated with IFN-γ response to PHA. Regarding TH_2 cytokines (Figure 2B, 2C), before vaccination correlations were weaker than that observed for IFN-γ. After vaccination, at 5 months and 1 year of age, the IL-5 and IL-13 production in response to PPD and PHA were correlated to a better extent. However, as for IFN-γ at 2 years of age the TH_2 responses to PPD and PHA were no longer correlated.

Innate immune responses before and after BCG vaccination: early IL-10 and TNF-α production in response to PPD and LPS

We were interested in the pro and anti inflammatory innate immune responses and therefore measured IL-10 and TNF-α in day 1 supernatants following stimulation of whole blood with PPD (stimulates TLR2, unpublished data) and LPS (stimulates TLR4). Compared to T0, no increase and even a tendency for a gradual decrease over time was observed for IL-10 response to PPD (T5: $p>0.05$, T12: $p>0.05$, T24: $p<0.001$). Similarly, TNF-α response to PPD decreased over time up to 2 years of age (T5: $p>0.05$, T12: $p=0.001$, T24: $p<0.001$) (Figure 3A). When responses to a classic innate stimulus, LPS, was examined, patterns similar to innate PPD responses were seen: no increase in IL-10 and TNF-α responses after vaccination, and a significant decrease in both cytokines between T0 and T24 ($p<0.001$) (Figure 3B). It was also noted that the spontaneous production of IL-10 and TNF-α in day 1 supernatants did not change after vaccination (Table 2).

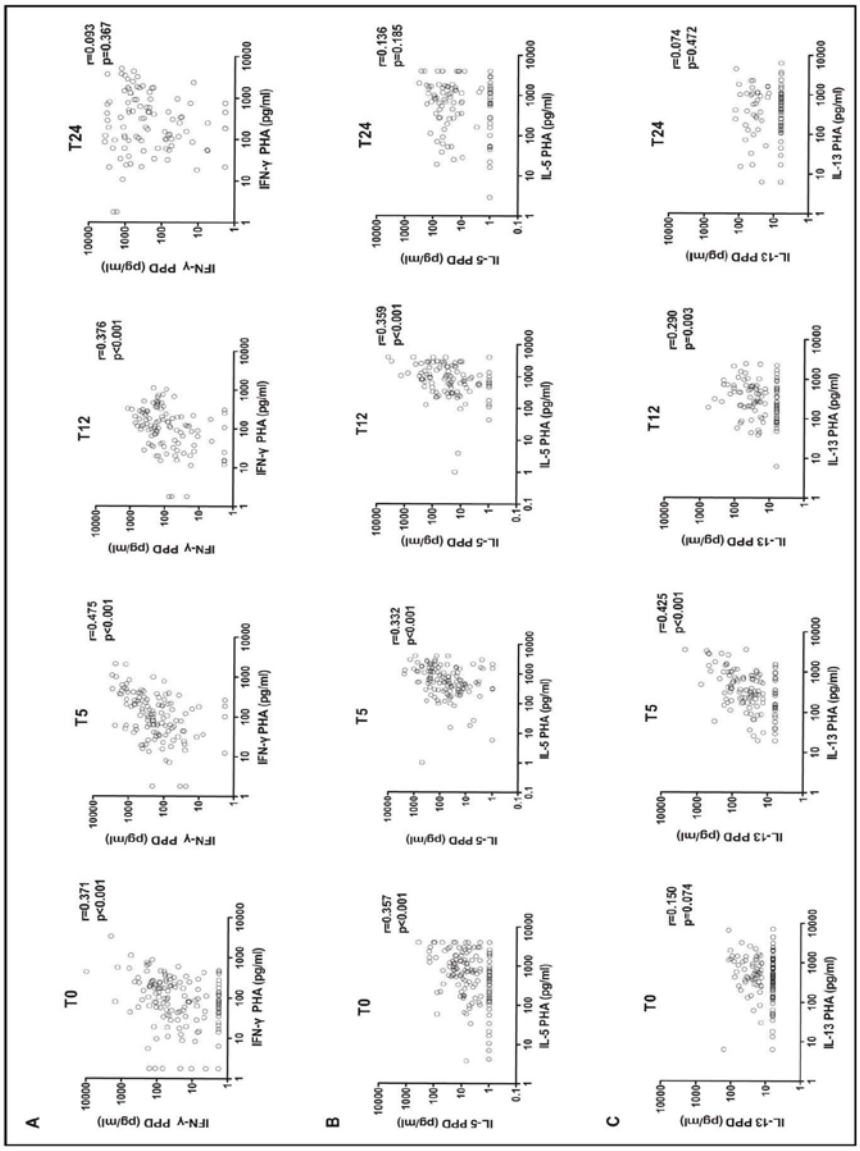

Figure 2. Correlations between PPD and PHA-induced cytokine responses showing IFN-γ (A), IL-5 (B) and IL-13 (C) before BCG vaccination (T0), at 5 months of age (T5), at 1 year of age (T12), and at 2 years of age (T24). r = Spearman's correlation coefficient, p = *p*-value.

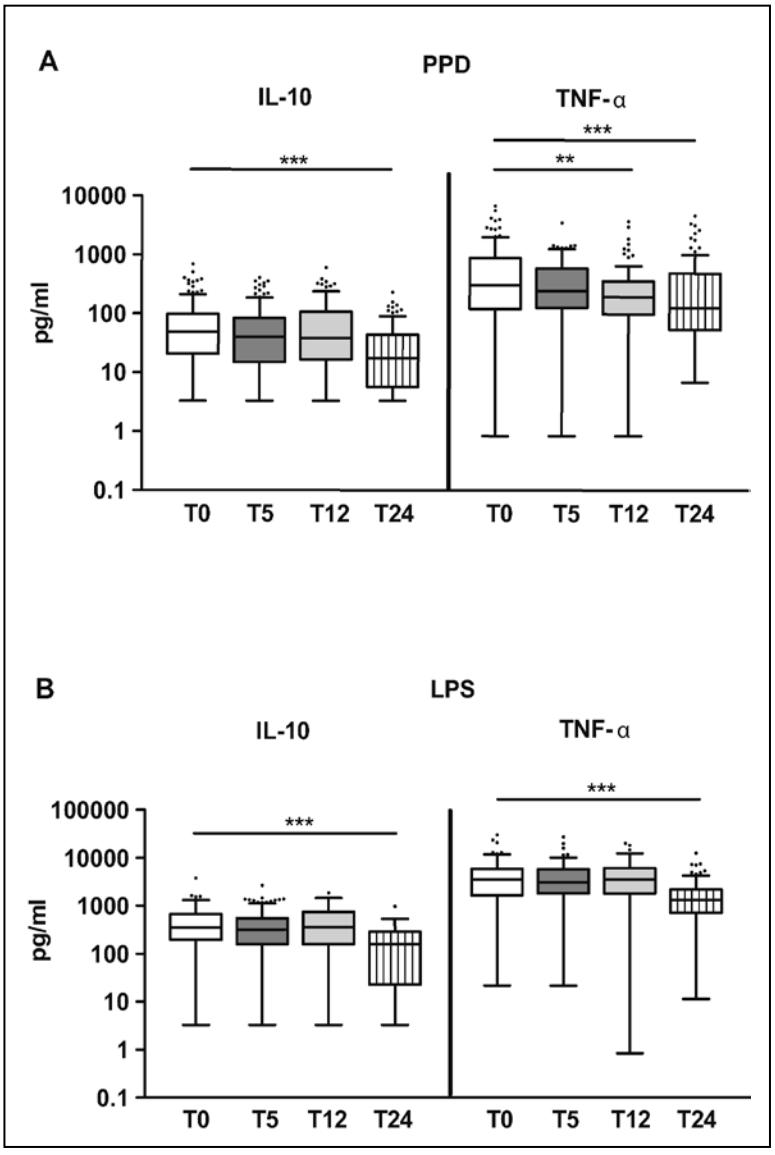

Figure 3. Innate IL-10 and TNF-α cytokine responses to PPD (A) and LPS (B). Levels of cytokines at T0 (before vaccination, n=147 for PPD and n=101 for LPS), T5 (5 months of age, n=120 for PPD and n=117 for LPS), T12 (1 year of age, n=105 for PPD and n=103 for LPS) and T24 (2 years of age, n=98 for PPD and n=97 for LPS).The horizontal lines in the bars represent the median, with the lower 25% and upper 75% percentiles and extended whiskers the 10% and 90% percentiles. Mann-Whitney test: *0.05 > $p \geq 0.01$; **0.01 > $p \geq 0.001$; ***0.001 > p.

Table 2. Cytokine production in unstimulated (medium) and in PHA, PPD, LPS-stimulated cultures.

Cytokine	Time point	n	Medium	n	PHA	n	PPD	n	LPS
IL-10	**T0**	146	3.3 (3.3 – 19.1)	147	84.8 (42.6 – 144.4)	147	48.6 (20.9 – 98.7)	101	348.0 (195.0 – 664.7)
	T5	119	3.3 (3.3 – 23.4)	120	56.4 (31.5 – 117.2)	120	40.3 (15.0 – 83.9)	117	325.4 (161.5 – 551.9)
	T12	105	3.3 (3.3 – 26.1)	105	58.3 (30.4 – 103.4)	105	38.6 (16.7 – 107.0)	103	357.8 (159.2 – 742.1)
	T24	98	3.3 (3.3 – 3.3)	98	64.7 (40.4 – 97.5)	98	17.5 (5.7 – 43.9)	97	159.2 (22.5 – 283.9)
TNF-α	**T0**	146	0.84 (0.84 – 9.7)	147	204.6 (63.9 – 517.3)	147	308.9 (122.2 – 925.6)	101	3528.8 (1651.5 – 5808.7)
	T5	119	0.84 (0.84 – 4.7)	120	223.0 (78.5 – 492.8)	120	243.9 (126.1 – 587.8)	117	3054.3 (1821.2 – 5671.1)
	T12	105	0.84 (0.84 – 5.2)	105	207.9 (87.8 – 458.5)	105	193.5 (98.5 – 356.2)	103	3553.2 (1794.0 – 6075.0)
	T24	98	5.4 (0.84 – 24.0)	98	176.6 (58.7 – 448.3)	98	125.8 (53.3 – 484.5)	97	1312.9 (709.2 – 2173.2)
IFN-γ	**T0**	142	1.8 (1.8 – 21.8)	143	92.2 (35.2 – 239.3)	143	38.2 (4.3 – 105.6)		
	T5	119	7.9 (1.8 – 40.6)	119	110.0 (41.2 – 265.6)	119	204.3 (72.1 – 504.1)		
	T12	103	6.6 (1.8 – 31.0)	103	116.9 (41.3 – 258.7)	103	132.1 (40.0 – 245.6)		
	T24	97	1.8 (1.8 – 1.8)	97	283.7 (89.4 – 840.8)	97	355.3 (63.0 – 983.5)		
IL-5	**T0**	142	2.4 (1.0 – 8.6)	143	713.0 (223.1 – 1636.0)	143	5.96 (1.0 – 16.5)		
	T5	119	2.4 (1.0 – 7.5)	119	554.9 (295.7 – 1421.4)	119	64.7 (19.3 – 226.6)		
	T12	103	1.0 (1.0 – 6.1)	103	825.4 (430.3 – 2000.0)	103	27.2 (8.4 – 104.4)		
	T24	97	1.0 (1.0 – 8.1)	97	793.5 (262.2 – 1575.9)	97	19.9 (1.0 – 54.5)		
IL-13	**T0**	142	6.3 (6.3 – 6.3)	143	497.5 (254.6 – 1164.6)	143	6.3 (6.3 – 20.3)		
	T5	119	6.3 (6.3 – 13.4)	119	331.3 (157.0 – 803.0)	119	31.0 (14.0 – 106.6)		
	T12	103	6.3 (6.3 – 15.4)	103	272.5 (150.5 – 645.6)	103	20.8 (6.3 – 48.8)		
	T24	97	6.3 (6.3 – 6.3)	97	435.9 (139.3 – 1033.4)	97	6.3 (6.3 – 33.2)		

T0: before vaccination (2 months of age), T5: 5 months of age, T12: 1 year of age, T24: 2 years of age. All cytokine levels are expressed in median and interquartile range.

The relationship between maternal parasitic infection status and the cytokine responses to PPD and LPS before vaccination and at different ages after BCG vaccination

Neither filarial infection (data not shown) nor intestinal helminth infection status of mothers had a clear effect on the response of children to PPD in terms of TH_1, TH_2, pro or anti-inflammatory cytokines either at pre or at different ages after vaccination (Table 3).

Interestingly, children born to mothers infected with intestinal protozoa in which *Blastocystis hominis* was the predominant species, had consistently lower TNF-α production in response to PPD at T0 ($p<0.01$), T5 ($p=0.020$), T12 ($p=0.011$) and T24 ($p<0.01$) compared to the children born to mothers with no protozoan infections. The same difference was also observed in IL-10 response to PPD at T0 ($p<0.01$), T5 ($p<0.01$), and T12 ($p=0.071$). Regarding innate responses to LPS, only at T0 children from infected mothers showed significantly lower IL-10 production compared to those from non-infected mothers ($p=0.029$). When TH_1 and TH_2 cytokines as adaptive immune responses were considered, the IFN-γ response to PPD at T5 ($p=0.036$) and the IL-13 response to PPD at T0 ($p=0.032$) were significantly lower in children born to mothers infected with intestinal protozoa (Table 3). Similar results were found when we compared children from mothers positive for protozoan infection only, with those from mothers negative for both intestinal helminth and protozoan infection (data not shown). However when the children from mothers co-infected with intestinal helminths and protozoa were compared to those from mothers negative for both, the differences became less significant especially after vaccination (Table S1). Although all findings show that maternal intestinal parasites may influence the degree of child's cytokine production, the pattern of change in any cytokine pattern over time was not affected (data not shown).

Table 3. Cytokine responses to PPD in children born to mothers with or without intestinal parasitic infection.

Cytokine	Time	n	IH+	n	IH-	*p*	n	IP+	n	IP-	*p*
IL-10	T0	51	39.0 (14.9 – 83.6)	88	50.6 (25.4 – 103.7)	0.104	39	25.6 (12.5 – 49.4)	100	62.5 (25.6 – 116.3)	**0.002**
	T5	39	30.6 (13.3 – 66.3)	75	48.3 (15.2 – 93.6)	0.303	32	17.2 (10.1 – 45.7)	82	52.8 (19.2 – 95.4)	**0.002**
	T12	34	34.7 (14.5 – 124.4)	64	45.2 (20.4 – 120.8)	0.485	23	27.8 (12.1 – 81.3)	75	49.3 (22.5 – 124.9)	0.071
	T24	35	22.1 (10.8 – 57.9)	62	15.2 (3.3 – 37.5)	0.102	27	20.4 (3.3 – 45.1)	70	17.4 (6.5 – 44.0)	0.942
TNF-α	T0	51	200.5 (84.8 – 885.5)	88	335.7 (149.0 – 930.1)	0.228	39	199.5 (77.0 – 502.5)	100	359.4 (148.6 – 1093.7)	**0.005**
	T5	39	184.0 (119.1 – 537.8)	75	287.6 (126.1 – 682.1)	0.348	32	158.9 (78.4 – 393.3)	82	282.2 (136.6 – 784.3)	**0.020**
	T12	34	237.4 (126.3 – 379.0)	64	177.1 (86.3 – 383.8)	0.438	23	124.1 (42.3 – 195.3)	75	243.0 (119.8 – 484.8)	**0.011**
	T24	35	160.2 (40.3 – 826.4)	62	119.7 (55.2 – 401.9)	0.583	27	60.2 (30.8 – 139.0)	70	163.8 (80.0 – 540.6)	**0.002**
IFN-γ	T0	51	21.3 (1.8 – 93.5)	85	50.2 (7.4 – 111.4)	0.084	39	18.3 (1.8 – 102.6)	97	48.4 (7.1 – 116.0)	0.131
	T5	38	159.5 (69.9 – 379.1)	75	210.3 (71.3 – 601.0)	0.470	31	140.0 (52.2 – 319.2)	82	234.8 (87.9 – 685.9)	**0.036**
	T12	34	114.5 (40.2 – 263.1)	63	158.1(42.5 – 253.9)	0.728	22	163.9 (58.0 – 288.0)	75	132.1 (34.1 – 243.8)	0.453
	T24	35	563.9 (128.4 – 1143.0)	61	273.8 (51.1 – 900.0)	0.104	27	171.0 (49.6 – 1057.5)	69	360.8 (77.6 – 983.6)	0.483
IL-5	T0	51	5.1 (1.0 – 16.4)	85	6.5 (1 – 16.8)	0.681	39	6.6 (1.0 – 17.1)	97	6.1 (1.0 – 16.5)	0.726
	T5	38	61.5 (12.4 – 276.8)	75	66.5 (22.9 – 213.6)	0.784	31	47.9 (16.9 – 242.7)	82	69.2 (20.6 – 228.7)	0.890
	T12	34	57.9 (15.8 – 161.6)	63	22.3 (7.3 – 72.1)	**0.026**	22	28.3 (10.9 – 81.9)	75	28.2 (8.4 – 133.0)	0.990
	T24	35	19.9 (1.0 – 63.6)	61	18.9 (1 – 54.5)	0.618	27	13.5 (1.0 – 46.1)	69	22.1 (1.0 – 66.3)	0.185
IL-13	T0	51	6.3 (6.3 – 18.9)	85	6.3 (6.3 – 20.4)	0.980	39	6.3 (6.3 – 15.3)	97	6.3 (6.3 – 22.8)	**0.032**
	T5	38	37.7 (6.3 – 113.6)	75	30.5 (14.1 – 104.9)	0.612	31	25.3 (6.3 – 86.9)	82	33.0 (16.8 – 116.7)	0.190
	T12	34	22.1 (6.3 – 58.7)	63	20.8 (6.3 – 48.8)	0.512	22	9.8 (6.3 – 46.8)	75	24.1 (6.3 – 51.2)	0.263
	T24	35	6.3 (6.3 – 42.6)	61	6.3 (6.3 – 31.7)	0.863	27	6.3 (6.3 – 22.3)	69	6.3 (6.3 – 34.2)	0.256

IH: intestinal helminth, IP: intestinal protozoa. T0: before vaccination (2 months of age), T5: 5 months of age, T12: 1 year of age, T24: 2 years of age. *P* values in bold: significant if < 0.05. All cytokine levels are expressed in median and interquartile range.

Table S1. Cytokine responses to PPD in children born to mothers either co-infected with intestinal helminths and protozoa or negative for both.

