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Summarising discussion

In this thesis, immunological differences between populations have been investigated. Various aspects have been studied starting from differences in immunoglobulin G (IgG) glycosylation, continuing with immune profiling, and ending with immune responses to malaria infection in volunteers. During these studies, populations residing in Europe and those from low to middle-income countries have been compared for their immunological differences.

IgG glycosylation

In **Chapter 2**, the changes in IgG Fc N-glycosylation upon vaccination of European adults and African children were studied. Vaccination had no effect on glycosylation of total IgG1, but vaccine-specific IgG1 galactosylation and sialylation increased and the level of bisecting *N*-acetylglucosamine decreased after vaccination. Therefore, dependent on the time point after vaccination, the glycosylation of the produced antibodies will be different, which could influence the efficacy of the vaccine. As both galactosylation and sialylation are considered to have anti-inflammatory effects[1], their increase after vaccination might seem disadvantageous. However, high galactosylation is also associated with antibody-dependent cellular cytotoxicity (ADCC) through enhanced binding to FcγRIIIa receptors, which has been shown to be beneficial in case of influenza[2,3]. Furthermore, these changes in glycosylation were shown to result in the production of higher affinity antibodies and therefore better vaccine efficacy[4]. Thus, IgG glycosylation adds another level of optimisation in the development of an appropriate immune response, and is therefore an important factor to consider in vaccine design.

In **Chapter 3**, the question was addressed whether IgG Fc N-glycosylation varies in populations with distinct environmental exposures in different parts of the world. Total IgG1 galactosylation levels were generally lower in lower-income countries, in more rural communities, and in asthmatic children and were negatively correlated with IgE and C-reactive protein (CRP) levels and the prevalence of parasitic infections. Overall, this indicates that IgG1 galactosylation levels could be used as a biomarker for immune activation when studying different populations. This could be a useful tool for studies investigating immunological changes in the context of urbanisation, such as those described in this thesis. Indeed, the rural and semi-urban areas in Gabon and the European countries analysed in Chapter 3 are the same as those in Chapter 2, 4, 6, and 7 of this thesis, where many immunological differences were found between these populations. This shows that in future studies, the relative level of immune activation or urbanisation could be determined by comparing their IgG glycosylation levels.

As we have linked low IgG galactosylation to immune activity in Chapter 3, the increasing galactosylation after vaccination as seen in Chapter 2 might be surprising. However, as antibody glycosylation has been found to be determined by the local milieu of B cells[5], the

composition and dosing of the vaccine and the route of administration might result in a different microenvironment as compared to immune activity due to natural exposure. For example, the harmless nature of a vaccine will probably result in less damage and therefore less danger signals as compared to pathogenic infections. Although not specifically assessed, in our study, the Begrivac flu vaccine, which did not contain adjuvants, the Focetria flu vaccine containing MF59 as adjuvant, and the tetanus vaccine containing aluminium phosphate as adjuvant, all resulted in similar glycosylation changes. However, in a study using a trivalent influenza vaccine, antigen-specific glycosylation was specifically compared and found to be different between antibodies generated against the different influenza strains[6]. Furthermore, in a study comparing two human immunodeficiency virus (HIV) vaccines against the envelop glycoprotein gp120, the glycosylation profiles differed between the vaccine which was based on a combination of adenovirus vectors and the recombinant vaccine with alum as adjuvant, suggesting that not only the antigen determines the glycosylation but also the other components of the vaccine[7]. It is therefore important to study which adjuvants and types of antigens and vaccines are inducing specific glycosylation patterns that result in the best protection by a vaccine. So far, changes in antibody glycosylation after natural infection have not been studied extensively, but the lower total antibody galactosylation of Africans in our study and their expected higher exposure to microorganisms suggest that natural infection results in reduced galactosylation. This assumption is in accordance with the lower galactosylation seen in subjects with acute HIV infection as compared to healthy controls[8]. Thus, unless proven otherwise, vaccination and natural infection might result in opposing galactosylation changes, which are expected to affect antibody efficacy. However, these differences could also reflect a change in glycosylation between first exposure (priming) and a memory response (boosting or reencounter). It remains to be determined whether specific IgG glycosylation in vaccinated individuals will change from the anti-inflammatory vaccination profile which was beneficial for affinity maturation to a more pro-inflammatory profile during a memory response when challenged with the pathogen through natural infection.

