Development of the human fetal immune system: novel insights from high-dimensional single-cell technologies

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Chapter 6

Summarising discussion

It has long been thought that the human fetal immune system develops in a sterile womb without exposure to foreign antigens. Therefore, the developing immune system is considered to be immature. However, due to the challenge to obtain fetal tissues, much of our understanding of the developing fetal immune system is based on studies performed with cord blood collected from preterm or term babies. Moreover, in recent years, several lines of evidence have challenged the dogmas about both the sterile environment in utero and the immature status of the developing fetal immune system. With the introduction of advanced single cell analysis methods, novel opportunities have become available to characterize the heterogeneity and composition of the immune system with unprecedented resolution. In this thesis, I have taken advantage of this opportunity to analyze the human fetal innate and adaptive immune compartments in the spleen, liver and intestine during the second trimester. The results demonstrate an early-life immune compartmentalization across the tissues before birth. By applying advanced computational and visualization tools I was able to identify putative differentiation trajectories in the developing intestine, results that were corroborated by functional analysis in vitro.

Diversity in the innate immune compartment and identification of a novel ILC subset

To explore the heterogeneity and development of the immune system in the human fetal intestine, we used mass cytometry with a 35-antibody panel that was designed to capture heterogeneity in both the innate and adaptive compartments. In **chapter 2** we focused on the innate lymphoid cell (ILC) compartment. Here, 34 phenotypically distinct innate lymphoid cell clusters were distinguished, including previously identified NK and $CD127^+$ ILC subsets as well as several previously unrecognized clusters, providing evidence for extensive heterogeneity in the innate compartment. In particular, we identified a $Lin^-CD7^+CD127^-CD45RO^+CD56^+$ subset that by unbiased hierarchical clustering was positioned in between the known NK cell and $CD127^+$ ILC subsets, suggestive of potential developmental relationships with both the NK cells and $CD127^+$ ILCs.

The differentiation of ILCs has been well studied in mice whereas there are fewer studies on human ILC development[1, 2]. Also, a recent report demonstrated that human ILC development was distinct from the established murine model[3], which highlights the need to conduct research directly on human samples. The differentiation of cells or transition from one subset to another usually exhibits stepwise changes in the transcriptional program and protein expression profiles. t-SNE is exceptionally well suited to integrate such gradual changes for many

markers simultaneously and in Cytosplore we were able to visualize the t-SNE computation over time. Therefore, we made use of this approach to probe potential cell differentiation trajectories in the fetal intestine (**Chapter 2 and 3**). This provides compelling evidence that in all 7 intestinal samples the int-ILCs clustered in between and connected to both the ILC2s, ILC3s and NK cells. By designing a minimal flow cytometry panel based on the mass cytometry data we were able to identify and isolate the int-ILC by conventional flow cytometry. Furthermore, in functional assays we validated that int-ILCs can generate both NK cells and ILC3s in vitro (**Chapter 2**). Consistent with our findings, Chen et al. identified an intermediate ILC subset in human tonsil that could give rise to NK cells and ILC3s[3]. Similar to our $CD117^{+}CD94^{-}$ int-ILCs, this subset lacked the expression of CD34, CD127 and CD94, but expressed CD56 and CD117. Also, Chen et al. showed that the $CD56^{-}$ counterpart of this intermediate ILC could give rise to ILC2s. As ILC2s were prominent in the fetal intestine (about 5% of total ILCs) and were found to be connected with int-ILCs, we investigated whether the int-ILCs could differentiate into ILC2s as well. As we observed a sub-population of $CD8a^{-}$ int-ILCs expressing high level of GATA3, a masker transcriptional factor for ILC2 function and differentiation, we flow purified these $CD8a^{-}$ int-ILCs and stimulated them with ILC2 stimuli (IL-7, SCF, IL-2, IL-4, IL-25 and IL-33) in an OP9-DL1 co-culture system. However, under these conditions the cell surface phenotype of the int-ILCs remained unchanged. Further research is thus needed to clarify the putative relationship between int-ILC and ILC2.

ILC plasticity has been extensively addressed in both mouse and human, where certain ILC subsets will convert into other subsets after the appropriate cytokine stimuli[4, 5, 6]. As shown in **chapter 2**, all ILC subsets except ILC1s were identified in the human fetal intestine. Moreover, we observed a clear trajectory between ILC2s and ILC3s, suggesting that ILC2s may convert into ILC3s in utero as there is a virtual absence of ILC2s in the intestine after birth[7]. Furthermore, we noticed that the $CD8a^{-}$ int-ILCs-derived ILC3s and NK cells could partly revert their phenotype upon exposure to appropriate cytokine stimuli, suggesting that the int-ILCs may represent an intermediate subset between two plastic lineages. Importantly, the majority of the ILC subsets defined in the intestine including int-ILCs were not found in the spleen and liver as shown in **chapter 4**, indicative of specific functions in the mucosa. The factors in the tissue microenvironment that promote the differentiation of int-ILC into NK cells and ILC3s need to be further investigated in the future.