Cytokine	Time point	n	IH+ IP+	n	IH- IP-	*p*
IL-10	**T0**	20	25.6 (10.4 – 65.3)	69	64.3 (30.2 – 144.2)	**0.011**
	T5	16	23.8 (11.9 – 45.7)	59	53.4 (19.6 – 114.9)	**0.037**
	T12	11	29.2 (12.1 – 123.2)	52	53.7 (23.9 – 124.8)	0.258
	T24	15	23.5 (3.3 – 58.5)	50	15.2 (3.3 – 38.3)	0.463
TNF-α	**T0**	20	214.1 (72.0 – 826.1)	69	475.4 (168.4 – 1115.8)	0.050
	T5	16	148.8 (78.4 – 504.1)	59	334.9 (138.4 – 790.6)	0.125
	T12	11	153.9 (100.3 – 425.5)	52	223.4 (109.0 – 509.6)	0.390
	T24	15	70.0 (24.0 – 373.5)	50	158.3 (76.3 – 524.0)	0.143
IFN-γ	**T0**	20	10.4 (1.8 – 40.7)	66	50.9 (9.9 – 117.6)	**0.026**
	T5	15	157.2 (60.8 – 319.2)	59	258.3 (88.7 – 678.7)	0.102
	T12	11	127.6 (59.6 – 344.7)	52	133.6 (40.7 – 246.0)	0.690
	T24	15	466.8 (49.6 – 1057.5)	49	307.1 (55.4 – 900.0)	0.800
IL-5	**T0**	20	4.1 (1.0 – 10.4)	66	6.4 (1.0 – 16.5)	0.697
	T5	15	41.3 (12.4 – 242.7)	59	64.7 (20.9 – 203.2)	0.568
	T12	11	34.0 (14.0 – 102.9)	52	22.8 (6.6 – 98.7)	0.319
	T24	15	13.5 (1.0 – 49.7)	49	21.3 (1.0 – 60.7)	0.524
IL-13	**T0**	20	6.3 (6.3 – 17.7)	66	6.3 (6.3 – 23.2)	0.234
	T5	15	21.7 (6.3 – 106.6)	59	30.5 (14.1 – 108.2)	0.339
	T12	11	13.8 (6.3 – 105.7)	52	23.0 (6.3 – 50.6)	0.144
	T24	15	6.3 (6.3 – 22.3)	49	6.3 (6.3 – 31.7)	0.392

IH: intestinal helminth, IP: intestinal protozoa. T0: before vaccination (2 months of age), T5: 5 months of age, T12: 1 year of age, T24: 2 years of age. All cytokine levels are expressed in median and interquartile range. *P* values in bold: significant if < 0.05.

Immune responses to PPD and the presence or absence of BCG scar at 4 years of age

Of sixty six children with complete cytokine data at all time points, there were 58 children with BCG scar measurement at 4 years of age. Since BCG vaccination increased the IFN- γ, IL-5 and IL-13 responses to PPD but not the innate responses as shown in IL-10 or TNF-α responses to PPD, we show the production of IFN- γ and IL-5 in 15 vaccinated children with no BCG scar (< 2mm) and 43 children with a positive BCG scar ($\geq$ 2 mm) (Figure 4A, 4B) . It is clear that some of the children with no BCG scar were still producing cytokines in response to PPD. The cytokine production at 5 months of age, correlated significantly with BCG scar size measured at 4 years of age: IL-5 response to PPD ($r=0.590$, $p<0.001$) and a very weak correlation with IFN-γ to PPD ($r=0.292$, $p=0.026$). The IL-13 responses were also significantly correlated with BCG scar size ($r=0.427$, $p=0.001$). We also used multiple regression analysis to be able to adjust for confounders. For this we used data of 61 children that had cytokine data at T5 and a positive BCG scar. IL-5 responses to PPD at 5 months of age stayed significant after adjustment for gender, age at BCG vaccination, birth weight, season at birth, place of residence, maternal intestinal helminth and protozoan infection status. IL-5: estimate (SE) = 0.049 (0.019), $p=0.012$, $p_{adjusted}=0.012$. However, IL-13 response to PPD became less significant after adjustment. IL-13: estimate (SE) = 0.057 (0.021), $p=0.010$, $.p_{adjusted} = 0.051$.

The cytokine responses to PPD at older age, thus longer after BCG vaccination were weakly associated with BCG scar size at 4 years of age (data not shown).

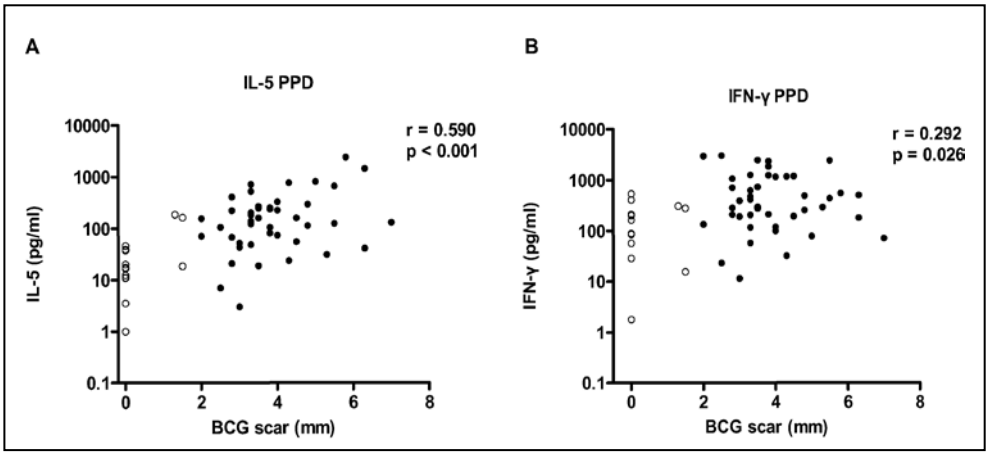

Figure 4. Correlations between PPD-stimulated adaptive cytokine responses and BCG scar size at 5 months of age. IL-5 (A) and IFN-γ (B) responses to PPD and BCG scar size. Open dots represent individuals with BCG scar < 2 mm, closed dots represent those with BCG scar >= 2 mm. N = 58 children. r = Spearman's correlation coefficient, p = *p*-value.

Discussion

The present study shows that BCG not only increases specific adaptive responses in terms of TH_1 and TH_2 cytokines in response to mycobacterial antigens but it also affects non-specific polyclonal responses. However, we did not find any evidence for its ability to influence pro and anti inflammatory innate immune responses as assessed by early IL-10 and TNF-α production in response to PPD or by LPS.

BCG vaccination enhanced TH_1 and TH_2 cytokine responses to mycobacterial antigen at 5 months of age (around 20 weeks after vaccination) and although the elevated TH_1 responses to PPD were maintained up to 2 years of age, the TH_2 responses to PPD waned. In line with our results, several studies in Gambian and United Kingdom infants [16,17] showed an increase in both TH_1 and TH_2 responses to PPD measured at 2 – 3 months after BCG vaccination. With respect to the sustained TH_1 responses, Lalor and coworkers observed that in UK infants IFN-γ levels in response to PPD decreased from 3 months to 12 months after vaccination [18]; however in our study, IFN-γ responses to PPD remained high at least until 2 years of age. This might indicate a higher

exposure to environmental mycobacteria in our study population, which could help maintain the PPD-specific TH_1 memory cells. Moreover, in our study 69% of the infants were producing IFN-γ to PPD before vaccination, which again may reflect the high exposure of these infants to mycobacterial derived products in utero and after birth. Early priming to mycobacteria recorded as cord blood lymphocyte responses to PPD, has been shown in newborns from an area in Kenya highly endemic for tuberculosis, while in the same study US infants did not response to PPD [19]. In agreement with a lack of early priming in areas where exposure to tuberculosis and to environmental mycobacteria is low, Lalor and colleagues found no PPD-induced IFN-γ in unvaccinated infants living in the UK [16]. Furthermore, in an older age group of schoolchildren, IFN-γ production to PPD prior to vaccination was higher in Malawi than in the UK where exposure to environmental mycobacteria is lower [20]. However, in addition to exposure to environmental mycobacteria, many other factors which were not accounted for in our study could influence the TH_1 responses, such as the nutritional status [21], genetic background [22], and strain of mycobacteria and its relatedness to *M. bovis* BCG or *M. tuberculosis* [20]. The interesting finding that TH_2 responses are not sustained, could indicate that the effect of BCG vaccination is different in magnitude or type from that seen upon natural exposure to mycobacteria, which is via the mucosa. It is also possible that IL-5 and IL-13 production is from cells that do not develop a memory response that can be boosted by re-exposure to mycobacteria.

With respect to adaptive immune responses, it was interesting to note that there was some enhancement of polyclonal responses as assessed by IFN-γ and IL-5 production stimulated by PHA. The correlations between responses to PHA and PPD were strongest at 5 months and 1 year of age but not at later age, which might suggest that BCG can have an effect on cellular immunity beyond that to mycobacterial antigen.

We found no significant increase in IL-10 and TNF-α responses to PPD following BCG vaccination. It should be noted that IL-10 and TNF-α were measured at day 1 post stimulation to give us an insight into early innate responses, which are highest in day 1 supernatants. It has been shown that mycobacteria and its components [23] as well as PPD (our unpublished data indicates that PPD activates TLR2 transfectants) can stimulate innate immune responses through engagement of Toll-Like Receptors such as TLR2 and for some, TLR4, on antigen presenting cells.

Almost all children produced IL-10 and TNF-α in response to PPD prior to vaccination and this did not change after vaccination with PPD. The lack of an effect of BCG on innate IL-10 and TNF-α production was confirmed by analyzing responses to LPS. The question whether BCG affects the maturation of innate immune responses in vivo needs to be answered when considering the non-specific beneficial effects of BCG. Our results of BCG vaccination here would argue against any change in the innate IL-10 and TNF-α responsiveness to PPD. However for a formal proof, it is essential to identify which cells are producing the IL-10 and TNF-α and study their dynamics at regular intervals after BCG vaccination, in addition to assessing the expression levels of TLRs and their downstream signaling after delivering the BCG vaccine.

In contrast to the study of Malhotra and coworkers [11], we did not find differences between cytokine responses to PPD after BCG vaccination in children born to helminth-infected mothers and those born to helminth-free mothers. The reason for this discrepancy is not clear. One possibility is that instead of peripheral blood mononuclear cells we used whole blood cultures which would mean that relatively lower numbers of PPD-specific memory cells were stimulated.

In our study, *B. hominis* was found to be the predominant species infecting pregnant mothers, as has been found in Indonesian adults working in Taiwan [24]. Similar to other intestinal protozoa, the presence of *B. hominis* can be associated with poor hygiene and contamination of water and food [25]. So far there have been few studies investigating the early priming of human immune responses by intestinal protozoa and the impact on responses to bystander antigens. One study by Kirch and coworkers showed the production of IgA against *Entamoeba histolytica* antigen by cord blood mononuclear cells of neonates born to seropositive mothers, implying that in utero sensitization by antigens from this intestinal protozoa can occur [26]. Here we show that the presence of intestinal protozoan infection with *B. hominis* as the predominant species in pregnant mothers can dampen the innate and adaptive responses to PPD. Earlier studies have shown that *B. hominis* infection can be associated with impaired intestinal permeability [27] as well as lower total leucocyte and neutrophil count [28]. Whether these may explain the effect seen on the cytokine production in the present study, for *B. hominis* alone or in combination with other pathogens/ factors, would need to be investigated further.

Finally, many studies examining the mechanisms behind tissue fibrosis and remodeling have indicated the involvement of TH_2 responses in pathogen or chemical induced injury [29,30], which by stimulating collagen formation might initiate the repair processes [31]. However the role of TH_2 responses in scar formation induced by BCG has not been studied before. Here we show a strong association between IL-5 or IL-13 responses to PPD early after BCG vaccination and scar formation at 4 years of age when scar formation is thought to be stabilized. A study done by Elliott and colleagues found that IL-5 response to culture filtrate protein of *M. tuberculosis* was correlated with BCG scar size at one year of age especially in infants from hookworm infected mothers [13]. Here we extend the analyses, showing that TH_2 responses are indeed correlated with the scar size when it has stabilized at 4 years of age. Although this might be considered a reflection of an overall strong immune responses to BCG vaccination, the fact that there was not a strong correlation with PPD stimulated IFN-γ responses after vaccination, would argue for a selective role of type-2 cytokines stimulated by BCG in scar formation. This could mean that studies assessing scar size are looking more at an early TH_2 response induced by BCG rather than generally accepted TH_1 responses. Our finding needs to be confirmed in other studies to allow any firm conclusions to be drawn.

In summary, this study has demonstrated the induction of both antigen specific TH_1 (long term) and TH_2 (short term) cytokine responses, with BCG scar formation associated more strongly with a TH_2 cytokine response early after vaccination. Although, the innate IL10 and TNF-α responses were not affected by BCG vaccination, there was some indication of enhancement of adaptive responses beyond PPD after vaccination. These studies need to be taken further by a more in- depth analysis of the immunological changes at the innate and the adaptive immune system in order to be able to understand the non-specific effects of BCG on mortality and morbidity found in epidemiological studies.

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Chapter 4

Determinants of the relationship between cytokine production in pregnant women and their infants

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Abstract

Exposure to environmental factors during fetal life and infancy is thought to play an important role in the early development of innate and adaptive immunity. The immunological relationship between mother and infant and the effect that environmental exposures have during pregnancy and early childhood has not been studied extensively.

Here the production of cytokines was measured in 146 pairs of mothers and their 2 month-old infants. The effect of place of residence, socio-economic variables, parasitic infections as well as maternal and child characteristics on measured cytokine production was determined. Mothers producing high levels of IL-10, IFN- γ and IL-5 were more likely to have infants who also produced high levels of these cytokines either spontaneously (OR 2.6(95%CI 1.2-5.4), OR 2.9(CI 1.3-6.6), OR 11.2(CI 4.6-27.2), respectively) or in response to PHA (IL-10: OR 3.0(CI 1.4-6.6), IFN-γ: OR 2.0(CI 1.0-4.2), respectively) even after adjustment for potential confounding variables. This was not the case for TNF-α. In response to LPS, place of residence was a strong determinant of infant IL-10 (OR 0.2(CI 0.1-0.9)) and TNF-α (OR 0.3(CI 0.1-0.9)) production. Maternal protozoan infections was independently associated with reduced infant IL10 in response to PHA and to LPS as well as reduced TNF-α and IFN-γ in response to PHA. These results indicate strong relationship between maternal and infant's cellular immune responses even after taking into account many environmental influences that could affect infant's response directly or indirectly through uterine microenvironment. However, place of residence and intestinal infections may still directly affect the immune responses of the infant. Taken together, the study provides evidence for imprinted cytokine responses of an infant which may have implications for their reaction to incoming antigens, warranting further investigation into the role that genetics or epigenetics play in shaping the cytokine response by an infant to self or external antigens.

Introduction

In utero environment has evolved to ensure that the semi-allogeneic fetus can grow optimally, with placenta as an immunological barrier between maternal and fetal circulation. It is known that maternal nutrient imbalance or exposure to allergens or pathogens may modulate the immune responses of the fetus. The capacity of cord blood mononuclear cells (CBMC) of neonates born to mothers infected with filarial parasite [1-3], intestinal helminth [4] or malaria [5,6] to mount parasite-specific cellular and humoral immune responses is taken as evidence for sensitization of fetal immune cells during the gestational period. The higher CBMC proliferative responses to birch pollen from babies born to mothers exposed to birch pollen during months 5-7 of pregnancy [7,8] is an indication of early priming to allergens. Furthermore, maternal smoking during pregnancy results in higher cotinine levels in cord blood; this condition is associated with attenuated neonatal innate immune responses and may have an impact on the maturation of antigen presenting cells [9]. In utero exposure to maternal diet such as fish oil supplementation during pregnancy could induce an immunoregulatory effect on infant cytokine production with [10] or without the presence of stimulus such as allergens [11]. Some cross-sectional studies on atopic disorders have shown a correlation between T helper (Th) 1 or Th2 cytokines produced by mothers and their corresponding cord blood cells [12] or produced by their 2 year-old children [13], but the analyses did not consider the role played by environmental factors. It is known that environmental factors can affect fetal life and may have long-term implications for susceptibility or resistance to infections [14], development of metabolic syndromes and cardiovascular diseases [15-17], or asthma and allergy [18].

In the present study we have investigated in Indonesia where environmental exposures are highly varied, the relationship between maternal and infant's cellular immune responses at early life before the start of vaccinations. This would circumvent the problems when studying cord blood responses, namely the effect that physiological stress caused during birth might exert and the possible cross contamination with maternal blood. The specific aims of this study were twofold: a) to assess how close the relationship is between cytokine responses in pregnant women and their children and b) to evaluate the associations between environmental

factors and maternal characteristics that in turn affect cytokine responses of their children. To this end, a conceptual framework was proposed to define the relationship between environmental factors and maternal characteristics and the infant's immune system. This framework was used to then guide the inclusion of the influential variables in the multiple logistic regression model.

Methods

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. The study was approved by Ethics Committee of Faculty of Medicine, University of Indonesia. All mothers were provided written informed consent for the collection of samples from themselves and their children and for subsequent analysis.

Study population

The present study was part of a birth cohort study examining the immune responses of children born to helminth infected mothers in Bekasi District, located approximately 30 km from the capital city Jakarta, Indonesia. Between 2002 and 2004, pregnant mothers and their infants were recruited from two adjacent villages, Jati Sampurna (JS) and Jati Karya (JK). These villages are in a peri-urban area, with a mixture of farmers and small traders. All pregnant mothers in second and third trimester from the villages were invited via midwives to participate in the study. Demographic and socio-economic data, as well as maternal characteristics during pregnancy were collected by questionnaires. Gestation age at the time of blood collection was estimated from the last menstrual date and confirmed by palpation and measurement of fundal height. Information about child gender and birth weight was obtained from the mothers during house-to-house visits.

Parasitological examination from maternal blood and stool

For determination of microfilaremia, one ml of maternal venous blood collected between 8 – 11 pm was filtered through 5 µm pore membrane (Millipore, Billerica, MA, USA). Circulating *Wuchereria bancrofti* antigen in

maternal blood was detected by using immunochromatographic test (ICT) in a card format (Binax, Scarborough, ME, USA), according to the manufacturer's recommendation. Stool samples were collected and preserved with formalin (10%), then transferred to the laboratory at the Department of Parasitology, University of Indonesia, and examined for the presence of intestinal helminth eggs and protozoan infections.

