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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