To extend the understanding of ILCs, the composition of ILCs in the fetal spleen and liver were determined in **chapter 4** and additional heterogeneity was

observed in several NK clusters compared with the fetal intestine, likely reflecting distinct functions in each tissue microenvironment. Furthermore, several new clusters were identified with this unbiased data-driven analysis approach, such as the CD8a^{+} ILC3s. This raises the issue of the biological significance of these and other clusters identified by high-dimensional mass cytometry. Therefore, it is important to validate these clusters and distinguish them functionally from previously identified subsets in future studies. However, our systemic and detailed studies of ILCs in fetal tissues have already advanced the understanding of ILC biology in human.

Memory formation in the human fetal intestine

One of the main features of adaptive immunity is the formation of immunological memory, where T cells play a critical role. Naive T cells recirculate through lymph nodes where they encounter peptide epitopes presented by major histocompatibility complex (MHC) class molecules on professional APCs such as DCs. After recognition of a given peptide-MHC complex, naive T cells proliferate and differentiate into effector cells, which migrate to the infection site and clear the pathogen. Thereafter the majority of the effector cells die, however, a small proportion survive as memory cells, which mediate the anamnestic immune responses upon encounter with the same pathogen. In the current thesis, we dissected the CD4^{+} T cell compartment in the human fetal intestine using an array of advanced single cell technologies, which revealed the presence of three major populations: T_N, T_M and T_{regs} (**Chapter 3**). Furthermore, with an array of computational tools including diffusion map, principal component analysis (PCA), Vortex and developing t-SNE, a linear differentiation trajectory was revealed. In this trajectory T_N cells clustered next to CD161$^{-/low}$ T_{CM} and CD161$^{-/low}$ T_{EM} cells, and the latter connected to CD161^{+} T_{EM} cells in both single cell mass cytometry and single cell RNA-seq datasets, consistent with CD4^{+} T cell memory formation in the fetal intestine. Moreover, pseudotime analysis revealed an up-regulation of TCR signaling associated transcripts at the end of T_N-T_M trajectory, which further underpinned the formation of memory T cells in the fetal intestine. These results are in line with a previous study that revealed a similar linear differentiation model of memory formation in circulating CD4^{+} T cells based on genome-wide profiles of DNA methylation, histone modifications and DNA accessibility[8]. A linear differentiation model was also observed in the human CD8^{+} T cell compartment[9, 10].

CD45RO is considered a marker for memory T cells, however, the majority of the CD4CD8 double-positive and 80% of the single-positive thymocytes are CD45RO^{+}

as well. Consequently, the occurrence of the isoform switching from CD45RO to CD45RA is considered one of the final steps in the maturation of T cells in the thymus[11]. This brings the possibility that the occurrence of CD45RO cells in the human fetal intestine might be due to thymic emigrants that have not switched from CD45RO to CD45RA. However, our mass cytometry analysis demonstrated that the T_M cells in the fetal intestine expressed CD161 and CD127, while these markers were not expressed by single-positive thymocytes in the fetus (Data not shown). Together with the abundance of CD45RA$^+$ T_N cells in cord blood and the observation that the fetal intestinal T_M cells readily produced cytokines, it is highly unlikely that the fetal intestinal T_M cells are derived from CD45RO thymocytes. However, the possibility that (a proportion) of the intestinal memory CD4$^+$ T cells originate from the mother still needs to be investigated through HLA typing or genotyping.

In the absence of foreign antigen exposure, the generation of memory-like T cells has been reported in mice. These antigen-inexperienced memory-like T cells harbored two main subsets: "innate memory" T cells and "virtual memory" T cells. The development of the former subset depends on IL-4 and thymic PLZF$^+$ cells, whereas the latter need IL-15 and CD8a$^+$ DCs[12]. However, these antigen-inexperienced memory-like T cells were found predominantly in the CD8$^+$ T cell compartment[12] and only few studies reported the formation of such T cells in the CD4$^+$ T compartment[13, 14]. While in man CD45RO$^+$ memory-like T cells have been identified in the fetal spleen[15, 16], these cells were able to proliferate upon stimulation with IL-2 but not after CD2 or CD3 cross-linking, indicative of an anergic state[16]. Therefore, these cells are distinct from the CD45RO$^+$ memory-like cells that we identified in the human fetal intestine (**Chapter 3 and 4**), as these readily produced IL-2, IFN-γ, IL-4, granzyme B and large amounts of TNF after stimulation.