Whole blood culture

The procedures of whole blood culture are based on optimized protocols developed during pilot studies. Heparinized venous blood obtained from pregnant mothers and their babies was processed within 6 hours after venipuncture. The whole blood was diluted 10 times as described before [19] and was cultured in duplicate, in the presence of phytohaemagglutinin (PHA; 2 µg/ml; Wellcome Diagnostics, Dartford, UK) as the mitogen, lipopolysaccharide (LPS; 100 ng/ml; Sigma-Aldrich chemie, Zwijndrecht, the Netherlands) as an innate immune stimulus, or without stimuli (medium only). The cultures were incubated for 1 day and 6 days in the presence of 5% CO2, at 37^{o}C. The collected supernatants were kept frozen in -20^{o}C until measurement. The concentrations of interleukin (IL)-10 and TNF-α were measured in day 1 supernatant, whereas IL-5, IL-13, IFN-**γ** were measured in day 6 supernatant. Paired samples of mother and infant were analyzed altogether in the same plate, in order to minimize variation.

Covalent coupling of capture antibodies to beads

The beads used to determine cytokine levels were prepared by using reagents described in Table 1.

Table 1. Recombinant proteins and antibodies used in multiple bead-based assay*

Recombinant protein			Capture Ab			Detection Ab		
Cytokine	**Cat. No.**	**Source**	**Clone**	**Cat. No.**	**Source**	**Clone**	**Cat. No.**	**Source**
IL-10	M191003	Sanquin	**IL10-5**	M9210	Sanquin	**IL10-2**	M9216	Sanquin
TNF-α	PHC3015	BS	**TNFα-7**	M9179	Sanquin	**TNFα-5**	M9218	Sanquin
IL-13	94/622	NIBSC	**IL13-1**	M9186	Sanquin	**IL13-2**	M9217	Sanquin
IFN-γ	PHC4031	BS	**MD5**	M9159	Sanquin	**MD2**	M9219	Sanquin

*All reagents listed were obtained from the sources indicated from the following abbreviations: Sanquin = Stichting Sanquin Bloedvoorziening (Amsterdam, The Netherlands); BS = BioSource (Nivelles, Belgium); NIBSC = National Institute for Biological Standards & Controls (Potters Bar, UK), with catalogue numbers (Cat. No.) for each reagent given.

Each of four different capture monoclonal Abs was covalently coupled to four different carboxylated bead sets (Luminexcorp, Austin, TX, USA) as described elsewhere [20,21]. For each set, 2.5 x 10^6 beads were added with PBS buffer to lower the viscosity. The beads were centrifugated at 15000 *g* for 2 min and washed twice with activation buffer (0.1 M NaH_2PO_4, pH 6.2), and finally re-suspended in 80 µL activation buffer. *N*-hydroxy-sulfosuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) (both from Pierce, Thermo Fischer Scientific, IL, USA) were used to activate the beads. This bead mixture was incubated and shaken for 20 min at room temperature. The activated beads were washed twice with 0.05 M 2-(N-morpholino) ethane sulfonic acid (MES, pH 5.1), added with capture antibodies (50 µg for IL-10, IL-13, TNF-α; and 100 µg for IFN-γ) and incubated for 2 hrs. The beads were washed twice with PBS/0.5% Tween 20 and were re-suspended in PBS with 10 mg/ml BSA and 0.05% sodium azide, then added with 100 µl 20% sucrose. Finally, the beads were counted using a hemocytometer to get a concentration of 10^6 per ml. Goat F(ab')2 anti-mouse Ig conjugated to R-phycoerythrin (Southern Biotech, Birmingham, Alabama, USA) was used to estimate the density of the monoclonal Abs coupled to the beads.

Determination of cytokine production by multiplex bead-based assay

The optimizations of multiple bead-based assay and the cytokine measurements were performed as described [21]. Briefly, single and multiple bead based assays were performed to determine the optimal concentration of the detection antibody, incubation times and reporter signal. From these assays, a bead mixture of IL-10 and TNF-α for day 1 supernatant, IL-13 and IFN-γ for day 6 supernatant were generated freshly before use and mixed with biotinylated detection antibodies (IL-10, 500 ng/ml; TNF-α, 500 ng/ml; IL-13, 25 ng/ml, IFN-γ, 100 ng/ml). Standard curves from each recombinant protein were prepared from three-fold dilution steps in HPE buffer (CLB Sanquin, Amsterdam, The Netherlands) supplemented with 2% sucrose (HPE/S). Samples (diluted twice with HPE/S) and standards in a final volume of 40 µl per well were placed in a 96-well round-bottomed microplates (Nunc, Roskilde, Denmark). Next, 10 µl of the mixture of beads was added to each well and incubated under continuous shaking overnight in the dark. Beads were washed twice with PBS/0.05% Tween 20. The reporter signal, streptavidine PE (Becton

Dickinson, San Jose, CA, USA), was added and the bead mixture was incubated for 30 min under continuous shaking. Before reading, the beads were washed once with PBS/0.05% Tween 20 and were reconstituted in a final volume of 70 µl HPE/S.

Mean fluorescent intensity from all cytokines was measured using Luminex IS 100 (Luminexcorp, Austin, TX, USA) and data were analyzed by Star Station software analysis (Applied Cytometry, Sheffield, UK). The measurements were done once, and blank values were substracted from all readings. The minimum detection limit was determined by adding two standard deviations to the mean of mean fluorescence intensity from 30 blanks assayed separately. The detection limits for IL-10, TNF-α, IL-13 and IFN-γ were 6.5 pg/ml, 1.7 pg/ml, 12.5 pg/ml, and 3.6 pg/ml, respectively.

IL-5 ELISA

IL-5 was measured by ELISA as described previously [22]. Matched antibody pairs, consisting of purified rat anti-mouse/human IL-5 monoclonal antibodies and biotinylated rat anti-human IL-5 monoclonal antibodies were purchased from Becton Dickinson Biosciences Pharmingen, San Jose, CA, USA. Recombinant IL-5 protein was used as standard (Genzyme, Cambridge, UK). The detection limit for IL-5 ELISA was 2 pg/ml.

Statistical analyses and conceptual framework

All cytokine levels below detection limit were given half of the threshold value. Raw cytokine productions were used for analysis, since the results showed the cytokine responses to antigen stimulation not only higher or the same, but also lower than spontaneous cytokine productions.

Mothers were classified into high producers (H) or low producers (L) based on median cytokine levels. Since almost all cytokine data from mothers and infants were not normally distributed, the Mann-Whitney *U*-test was used to compare levels of cytokine production in infants born to high or low producer mothers. Pearson Chi-Square test was used to find association between two dichotomous variables such as between place of residence and cytokine producer status or between maternal education and the use of cooking fuel.

We used multivariable logistic regression model to investigate the association between mother's cytokine production and infant's cytokine production. The outcome for logistic regression model was infant's cytokine

which was grouped into: high producer and low producer, based on the median. Mother's cytokine production was treated as exposure variable. Other variables, such as demographic and socio-economic factors, maternal characteristics, maternal parasitological data and child characteristics, were treated as potential confounders.

The original plan for the logistic regressions was based on a conceptual framework (Figure 1) of the proposed causal pathways [23,24].

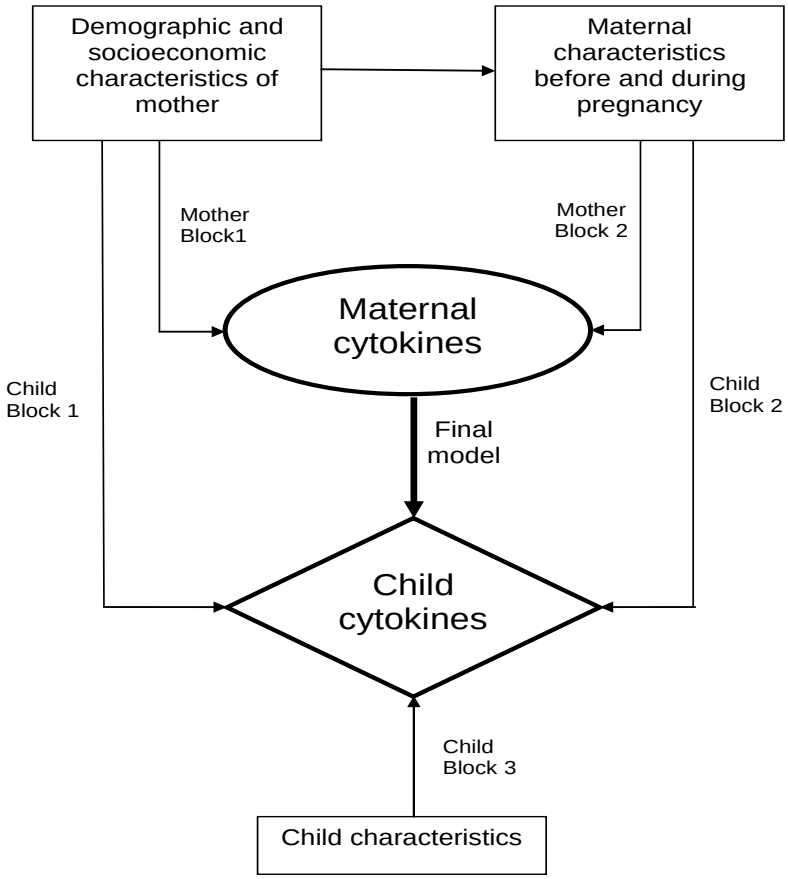

Figure 1. Conceptual framework for logistic regression analysis of the relationships between maternal and infant cytokine responses. Mother Block 1 consists of univariate and multivariate logistic regression models for maternal demographic and socio-economic data, such as place of residence, nativity, education, material of house, water supply, cooking fuel. The outcome variable is maternal cytokine producer status. Mother Block 2 consists of univariate and multivariate logistic regression models for maternal characteristics, such as number of age, number of pregnancies, parasitological data. The outcome variable

is maternal cytokine producer status. Child Block 1 consists of univariate and multivariate logistic regression models for maternal demographic and socio-economic data, such as place of residence, nativity, education, material of house, water supply, cooking fuel. The outcome variable is child cytokine producer status. Child Block 2 consists of univariate and multivariate logistic regression models for maternal characteristics, such as number of age, number of pregnancies, parasitological data. The outcome variable is child cytokine producer status. Child Block 3 consists of univariate and multivariate logistic regression models for child characteristics, such as birth weight, mode of delivery, breast feeding. The outcome variable is child cytokine producer status.

Since maternal – infant immune relationships is the central question of this analysis, we initially performed a simple logistic regression analysis to obtain crude odds ratios (ORs) of the effect of level of each cytokine production in the mother on the level of the same cytokine production on the infant. We then selected potential confounding variables by identifying environmental factors and maternal characteristics that were associated with level of cytokine production in both pregnant mothers and their offspring. A variable was said to be a potential confounder if the introduction of that variable into the model lead to a change in the OR of the association between maternal cytokine and child cytokine of more than 10 percent. Besides confounding effect of other variables, we also tested for interaction between variables.

Environmental factors included were demographic and socio-economic variables (Table 2): material of house (categorized into two groups with semi wood/brick was added to wood as the reference); water supply (pipe users were included in the group of pump users and well users were used as the reference). Maternal characteristics during pregnancy included were age (above or below the mean), number of pregnancies, gestational age at the time of blood collection and maternal parasitological status (two groupings, based on circulating filarial antigen/ ICT positivity and on intestinal parasitological status, i.e. the presence of intestinal helminth and/ or intestinal protozoan infection). Selection of potential covariates for logistic regression with the binary outcome producer status of mother was through 2 blocks of separated logistic regression analysis, which were of demographic and socioeconomic characteristics of mother, maternal characteristics before and during pregnancy. In each block, variables with p value less than 0.25 were treated as covariates and a multivariable logistic regression was done with all potential covariates. All

variables with p value less than 0.05 in multiple logistic regression for potential covariates in each block were included for the next analysis. A final logistic regression analysis was done with all covariates which showed p value less than 0.05 in the previous analysis. As the final model, only variables with p value less than 0.05 were retained in the model.

The same steps were applied to identify the variables which influenced the infant production of cytokines, logistic regressions with the binary outcome producer status of the child, and exposures were grouped into the two previous blocks, environmental factors and maternal characteristics during pregnancy and a third block of child characteristics: gender, birth weight, mode of delivery and breast feeding status: exclusive breast-feeding (receiving only breast milk for at least 6 months), partial breast-feeding (receiving breast and formula milk), or no breast-feeding. The variables from first and second child blocks were considered as more distal determinants than the third child block [24], so the selected variables from child block 1 and 2 were modeled together and later on the selected variables from this model were added to the selected variables from child block 3 in a new regression model. A final model for infant's cytokine was created with maternal cytokine producer status and confounding factors for maternal and child cytokine production. In this paper we will present only the table of crude OR for maternal cytokines and the table of adjusted OR in final model for maternal cytokines and potential confounding factors. Additional results (other than the two tables presented here) are available from the corresponding author. Information on child gender and age at the time of blood collection was collected but not included in the models since these child characteristics had no influence on maternal cytokines and maternal-infant cytokine relationships. Gestational age at time of blood collection which might have influence on maternal cytokines but not on child cytokines was not included in the models.

All statistical analyses were performed using SPSS version 15. Hosmer-Lemeshow Goodness-of-Fit test was done at final step for each cytokine/stimuli, to ensure that the final model adequately fit the data.

Results

Study subjects

One hundred and seventy mothers in second and third trimester of pregnancy donated their blood for immunological studies and subsequently after birth one hundred and forty six infants between 1 to 17 weeks old (before any vaccination) participated in the study. Twenty four infants could not be included in the study due to refusal of parents to donate their infant blood or due to infant death, being sick, moving outside the study area, or being untraceable. The analysis of maternal and infant relationships was done for 146 pairs of mother and child for spontaneous or mitogen-induced cytokine production, and 74 pairs of mother and child for LPS-induced cytokine production. The reason for lower number for LPS is the late arrival of this stimulus, at a time point when the study has already started.

Table 2 shows the characteristics of the study population and includes the demographic and socioeconomic details along with the pregnancy and infection status of the mothers as well as the relevant child data. The median age of the infants at the time of blood collection was 4.6 weeks (IQR= 2.1 – 8.2 weeks) and the proportion of girls was 51%. For pregnant women, the median gestational age at the time of blood collection was 28 weeks (IQR= 24 – 32 weeks) with 60% of samples collected in the third trimester and the rest in the second trimester of pregnancy. Most births (97%) were vaginal delivery and most infants (85%) were breastfed. The majority of the mothers (67%) had a low education level. The water sources in 71% of the study population were from hand pumps, 28% from wells. Since there was no data about the water sanitation, we were not able to compare which of these two water sources was considered to be more hygienic. Maternal filarial infection as determined by circulating antigen was 24% while 35% and 27% of mothers were infected with intestinal helminths and protozoa, respectively.

Table 2. Characteristics of pregnant mothers and their infants included in the logistic regression models

Demographic and socio-economic data		Maternal parasitological data	
Maternal residence		**Filarial infection**	
Jati Sampurna village	86/170 (51%)	Microfilaria positive	8/170 (5%)
Jati Karya village	84/170 (49%)	ICT positive	41/170 (24%)
Native	119/169 (70%)	**IH infection**	
Non-native	50/169 (30%)	*Ascaris lumbricoides*	24/161 (15%)
Maternal education		*Trichuris trichiura*	12/161 (7%)
Never schooled or primary school	113/169 (67%)	Hookworm	28/161 (17%)
Higher education	56/169 (33%)	Any IH infection	57/161 (35%)
Maternal occupation		**Any helminth infection***	79/161 (49%)
Unemployed	156/169 (92%)	**IP infection**	
Others (trader, employee)	13/169 (8%)	*Blastocystis hominis*	36/161 (22%)
Material of house		*Entamoeba histolytica / dispar*	6/161 (4%)
Wood	44/169 (26%)	*Endolimax nana*	5/161 (3%)
Brick	121/169 (72%)	*Entamoeba coli*	1/161 (1%)
Semi wood/brick	4/169 (2%)	*Iodamoeba butschlii*	2/161 (1%)
Water supply		Any IP infection	44/161 (27%)
Well	47/169 (28%)	**Status of IH and IP infections**	
Pump	120/169 (71%)	No IH or IP infection	82/161 (51%)
Pipe	2/169 (1%)	IH infection only	35/161 (21%)
Cooking fuel		IP infection only	22/161 (14%)
Wood	22/167 (13%)	Co-infection of IH and IP	22/161 (14%)
Kerosene	125/167 (75%)		
Gas	20/167 (12%)		
Maternal characteristics			
Number of pregnancies			
Primigravid	56/169 (34%)		
Multigravid	113/169 (66%)		
Mean maternal age, years (SD)	25.5 (5.9)		
< 25 yrs	84/169 (50%)		
≥ 25 yrs	85/169 (50%)		
Child characteristics			
Median birth weight, g (IQR)	3200 (3000-3500)		
Mode of delivery			
Vaginal	138/142 (97%)		
Caesarian section	4/142 (3%)		
Breast feeding			
Exclusive breast feeding	101/119 (85%)		
Partial breast feeding	13/119 (11%)		
No breast feeding	5/119 (4%)		

* any helminth infection: either single or mixed infections of intestinal helminth and filaria
IH = Intestinal helminth, IP = Intestinal protozoan

Relationship between maternal and infant cytokine responses

Maternal cytokine responses, spontaneous (to medium), to PHA and to LPS are given in Figure 2. The pattern of maternal IL-13 production in response to various stimuli was similar to IL-5 (data not shown). As indicated in Methods, the median cytokine production was used to stratify mothers into high and low cytokine producers.

