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Overlapping patterns and unique differences: a study into immunological variation within and between populations

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Chapter 7

Summarizing Discussion

Adapted from: Immunological factors linked to geographical variation in vaccine responses

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LESSONS LEARNED

In this thesis immunological differences between populations and their implications for responses to immune perturbations and health outcomes have been studied. Comparing the immune system of those from different socio-economic backgrounds within an urban area and between urban and rural residents of LMICs, has taught us a number of valuable lessons. Firstly, even within a single urban center, differences in the immune system and the response to immune perturbations such as allergen or bacteria, can be detected among children with different socio-economic backgrounds. Importantly, these differences can be studied using minimally invasive sample collection methods that require few laboratory facilities making them suitable for studies in LMICs. Secondly, studying immune profiles in relation to socioeconomic status (SES), rural/urban residency and lifestyle revealed differences in the immune system, including the level of activation and skewing of the immune system towards type 1 or type 2 and regulatory immune responses. These differences highlight the impact of environmental and lifestyle factors on the immune system. Finally, examining the response to yellow fever (YF) vaccination in rural and urban Tanzanians and Dutch, showed differences in antibody responses where most Dutch were high responders, while the proportion high and low responders in urban versus rural Tanzanians did not significantly differ. In terms of cellular immune profiles, again there were large differences between Dutch and Tanzanians and more modest differences between urban and rural Tanzanians. We found indications that baseline features of the innate immune system may be associated with the response to YF vaccine. These findings contribute to our understanding of the immunological characteristics underlying the differences in disease prevalence and vaccine responses and will help design interventions needed to reverse the alterations of the immune system that negatively impact health outcomes.

Differences in the response to immune perturbations in high and low SES children.

Although it is known that large wealth inequalities exist within urban areas of LMICs, which can impact an individual's health, few studies have compared the immune system of people with different SES. Therefore, we studied several aspects of the immune system of children from different socioeconomic backgrounds within the urban center of Makassar, Indonesia in Chapter 2, 3, and 4 of this thesis. Importantly, SES per se does not directly alter the immune system, but it is associated with differential exposure to environmental factors which thereby can impact the immune system. Key environmental factors that have been shown to impact the immune system,

include nutrition [1], exposure to pathogens [2] and the gut microbiome composition [3]. Moreover, differences in the gut microbiome have been observed in children from high and low SES in Makassar [4]. However, untangling the effects of these single factors on the immune system has been proven challenging as exposure to these factors often coincides. Therefore, the choice has been made to focus on studying the immune system of children from different SES to understand the downstream impact of the environmental exposures in shaping the immune system and how it potentially affects perturbations.

As the study population consisted of school-aged children in Indonesia, minimally invasive sample collection methods that do not require extensive laboratory facilities were employed and proven successful, illustrating the feasibility of such studies in low- and middle income countries and in vulnerable populations such as children.

In **Chapter 2**, we show that nutritional status varies greatly among schoolchildren from different socio-economic backgrounds in Makassar. A substantial number of children were malnourished as 21.6% were underweight and 14.8% overweight, confirming the double burden of malnutrition that this population is facing. Almost all underweight children attended low SES schools, while children in the overweight group had parents who on average had higher education. Underweight was associated with lower eosinophil counts, but not with total Immunoglobulin E (IgE) levels or skin prick test (SPT) reactivity while overweight was positively associated with SPT reactivity. This aligns with the findings of an earlier study among high and low SES children in Makassar, that found higher prevalence of allergic sensitization determined by SPT in high SES children and showed that SPT was positively associated with wheeze, a clinical symptom of asthma in high, but not in low SES children [5]. Moreover, our finding that both underweight and overweight conditions are associated with a T helper 2 (Th2) marker, may indicate that both forms of malnutrition might pose a risk for the development of allergy. This is in line with a previous study reporting a U-shaped association between body mass index and the prevalence of wheeze and allergic asthma, indicating that both over- and underweight children are more likely to develop allergic disorders [6]. Together, our findings demonstrate that malnutrition is common among school-aged children and that nutritional status, which is largely determined by SES, influences the immune responses to allergens. Interventions improving the nutritional status of children are essential to prevent the increase of allergic disorders in Indonesia.