Migratory DCs were found to be virtually absent before week 16 of gestation in the human fetus[17]. Consistent with this previous report[17], our single-cell RNA-seq analysis of fetal intestinal cells identified a cluster of APCs, which displayed high expression levels of genes encoding HLA-DR, CD74 (HLA-class II invariant chain), CD80, CD86 and chemokine receptor CCR7. As CCR7 mediates DCs migration to lymph nodes in adults[18], this would be compatible with priming of naive T cells in the mesenteric lymph nodes followed by homing of the resulting memory T cells to the lamina propria after week 16 of gestation. Also, we observed clonal expansion of intestinal T_M cells. Together, our data in **chapter 3** provide strong evidence for the formation of the bona fide memory CD4$^+$ T cells in the developing human fetal intestine in utero.

Nature of the antigens

The formation of memory T cells in the human fetal intestine (**Chapter 3**) suggests that this occurs due to exposure to (foreign) antigens. Strikingly, a marked clonal overlap in CDR3 amino acid repertoires was observed between memory $CD4^+$ T cells isolated from different fetal intestinal samples, suggesting exposure to similar antigen(s).

It is well established that maternal cells can cross the placental barrier and reside in the human fetal tissues from the second trimester onward during pregnancy, a phenomenon defined as "microchimerism" that can persist throughout adulthood[19]. In addition, it has been reported that non-inherited maternal antigens (NIMAs) promote the differentiation of human fetal $CD4^+$ T cells[19], presumably due to the direct contact between the fetal immune system and the semi-allogeneic maternal cells. Thus, some of the memory $CD4^+$ T cell responses in the fetus may result from this. However, as the memory formation was most pronounced in the fetal intestine and not in the fetal spleen and liver, it is highly unlikely that this is solely due to exposure to NIMAs.

It is now well established that the microbiota plays an important role in the development and shaping of the function of the immune system. However, in fetal life the infant has been thought to be protected from exposure to foreign antigens[20], a dogma that has been challenged by the detection of microbiota in the placenta[21], amniotic fluid[22] and meconium[23]. Although it is highly unlikely that there will be an abundance of viable microbes that pass the placental barrier, it is conceivable that microbial-derived antigens derived from the maternal intestinal microbiota will reach the amniotic fluid and as such reach the lumen of the fetal intestine where they could prime $CD4^+$ T cell responses. In addition, it has been reported that infection with cytomegalovirus is one of the most common causes of congenital infection and that cytomegalovirus-specific T cell responses can be detected in early life[24, 25], which might also lead to the priming of T cell responses in fetal life. In addition, allergen-specific T cells have been detected in cord blood[26, 27] and antigen-specific fetal T cells have also been detected in children whose mothers were infected with human immunodeficiency virus (HIV)[28], hepatitis C virus (HCV)[29] and malaria[30]. However, in our current studies, we used materials from fetuses obtained from mothers with a healthy pregnancy making it unlikely that the formation of memory T cells is driven by HIV, HCV or parasites. Rather we speculate that the memory $CD4^+$ T cells in the fetal intestine are specific for microbial antigens derived from the mother. Therefore, we are currently investigating if intestinal T cell lines can be generated from fetal samples that are

specific for such microbial antigens. For this purpose, HLA-typed fetal intestinal cells are cultured with lysates of relevant intestinal bacterial strains and propagated with cytokines and repeated antigen stimulation in the presence of HLA-matched antigen presenting cells. The specificity of such primary cultures and T cell clones derived thereof will subsequently be determined in cell proliferation and cytokine secretion assays. It is tempting to speculate that the composition of the microbiota of the mother may determine the imprinting of the mucosal immune system in the fetus already at this early age and that this may have consequences later in life.

Although the frequency of Tregs identified in our studies (**Chapter 3 and 4**) is lower than expected, we did observe clear clonal expansions in the CD45RO$^+$ T_{regs} compartment and a separate differentiation trajectory of the T_{regs} compared with T_M cells in the intestine (**Chapter 3**). Thus, the relationship between these cell subsets and the nature of the antigens that drive the expansion of the CD45RO$^+$ T_{regs} warrants further investigation.