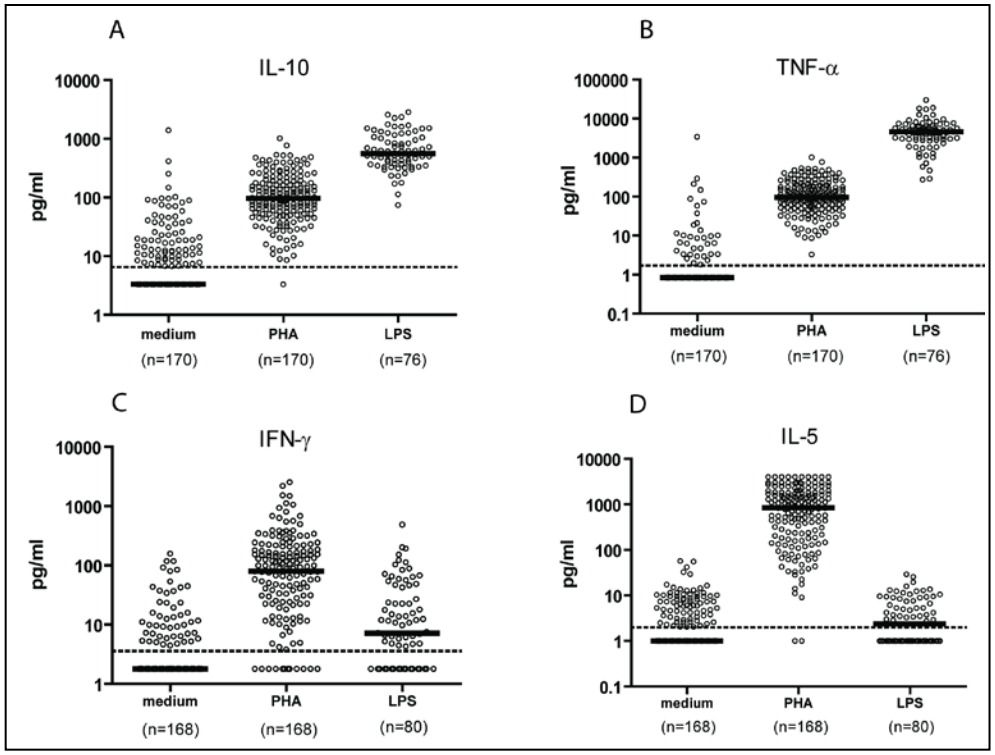

Figure 2. Maternal cytokine production. Solid lines represent median levels of each cytokine; broken lines represent the detection limits of each cytokine. Each dot represents one individual. The number of non-detectables are given in parenthesis: (A) IL-10 medium, PHA, LPS (93, 1, 0); (B) TNF-α medium, PHA, LPS (135, 6, 0); (C) IFN-γ medium, PHA, LPS (119, 22, 28); (D) IL-5 medium, PHA, LPS (91, 2, 37).

As a whole, the comparison between cytokine levels of infants born to high and low producer mothers revealed that infants born to high producer mothers had significantly higher IL-10, IL-5 and IFN-γ responses (Figure 3). This was true either for spontaneous or LPS stimulated

cytokines. Although TNF-α responses showed a similar trend, the difference between infants born to mothers with a high or a low TNF-α production was not statistically significant. Similarly, we found IL-10 and IFN-γ to PHA was higher in infants born to high producer mothers compared to those born to low producer mothers.

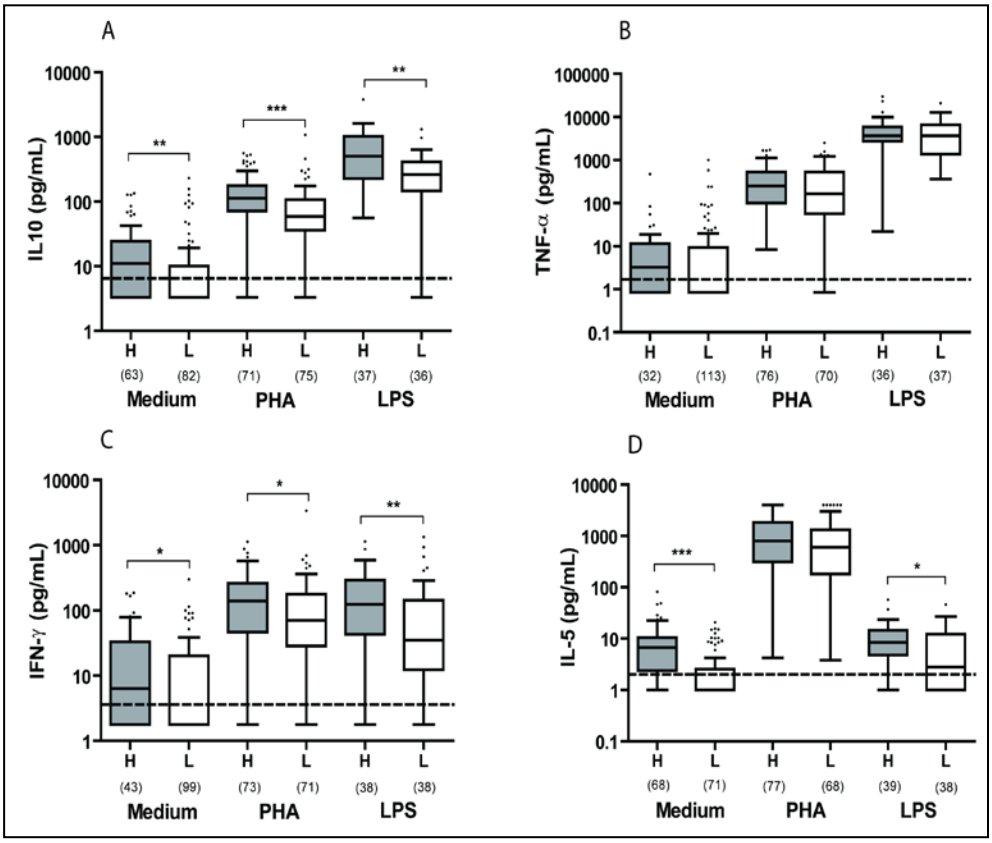

Figure 3. Comparisons of cytokines in infants born to High producer (H) or to Low producer (L) mothers. A: IL-10, B: TNF-α, C: IFN-γ, D: IL-5. The line within the box represents the median (50th percentile), with the lower and upper borders representing the interquartile range (25th and 75th percentiles). The whiskers extend to the 10th and 90th percentiles. The closed dots represent values above the 90th percentiles. Detection limit of each cytokine is shown as a broken line. *0.05 > $p \geq$ 0.01; **0.01 > $p \geq$ 0.001; ***0.001 > p.

Table 3 shows the increase in likelihood of a child being a high producer of each cytokine either spontaneously or in response to PHA and LPS when the mother is a high producer of the corresponding cytokines.

Table 3. Crude odds ratios for high cytokine production by a child according to maternal cytokine production

Maternal Cytokine	Medium		PHA		LPS	
	Crude OR (95% CI)	*p*-value	Crude OR (95% CI)	*p*-value	Crude OR (95% CI)	*p*-value
IL-10						
Low producers	reference		reference		reference	
High producers	2.9 (1.5-5.8)	0.002	4.2 (2.1-8.3)	<0.001	3.3 (1.3-8.5)	0.02
TNF-α						
Low producers	reference		reference		reference	
High producers	1.8 (0.8-4.0)	0.14	1.7 (0.9-3.4)	0.10	0.7 (0.3-1.8)	0.49
IFN-γ						
Low producers	reference		reference		reference	
High producers	2.6 (1.2-5.4)	0.01	1.9 (1.0-3.7)	0.06	3.0 (1.2-7.8)	0.02
IL-5						
Low producers	reference		reference		reference	
High producers	8.4 (3.9-17.9)	<0.001	1.7 (0.9-3.3)	0.11	2.7 (1.1-6.9)	0.03

Model for infant's cytokine production

Table 4 summarizes final logistic regression model for each cytokine after including maternal cytokine producer status, potential confounding factors from block 1 and 2 of maternal cytokines and potential confounding factors from block 1, 2 and 3 of child cytokines (see Methods). The findings for each cytokine are given below:

Model for infant IL-10 production

Mothers with higher spontaneous IL-10 production had children with higher spontaneous IL-10 release (OR 2.6 (95%CI 1.2-5.4)) (Table 4). The value is very similar before and after controlling for environmental measures, indicating that none of the factors examined confounded the relationship between mother and child on spontaneous IL-10.

Mothers with high IL-10 in response to PHA had children with high IL-10 in response to PHA (OR 3.0 (95%CI 1.4-6.6)), but the value is much lower than before controlling for environmental measures; this was most marked when controlling for maternal intestinal protozoa, indicating that intestinal protozoan infection is an independent predictor of a child's IL-10 production in response to PHA and a confounder of this relationship.

Table 4. Multivariable analysis giving adjusted odds ratios for high cytokine response of a child according to maternal cytokine responder status and maternal characteristics

	Medium		PHA		LPS	
	Adjusted OR (95% CI)	***p*-value**	**Adjusted OR (95% CI)**	***p*-value**	**Adjusted OR (95% CI)**	***p*-value**
Maternal IL-10						
Low producers	reference		reference		reference	
High producers	2.6 (1.2-5.4)	0.01	3.0 (1.4-6.6)	0.005	1.6 (0.5-5.9)	0.45
Village						
JS			reference		reference	
JK			0.7 (0.3-1.5)	0.38	0.2 (0.1-0.9)	0.03
Education						
Low			reference			
High			0.7 (0.3-1.7)	0.44		
Cooking fuel						
Wood	reference					
Kerosene	1.3 (0.4-3.7)	0.68				
Gas	0.5 (0.1-2.3)	0.34				
Status of IH and IP infections						
Negative	reference		reference		reference	
IH only	0.5 (0.2-1.4)	0.20	0.7 (0.3-1.7)	0.39	0.5 (0.1-2.3)	0.38
IP only	0.5 (0.2-1.4)	0.47	0.3 (0.1-0.9)	0.04	0.1 (0.03-0.6)	0.01
IH + IP	0.8 (0.3-2.3)	0.71	0.5 (0.2-1.7)	0.29	0.2 (0.1-0.9)	0.03
Maternal TNF-α						
Low producers	reference		reference		reference	
High producers	1.7 (0.7-4.1)	0.20	1.9 (0.9-4.0)	0.11	0.5 (0.2-1.5)	0.23
Village						
JS			reference		reference	
JK			1.7 (0.8-3.8)	0.16	0.3 (0.1-0.9)	0.03
Native			reference			
Non native			1.9 (0.8-4.5)	0.17		
Education						
Low	reference		reference			
High	0.4 (0.2-0.9)	0.04	0.7 (0.3-1.7)	0.44		
Cooking fuel						
Wood	reference					
Kerosene	2.6 (0.8-7.8)	0.10				
Gas	17.1 (3.0-98.1)	0.001				

OR = Odds ratio; Bold = significant association
JS = Jati Sampurna, JK = Jati Karya, IH = Intestinal helminth, IP = Intestinal protozoan

Village of residence and maternal education, which were associated with high IL-10 production of a child in response to PHA were no longer significant after adjustment for mother's IL-10 production against PHA.

Mothers with high IL-10 in response to LPS had children with high IL-10 in response to LPS, but the magnitude of association was much smaller and no longer significant when maternal intestinal parasitic infections and village of residence were adjusted for. Both mothers (Chi-Square test, $p<0.001$) and infants born to mothers from JK village had lower IL-10 in response to LPS (OR 0.2 (95%CI 0.1-0.9)). Since there is biological plausibility for place of residence influencing level of IL-10 production but level of IL-10 production can not influence place of residence then the direction of this association must be place of residence causing levels of IL-10 production rather than the other way round. Since the relationship between levels of production between mother and child disappears when village is controlled for, the only plausible explanation is that village of residence was influencing both maternal and child cytokine levels. Having intestinal protozoan infection alone or mixed with intestinal helminths was independently associated with lower levels of infant's IL-10 production in response to LPS (OR 0.1(95%CI 0.03-0.6), OR 0.2(95%CI 0.1-0.9), respectively).

Model for infant TNF-α production

There were no significant associations between maternal and infant TNF-α production (Table 4). However, several maternal factors such as education and cooking fuel had significant direct effect on infant spontaneous TNF-α production. Higher education of mother was associated with lower spontaneous TNF-α production by her child (OR 0.4 (95%CI 0.2-0.9)). Using gas (OR 17.1(95%CI 3.0-98.1)) and kerosene (OR 2.6(95%CI 0.8-7.8)) as cooking fuel was positively associated with spontaneous TNF-α release in children. Mothers with higher educational levels were more likely to cook using gas stove than wood (Chi-Square test, $p<0.001$).

Intestinal protozoan infection of mothers was significantly associated with lower TNF-α responses to mitogen in infants (OR 0.2(95%CI 0.04-0.6)). Other variables were not significant anymore after adjustment. TNF-α production of infant in response to LPS was not associated with any of maternal factors, except for residence, where infants born and living in JK had significantly lower levels than those born in JS (OR 0.3(95%CI 0.1-0.9)).

Table 4 (continued). Multivariable analysis giving adjusted odds ratios for high cytokine response of a child according to maternal cytokine responder status and maternal characteristics

	Medium		PHA		LPS	
	Adjusted OR (95% CI)	***p*-value**	**Adjusted OR (95% CI)**	***p*-value**	**Adjusted OR (95% CI)**	***p*-value**
Status of IH and IP infections						
Negative			reference			
IH only			1.3 (0.5-3.4)	0.54		
IP only			0.2 (0.04-0.6)	0.008		
IH + IP			0.7 (0.2-2.1)	0.56		
Maternal IFN-γ						
Low producers	reference		reference		reference	
High producers	2.9 (1.3-6.6)	0.01	2.0 (1.0-4.2)	0.05	2.8 (1.03-7.8)	0.04
Village						
JS	reference				reference	
JK	0.4 (0.2-0.9)	0.02			0.2 (0.1-0.6)	0.003
Education						
Low	reference					
High	0.4 (0.2-0.9)	0.03				
Occupation						
Unemployed			reference			
Others			0.7 (0.2-2.8)	0.62		
No. of pregnancies						
Primigravid	reference					
Multigravid	0.5 (0.2-1.2)	0.11				
Status of IH and IP infections						
Negative			reference			
IH only			0.9 (0.4-2.1)	0.79		
IP only			0.2 (0.1-0.7)	0.01		
IH + IP			0.6 (0.2-1.7)	0.37		
Maternal IL-5						
Low producers	reference		reference			
High producers	11.2 (4.6-27.2)	<0.001	1.7 (0.8-3.3)	0.15		
Village						
JS	reference					
JK	1.3 (0.6-2.9)	0.51				
Maternal age						
< 25 yrs	reference					
≥ 25 yrs	2.8 (1.2-6.9)	0.02				
Status of IH and IP infections						
Negative			reference			
IH only			2.2 (0.9-5.3)	0.08		
IP only			1.2 (0.4-3.2)	0.76		
IH + IP			1.5 (0.5-4.1)	0.43		

OR = Odds ratio; Bold = significant association
JS = Jati Sampurna, JK = Jati Karya, IH = Intestinal helminth, IP = Intestinal protozoan

Model for infant IFN-γ production

Maternal spontaneous IFN-γ response was significantly associated with child cytokine response (OR 2.9(95%CI 1.3-6.6)), after adjustment for residence (OR 0.4(95%CI 0.2-0.9)) and educational level (OR 0.4(95%CI 0.2-0.9)). Residence factor increased the crude OR for maternal-infant relationship in spontaneous IFN-γ by 19% (adjusted OR 3.1(95%CI 1.4 – 6.8)). As seen for maternal IL-10 response to LPS, maternal spontaneous IFN-γ production was the mediator between residence factor and infant spontaneous IFN-γ. Number of pregnancies, which was significantly associated with maternal IFN-γ production, lost significance in the final child model.

The relationship between maternal and child's IFN-γ was less significant in response to PHA (OR 2.0(95%CI 1.0-4.2)). Maternal intestinal protozoan infection had a stronger effect on child cytokine production (OR 0.2(95%CI 0.1-0.7)) than maternal IFN-γ. In responses to LPS, maternal IFN-γ production was a significant determinant of the infant's IFN-γ production (OR 2.8(95%CI 1.0-7.8)) although the effect of residence was stronger (OR 0.2(95%CI 0.1-0.6)). Since the level of IFN-γ production in response to LPS was considered low in mothers and infants (Figure 2, 3), this maternal-infant association could partly reflect the association found in the production of spontaneous IFN-γ. Indeed, the association between maternal and infant IFN-γ was no longer significant when net LPS-stimulated IFN-γ (spontaneous IFN-γ subtracted from LPS-stimulated IFN-γ) by mothers and infants was used in the regression model (data not shown).

Model for infant's IL-5 production

For spontaneous IL-5 release, there was a significant association between maternal and infant responses (OR 11.2(95%CI 4.6-27.2)). Younger age of mother was associated with higher spontaneous IL-5 production (data not shown) but not with infant's IL-5; however in the final model (Table 4) including maternal age lead to changes in the the maternal-infant relationship. The crude OR for maternal-child spontaneous IL-5 (Table 3) increased by 39% when adjusted for maternal age (OR 11.7(95%CI 4.9-28.1)), showing that maternal age had an indirect effect on infant IL-5 through maternal IL-5 as the mediator. Maternal residence was no longer significantly associated with infant's spontaneous IL-5 after adjustment with maternal cytokine and maternal age.

Maternal IL-5 responses to PHA had no significant effect on child's IL-5 to PHA, however there was a tendency for intestinal helminth infections of mother to be associated with higher IL-5 responses to PHA of infants (OR 2.2(95%CI 0.9-5.3)).

Discussion

This study indicates that maternal cytokine responses are important determinants of the corresponding cytokines in infants during early life. This was particularly the case for spontaneous production of cytokines. Spontaneous production of IL-10, IFN-γ and IL-5 by two month old infants was strongly determined by maternal cytokine and was not influenced by any other environmental variables recorded in our study. Relationship between maternal cytokine responses to PHA and the corresponding infant's cytokine production was also found in the production of IL-10, and to lesser extent of IFN-γ. The findings, especially for IL-10 production, that infants up to 17 weeks still inherited a similar intrinsic capacity to produce this cytokine as their mothers is supported by the findings of a cross-sectional study of allergic and non-allergic mothers in Europe, showing that the production of IL-10 and IFN-γ in response to medium (spontaneous production) and after PHA stimulation were correlated between mothers and their 2 year-old children irrespective of maternal atopic status [13] and measured environmental factors such as month of birth, length of breastfeeding, smoking parents, having pets at home, number of sibling, and day care attendance. We show that area of residence is a strong confounder for infant IL-10 and TNF-α response to LPS. LPS as a Toll-like receptor 4 ligand and a major component of Gram-negative bacterial cell wall is a strong stimulus for innate immune responses. The lower responses to LPS in JK infants was interesting as the JK village had higher prevalence of intestinal parasite infections (data not shown) which would suggest lower standards of hygiene and in turn higher chance of exposure to bacterial pathogens. It is known that continuous exposure to high microbial or parasitic stimuli may result in down-regulation of the TLR function as shown by some *in vitro* studies with human epithelial cell lines [25, 26] or in studies of school children living in rural areas of some European [27] or African [28] countries. Interestingly, comparison of TLR expression on cells of the immune system between urban European neonates and Gabonese neonates who in a semi urban area are exposed

to high burden of infections, indicated a significantly lower expression TLR-2 on Gabonese cells, suggesting that there is a very early down regulation of TLRs possibly as a result of in utero exposure to micro organisms and parasites [29]. We noted that maternal IL-10 and TNF-α production in response to LPS (during pregnancy) was lower in JK compared to JS (data not shown) as was those of their infants. Indeed the relationship between maternal and infant responses to LPS was primarily accounted for by place of residence. We also realized that the environmental factors included in the analysis of our study may not be complete, and residence may represent several environmental parameters not measured in our study such as exposure to pets or livestock, maternal nutritional status or access to sanitation before and during pregnancy. With respect to the latter a recent study in Brazil, found high spontaneous IL-10 responses in children without access to safe drinking water or sewage system [30].