From a small subset of the children included in the study presented in chapter 2, nasosorption samples were collected to study the bacterial colonization and cytokine response in the nasal mucosa as described in **Chapter 3**. In the low SES setting, we found an increased nasal mucosal bacterial load of *H. influenzae* and *S. pneumoniae*, species of bacteria that can cause lower respiratory tract infections (LRTIs). Increased density of *H. influenzae*, *S. pneumoniae*, and *M. catarrhalis* has been associated with febrile acute respiratory infections in a study among Tanzanian children and the carriage and density of these bacteria was shown to be a good predictor for clinical pneumonia [7]. Our results showed that the densities of these bacteria were positively correlated with Interleukin (IL)-1β and IL-6 levels in the nasal fluid in both SES groups. After adjusting for bacterial density, IL-6 levels were increased in high SES children compared to low SES colonized by *H. influenzae* and *S. pneumoniae*, indicating a stronger cytokine response in high SES children upon colonization. The pro-inflammatory cytokine IL-6 is known to promote the differentiation of naïve T cells into T helper 17 (Th17) cells, a T cell subset that plays a critical role in host defense against pathogens at mucosal sites [8]. A previous study also found higher levels of IL-6 and IL-17A, and increased numbers of Th17 cells were observed in the adenoids of children carrying *S. pneumoniae* compared to S. *pneumoniae*-negative children [9]. Additionally, depletion of systemic IL-17A in mice resulted in a loss of pneumococcal clearance, underscoring the importance of the Th17 response in the control of bacterial colonization [10]. Thus, the lower levels of IL-6 and the higher densities of *S. pneumoniae* and *H. influenzae* observed in low SES children in our study suggest reduced bacterial clearance in these children, which may put them at a greater risk for the development of LRTIs.

In **Chapter 4**, yet another minimally-invasive but powerful method has been employed to investigate the immune system: collection of whole blood using a finger prick. Blood was collected from a small subset of children of the cohort described in Chapter 2, which did not overlap with the subset described in Chapter 3. Blood drawing using finger prick is perceived as less painful compared to venous blood collection and is considered less invasive. Whole blood samples were lysed, fixed, and thereafter stored at -80 °C until further use. In contrast to the isolation of peripheral blood mononuclear cells (PBMCs), for which sterile conditions and liquid nitrogen storage is needed, fixation of whole blood requires few laboratory facilities and can thus be performed in settings with limited resources such as in LMICs. Using mass cytometry (CyTOF) we were able to retrieve information from a total of 5.8 million peripheral immune cells and could find rare subsets such as Innate Lymphoid Cells (ILCs). The immune system of children can thus be studied using small amounts of whole blood collected from fingertips. As

this type of sample collection is minimally invasive and does not require extensive laboratory facilities, this method is suitable and promising for immune profiling from diverse geographical areas and populations in a standardized manner.