European and African immune profiles

In **Chapter 4**, innate immune responses between European and semi-urban and rural African children were examined by comparing cytokine responses after *in vitro* stimulation of various classes of pattern-recognition receptors (PRR). Responses to toll-like receptor (TLR) ligands differed between Europeans and Africans, but not to non-TLR ligands or when synergy was assessed after stimulation with a combination of both. Upon stimulation with FSL-1 (TLR2/6), Pam3 (TLR2/1), and lipopolysaccharide (LPS) (TLR4), Africans had a stronger pro-inflammatory response as compared to Europeans, but a weaker pro-inflammatory response

upon polyinosinic-polycytidylic acid (poly(I:C)) (TLR3) stimulation, suggesting population- and pathogen-specific modifications of innate immune responses. Next, the adaptive immune profile of European and urban and rural African young adults was studied. As reported in **Chapter 5**, Africans had higher percentages of Th1, Th2, Th22, memory $CD4^+$ T cells, and memory B cells as well as higher activation levels of these cells than Europeans, while urban Africans were more similar to Europeans than rural Africans. In **Chapter 6**, both innate and adaptive immune responses were compared between European and urban and semi-urban African young adults. Compared to Europeans, $CD4^+$ T cells of Africans generated stronger and more pro-inflammatory responses, although their Th2 cells were hyporesponsive. Signalling in monocytes was reduced in Africans, but this was not reflected by differences in activation or cytokines responses of the monocytes. Urban Africans generally showed intermediate responses as compared to Europeans and semi-urban Africans.

Together, these chapters show large immunological differences between not only Europeans and Africans, but also between urban and rural Africans. Both innate and adaptive immune responses were found to be affected. Although a different genetic background might be partly responsible, the differences amongst urban and rural Africans show that environmental exposure plays an important role. Other studies have indeed shown that non-hereditable factors are responsible for a large part of immunological differences between populations[9,10]. A system-level analysis of twins found that 77% of parameters, including cell population frequencies and cytokine levels and responses, were dominated and 58% were almost completely determined by non-heritable factors[9]. The increase in variation of these parameters with age suggest a cumulative effect of environmental exposure[9]. Also vaccine responses seemed to be affected by previous exposure to vaccinations and infections, as the response to influenza vaccines were entirely determined by non-heritable factors, while responses to vaccines against diseases with little pre-exposure, such as for measles and hepatitis B virus, were largely heritable[9,11-13]. Exposure to infectious agents are known to result in trained immunity of innate immune cells as well as memory amongst adaptive immune cells, but besides infectious agents, exposure to different (compositions of) microbiota, diet, pollutants, and other environmental factors could play a role too[14,15]. For example, skin microbiota composition was found to differ between urban and rural Finnish children, and was influenced by age due to behavioural changes affecting contact to environmental microbiota[16]. The differences in skin microbiota were correlated with allergic sensitization of these children as well[16]. Hence, the immunological differences observed between human populations with different environmental exposure are likely to result in different responses to infections, allergens, and self-antigens as well as to vaccinations and treatments. These differences might also be able to explain the increased susceptibility of Europeans and other Western or urbanised populations for allergic and chronic inflammatory diseases. Therefore, further population-based studies are required to understand the mechanisms underlying

the differences in immune responses. This knowledge could then be applied to optimise population-specific preventative and curative treatments.

The European and urban and rural African groups did not always follow a clear urban/rural gradient in the assays of these chapters. As a twin study showed that innate immunity is mostly affected by the environment and adaptive immunity mostly by genetics, the largest differences between the urban and rural African groups are expected to be found amongst assays studying the innate immune system[10]. However, in Chapter 4, the innate cytokine responses to *in vitro* stimulation were not significantly different between children from semi-urban Lambaréné and rural Zilé in Gabon, while in Chapter 5, significant differences were often detected in the adaptive immune system between the urban and rural Senegalese adults. The environmental differences between the two groups in Gabon might have been too small to result in detectable immunological differences, while the environmental differences between the urban Senegalese group, consisting of laboratory personnel from a university hospital in the capital Dakar, and the rural Senegalese group, consisting of farmers from a village in northern Senegal, seem to have been larger. In Chapter 6, both innate and adaptive immune responses have been assessed and no significant differences were observed in adaptive immune responses between the urban and semi-urban Gabonese, although the urban Gabonese sometimes appeared more similar to the Dutch adults. However, when signalling in monocytes was compared, urban Africans were significantly more similar to Europeans than to their semi-urban counterparts. Thus, this confirms that the environment has a larger impact on the innate than the adaptive immune system.