TNF-α as a pro-inflammatory cytokine was shown to have no strong associations between mother and child. This may simply suggest that the environment in the first several months of an infant's life has a strong effect on the immune system. For example, infections such as rotavirus which are prevalent very early in infants may alter the TNF-α responses [31]. However, in the case of spontaneous TNF-α, maternal education and cooking fuel seem to independently affect infant cytokine responses.

We used crosstabs to find the association between education and the use of cooking fuel. The result showed that higher education may be associated with higher economic status, which explains why this group used more gas stove than wood. The finding that cooking fuel only affected the child's spontaneous TNF-α release but not maternal cytokine may indicate that child immune system is more vulnerable to this kind of environmental exposure.

With respect to maternal infections, the final model of multiple logistic regression in our study showed that intestinal parasitic infections especially protozoa influenced the relationship between maternal and infant IL-10 and IFN-γ production in responses to PHA. *Blastocystis hominis* was the most prevalent species of protozoa found in our pregnant subjects. The presence of *B. hominis* could be an indicator for environmental contamination [32]. However as the effect was on PHA and unlike the village effect which was on LPS stimulated cytokine responses, the protozoa may be affecting adaptive rather than innate immune responses. To our knowledge this is the first study that has found an interaction

between intestinal protozoan infection and cytokine production in pregnant mothers and their infants which needs to be studied further.

In conclusion this study provides evidence for strong associations between maternal and infant cytokine responses in geographical areas where environmental exposures are highly varied such as in Indonesia. However, the mechanisms behind the strong associations have not been elucidated and form the basis for future studies. It is possible that maternal cytokine responses specifically drive the infant cytokine responses, either by crossing or transmitting signals through the maternal-fetal interface. There is so far no evidence for such direct cross talk between mother and fetus. It is also possible that as yet unidentified environmental factor affects both maternal and infant cytokine responses leading to the correlations observed. Another possibility lies in the genetic link between mother and infant. Whether such cytokine imprinting affects infant's responses to vaccinations or incoming infections needs to be studied in longitudinal manner along with possible associated genetic or epigenetic modifications.

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Chapter 5

Maternal and child cytokine relationship is not altered by cytokine gene polymorphisms: A longitudinal study of young children in Indonesia

Manuscript in preparation for submission

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Abstract

Background: The development of immune responses in early life is influenced by the interaction between environmental and genetic factors. Our previous study on young infants showed a close association between maternal and child's cytokine responses. We have now addressed the question how this association evolves over time and the contribution of genetic polymorphisms to this association.

Methods: TH_1 (IFN-γ), TH_2 (IL-5, IL-13), pro- (TNF-α) and anti-inflammatory (IL-10) cytokines in mitogen-stimulated whole blood culture were measured from mothers during pregnancy and from their children aged 2, 5, 12, 24, and 48 months. Cytokine gene polymorphisms were determined (or investigated) from blood samples of paired mothers and children.

Results: High production of maternal IL-10, TNF-α and IFN-γ was significantly associated with higher levels of the corresponding cytokines in their children at 2 months of age (baseline/T0), however these associations decreased in magnitude with time. Using an additive genetic model, maternal IFN-γ gene polymorphism, rs3181032, was associated with child cytokine levels at T0 (β: -0.30, 95%CI: -0.58, -0.01) but this relationship disappeared with time (β: 0.01, 95%CI: 0.002, 0.03). Maternal IL-10 rs4579758 was not associated with child's cytokine at T0, however this association changed over time to become positively and significantly associated child's cytokine (β: 0.005, 95%CI: 0.0002, 0.01). The child's genotype for rs13215091 had a significant effect on TNF-α (β: -0.01, 95%CI: -0.02, -0.004) at later ages but not at T0. In the final models including measured gene polymorphisms, maternal cytokines were the strongest determinant of child cytokines.

Conclusion: Independently from gene polymorphism, child's cytokine production was significantly associated with maternal cytokine production at 2 months of age. Maternal cytokine during pregnancy, which could be a proxy for environmental factors for the child showed its highest impact at early age, without any influence from genetic factors.

Keywords: cytokines, single nucleotide polymorphism, pregnant mother, early life

Introduction

There has been an increasing number of studies looking at immune responses in early life and its modulation by environmental factors, which may predispose an individual to certain diseases in later life. Several studies have shown parallels in cellular immune responses between mothers and their children [1–5]. The mechanisms behind this have not been elucidated. Moreover, all these studies were conducted in developed countries, where infectious diseases are better controlled while allergic and autoimmune diseases are increasingly affecting the population. Specifically, the relationship between in utero exposure, early immune profiles after birth and the development of immune responses in early childhood has not been examined comprehensively in populations where chronic parasitic infections are endemic. In this regard, our previous study in a helminth-endemic area found that maternal cytokines (IL-10, IFN-γ) were associated with infant's cytokines at the age of 2 months, even after taking into account several environmental factors including maternal parasitic infections [6], in agreement with the findings from the studies in industrialized countries where maternal and child cytokine responses appear to be tightly linked.

Interaction between environment and genetic factors will determine the phenotypic outcome of an individual. To our knowledge, there has been no study looking at the changing pattern of the mother-child cytokine relationship over time and whether genetic factors can modify this relationship. To investigate this, we measured child' cytokine responses to mitogen at 5 time points, starting around 2 months and up to 4 years of age and examined the relationship with maternal cytokine responses during pregnancy. We genotyped single nucleotide polymorphisms (SNPs) from mothers and children and asked whether they modified the cytokine relationship of mother and child.

Methods

Study population

This study is part of the longitudinal study of children living in a peri-urban area in Bekasi Distric, West Java province, Indonesia. Children were followed up at 5 time points: 2 (T0), 5 (T5), 12 (T12), 24 (T24) and 48 (T48) months of age. Among all participants, there were 126 pairs of pregnant

mothers in second or third trimester and their children who had both cytokine measurements and genotyping data.All mothers provided written informed consent for themselves and their children. This study was approved by the Ethical Committee Faculty of Medicine University of Indonesia.

Whole blood culture and cytokine measurement

Whole blood culture and stimulation was performed as described previously [6]. Briefly, heparinized venous blood was diluted 1:10 with RPMI-1640 medium (added with 1mM pyruvate and 2mM glutamate), followed by incubation with phytohaemmaglutinin (PHA; 2 µg/ml; Wellcome Diagnostics, Dartford, UK) in 37^{o}C and 5% CO_2. The concentrations of interleukin (IL)-10 and TNF-α were measured in day 1 supernatant, whereas IL-5, IL-13, IFN-γ were measured in day 6 supernatant. Supernatants were kept frozen at -20^{o}C and later on were thawed for the measurement with in-house multiplex bead-based assay (Luminex IS 100, Luminexcorp, Austin, TX, USA) for IL-10, TNF-α, IL-13 and IFN-γ , while ELISA was performed for measurement of IL-5 [6]. The detection limits for IL-10, TNF-α, IL-13, IFN-γ and IL-5 were 6.5 pg/ml, 1.7 pg/ml, 12.5 pg/ml, 3.6 pg/ml and 2 pg/ml, respectively. All cytokine levels below detection limit were given half of the threshold value.

DNA purification and genotyping

Genomic DNA was purified from 200 ul of whole blood samples from pregnant mothers and their children which was kept frozen at -20^{o}C, using QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The purified DNA was quantified using NanoDrop^{TM} 1000 Spectrophotometer (Thermo Fischer Scientific).

The source of cytokine gene polymorphisms for Indonesian living in West Java were derived from Malays population in Singapore Variation Genome Project (SGVP) database (http://www.statgen.nus.edu.sg/~SGVP)[7], with some additional single nucleotide polymorphisms (SNP)s which were not found in the database but were associated with phenotypes in Asian or other population. A set of gene polymorphisms spanning from 20 kb upstream and 10 kb downstream from each cytokine gene region were included in the genotyping.Pairwise tagging SNPs were obtained from Haploview's Tagger program (http://www.broad.mit.edu/mpg/tagger), with selection based on

minor allele frequency (MAF) $\geq$ 5% and r2 threshold = 0.8. In total there were 10 SNPs for IL-10, 4 SNPs for TNF-α, 6 SNPs for IFN-γ, 6 SNPs for IL-5, and 8 SNPs for IL-13. In addition to these cytokine genes, 4 SNPs of RAD50 genes were added since they occupy the region between IL-4, IL-5 and IL-13 genes (TH_2 cytokine locus) in chromosome 5q31. RAD50 gene encodes a DNA repair enzyme; it was recently found to have locus control region at its 3'end and is shown to be associated with asthma and eczema [8,9]. Genotyping of all SNPs were performed using Sequenom MassARRAY iPLEX Platform. Quality control was performed by including $\pm$ 10% of successfully genotyped samples and positive controls in the repeated measurement of failed samples. At the end all SNPs were genotyped successfully with call rates $\geq$ 90%. After exclusion of 2 mother-child pairs who had call rates < 95% and 5 pairs with Mendelian inconsistencies, data were available for a total of 119 pairs.

Statistical analysis

Cytokine levels displayed skewed distributions, therefore log (base 10) transformation was used for all cytokines except for IL-5 for which a square-root transformation was used. To investigate the association between mother and child's cytokine productions, we divided maternal cytokines based on median levels into high or low producer mothers [6] and by using this category we compared child cytokine levels at each time point. Minor allele frequency (MAF), deviations from Hardy-Weinberg Equilibrium (HWE) and pairwise linkage disequilibrium (LD) were calculated for each mother or child's SNP using Haploview software (http://www.broadinstitute.org).

First we modeled the association between maternal cytokines and child cytokines. Next we modeled the maternal cytokines together with maternal or child genotype. We used linear mixed models to study the effect of maternal cytokines and maternal or child genotypes over time on child cytokines. Using a likelihood ratio test, we tested whether the simplerfirst order autoregressive heterogenous (for IL-10, TNF-α) structrure could be used to model correlation over time instead of the unstructured (for IFN-γ, IL-5, IL-13) covariance structure. Each genotype was coded 0, 1, 2 for the increasing number of minor allele and all SNPs were tested using an additive genetic model. First we used model with main effects and one interaction term between time and SNP (i.e. linear change over time) for all SNPs. For significant SNPs, we continued with a larger model where time was included as a categorical variable to obtain more insight of the effect of the SNP over

time. Bonferroni corrections were done for multiple testing, where a *p* value was considered significant if < 0.0028. All models were adjusted for child gender as an *apriori* factor for child's cytokine responses [10]. The statistical analyses were performed using IBM SPSS version 20.

Result

Characteristics of mother-child pairs

The flow diagram of the entire study was described elsewere (*Djuardi et al*-**Chapter 2**). There were 119 mothers and children included in the present study, with 54% (64/119) children being males. Among 107 children with data on breastfeeding, 83 children (78%) received breastfeeding exclusively for 6 months, followed by 21 (19%) children who were partially breastfed (mixed with formula milk) and 3 (3%) children who were not breastfed. While all 119 mother and child pairs had genotype data, the number of children with cytokine data was different at each time point: 111 at T0, 95 at T5, 90 at T12, 88 at T24, 86 at T48. The median and interquartile range (IQR) of maternal cytokine levels were as follows: IL-10 (91.7, IQR:46.9 – 167.1 pg/mL), TNF-α (274.2, IQR:54.7 – 915.6 pg/mL), IFN-γ (152.5, IQR:43.7 – 425.5 pg/mL), IL-5 (859.8, IQR:295.0 – 1673.8 pg/mL) and IL-13 (243.5, IQR:75.7 – 627.2 pg/mL).

Minor allele frequency (MAF) and p-values for testing the null hypothesis that Hardy-Weinberg Equilibrium (HWE) holds forall SNPs of the mother-child pairs are shown in Supporting Table 1. All genotyped SNPs were in HWE with exception of the two IL-5 gene polymorphism, rs4143832 and rs17690122 (both in perfect linkage disequilibrium/r^2=1), which slightly deviated from HWE (p=0.039). The other SNPs which showed perfect LD were rs1878672 and rs1800896 in IL-10 gene. Several SNPs which were in high LD (r^2>0.8) as indicated in Supporting Table 1. The five SNPs which were in perfect LD or high LD were excluded from the analyses.

Association between maternal and child cytokines over time

Table 1 shows that high cytokine producer mothers were associated with higher child cytokine production at T0 or 2 months of age, this is true for IL-10 (estimate: 0.31; 95%CI: 0.19, 0.43), IFN-γ (estimate: 0.26; 95%CI: 0.03, 0.49) and to a lesser extent for TNF-α (estimate: 0.19; 95%CI: 0.02, 0.36). With increasing age, the mother-child relationship for IL-10 disappeared

(interaction between maternal IL-10 with time, p = 0.002). TH_2-type cytokine productions (IL-5, IL-13) were not significantly associated between mother and child at baseline or over time (Table 1).

Table 1. Effect of maternal cytokine on child's cytokine production over time.

	Estimate (95% CI)§	P value
IL10		
Maternal cytokine	0.31 (0.19, 0.43)	**<0.0001**
Time*maternal cytokine	-0.009 (-0.01, -0.003)	**0.002**
TNF-α		
Maternal cytokine	0.19 (0.02, 0.36)	**0.030**
Time*maternal cytokine	-0.006 (-0.01, 0.002)	0.146
IFN-γ		
Maternal cytokine	0.26 (0.03, 0.49)	**0.027**
Time*maternal cytokine	-0.009 (-0.02, 0.002)	0.093
IL-5		
Maternal cytokine	1.51 (-2.58, 5.60)	0.466
Time*maternal cytokine	0.15 (-0.04, 0.34)	0.121
IL-13		
Maternal cytokine	0.07 (-0.08, 0.23)	0.348
Time*maternal cytokine	0.0002 (-0.008, 0.009)	0.954

Time units are 2, 5, 12, 24 and 48 months. Bold: *p* value < 0.05
§adjusted for child gender. *interaction with time

Association between maternal cytokines or genetic factors and child's cytokines over time

IL-10 and TNF-α

The estimated effect of maternal IL-10 and maternal or child's genotype on child's IL-10 production are shown in Table 2. Here only genotypes which revealed significant associations are presented. In this model, at 2 months of age the estimates for mother-child cytokine relationship were similar to the estimates in the previous model without genetic factors (Table 2 & Figure 1A; estimate: 0.31; 95%CI: 0.18, 0.43), indicating that gene polymorphisms

Table 2. Effect of maternal cytokine, maternal and child's genotype on child's PHA-induced cytokine production over time.

Child cytokine	Variable	Estimate (95% CI)§	P value
IL10	Maternal cytokine	0.31 (0.18, 0.43)	**<0.0001****
rs4579758	Maternal genotype	-0.05 (-0.16, 0.05)	0.321
	Child's genotype	-0.009 (-0.11, 0.10)	0.866
	Time*maternal cytokine	-0.008 (-0.01, -0.003)	**0.004**
	Time*maternal genotype	0.005 (0.0002, 0.01)	**0.039**
	Time*child's genotype	0.001 (-0.003, 0.006)	0.616
TNF-α	Maternal cytokine	0.20 (0.03, 0.37)	**0.024**
rs13215091	Maternal genotype	-0.005 (-0.21, 0.20)	0.961
	Child's genotype	0.17 (-0.02, 0.37)	0.080
	Time*maternal cytokine	-0.006 (-0.01, 0.001)	0.108
	Time*maternal genotype	0.004 (-0.005, 0.01)	0.354
	Time*child's genotype	-0.01 (-0.02, -0.004)	**0.005**
IFN-γ	Maternal cytokine	0.26 (0.04, 0.48)	**0.023**
rs3181032	Maternal genotype	-0.30 (-0.58, -0.01)	**0.039**
	Child's genotype	0.26 (-0.10, 0.61)	0.152
	Time*maternal cytokine	-0.009 (-0.02, 0.001)	0.077
	Time*maternal genotype	0.01 (0.002, 0.03)	**0.024**
	Time*child's genotype	-0.0008 (-0.02, 0.01)	0.919
IL-5	Maternal cytokine	1.46 (-2.59, 5.51)	0.476
Rs4143832	Maternal genotype	4.32 (-1.27, 9.91)	0.129
	Child's genotype	-4.93 (-9.60, -0.27)	**0.038**
	Time*maternal cytokine	0.17 (-0.02, 0.36)	0.075
	Time*maternal genotype	-0.24 (-0.49, 0.02)	0.066
	Time*child's genotype	0.14 (-0.08, 0.35)	0.210
IL-5	Maternal cytokine	1.02 (-3.07, 5.10)	0.622
Rs739719	Maternal genotype	-2.10 (-5.76, 1.56)	0.259
	Child's genotype	4.17 (0.12, 8.23)	**0.044**
	Time*maternal cytokine	0.14 (-0.05, 0.34)	0.143
	Time*maternal genotype	-0.02 (-0.19, 0.15)	0.799
	Time*child's genotype	-0.02 (-0.21, 0.17)	0.849

§adjusted for child gender. Bold: p value < 0.05. ** significant after Bonferroni's correction (p<0.0028)

did not modify the relationship between maternal and child cytokines. This result was similar across all models of SNPs (data not shown). In contrast to maternal cytokine status, maternal or child's IL-10 gene polymorphism were not significantly associated with child's cytokine at T0. There was a tendency for children born to mothers with more minor alleles in rs4579758 to have significantly higher production of IL-10 over time (Table 2 & Figure 2A). Child's genotype had no effect on IL-10 production over time.

In a similar manner to IL-10 but weaker, the production of child's PHA-induced TNF-α at 2 months of age was associated with maternal cytokine producer status during pregnancy (Table 2 & Figure 1B; estimate: 0.20, 95%CI: 0.03, 0.37) with decreasing trend over time. Although at the beginning there was a positive trend for association between polymorphism of child's rs13215091 and TNF-α levels (Table 2 & Figure 2B; estimate: 0.17; 95%CI: -0.02, 0.37), the direction of association reversed over time with significantly stronger effect exerted by child's decreasing number of minor allele (estimate: -0.01; 95%CI: -0.02, -0.004). For the other 3 SNPs of TNF-α maternal and child s genotype did not show significant associations with child's cytokine at baseline and over time (data not shown).