Immune profiles differ across location, socioeconomic status and lifestyle

Studying the immune profiles in whole blood samples using mass cytometry revealed differences in the immune profiles among high and low SES as described in **Chapter 4**. Low SES children had significantly higher frequencies of CD11c+ B cells, often referred to as atypical B cells, and increased expression of C-C chemokine receptor type 7 (CCR7), a homing receptor present on activated B cells. This implies recent antigen encounter and a higher pathogen burden in low SES children. Enrichment of CCR7+ and CD11c+ B cells has also been observed in rural Senegalese compared to both urban Senegalese and urban Dutch volunteers [11]. Further analysis of these B cells revealed that they had a pro-inflammatory profile evident from increased fatty acid synthesis and production of Tumor Necrosis Factor (TNF) upon stimulation. Frequencies of these pro-inflammatory B cells were negatively correlated with Immunoglobulin G2 (IgG2) galactosylation, a post-translational modification that enhances the anti-inflammatory properties of antibodies [11]. In our dataset, further examination of the CD4+ T cell compartment revealed a trend towards a higher frequency of Th1 cells in high SES. Whereas in the low SES we observed an expansion of CD161+ Th2 cells, a subset of Th2 cells that is considered more mature and reported to be elevated in rural areas where helminth infections are highly prevalent [2]. Moreover, within the regulatory T cell (Treg) population, a cluster characterized by the expression of CCR7, Cytotoxic T-lymphocyte associated protein 4 (CTLA-4,) Inducible T-cell costimulatory (ICOS) and CD38 was found to be increased in low compared to high SES children. Interestingly, Tregs expressing CTLA-4 have been reported to have increased regulatory activity [12] and CTLA-4 expression on T cells was found to diminish after anthelminthic treatment [13]. Finally, changes in the myeloid compartment could be seen as the number of CD163+ monocytes dimly expressing Human Leukocyte Antigen DR (HLA-DR) was increased in low compared to high SES children. The expression of HLA-DR is a hallmark of myeloid derived suppressor cells, a subset that expands upon chronic infections such as helminth infestations [14] and that have been associated with reduced vaccine responses [15]. However, studies that further characterize and assess the functionality of this subset are needed to confirm the identify of these cells and validate their suppressive activity. Altogether, our findings suggest that the immune profile of low SES children has a more regulatory

and Th2 phenotype and an expanded B cell compartment characterized by expression of CD11c and CCR7, whereas in the high SES there is a trend towards a shift to a Th1 phenotype. As such differences, which have been reported previously when comparing rural and urban populations, may lead to altered immune responses, our findings highlight the need to account for SES when studying the variation in the immune function.

In **Chapter 5**, we profiled the immune system of 100 healthy Tanzanians living across four areas with different levels of urbanization. Cluster analyses resulted in 80 distinct immune cell clusters, of which nine clusters were significantly different between the four locations. Five of these clusters, belong to the CD4 T cell lineage. Th2 cells represented the strongest rural signal, with highest frequencies in rural locations compared to urban. Moreover, two clusters of Tregs cells expressing CTLA-4 were increased in rural-living individuals, which was most pronounced in those originating from Mwanga district, the area with the most rural features, such as limited access to tap water and low housing quality (e.g. soil floor). Expansion of Th2 cells and Tregs has previously been observed in rural Indonesians and has been associated with helminth infection, as their frequency decreased upon deworming treatment [2]. In our study, only very few individuals were found to be infected with helminths. However, the expansion of Th2 and Tregs may represent an immunological footprint induced by past helminth infections even during childhood, as has been suggested by others [16, 17]. Given the current age of our study participants, high burden of previous helminth infections is likely in individuals from Mwanga, as a high prevalence of schistosomiasis was found in school-aged children from this district in 2005 [18].

Although geographical location was found to be a great contributor to differences in immune profiles between individuals in our study, considerable variation in immune signatures within living locations was observed. To better capture the urbanization level, we developed a lifestyle score based on household assets, housing condition, and recent dietary history. A higher lifestyle score corresponded to a more urbanized lifestyle while those with lower scores have a more rural lifestyle (e.g. poorer housing conditions and fewer assets). Studying the association between the lifestyle score and immune cell frequencies showed that 13 clusters were associated with lifestyle score, of which eight were not previously linked to living location. Of these eight, three were CD8+ T cell subsets and showed a positive association with lifestyle. These cells represented CD8+ T effector memory (Tem) cells expressing CD161 and two subsets of CD8+ naïve T cells which differed in their expression of CD127 and CD28. Although we lack