The immunological differences between Europeans and Africans described in Chapter 4 were based on comparisons of schoolchildren while those in Chapter 5 and 6 were based on young adults. To learn more about the basis of these differences, various populations should be studied from birth to older age. Then, effects of genetic and *in utero* exposures can be determined at birth, while the effect of environmental exposure can be studied over time. Therefore, in addition to the data presented in Chapter 6, cord blood of newborns was collected from the same European and African regions as the young adults and will be analysed in the future. The analysis of the differences between European and urban and rural African cord blood, as well as between cord blood and young adults, should provide further insight into the factors that determine immunological differences.

Although these three chapters analysed separate aspects of the immune system and immunological responses, there is some overlap between the assays. In Chapter 4, whole blood was stimulated for 24 hours with LPS, which resulted in higher tumour necrosis factor (TNF) and similar interleukin-10 (IL-10) levels in the supernatant of African as compared to European children. However, in Chapter 6, 24-hour LPS stimulation of isolated monocytes did not show differences in the percentage or median fluorescent intensity (MFI) of TNF^+ and $IL\text{-}10^+$ monocytes between Africans and Europeans. Among other reasons, it could be that the number of monocytes was not equal between Europeans and Africans in the whole blood

assay or that TNF was also produced by other LPS-responsive cells, such as dendritic cells. This highlights that although whole blood assays are more easily applied in the field, the large differences in cell composition between Europeans and Africans require separate culturing of isolated cells and/or single-cell analysis such as by flow cytometry.

Natural immunity to malaria

After determining the European and African immune profiles, we addressed the question whether the *ex vivo* and *in vitro* differences in the immune response can be seen *in vivo*. In **Chapter 7**, the complete immune profile of Europeans and Africans was studied together with their immune response to controlled human malaria infection. Europeans and Africans, as well as relatively resistant and susceptible Africans, had a distinct immune signature, and also responded differently to infection. Whereas Europeans all became infected and developed symptoms rapidly, Africans, in general, controlled their infection better. However, it was interesting to note that even though there were Africans who had the same level of parasitemia as the Europeans, they did not develop symptoms as readily as Europeans. Thus, the immune system of Europeans and Africans was not only mapped at unprecedented detail, but the immune response to human malaria infection was also reported more comprehensively and in an unbiased manner than before.

The immune signatures for Europeans and Africans found in Chapter 7 were in accordance with the differences found in the preceding chapters, but also showed that the differences went beyond $CD4^+$ T cells, B cells, and monocytes, as differences in frequencies of subsets of $CD8^+$ T cells, natural killer T (NKT) cells, γδ T cells, and innate lymphoid cells (ILCs) were seen as well. Besides the different immune cell composition, it was observed that the individual cells themselves were different between Europeans and Africans. The African immune cells often responded more strongly and appeared more experienced based on memory, activation, and exhaustion markers, while also *in vitro* hyporesponsiveness amongst Th2 cells was noted. We confirmed that all of these *ex vivo* and *in vitro* differences seen in earlier chapters could culminate into differences in *in vivo* responses to an identical challenge. The earlier response of both innate and adaptive cells of Africans as compared to Europeans after *Plasmodium falciparum* sporozoite inoculation suggests that this was due to pre-exposure of Africans to the parasite. However, it is not clear how the generally stronger *in vitro* response of African $CD4^+$ T cells and Th2 hyporesponsiveness observed in Chapter 6 compare to other *in vivo* immune responses. Studies comparing different human populations are scarce and have sometimes given conflicting results. For example, $CD8^+$ T cell and B cell responses to yellow fever vaccination were lower in African than European individuals, which is in contrast to what would be expected from our *in vitro* data[17]. Furthermore, when comparing semi-urban

and rural African children from the same area as analysed in Chapter 6, the rural children had lower antibody responses to influenza vaccination but higher antibody responses to tetanus vaccination than the semi-urban children[18,19]. Thus, immunological differences between populations seem to result in antigen-specific differences in immune responses instead of overall hypo- or hyperresponsiveness. This indicates that the complexity of immune responses can depend on many factors, which can make specific predictions difficult. Although one thing that seems to be consistent is that cytokine responses against both vaccines showed a Th2 bias in the rural children[18,19].