IFN-γ, IL-5 and IL-13

The production of TH_1-type responses (IFN-γ) in children after adjustment for genotypes was associated with maternal cytokine producer status at baseline (Table 2 & Figure 1C; estimate: 0.26; 95%CI: 0.04, 0.48) and this association did not change over time (estimate: -0.009; 95%CI: -0.02, 0.001). Among 6 IFN-γ polymorphisms, the only genotype found to be significantly associated with child's cytokine was rs3181032 of mother, which is an IFN-γ gene polymorphism located in promoter region. Mothers with increasing number of this SNP's minor allele were more likely to have children producing lower IFN-γ levels at T0 (Table 2 & Figure 2C; estimate: -0.30; 95%CI: -0.58, -0.01), however over time the direction of the association reversed (estimate: 0.01; 95%CI: 0.002, 0.03).

Among all genotyped IL-5 SNPs, only 2 child's IL-5 polymorphisms, rs4143832 and rs739719, were associated with child's cytokine production at T0 (Table 2 & Figure 2D, E; estimate: -4.93; 95%CI: -9.60, -0.27 and estimate: 4.17; 95%CI: 0.12, 8,23, respectively) with no significant difference of slopes with time. None of IL-13 or RAD50 gene polymorphisms were associated with child's IL-13 or with IL-5 and IL-13 levels, respectively (data not shown).

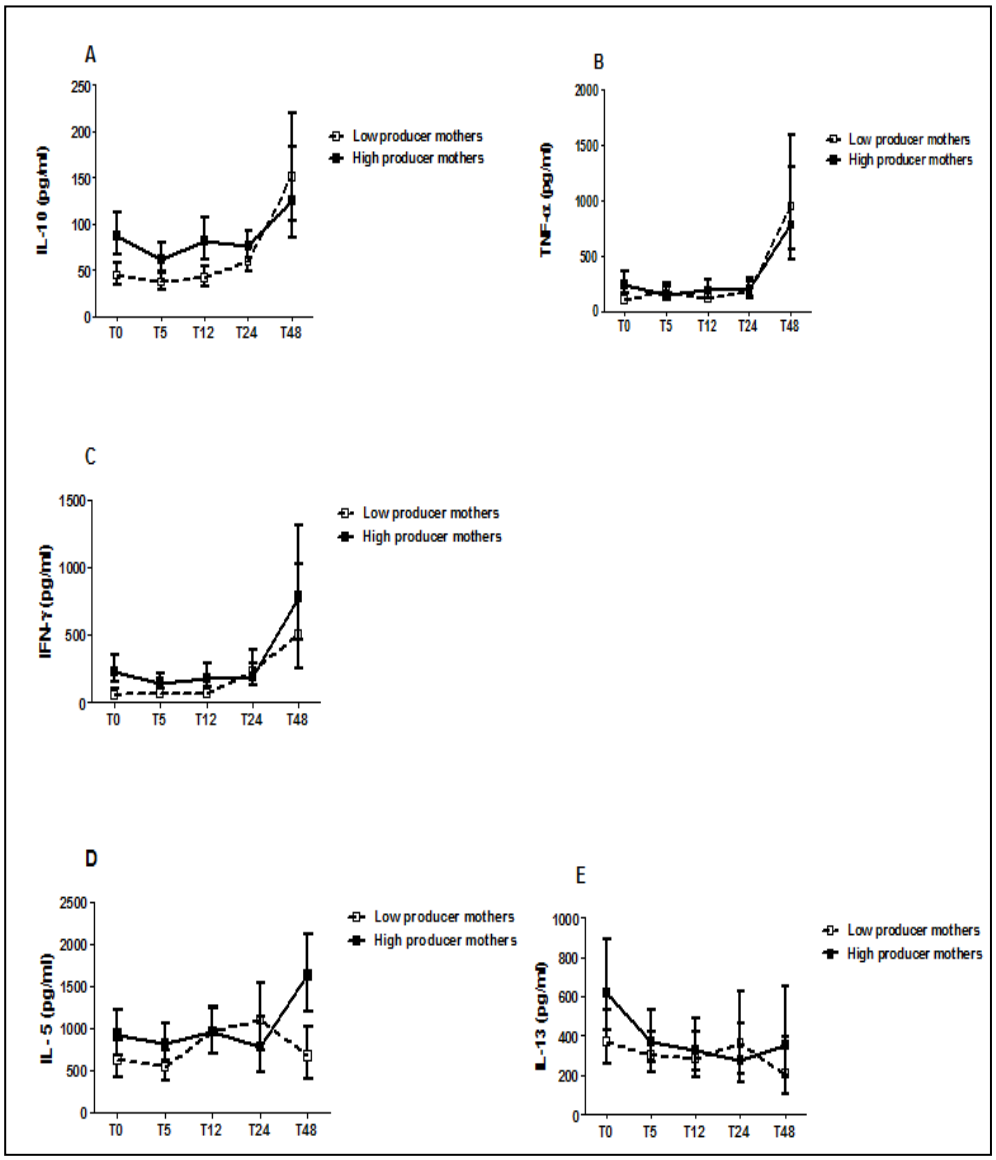

Figure 1. Child's PHA-induced cytokines over time, based on maternal cytokine producer status during pregnancy. Error bars represent geometric means of (**A**) IL-10, (B) TNF-α, (**C**) IFN-γ, (**D**) IL-5 levels, and mean of square of (**E**) IL-13 levelsof children at the age of 2 months (T0), 5 months (T5), 12 months (T12), 24 months (T24) and 48 months (T48). Closed dots are children born to high producer mothers, while open dots are children born to low producer mothers. All values were adjusted for mother and child's gene polymorphism, village of residence and child's gender.

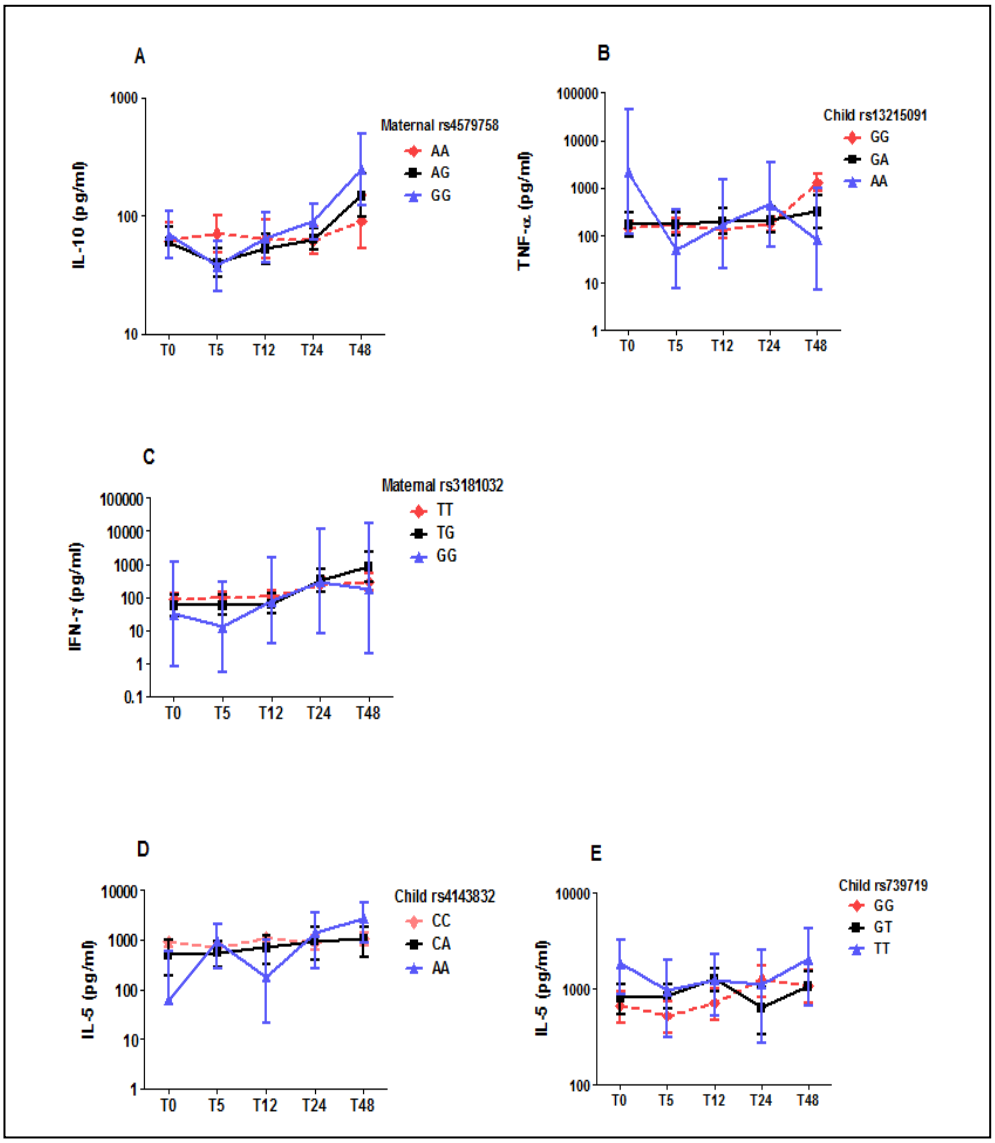

Figure 2. Child's PHA-induced cytokines over time, based on maternal or child genotypes. Only SNPs which showed significant associations in the multivariate models are shown.Error bars represent geometric means of (A) IL-10, (B) TNF-α, (C) IFN-γ and (D, E) IL-5 levels of children at the age of 2 months (T0), 5 months (T5), 12 months (T12), 24 months (T24) and 48 months (T48). Red dots/interrupted lines indicate the children having or born to mothers with major homozygote alleles, while black dots/solid lines for heterozygotes and blue dots and solid lines for minor homozygotes. All values were adjusted for maternal cytokines, village of residence and child's gender.

Discussion

In the present study we showed that genetic polymorphisms do not explain the strong association between maternal cytokine production during pregnancy with child's cytokine production in the first year of life. We also found that most of the mother-child cytokine relationships became weaker over time (IL-10, IFN-γ, and TNF-α) with the exception of IL-5. The association between maternal and child IL-5 production became significant when the child reached 4 years of age. These findings indicate that the strong association between cytokine responses of a pregnant mother and her child is not directly due to genetic factors but rather results from similar immune conditioning during gestational period extending into early childhood.

Earlier studies had found associations in cytokine production between mother (pre- or postpartum) and child at one or two time points, such as at birth in cord blood [1,2,5], at a time when infant was 3 months [3], 1 year [5,11] or 2 years of age [4]. The mother-child cytokine relationship was not always apparent directly after birth. For example, the birth cohort study by Halonen and coworkers showed no correlation of mitogen-stimulated IFN-γ and IL-13 between pregnant mother and fetus but instead with the child at 3 months of age [3]. Similarly, in another study with the lipopolysaccharides-induced IL-10 and TNF-α production, there was a significant association between pregnant mother and the child at the age of 1 year but not with cord blood [11]. Our results are in agreement with the previous two studies in that maternal IL-10 or IFN-γ production during pregnancy was positively associated with child's corresponding cytokines up to 1 year of age but with longer observation, the association was no longer present (Figure 1). This particular finding regarding IL-10 or IFN-γ may reflect intra uterine and nursing effect on child's developing immune system. While no trans-placental transfer of maternal cytokines in human are believed to occur [12]; the components of uterine microenvironment may modulate the fetal naïve immune cells. This might explain why maternal IL-10 during pregnancy showed the strongest association with child's IL-10. After birth, the maturating immune system of infants is believed to get compensations from breast milk which contains maternal humoral and cellular immune components including cytokines, chemokines and immune cells. Since the majority (97%) of the infants/children in our study was breastfed, breast milk may also contribute to the transfer of maternal immunological information to infants in this population. Both IL-10

and IFN-γ are present in breast milk [13,14]. In the case of IL-10 this is thought to be a continuation of immune regulation during gestation to avoid rejection of fetal allograft, while for IFN-γ the nursing can be the best way to complement the infant's immune system whose capacity to produce TH_1-type responses are less than in adults [15]. Interestingly, high levels of TNF-α are present in early milk but become almost undetectable after 1 month (reviewed in [14]). This might explain the weak association we observed in TNF-α production between mother and child in the first year of life. Interestingly, the pattern of mother-child relationship in IL-5 responses was different from other cytokines, in that the significant association was found only when the children reached 4 years of age.

It seems that at early age, the capacity of infant's immune cells to produce pro- and anti-inflammatory cytokines is more influenced by maternal cytokines, than gene polymorphisms which seem to have more influence at later age. On the other hand, we found that the production of TH_1-type cytokine at early age was independently associated with maternal cytokine and gene polymorphisms. Previous studies in twins showed that cytokines can have a low to high proportion of heritability, ranging from 30 – 75% for IL-10 [10,16,17], 40 – 85% for IFN-γ [10,18] and 17 – 80% for TNF-α [10,16–19]. The participants of these studies were adults. Our cohort study is unique in showing the effect of maternal cytokines on child cytokines in early age may overrule or mask the genetic effect during the maturation of child immune responses. This notion is supported by the finding in a twin study that the genetic effect on serum TNF-α increased with age [17].

In our previous study investigating factors that determined child cytokine production at 2 months of age, maternal TNF-α was not significantly associated with child TNF-α ($p=0.1$). However, the present study which analyzed a subset of whom there was both cytokine and genotype data showed a significant association ($p<0.05$). The discrepancy is not due to the method of analysis (child high/low producer status vs child continuous cytokine levels as the outcome), but rather in the different characteristics of participants. The participants from the present study were more often from Jati Karya village (71%), compared to the previous one (49%), which might contribute to the slight difference.

It is important to note that the significant associations between child cytokine production with SNPs detected in this study might also be caused by other unmeasured SNPs that were located in a larger distance but still in

LD. We realize that using single nucleotide analysis in this study may not entirely represent the genetic effect on child's cytokine responses since there are unmeasured genetic variations besides the measured SNPs, which may reveal a cytokine relationship between mother and child, such as copy number variation, haplotype, microsatellite alleles, as well as epigenetic processes (DNA methylation, histone modifications, small non-coding RNA). Nevertheless using the tagging SNPs we expected to limit the number of SNPs to be tested by covering those not genotyped which were in high LD with the tagging SNPs in the same gene [20].

Since the aim of the study was more focused on mother-child cytokine relationship, we consider the SNP analysis in relation to the child' cytokine responses exploratory. Therefore the weak associations ($p < 0.05$) found between genotypes and cytokine production before correction for multiple testing were not considered immediately as not significant. The confirmation of the gene association resultswould need replication with larger sample size in similar population/ race.

In conclusion, the close relationship of mother and child cytokine production, especially IL-10 and IFN-γ, was prominent in early life, before 1 year of age. This immunological relationship was independent of cytokine gene polymorphisms, suggesting that infant's cytokine responses were more influenced by the environment shared with the mother during intra uterine and breastfeeding period. Different cytokines can have different interaction with maternal cytokine and maternal/child genetic factors at certain time points, depending on the maturation of child immune responses and the challenges from the environment. Furthermore, whether the cytokine profile of children born to high or low producer mother is associated with clinical outcomes or only reflects physiological variation, needs to be further investigated.

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Supporting Table 1. Genotypes of 119 pairs of mothers and children

Gene	Polymorphism	Region	Position	Mother			Child			MAS
				N (%)	MAF	HWE, *P* value	N (%)	MAF	HWE, *P* value	MAF
IL-10	rs4579758									
	AA	intergenic	204997334	43 (36.4)	0.432	0.085	43 (36.1)	0.420	0.324	0.376
	AG			48 (40.7)			52 (43.7)			
	GG			27 (22.9)			24 (20.2)			
	rs4390174									
	AA	intergenic	204999074	47 (39.5)	0.395	0.240	48 (40.3)	0.374	0.699	0.287
	AG			50 (42.0)			53 (44.6)			
	GG			22 (18.5)			18 (15.1)			
	rs1878672*θ									
	CC	Intron	205010336	107 (89.9)	0.055	0.585	107 (89.9)	0.055	0.585	0.090
	CG			11 (9.3)			11 (9.3)			
	GG			1 (0.8)			1 (0.8)			
	rs1554286§									
	TT	Intron	205010856	54 (45.4)	0.307	0.261	62 (52.1)	0.294	0.306	0.376
	TC			57 (47.9)			44 (37.0)			
	CC			8 (6.7)			13 (10.9)			
	rs3021094									
	CC	Intron 1	205011575	34 (28.6)	0.437	0.247	34 (28.6)	0.454	0.745	0.449
	CA	5'upstream		66 (55.4)			62 (52.1)			
	AA			19 (15.0)			23 (19.3)			
	rs1800872§									
	AA	Intron	205013030	63 (52.9)	0.265	0.739	68 (57.2)	0.248	0.873	0.371
	AC	5'upstream		49 (41.2)			43 (36.1)			
	CC			7 (5.9)			8 (6.7)			
	rs1800896*θ									
	AA	5'upstream	205013520	107 (89.9)	0.054	0.585	107 (89.9)	0.055	0.585	0.090
	AG			11 (9.3)			11 (9.3)			
	GG			1 (0.8)			1 (0.8)			
	rs10494879θ									
	CC	Intergenic	205018827	103 (86.6)	0.071	0.918	105 (89.0)	0.059	0.672	0.108
	CG			15 (12.6)			12 (10.2)			
	GG			1 (0.8)			1 (0.8)			
	rs4072227									
	TT	Intergenic	205024181	56 (47.1)	0.328	0.443	56 (47.1)	0.315	1.000	0.315
	TC			48 (40.3)			51 (42.8)			
	CC			15 (12.6)			12 (10.1)			
	rs885334									