markers to confirm this, the cluster of CD161+ CD8+ T cells might represent mucosal-associated invariant T cells (MAIT) cells, a subset that mediates the local response to infections by production of pro-inflammatory cytokines. The expansion of such cells in the urban-living population may indicate stronger responses to antigens in those living in urban compared to rural environments. Indeed, an earlier study in this same region of Tanzania, showed increased pro-inflammatory cytokine responses upon stimulation with different bacterial, fungal and Toll-like receptor (TLR) agonists *ex vivo* in individuals from urban Moshi compared to rural Moshi [19]. The enrichment of naïve CD8+ T cells found in the urban individuals in our study may also contribute to stronger immune responses in urban-living individuals. This is supported by a previous study reporting that Bangladeshi compared to American children had reduced numbers of naïve CD8+ T cells and that the immune system of these Bangladeshi children was broadly defective in their ability to respond to stimulation [20]. Together, these results show that immune profiles vary across location, SES and lifestyle. Further exploration of immunological and clinical consequences is needed to validate and strengthen these findings. Moreover, this work, together with that from others, emphasizes the importance of lifestyle and SES in immune variations among populations.

Response to yellow fever vaccination among different populations

Although differences in the immune system between urban and rural populations have been described, to what extent these differences result in altered vaccine responses remains unknown. To investigate this, in **Chapter 6** rural and urban Tanzanians and Dutch were vaccinated with the YF vaccine and followed up for 6 months. The findings showed that almost all Dutch vaccinees were high responders according to their level of IgG specific to the non-structural protein 1 of the yellow fever virus (anti-NS1 IgG) at day 178 post-vaccination, whereas in both the rural and urban Tanzanian cohorts only half of participants demonstrated a high response. This aligns with a previous study, showing that although neutralizing antibody (nAb) titers reached protective levels in both Ugandan and the Swiss, the titers were significantly higher in Swiss [21]. Unlike what we hypothesized, the proportion of high responders did not differ between the urban and rural Tanzanians. In a recent study in Uganda into population differences in vaccine responses (POPVAC), urban individuals demonstrated significantly higher nAb upon YF vaccination than helminth-endemic rural individuals 28 days post-vaccination and from malaria-endemic rural individuals 1 year after vaccination [22]. As neither malaria nor helminth infections were highly prevalent in our study area in Tanzania, exposure to these pathogens might account for the different findings in our study and the POPVAC study in

Uganda. However, in the POPVAC study preventive treatment for malaria or intensive praziquantel administration against *Schistosoma mansoni* did not improve the response to YF vaccine [23, 24]. Therefore, again, immune imprinting resulting from past infections rather than active infections, might be responsible for the observed reduced response to vaccination. Another possibility could be differential exposure to other environmental factors, including those that are tightly linked to these infections (e.g. malnutrition and/or gut microbiome composition).

To further understand the immunological mechanisms underlying variation in the response to yellow fever vaccination, the baseline innate immune profiles of high and low responders were compared. As the Dutch innate immune profiles at baseline were very distinct from that of Tanzanians and since Dutch individuals were more likely to be high responders, comparison of high and low responders was performed separately for the Dutch and the Tanzanian cohort. When PBMCs from low and high vaccine responding Tanzanians were cultured, a reduced frequency of cDC2s and expansion of CD1c- CD141- DCs was observed in low responders. These CD1c-CD141- DCs are known to be enriched for type 1 interferon signaling and to share signature with CD16+ monocytes [25]. Although the frequency of CD1c-CD141- DCs was increased in low compared to high responders, the IFN-alpha production by these cells was found to be significantly decreased, indicating a hampered type 1 interferon response in low responding individuals. Expansion of CD1c- CD141- DCs defective in their IFN signaling has previously been linked to reduced responses upon SARS-CoV-2 infection and this subset was shown to have high mitochondrial metabolism with reduced ability for immune cross-talk [26]. Given the large proportion of cells referred to as CD1c- CD141- DCs in the current study and the close resemblance of these cells with other cell types such and CD16+ monocytes, it is likely that the cell population identified in the current study comprises of multiple cell subsets. Therefore, additional analysis such as sub-clustering of this population is needed to further characterize these cells. Moreover, studying the transcriptome of these cells, by employing single cell RNA analysis, would give us insight in the pathways involved and how these can be modulated to restore their contribution to vaccine responses.