Through this work, we also looked to better understand which responses are responsible for naturally acquired immunity against *P. falciparum*. Africans with varying degrees of immunity to malaria infection were compared and $CD4^+$ T cells (Th1 and Th17), effector memory $CD8^+$ T cells, $CD8^+$ NKT cells, a number of γδ T cell subsets, and plasmacytoid dendritic cells were found to be associated with immunity, while ILC2s and intermediate monocyte seemed to merely associate with susceptibility. Correlations between certain cell subsets also imply that some of these subsets are induced by the same mechanism or that they induce each other. Therefore, it might be necessary for malaria vaccine development to not only consider targeting adaptive cells, as innate immune cells might be required for optimal protection as well. Furthermore, these results show that although a part of the protective immune response might be taking place in inaccessible locations such as the liver, peripheral blood from volunteers does contain valuable information which should not be neglected.

In addition to controlled infection, in **Chapter 8**, longitudinal changes in γδ T cells and $CD4^+$ T cells were studied amongst children in Indonesia where malaria is endemic. Asymptomatic malaria infection was associated with higher percentages of γδ T cells and Th1 and Th17 cells, while TNF, interferon-γ (IFN-γ), and IL-17 responses to *P. falciparum*-infected red blood cells (PfRBCs) were similar and IL-5 and IL-13 responses lower. Furthermore, infected children had more PD-1^+ $CD4^+$ T cells, more Tregs expressing TNF receptor II (TNF-RII), and higher IL-10 responses to PfRBCs. As many changes in frequencies and immunoregulation of γδ T cells and $CD4^+$ T cells were long-lasting, subsequent infections could be affected. Our study was not large enough to be able to test if the frequencies and phenotype of these T cell subsets could predict whether children would become infected or remain uninfected, but such a comparison would provide further proof about the importance of these subsets in immunity.

The γδ T cells and $CD4^+$ T cells, associated with natural malaria infection in Indonesian children in Chapter 8, also responded to controlled infection in the African young adults in Chapter 7. First of all, the number of γδ T cells was higher in asymptomatic infected than in uninfected Indonesian children. In Africans, an increase in their numbers after infection was associated with relative resistance against parasitaemia. Secondly, the number of Th1 and Th17 cells was higher in asymptomatic infected than in uninfected Indonesian children. In Africans, these subsets and $CD4^+$ T cells in general responded upon infection as well, but the dynamics of these responses differed between relatively susceptible and resistant Africans.

Th2 cells on the other hand, were not associated with infection or resistance in the two studies. However, the downregulation of Th2 cytokines in the asymptomatically infected Indonesian children is remarkable in view of the hyporesponsiveness of Africans as compared to Europeans in Chapter 6. Although these Africans were not infected with *Plasmodium* at the time of the study, their exposure to the parasite throughout life might have resulted in long-lasting changes, as had been described for chronic helminth infections[3,20,21]. Continuing the comparison of Chapter 7 and 8, Tregs numbers were lower in asymptomatic infected than uninfected Indonesian children, and in Africans, a decrease in Tregs after infection was associated with resistance. Thus, while changes in γδ T cells and $CD4^+$ T cells were noted in Indonesian children upon infection, the comparisons made in Chapter 7 show that these cells were probably also contributing to resistance against malaria parasites, further strengthening evidence in literature on the involvement of these cells in immunity[22-24].

Conclusions

By studying immunological differences between populations, this thesis offers a new scope to enhance our understanding of the geographical differences in vaccine efficacies and prevalences of inflammatory diseases. Although the impact of the immunological differences requires further investigation and with larger numbers of participants, this thesis shows that the composition of both the innate and adaptive immune system differs between populations. Not only genetic differences, but especially environmental exposure seems to shape the immune system and could potentially alter immune responses to antigens encountered. Therefore, it might be necessary to develop population-specific vaccines and treatments against infectious, allergic, or chronic inflammatory diseases.

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