	GG									
	GA	Intergenic	205029039	32 (26.9)	0.483	1.000	33 (27.7)	0.487	0.618	0.466
	AA			59 (49.6)			56 (47.1)			
				28 (23.5)			30 (25.2)			
NFKBIL1	rs13215091									
	GG	3'downstream	31636669	95 (79.8)	0.109	0.822	87 (73.1)	0.143	1.000	0.169
	GA			22 (18.5)			30 (25.2)			
	AA			2 (1.7)			2 (1.7)			
TNF-α	rs2844482									
	CC	5'upstream	31647746	59 (49.6)	0.282	0.408	69 (58.0)	0.235	1.000	0.230
	CT			53 (44.5)			44 (37.0)			
	TT			7 (5.9)			6 (5.0)			
LTA	rs1800683									
	GG	5' UTR,	31648050	58 (48.7)	0.324	0.187	56 (47.1)	0.298	0.385	0.326
	GA	upstream,		45 (37.8)			55 (46.2)			
	AA	intron		16 (13.5)			8 (6.7)			
LTA	rs2239704									
	CC	5' UTR,	31648120	45 (37.8)	0.387	1.000	31 (26.1)	0.458	0.217	0.419
	CA	upstream,		56 (47.1)			67 (56.3)			
	AA	intron		18 (15.1)			21 (17.6)			
IFN-γ	rs10878763†									
	GG	3'downstream	66829965	98 (82.4)	0.088	0.761	96 (80.7)	0.105	0.738	0.148
	GT			21 (17.6)			21 (17.6)			
	TT			0 (0)			2 (1.7)			
	rs2069718									
	TT	Intron 3	66836429	47 (39.5)	0.378	0.811	46 (38.6)	0.399	0.308	0.327
	TC			54 (45.4)			51 (42.9)			
	CC			18 (15.1)			22 (18.5)			
	rs2069705									
	TT	5'upstream	66841278	31(26.1)	0.487	1.000	30 (25.2)	0.483	0.498	0.420
	TC			60 (50.4)			55 (46.2)			
	CC			28 (23.5)			34 (28.6)			
	rs3181032									
	TT	5'upstream	66842442	88 (74.0)	0.134	0.685	100 (84.0)	0.080	0.917	0.093
	TG			30 (25.2)			19 (16.0)			
	GG			1 (0.8)			0 (0)			
	rs10784683†									
	GG	Intron	66856790	97 (81.5)	0.092	0.687	97 (82.9)	0.085	0.824	0.125
	GA			22 (18.5)			20 (17.1)			
	AA			0 (0)			0 (0)			
	rs12146822									

	GG	?	66857933	47 (39.5)	0.395	0.240	42 (35.3)	0.412	0.864	0.453
	GA			50 (42.0)			56 (47.1)			
	AA			22 (18.5)			21 (17.6)			
IL-5	rs4143832*									
	CC	intergenic	131890876	92 (77.3)	0.118	1.000	94 (79.0)	0.126	**0.039**	0.101
	CA			26 (21.9)			20 (16.8)			
	AA			1 (0.8)			5 (4.2)			
	rs2706399									
	AA	3'UTR	131895601	77 (64.7)	0.189	0.710	68 (57.2)	0.227	0.173	0.258
	AG	Intergenic		39 (32.8)			48 (40.3)			
	GG			3 (2.5)			3 (2.5)			
	rs17690122*									
	AA	3'UTR	131895734	92 (77.3)	0.118	1.000	94 (79.0)	0.126	**0.039**	0.101
	AG	Intergenic		26 (21.9)			20 (16.8)			
	GG			1 (0.8)			5 (4.2)			
	rs743562									
	CC	3'downstream	131900282	59 (49.6)	0.282	0.408	51 (42.9)	0.340	0.950	0.343
	CT			53 (44.5)			55 (46.2)			
	TT			7 (5.9)			13 (10.9)			
	rs739719									
	GG	3'downstream	131900764	56 (47.1)	0.319	0.834	61 (51.3)	0.269	0.349	0.253
	GT			50 (42.0)			52 (43.7)			
	TT			13 (10.9)			6 (5.0)			
	rs2069812									
	TT	5'upstream	131907815	48 (40.3)	0.357	0.826	41 (34.5)	0.416	0.989	0.393
	TC	Intron		57 (47.9)			57 (47.9)			
	CC			14 (11.8)			21 (17.6)			
RAD50	rs17772565									
	CC	Intron	131980304	31 (26.0)	0.471	0.524	48 (40.3)	0.366	1.000	0.410
	CT	5'upstream		64 (53.8)			55 (46.2)			
	TT			24 (20.2)			16 (13.5)			
	rs17772583									
	AA	Intron 20	131981409	82 (68.9)	0.164	0.708	74 (62.2)	0.202	0.482	0.185
	AG	5'upstream		35 (29.4)			42 (35.3)			
	GG			2 (1.7)			3 (2.5)			
	rs2237060									
	AA	Intron 21	131998784	97 (81.5)	0.101	0.657	88 (73.9)	0.130	0.211	0.124
	AC			20 (16.8)			31 (26.1)			
	CC			2 (1.7)			0			
	rs2040704†									

	AA	Intron 22	132001076	72 (60.5)	0.214	0.648	68 (57.1)	0.244	1.000	0.180
	AG			43 (36.1)			44 (37.0)			
	GG			4 (3.4)			7 (5.9)			
IL-13	rs1881457θ									
	AA	Intronic	132020308	68 (57.1)	0.244	1.000	66 (55.5)	0.248	0.737	0.213
	AC	5'upstream		44 (37.0)			47 (39.5)			
	CC			7 (5.9)			6 (5.0)			
	rs1800925†θ									
	CC	Intronic	132020708	74 (62.2)	0.206	0.820	70 (58.8)	0.231	1.000	-
	CT	5'upstream		41 (34.4)			43 (36.1)			
	TT			4 (3.4)			6 (5.1)			
	rs2066960									
	CC	Intron 1	132022334	71 (59.7)	0.214	0.302	72 (60.5)	0.218	0.983	-
	CA			45 (37.8)			42 (35.3)			
	AA			3 (2.5)			5 (4.2)			
	rs2069744									
	CC	Intron 1	132022568	82 (68.9)	0.160	0.311	90 (75.6)	0.130	1.000	0.079
	CT			36 (30.3)			27 (22.7)			
	TT			1 (0.8)			2 (1.7)			
	rs1295686‡									
	GG	Intron 3	132023742	49 (41.2)	0.387	0.137	46 (38.7)	0.403	0.213	0.331
	GA			48 (40.3)			50 (42.0)			
	AA			22 (18.5)			23 (19.3)			
	rs20541‡									
	CC	Exon 4	132023863	47 (39.5)	0.395	0.240	42 (35.3)	0.412	0.864	0.343
	CT			50 (42.0)			56 (47.1)			
	TT			22 (18.5)			21 (17.6)			
	rs2069757									
	GG	Intron	132026312	70 (58.8)	0.235	0.979	73 (61.3)	0.210	0.732	0.135
	GA	3'downstream		42 (35.3)			42 (35.3)			
	AA			7 (5.9)			4 (3.4)			
	rs2243218									
	GG	3'UTR	132029923	91 (76.5)	0.118	0.330	91 (76.5)	0.122	0.925	0.057
	GA	5'upstream		28 (23.5)			27 (22.7)			
	AA			0			1 (0.8)			

dbSNP: the Single Nucleotide Polymorphism database, MAF: minor allele frequency, HWE: Hardy-Weinberg equilibrium, MAS: Malay in Singapore.
Pairwise linkage disequilibrium within Haploview:
* r^2=1 † ‡ r^2>0.8; rs 2040704 in RAD50 is in strong LD with rs1800925 in IL-13.
θ r^2 for mother >0.7, child >0.8. § r^2 for mother >0.8, child >0.7.

Chapter 6

General Discussion

Development of immune responses in early life

The immune system of a child soon after birth will face various stimuli from both harmful and non-harmful environments. The preparation for the challenges starts during fetal life through the interactions between maternal and child immune components with placenta as the mediator. Several studies have been carried out, mainly in western populations, where antenatal influences such as maternal diet or smoking, were shown to affect cord blood responses [1]. All these environmental influences during early life may contribute significantly to shaping the immune responses of the child in later life against subsequent environmental stimuli such as pathogens, vaccine antigens, allergens and self-antigens. When the living conditions change, such as the elimination of pathogens through improved hygiene and sanitation as well as administration of antibiotics, then the immune pre-conditioning received in utero may alter. It is thought that the alteration might lead the immune responses to become hypersensitive against non-harmful stimuli such as allergens or self-antigens. This might be one of the explanations for the health profile seen in western countries, where the prevalence of infectious diseases is decreasing while the prevalence of allergies and autoimmune diseases is increasing. Recently more attention has been given to the critical period during early life when the immune system is still plastic in responding to environmental changes and therefore may open possibilities to modify the immune system for a healthier childhood, adulthood and old age.

In populations of developing countries where pathogens are still abundant, the relationship between in utero exposure and the development of immune responses in early childhood has not been addressed comprehensively. In order to answer this, we have set up a birth cohort study following up pregnant mothers and their children living in a helminth-endemic area in Indonesia. We observed the development of child's innate and adaptive immune responses, by measuring cytokines from whole blood culture and antibodies in plasma samples. We hypothesized that children living in this area would be exposed to helminth antigens and other environmental factors which could influence the maturation of the immune response in the first 4 years of life.

The development of polarized immune responses in developing countries

The immune system in early age, especially after birth and infancy, has been shown to be TH_2-biased [2] and immature in terms of the innate and adaptive immune system [3,4]. It is therefore thought that during this developmental period the child is more susceptible to bacterial and viral infections than an adult. The increasing capacity to produce cytokine responses with age can be variable as can be the degree of skewing, depending on the environmental setting. In order to provide the best health interventions for a young child, it is important to characterize the development of the immune system in early life and to chart how environmental factors in different areas can affect it.

In developing countries, in areas outside the urban centers, prevalence of many infections, in particular parasitic infections is high. Of interest are helminth infections, which in contrast to bacterial, protozoan and viral infections, skew immune responses toward TH_2. The development of TH_2 responses in early life has been studied in the context of allergies in affluent countries but not in areas where helminths are highly prevalent. In **Chapter 2**, we found that TH_2-type immune responses (total IgE, mitogen-stimulated IL-5) increased with age as also reported in studies conducted in western countries [5–8]. Compared to what has been measured in affluent countries [9–11], the infants and young children in our study had much higher total IgE levels. Interestingly, maternal education, not maternal helminth infection, was the most prominent factor associated with child total IgE followed by socio-economic status (SES, in **Chapter 2** was defined by house material, cooking fuel and water supply) and village of residence. For PHA-induced IL-5, we found that maternal SES was significantly associated with production of this cytokine by her child. Although we might not have the sensitive tests to measured very low levels of infection, the data seem to suggest that helminth infections, which are known to carry molecules that skew immune responses toward TH_2 [12], are not the only environmental factors capable of inducing TH_2 responses. Besides hygiene, there might be other unmeasured factors in early life that could mediate the effect of maternal education, SES or village of residence on the child's immune responses, such as nutrition, stress, or antibiotic medication. The effect of maternal education or socio-economic factors on child TH_2-type responses increased in magnitude with time [**Chapter 2**], suggesting that besides the

maturation of the immune system with age, the environmental factors created/shared with the mother keep their influence on the developing immune system. It would be of importance to identify the specific factors other than helminths that could act as adjuvants for type-2 responses.

When analysing antigen specific responses, we showed that the child's specific immune responses against helminth antigens [**Chapter 2**] and Bacille Calmette-Guérin (BCG) [**Chapter 3**] increased with age. There have been several studies in areas endemic for parasitic infections, showing that maternal infection during pregnancy can prime fetal specific immune responses against parasite antigens [13–17]. In agreement with previous findings which were mostly in neonates, we showed that maternal filarial infection was significantly associated with increasing production of helminth-specific IL-5 over time in young children [**Chapter 2**]. Although it was not likely that these children were infected with filarial parasites (due to very young age and low prevalence of this infection in the population), the child might still have been exposed to the parasite antigens in utero and later to the bites of mosquitoes carrying these parasites in the same environment shared with the mother.

Vaccination is important to protect vulnerable populations such as infants against certain pathogens and it needs competent adaptive immunity to produce sufficient protection. BCG is a strong inducer of TH_1-type cellular immunity and confers protection for infants and young children against severe forms of tuberculosis (TB) such as meningitis and disseminated TB [18,19]. Moreover several studies showed other benefits beyond protection against TB, such as the decreasing mortality and morbidity to other diseases [20–22]. This bystander effect could be due to the vaccine's ability to enhance the maturation of innate and adaptive immune responses [23]. In **Chapter 3,** BCG vaccination was shown to induce the child's adaptive (TH_1- and TH_2-type) responses against PPD, an extract of *Mycobacterium tuberculosis*. While the levels of IFN-γ response to PPD were maintained till at least 2 years of age, IL-5 and IL-13 production decreased [**Chapter 3**]. The maintenance of TH_1 responses after vaccination was different from the study in vaccinated UK infants, which showed a decreasing TH_1 response between 3-12 months after vaccination [24]. The cause of this discrepancy is not clear but it could include continuous exposure to environmental mycobacteria in tropical countries or different BCG strains used for vaccination (Indonesia: Paris strain, UK: Danish strain). We found that TH_1- and TH_2-type cytokine

responses to PPD and to PHA were significantly correlated, suggesting that BCG could also induce polyclonal/ non-specific responses after vaccination up to 1 year of age.

Interestingly, the diameter of a positive BCG scar (> 2mm) at 4 years of age was correlated with TH_2-type (IL-5, IL13) responses to PPD and less so with TH_1-type responses [**Chapter 3**]. The presence of BCG scar is frequently associated with tuberculin reaction which is the gold standard for delayed type hypersensitivity testing and an indicator for type-1 cellular immunity. However, the association between cytokine responses to PPD and BCG scar or tuberculin testing has not always been found, as shown in a Gambian study [25]. The difference between our findings and those in the Gambia could be due to age of the vaccinees (within 24 hours after birth in Gambian infants vs 2 – 8.5 weeks in Indonesian infants), different time of scar measurement (in the Gambian study it was measured at 2 months after vaccination but in our study it was done at 4 years of age when the scar had stabilized), genetic background or degree of exposure to environmental mycobacteria. Despite the inconsistent association with cellular responses in vitro, the presence of a BCG scar has been shown to be associated with better survival in vaccinated infants compared to those who did not develop a BCG scar after vaccination [21,26]. Our study was not designed to assess the effect of BCG scar on survival.

Parasitic infections and immune hyporesponsiveness

We observed that the capacity of immune cells in mitogen-stimulated blood to produce cytokines was lower in infants born to mothers harbouring intestinal protozoa (mainly *Blastocystis hominis*) compared to those born to mothers free of these infections [**Chapter 4**]. The same was true when the child's innate and adaptive responses to mycobacterial antigen were examined [**Chapter 3**]. *B. hominis* infection is commonly found in developing countries [27] and can be associated with poor hygiene and sanitation [28]. The pathogenicity of this organism is still controversial while the majority of individuals harbouring it can be asymptomatic [28]. In vitro studies showed that *B. hominis* or its culture filtrate could induce pro-inflammatory cytokine production by colonic epithelial cell lines [29,30]. However, decreased pro-inflammatory responses during co-incubation with *Escherichia coli* was shown, suggesting that *B. hominis* could modulate immune responses in the presence of gut bacteria [30]. With regard to in

utero sensitization, a previous study has shown that mothers infected with intestinal protozoan *Entamoeba histolytica* can prime the fetal immune cells [31]; however, further investigation are needed to confirm this, and to answer what the consequences such a priming is.

What is important to note is that in these early responses, helminth infections of the mother are not associated with any down regulatory activity on the immune response of the child. Helminth infections during school age and in adults have often been reported to be associated with immune hyporesponsiveness [32–36], which is in contrast to our findings at early age. In a birth cohort study in Uganda, the treatment of infected pregnant women with praziquantel enhanced the maternal cellular and humoral responses to schistosome egg antigens [37,38], without affecting cord or infant/child specific immune responses to helminth antigens [39] or to vaccine antigens [40,41]. On the other hand albendazole treatment of maternal hookworm infections decreased the child's TH_2-type responses to tetanus toxoid compared to the placebo-treated infected group, which were found at 1 year [41] but not at 5 years of age [42]. The investigations on the underlying cellular immune profiles in Gabonese neonates born in areas endemic for helminths and malaria compared to European neonates, found that the Gabonese had a decreased frequency of regulatory T cells ($CD4^+CD25^{++}$) as well as expression of CTLA-4 (CD152) and Foxp3 in these cells [43]. The lower frequency of Treg in Gabonese neonates are not in line with the findings of expansion of regulatory T cells seen in cord blood from neonates born to mothers infected with malarial parasites (detected in the placenta) [44,45] as well as in adults with malarial parasitemia [46] or chronic helminth infections [33,47]. Altogether, these studies indicate that either technical differences whereby regulatory T cells are identified are causing the discrepancies or that regulatory cells develop at different windows of time depending on the type of infection or other additional environmental factors that a pregnant woman is exposed to. In any case, in the previous studies there has been no evidence for a suppressive effect of maternal helminth infection on the child's cytokine production following stimulation of cord blood. It is clear that more studies are needed to establish whether maternal helminth infection affects the child's immune system.

The influence of environmental factors on innate immune responses

In **Chapter 4** we showed that at 2 months of age (before vaccinations were given) village of residence was associated with the infant's innate cytokine (IL-10, TNF-α, IFN-γ) responses against the TLR4 ligand lipopolysaccharides (LPS) [**Chapter 4**]. LPS with endotoxin as its bioactive component can be found in gram-negative bacteria. In our study the residence can be a proxy for exposures to microbes or parasites in the environment. Although the two villages in our study area are located adjacent to each other, we found that the infants with lower innate immune responses were living in the village with higher prevalence of maternal helminth infections. The downregulation of innate immune responses (IL-6, IL-10, TNF-α) to LPS was also shown in cord blood mononuclear cells of Papua New Guinean neonates, when compared to the responses of Australian neonates [48]. In a different setting such as in European farming areas, gene expression of TLRs were increased in neonates [49] or school-age children [50] of farmers compared to those of non-farmers, and were associated with prenatal and postnatal contact with farm animals or consumption of raw milk. In contrast, a study of Gabonese vs Austrian neonates by Kohler and co-workers showed a lower TLR2 expression on monocytes and myeloid dendritic cells in Gabonese neonates [43]. It is interesting that there seems to be an agreement between the downregulation of innate responses when exposure to microorganisms and parasites is in developing countries but not when it is in an affluent region of the world. Therefore, the question is whether up or downregulation is simply a marker of an affected immune system rather than how it is affected. Another question that follows is whether the up or downregulation of the innate immune response is dependent on the additional environmental exposures. While the underlying mechanism of up- or downregulation of innate responses are still unclear, it is thought that the degree of exposure to endotoxin [51] and exposure to other microbial or parasitic agents may program child immune responses to be prepared for the future challenges of the same agents, while at the same time the regulatory mechanisms are enhanced to avoid excessive inflammation that can result from continuous exposure.

In our studies it was noted that although BCG can stimulate innate immune responses through engagement of Toll-like receptor (TLR) 2 and 4

[52,53], after vaccination we did not find an increased production of pro- and anti-inflammatory cytokines (TNF-α and IL-10) in response to PPD or LPS [**Chapter 3**], suggesting that the innate cytokine production in this study do not appear to be affected and therefore do not explain the reported non-specific effects of BCG on child morbidity and mortality.