Differences in the monocyte compartment were noted between the geographical locations and within the Tanzanian cohort between the high and low responders. A rural-urban gradient in the proportion of non-classical monocytes with highest frequencies in the rural Tanzanians was seen, whereas classical monocytes showed the opposite trend. This aligns with a previous study, showing that the proportion of intermediate and

non-classical monocytes was increased in Africans compared to Europeans, whereas classical monocytes were highest in Europeans [27]. Comparison of the low and high responders within the Tanzanian cohort showed increased frequency of classical monocytes in high responding Tanzanians, both *ex vivo* and upon culturing and when urban and rural were analyzed separately. The study of Muyanja *et al.* (2013) reported a negative association between the total monocyte counts and the YF nAb within the Ugandan cohort [21]. When both Ugandan and Swiss vaccinees were combined, the frequency of intermediate monocytes as percentage of total monocytes was reported to be negatively associated with YF nAb, but this was clearly driven by the distinct responses between geographical areas. Given the importance of panels used in cytometry, gating strategies, as well as the problem of downregulation of markers upon monocyte culturing, the difference in our study compared to the Ugandan study should be interpreted with caution. It is important that a more standardized characterization of the monocyte compartment is agreed upon, to allow the comparison of different studies and to unravel the role of monocytes in the response to (YF) vaccination.

Together, these results show that upon receiving the yellow fever vaccine Dutch are more likely to demonstrate a high response, whereas no difference was observed between rural and urban Tanzanians. Comparison of the low and high responders within the Tanzanian cohort showed differences in the myeloid compartment at baseline, therefore the state of the innate immune system pre-vaccination could be an important predictor of responses post-vaccination. Additional analysis is needed to further characterize the monocyte and dendritic cell subsets and to unravel what pathways are involved and how these can be modulated in order to induce high vaccine responses in all individuals.

DIRECTIONS FOR FUTURE RESEARCH

The studies described in this thesis, characterizing differences in the immune system in populations from different geographical areas and socioeconomic backgrounds, have provided us with insights that advance the field and give direction to forthcoming studies. Further exploration of the differences in vaccine responses between and within urban and rural areas is needed to enhance our understanding of the immunological mechanisms underlying vaccine hyporesponsiveness. Moreover, studying immune compartments other than the peripheral blood, by using newly developed models, will shed light on the local immune response and will enable us to test new vaccines and interventions *in vitro*. Finally, combining research efforts such as the integration of high-dimensional immunological datasets and harmonization

of (experimental) protocols, will help us to synergize and accelerate the identification of feasible targets for interventions needed to overcome vaccine hyporesponsiveness.

Adaptive immune responses upon vaccination in rural and urban areas

Since both innate and adaptive immune features have been found to be predictive for the YF response [21, 28], characterizing the adaptive compartment of the cohort described in Chapter 6 would be insightful. Examining the adaptive cellular immune subsets and cytokine responses at baseline in relation to the antibody response can provide us with additional predictive signatures for the response to YF vaccination. Also, combining innate and adaptive features together might increase our predictive power. Next to antibody responses, strong T cell responses are induced by the YF vaccine and contribute to long-term protection induced by this vaccine [28, 29]. Increased YF-specific CD8+ T cell responses were found in Swiss compared to Ugandan Individuals upon vaccination and these YF-specific T cell responses strongly correlated with the neutralizing antibody titers. Moreover, a previous study investigating the phenotype of Ebola-specific T cell responses after vaccination found that in Senegalese individuals a greater proportion of these cells expressed CD57 and Killer cell lectin-like receptor subfamily 1 (KLRG1), markers for immunosenescence, and that the frequency of these cells negatively associated with IFN-y and IL-2 response to Ebola Glycoprotein [30]. To study the frequency and phenotype of antigen-responsive cells in our study cohort, intracellular cytokines, activation-induced markers (AIMs) and general activation markers (e.g. KLRG1) could be measured upon *in vitro* stimulation with a peptide pool containing the YF protein as previously demonstrated [31]. In addition, antigen-specific B cell responses can be studied using tetramers labeled with streptavidin as successfully employed previously in the context of malaria [32], however finding a suitable protein for preparation of these tetramers has proven challenging (personal communication R. Murugan). The insights obtained by studying the response to YF in detail will be valuable for understanding vaccine hyporesponsiveness of both YF and other vaccines, as similar immunological mechanisms are thought to underly the reduced responses to vaccines.