In this respect it is interesting that Glanville et al. developed an elegant algorithm termed GLIPH (group of lymphocyte interactions by paratope hotspots), which can cluster TCRs that have a high probability of sharing specificity, taking both conserved motifs and global similarity of complementarity-determining region 3 (CDR3) sequences into account[31]. As fetal intestinal T cells will be exposed to a large repertoire of microbes upon birth, it will be of interest to apply this algorithm to our TCR-sequencing dataset as a means to identify commonalities in the TCR repertoire, which may point to the specific antigens that drive the formation of the T_M and CD45RO$^+$ T_{regs}.

Immune compartmentalization across and within tissues

It is well established that the anatomical tissue location of T cells is of great importance for their T cell function in both mice and man[32, 33]. Recently, Farber et al. demonstrated early-life compartmentalization of human T cells with abundant naive T cells in the pediatric blood and almost all tissues and abundant effector memory T cells in pediatric lung and small intestinal tissue only[34]. Here, we utilized high-dimensional mass cytometry to profile the immune landscape in fetal intestine, spleen and liver (**Chapter 4**) with a 35-antibody panel. In line with the previous study, fetal intestines harbored memory-like T cells, whereas most T cells in fetal spleens and livers displayed a naive-like phenotype, indicative of T cell compartmentalization as early as the second trimester. Moreover, mass cytometry analysis of fetal DCs derived from spleen, thymus, lung and intestine revealed substantial heterogeneity between these tissues, suggestive of different tissue

imprinting[17]. Very recently, Yudanin et al. have reported that the tissue location differentially impacted the composition of ILCs in human as well[35], supporting our finding of site-specific ILC signatures across fetal tissues. Our study in chapter 4 was one of the first to show the existence of the early-life compartmentalization of the immune cells across tissues within almost each immune lineage in utero, which suggests different immune responses in situ and further highlights the importance of tissue environment on the development of immune cells. Moving on from the fetus to the adult, our study in **chapter 5** also provides evidence for the regional specialization within the intestinal immune system in adults. Therefore, the integrated high-dimensional analysis provides a global and deeper understanding of the entire immune system and is in line with a local and tissue-specific immune signature.

Functionality of the clusters

The number of phenotypically distinct immune clusters has increased significantly due to the introduction of high-dimensional single-cell techniques such as mass cytometry and RNA-sequencing. This raises the question to what extent such clusters represent functionally distinct entities. In this thesis, clusters were distinguished by differential expression of at least one protein or gene. In **chapter 2 and 3**, 34 and 22 phenotypically distinct clusters were revealed in the intestinal ILC and $CD4^+$ T cell compartment based on the marker expression profiles, respectively. To allow further functional studies with the identified clusters, a minimal marker gating strategy was designed to identify and isolate clusters of interest by conventional flow cytometry. By applying this approach, we demonstrated that the int-ILCs can differentiate into NK cells and ILC3s in vitro and the differentiation trajectory and cytokine production profiles supported the observation of the generation of intestinal memory T cells in the fetus. Therefore, this proved to be an useful approach where mass cytometry is used as an exploratory tool leading to novel hypotheses that can be further tested in functional assays (**Figure 1**).

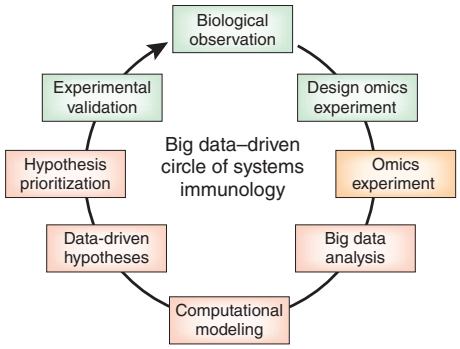

Figure 1 Big data–driven circle of systems immunology. Big dataset can be used to generate novel hypotheses in an unbiased data-driven fashion for further experimental validation using the classical approaches such as loss- and gain-of-function experiments, different murine models and other functional assays with flow cytometry. Adapted from Schultze (2015).