Mother-child relationship in cytokine production

The study in **Chapter 4** revealed that there was a strong association between the capacity of cells in whole blood of a pregnant mother to produce cytokines, particularly IL-10 and IFN-γ, and the capacity of cells in whole blood of her child at 2 months of age. Previous studies have shown the association between maternal and child cytokine responses soon after birth [7,54,55], in the first year of life [7,56,57], and at 2 years of age [58]. In our longer birth cohort, the mother-child relationship in the production of IL-10 and IFN-γ was decreasing with increasing age [**Chapter 5**]. We can assume that from in utero until at least the first year of life an infant has close immunological contact with the mother through intrauterine environment and breastfeeding. On the other hand, genetic factors such as gene polymorphisms (SNPs) could contribute to this cytokine relationship since the child shares half of maternal alleles. To investigate this we chose several tagging single nucleotide polymorphisms (SNPs) with a minor allele frequency of > 5%. We found that at earlier time points within the first 1 year of age, maternal cytokine responses were the most important factor associated with corresponding child cytokines with little influence from the genetic make-up. This mother-child cytokine relationship waned with time, and in later life (at 4 years of age) genetic factors contributed more to child cytokine responses. All these findings suggest that immunological interaction between mother and child is not necessarily directly due to genetic background. However to confirm the association with the cytokine gene polymorphisms, larger sample size would be needed and different approaches for genetic determinants (haplotype, copy number variation, microsatellite alleles) should be included.

Early exposure to helminths and potential risk of allergy in later life

The interaction between gene and environment is thought to shape the developing immune system with potential impact on health outcomes in later life (**Figure 1, Chapter 1**).

With regards to atopic sensitization, the prevalence of skin prick test (SPT) positivity at 4 years of age [**Chapter 2**] was similar to European countries. On the other hand, total IgE levels and the prevalence of positive allergen-specific IgE were much higher than those in affluent countries. While SPT and total/ specific IgE antibodies are strongly associated in western countries, we found no association between SPT and total IgE and many children with positive sIgE had negative SPT. The discordance between skin test reactivity and IgE as systemic marker for allergic sensitization is not uncommon; it has been seen in school-aged children and adult populations living in less affluent areas with poor sanitation and hygiene [59,60]. As already alluded to, helminth infections which can be highly prevalent in these areas, are potent inducers of Type-2 responses, both innate [61,62] and adaptive, and in addition lead to the induction of regulatory T cells [63] and B cells [64]. We found that filarial infection in pregnant mothers tended to decrease the child's risk to have positive SPT but interestingly also allergen-specific IgE a [**Chapter 2**], indicating that the early exposure to helminth antigens might suppress the child's responses to allergens both in terms of IgE and SPT. In the same pediatric population, lower maternal education was associated with increased total IgE and decreased skin test positivity at 4 years of age. The question is whether the inverse association between maternal helminth infection and atopic markers in our observational study could be extrapolated to allergic disorders in the child. This question has been addressed in an interventional randomized clinical trial with praziquantel administration to clear *Schistosoma mansoni* in infected pregnant mothers [65]. The study found an increased risk of eczema in the children born to mothers treated with praziquantel. Better powered studies with randomized clinical trials are needed to replicate our finding in filarial-endemic areas, as well as in multi-parasite endemic settings where not only clinical outcomes but also sequential sensitization to parasite antigens as well as allergens is examined in detail as a function of age.

Concluding remarks, gaps and future research

In early stages of life there is a dynamic and complex interaction between the developing immune system and the environment. This early period marks a time when a child is most vulnerable to infections and when immunological set points get established which might affect disease outcomes in later life.

Our longitudinal study has shown significant associations between maternal immune parameters and environmental factors during pregnancy with the child's general and specific immune responses, suggesting that preventions/ interventions aimed at mothers at pre and post natal periods may have a long lasting impact on the child's immune system and even on health outcomes later, such as on allergies. With the prevalence of child mortality being still high in low income countries [66], more birth cohort studies in different settings/ environment especially in pathogen-rich environments are needed to identify optimal health interventions. For example, if we consider the possibility of bystander effects imposed by parasitic infections during pregnancy on the child's immune responses to vaccinations and or microbial/viral infections, we can develop strategies to influence this. As an alternative, mammals with closest similarity of placental architecture to humans such as guinea pig and rhesus monkey [67,68] can be used as a model for studying, in molecular detail, the immunological interaction between pre and post natal environment and the health of the offspring.

The immaturity of the child's immune system is also a challenge for improving the efficacy of vaccines within EPI (the Expanded Program on Immunization) or when implementing new vaccines; therefore recent work has focused on finding new or to improve old vaccines, with adjuvants such as TLR ligands that can boost the maturation of dendritic cells, and on circumventing the interference of passive maternal antibodies during the first 6 months of age.

While breastfeeding is well recommended by WHO and local health policies, so far many of its unique components are not replaceable by formula milk. Therefore more research is needed to find the active agents in milk which can modulate the immune responses of infants who cannot be breastfed.

Lastly, with recent advances on epigenetic programming in early life, it would be interesting to expand our current research to epigenetic

processes (DNA methylation, histone modifications, small non-coding RNA) to understand the interaction between maternal and child cytokine responses at different stages of life. With the immense variation in environmental conditions, studies in developing countries would provide a great window of opportunity for this purpose.

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Summary

Early childhood is a critical period where the maturation of the immune system occurs while it receives various challenges from the environment shared with the mother. Children growing in an environment rich in micro-organisms and parasites are thought to have a different pattern of immune response compared to those children growing in a more hygienic environment. This difference might contribute to the lower prevalence of allergic and autoimmune disorders in developing countries compared to affluent countries. Within this context, a number of cohort studies mostly performed in developed countries have focused on finding the link between the pattern of immune responses in early life and health outcomes in later life. In this thesis, we studied child's innate and adaptive responses during the first 4 years of life in a helminth-endemic area in Indonesia.

Chapter 1 provides a general introduction to the development of immune responses in early childhood. It describes and reviews how the maternal environment and exposures (for example farming environment or parasitic infections) during pregnancy might affect the child's immune responses in later life. The immune system of a child, in turn, is an important determinant of responses to vaccinations, allergens or parasitic and other infections. This chapter also describes the aim, the study population and the longitudinal design of the study.

In **Chapter 2**, we measured the different immunological patterns. Put in a very simplified way, the immune system can be characterized by two types of immune responses: TH_1 and TH_2. We analyzed TH_2-type cytokines and antibodies in young children from 2 months to 4 years of age. TH_2 responses increased significantly with age and to higher levels compared to what was seen in children of developed countries. Maternal education and socioeconomic status influenced the production of child's general TH_2 responses, while maternal filarial infection was more associated with the child's helminth-specific TH_2 responses. Maternal education was also shown to affect the child's skin prick test reactivity to allergen more strongly compared to other factors such as maternal helminth infection. Although both helminth infection and allergy are known to induce TH_2-type immune responses, our findings show that measured maternal helminth infections were not the most prominent contributors to the development of TH_2 responses in early life, indicating that more attention should be paid to find out what specific stimuli can skew immune responses toward TH_2.

In **Chapter 3,** we compared the development of innate and adaptive responses in children before and after BCG vaccination. While innate responses (early cytokines taking part in innate responses: IL-10 and TNF-α) induced by PPD & LPS were not affected, both TH_1- and TH_2-type responses against PPD were increased after vaccination and only TH_1-type was sustained into later age. BCG vaccination was also shown to stimulate child's general adaptive responses in response to mitogen-stimulation of whole blood, which correlated with PPD-specific responses till 1 year of age. All these in vitro findings still need to be studied further in relation to the effect of BCG on the maturation of innate and adaptive immune responses in vivo, in order to explain the reported beneficial bystander effects of BCG on morbidity and mortality of children. Surprisingly we found that maternal intestinal protozoan infection (*Blastocystis hominis*) but no other measured factors during pregnancy, was associated with lower child's immune responses to mycobacterial antigen, again stressing the importance of exploring the contribution of a wide range of factors during early life for shaping immune responses later in life.

Chapter 4 described the relationship between maternal and child's cytokine responses at the age of 2 months, before any vaccination was given. The high production of IL-10 and IFN-γ in mitogen-stimulated whole blood from the mother was significantly associated with higher corresponding child's cytokine production. On the other hand the child's innate responses, represented by LPS-induced IL-10 and TNF-α, were more associated with area of residence than with maternal cytokines. We conclude that mother-child cytokine relationship at early infancy is seen in the adaptive responses but not in innate immune responses. Again maternal *B. hominis* infection was found to affect child's cytokine responses, warranting further research on the contribution of this particular parasitic infection on the developing immune system of the child starting from *in utero* period.

In **Chapter 5**, we followed the mother-child cytokine relationship up to 4 years of age and related this to gene polymorphisms as well as other factors. Our finding of strong mother-child cytokine relationship in early age was consistently found till 1 year of age, albeit in a decreasing strength. The genetic factors, either maternal or child's gene polymorphisms, were shown to be more associated with child's cytokines at later life. We concluded that during the first year of life the child's cytokine responses were more affected by cytokine responses of mothers, possibly through in utero preconditioning or breastfeeding which over rules the genetic

influences. Larger studies are needed to dissect this in more detail with sufficient power.

Chapter 6 discusses the findings in this thesis, together with previous studies in both developed and developing countries. Emphasis was put on the contribution of maternal parasitic infections and other environmental factors to the child's immune responses and atopy, as well as to mother-child cytokine relationship. At the end, the possible consequences of the findings and future research have been discussed.

Altogether, this thesis has provided a case for the importance of studying early life events not only in affluent countries but also in low to middle income countries where the changes in life style and environmental factors are the most dynamic and therefore informative for understanding development of diseases later in life.

Samenvatting

De vroege kinderjaren zijn een kritieke periode, waarin de rijping van het afweersysteem plaatsvindt terwijl het de verschillende uitdagingen vanuit de omgeving ondervindt, die gedeeld worden met de moeder. Er wordt gedacht dat kinderen die opgroeien in een omgeving die rijk is aan micro-organismen en parasieten andere patronen van afweerreacties hebben in vergelijking met kinderen die opgroeien in een meer hygiënische omgeving. Dit verschil zou kunnen bijdragen aan het minder vóórkomen van allergische en auto-immuun aandoeningen in ontwikkelingslanden dan in welvarende landen. Binnen deze context is een aantal cohortstudies uitgevoerd, veelal in ontwikkelde landen, gericht op het vinden van het verband tussen het patroon van de afweerreacties in de vroege levensjaren en gezondheidsuitkomsten in het latere leven. In dit proefschrift, hebben wij de aangeboren en verworven afweerreacties bestudeerd van kinderen tijdens de eerste vier levensjaren in een gebied waar worminfecties voorkomen in Indonesië.

Hoofdstuk 1 geeft een algemene inleiding over de ontwikkeling van afweerreacties in de vroege kinderjaren. Het beschrijft en vat samen hoe de omgeving (bijvoorbeeld een plattelandsomgeving) en de blootstelling bijvoorbeeld aan infecties met parasieten van de moeder tijdens de zwangerschap de afweerreacties van het kind op latere leeftijd kunnen beïnvloeden. Het afweersysteem van een kind is heel belangrijk voor reacties op vaccinaties, allergenen of parasitaire en andere infecties. Dit hoofdstuk beschrijft ook het doel, de onderzoekspopulatie en de longitudinale opzet van het onderzoek zoals beschreven in dit proefschrift.

In **hoofdstuk 2**, hebben we de verschillende patronen in afweerreacties gemeten. Op een zeer vereenvoudigde manier kan het afweersysteem worden gekenmerkt door twee soorten afweerreacties: T helper $(TH)_1$ en TH_2. We analyseerden TH_2 type cytokinen en antilichamen bij jonge kinderen van 2 maanden tot 4 jaar oud. De TH_2 reactie nam aanzienlijk toe met de leeftijd en was veel hoger in vergelijking met wat werd gezien bij kinderen in ontwikkelde landen. Het opleidingsniveau en de sociaal-economische status van de moeder beïnvloeden de productie van algemene TH_2 reacties van het kind, terwijl filaria infectie (een worminfectie) van de moeder meer geassocieerd was met TH_2 reacties van

het kind die specifiek gericht zijn tegen wormen . Het opleidingsniveau van de moeder bleek de huid-prik allergie-test van het kind sterker te beïnvloeden dan andere factoren. Hoewel het bekend is dat zowel worminfecties als allergie tot afweerreacties van het TH_2 type leiden, blijkt uit onze bevindingen dat de worminfecties van de moeder niet de meest prominente bijdrage leverden aan de ontwikkeling van TH_2 reacties in het begin van het leven. Dit geeft aan dat er meer aandacht moet worden besteed aan het uitzoeken van welke specifieke stoffen TH_2-type afweerreacties tot gevolg kunnen hebben.

In **hoofdstuk 3** vergeleken we de ontwikkeling van de aangeboren en verworven afweer bij kinderen voor en na BCG-vaccinatie (tegen tuberculose). Terwijl aangeboren reacties (gemeten met cytokines Interleukine-10 [IL-10] en Tumor Necrose Factor-α [TNF-α]) niet werden beïnvloed, waren zowel TH_1 als TH_2-type reacties tegen Purified Protein Derivatives (PPD, bestanddeel van tuberculose) na vaccinatie verhoogd. Alleen type TH_1 bleef tot op latere leeftijd verhoogd. Er werd ook aangetoond dat BCG-vaccinatie de verworven afweer van kinderen stimuleert in reactie op algemene stimulatie van bloed, wat in verband werd gebracht met de reactie tegen PPD tot de kinderen 1 jaar oud waren. Al deze *in vitro* bevindingen moeten nog verder worden onderzocht in verband met het effect van BCG op de rijping van aangeboren en verworven afweerreacties *in vivo*, teneinde de mogelijke positieve effecten van BCG op de morbiditeit en mortaliteit in kinderen te kunnen verklaren. Verrassend genoeg vonden we dat infectie van moeders met de parasitaire eencellige *Blastocystis hominis* in de darmen tijdens de zwangerschap is geassocieerd met een verminderde afweerreactie van het kind tegen tuberculose. Dit wijst nogmaals op het belang van het verkennen van de rol van een breed scala van factoren tijdens het vroege leven op het vormgeven van afweerreacties later in het leven.

Hoofdstuk 4 beschrijft de relatie tussen de cytokine reacties van de moeder en haar kind op de leeftijd van 2 maanden, voordat vaccinaties werden gegeven. De hoge productie van cytokines IL-10 en IFN-γ in algemeen gestimuleerd bloed van de moeder leidde tot hogere cytokine productie van haar kind. Anderzijds waren de aangeboren afweerreacties van het kind, in dit geval vertegenwoordigd door IL-10 en TNF-α na stimulatie met lipopolysacchariden (LPS, het belangrijkste bestanddeel van

de buitenmembraan van gram-negatieve bacteriën), hadden meer te maken met de woonomgeving dan met de afweerreacties van de moeder. We concludeerden hieruit dat er een relatie tussen de cytokines van moeder en kind in de vroege kindertijd wordt gezien in de verworven, maar niet in de aangeboren afweer. Wederom bleek *B. hominis* infectie van de moeder de afweerreacties van het kind te beïnvloeden. Dit rechtvaardigt verder onderzoek naar de bijdrage van deze specifieke parasitaire infectie op de ontwikkeling van het afweersysteem van het kind vanaf de periode in de baarmoeder.

In **hoofdstuk 5** volgden we de relatie tussen de cytokines van moeder en kind tot vier jaar oud, en hebben we gekeken naar genpolymorfismen (genveranderingen) en andere factoren. Onze bevinding omtrent de sterke moeder-kind cytokine-relatie op vroege leeftijd bleef inderdaad aanwezig tot de kinderen 1 jaar oud waren, zij het steeds minder sterk. De genetische factoren, ofwel de genpolymorfismen van moeder en kind, bleken meer geassocieerd te zijn met afweerreacties van het kind op latere leeftijd. We concludeerden dat tijdens het eerste jaar van het leven cytokinereacties van het kind primair beïnvloed werden door cytokinereacties van de moeder. Deze ontwikkeling lijkt gestuurd te worden door factoren in de baarmoeder of tijdens de borstvoeding, en lijken de invloed van genetische factoren te overstemmen. Grotere studies met voldoende deelnemers zijn nodig om deze relaties in meer detail te ontleden.

In **hoofdstuk 6** worden de bevindingen in dit proefschrift besproken in de context van eerdere studies in zowel ontwikkelde als ontwikkelingslanden. Nadruk werd gelegd op de bijdrage van parasitaire infecties van de moeder, en op de rol van omgevingsfactoren. Gezamenlijk vormen zij de basis van de ontwikkeling van de afweer en de allergische reacties van het kind. Tot slot worden de mogelijke gevolgen van deze bevindingen en enkele mogelijkheden voor toekomstig onderzoek besproken. Al met al, heeft dit proefschrift het belang aangetoond van het bestuderen van de gebeurtenissen in het vroege leven, niet alleen in welvarende landen, maar ook in lage tot midden-inkomenslanden waar de veranderingen in levensstijl en omgevingsfactoren het meest dynamisch zijn. Deze veranderingen zijn daarom zeer informatief voor het begrijpen van de ontwikkeling van ziekten later in het leven.

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Curriculum Vitae

Yenny Djuardi was born on May 21th 1972 in Jakarta, Indonesia. In 1990 she graduated from the Christian Senior High School (SMAK) I BPK Penabur, Jakarta, and started her medical education at Faculty of Medicine University of Padjadjaran, Bandung. She received her medical degree in 1997. In 1999 she started to work at Department of Parasitology Faculty of Medicine University of Indonesia (FMUI/FKUI), as a research assistant at Mycology Division under supervision of Prof.Dr. Jan Susilo, before she moved to work at the Filariasis Center in Helminthology Division. Since 2000 she has conducted several field studies in areas endemic for filariasis in different parts of Indonesia and performed data analysis under supervision of Dr. Taniawati Supali.

In 2001 she was involved in the first NWO-WOTRO funded research project on a longitudinal study of pregnant women and their babies up to 1 year of age. The work was conducted in collaboration with her senior colleague Dr. Heri Wibowo. During that period she visited LUMC several times to be trained in immunological laboratory work. In 2004 she enrolled as a PhD student at Leiden University Medical Center. She received a second NWO-WOTRO grant from 2005-2009 to continue to study the maturation of the immune system of children who were born during the first NWO-WOTRO study. The follow up of the children would continue until they reached 4 years of age. Her research project was carried out in the Department of Parasitology at FMUI and in the Department of Parasitology at LUMC. She spent most of her time at LUMC between 2009 and 2012 to analyze samples, to perform data analysis and to write manuscripts.

Yenny Djuardi has intermittently been a tutor for medical undergraduate students of FMUI, and after finishing her PhD, she will continue working at University of Indonesia as a lecturer and researcher.

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