Studying the immune responses in tissues

Most studies, including the majority of those in this thesis, study the immune system in peripheral blood, since this is easily accessible and multiple collections over time are possible. However, obviously, tissue-specific immune responses would be even more insightful and differences in the

local immune responses between populations have been demonstrated such as the study in Chapter 3 of this thesis. With the use of novel methods to collect tissue samples and by employing organoid models that resemble the tissue, tissue-specific immune responses can be further explored and studied in detail. To further characterize the nasal immune response, nasal curettage can be performed to obtain epithelial and immune cells. Studying the response to the influenza vaccine using this method showed that influenza-specific cellular responses upon vaccination can be characterized successfully; these responses were diminished in healthy adults colonized with *S. pneumoniae* [33]. Another type of sample collection that has been proven valuable in studying the tissue-specific response is the collection of fine needle aspirates of lymph nodes. By taking serial fine needle aspirates of a single lymph node, the germinal center response to a vaccine can be studied over time, which has provided unique insights in the case of mRNA-based vaccines [34]. Moreover, organoids can be derived from tonsil tissue cultures which can provide a representative of a secondary lymphoid organ in which the immune response to vaccines can be studied over time [35, 36]. Indeed, one tonsil can generate a great number of organoid cultures and this model has already been used to systemically study vaccine responses [35]. Interestingly, insights in the immunological mechanisms underlying vaccine responses can be obtained by modulating the culture by adding for example pathway inhibitors. Finally, the contribution of individual immune cell subsets can be studied by adding these cells, which are autologous as peripheral blood mononuclear cells can be isolated from the same donor. Currently, tonsils from different geographical locations (including rural and urban Tanzania) have been collected and tonsil organoids of this material are currently in culture to compare the response to vaccines (personal communication M. Coppola). Future studies should consider employing these new methods to see whether local tissue-specific immune responses also differ between populations and, if so, what the implications are for the overall response to immune perturbations including vaccines.

Combining research efforts

Recent advancements in methods to capture high-dimensional data have resulted in a growing quantities of data, complicating the data analysis but also offering new opportunities. Given the high cost and low throughput of high-dimensional methods such as CyTOF, the number of individuals in such studies are often limited, which hampers the ability to find subtle differences and to characterize rare subsets but also to draw generalizable conclusions. Although integrating high-dimensional datasets is challenging, recent work shows that multiple CyTOF datasets can be successfully integrated and analysis of this combined dataset has led to the identification

of common immunological differences across the rural-urban gradient [37]. As the level of success of integrating datasets is largely determined by the alignment of protocols, employing identical panels and taking along similar reference samples will facilitate integration of datasets. We are planning such analyses for the CapTan and aforementioned POPVAC study, which have both examined the response to YF in rural and urban populations (personal communication G. Nkurununngi). Moreover, efforts such as the recently founded Hypovax Global Knowledge Hub (hypovax.org) aim to establish a collaborative network and allow members to share expertise, methodologies, advanced technology platforms as well as data, to obtain a better understanding of population-level variations in responses to vaccines.

CONCLUDING REMARKS

By studying the immune system of individuals from rural and urban areas and with different socioeconomic backgrounds within a single country, this thesis has contributed to a greater understanding of the environmental and immunological factors underlying the variation in vaccine response and disease prevalence in different populations. We have shown that the environment impacts the immune system and thereby shapes the response to antigens. By further exploring the response to immune perturbations between populations, future studies may lead to development of interventions needed to reverse detrimental immunological alterations and thereby improving health outcomes.

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