Interestingly, the int-ILCs were a main contributor to the separation of non-affected inflammatory bowel disease (IBD) (control biopsies) and affected IBD biopsies that contained NK cells and ILC3s (**Chapter 5**). Thus, the int-ILC may give rise to NK cells and ILC3s in the intestine in the inflammatory context. Compared with the phenotype of intestinal ILCs in adult, fetal intestinal ILCs mainly expressed CD45RO, a marker expressed by memory T cells. As it has been reported that $CD45RO^+$ ILC3s produced higher amounts of IL-17, IL-22, GM-CSF, TNF and IL-8 than the $CD45RA^+$ ILC3s[36], the function of these $CD45RO^+$ ILCs need to be investigated in the future. Consistent with previously finding[37], we did not find IL-17A-producing $CD4^+$ T cells in the fetal intestine, indicating that the generation of such cells takes place at a later time point and is associated with the post-natal exposure to the microbiota. Of note, a subgroup of the memory-like $CD4^+$ T cells expressed CCR6 and CD117. As the former marker is associated with Th17 cell differentiation, these cells may be the precursor for the Th17 cells. Alternatively, the expression of CD117 may indicate that these cell represent a reservoir of memory cells that can rapidly expand when stem cell factor is released. Thus, the function of these cells warrants future investigation. In addition, the intestinal $CD4^+$ T cells produced not only Th1- and Th2-type cell cytokines, but also IL-2, granzyme B and TNF. Recently, it has been shown that low numbers of TNF^+CD4^+ T_{EM} cells promoted intestinal epithelial development, whereas high numbers impaired the epithelial development[38], pointing to a non-immune function of these cells. However, these cells can also mediate an intestinal inflammatory response in preterm babies that may contribute to the necrotizing enterocolitis[38]. Thus, tight regulation of these memory $CD4^+$ T cells is crucial already at a very early age, which may be reflected by the prominent T_{reg} compartment in the developing fetal intestine. Together, these data illustrate that studies on the early-life immune system can potentially contribute to the understanding of derailed immune-associated diseases later in life. With the identification of the 177 and 142 immune clusters in different fetal tissues and in the intestinal biopsy and PBMCs through the unbiased data-driven approach, respectively (**Chapter 4 and 5**), there is a clear need to further determine the functional significance of these clusters in future studies.

Spatial distribution of the immune subsets in tissue microenvironment

Next to cell types, the cellular organization and cell-cell interactions play an important role in the maintenance of the appropriate tissue function. The tissue architecture and relationships between cells are lost when using mass and flow cytometry, however, imaging-mass cytometry breaks this limitation, as it can measure up to 40 markers in a single tissue section at 1 µm resolution, which thus

allows for an in-depth analysis of tissue structure[39]. We were one of the first to use imaging-mass cytometry in the Netherlands. Using this promising technique, the co-localization of the $CD4^+$ T cells with HLA-DR^+CD163^+ APCs and $CD127^+$ ILCs was revealed in the fetal intestine, which suggests functional interactions between these types of cells in the developing fetal immune system (**Chapter 3 and 4**). With the identification of a large number of phenotypically distinct cell clusters in both the innate and adaptive immune compartment within and across tissues, we were able to visualize the early-life immune compartmentalization in the fetal tissues in situ by using imaging-mass cytometry (**Chapter 4 and Figure 2**). Therefore, imaging-mass cytometry provides an unique opportunity to determine immune heterogeneity and cell-cell interactions in situ, providing another layer of understanding of the immune system. Furthermore, imaging-mass cytometry makes it possible to investigate issues that were raised in observations made in the current thesis. For example, we observed that int-ILCs can differentiate into NK cells and ILC3s during co-culture with stromal cells (OP9-DL1) in vitro (**Chapter 2**). Consequently, better understanding of the neighborhood of int-ILCs may help to put this observation in the tissue context. Also, we expect that the determination of spatial location of the distinct $CD4^+$ T_M cell clusters and the interaction of these memory T cells with stromal cells and other innate or adaptive immune cells in the human fetal intestine will shed light on the cellular interactions underlying the generation of various $CD4^+$ memory T cells populations in utero. In this respect, it will also be very interesting to study the development of fetal lymphoid structures like Peyer's patches. Finally, the intestine has been considered as "the second brain" in our body and it has been reported that ILCs are adjacent to mucosal neurons[40, 41], so application of the imaging-mass cytometry on the human fetal intestine can provide more insights into the development of the mucosal neuronal network and its interactions with the developing immune system.

Imaging-mass cytometry data can be visualized by an overlay of individual makers as in traditional immune fluorescence staining. However, the limited number of markers that can be visualized simultaneously is not compatible with the complex multiplexed imaging-mass cytometry data. Up to recently, only few tools have been developed to analyze imaging-mass cytometry data, such as CellProfiles[42] and histoCAT[43], which mainly work at the cellular level. Therefore, the analysis of imaging-mass cytometry data is still a big challenge in this field. Besides the development of the Imacyte toolkit (accepted), which facilitates the analysis of imaging-mass cytometry data at the cellular level, in collaboration with the bioinformaticians in the LUMC and TU Delft we have extended this analysis to the pixel level (**Figure 2**) using another in-house developed software tool (Cytosplore Imaging, unpublished), which allows an in-depth exploration of the data at the

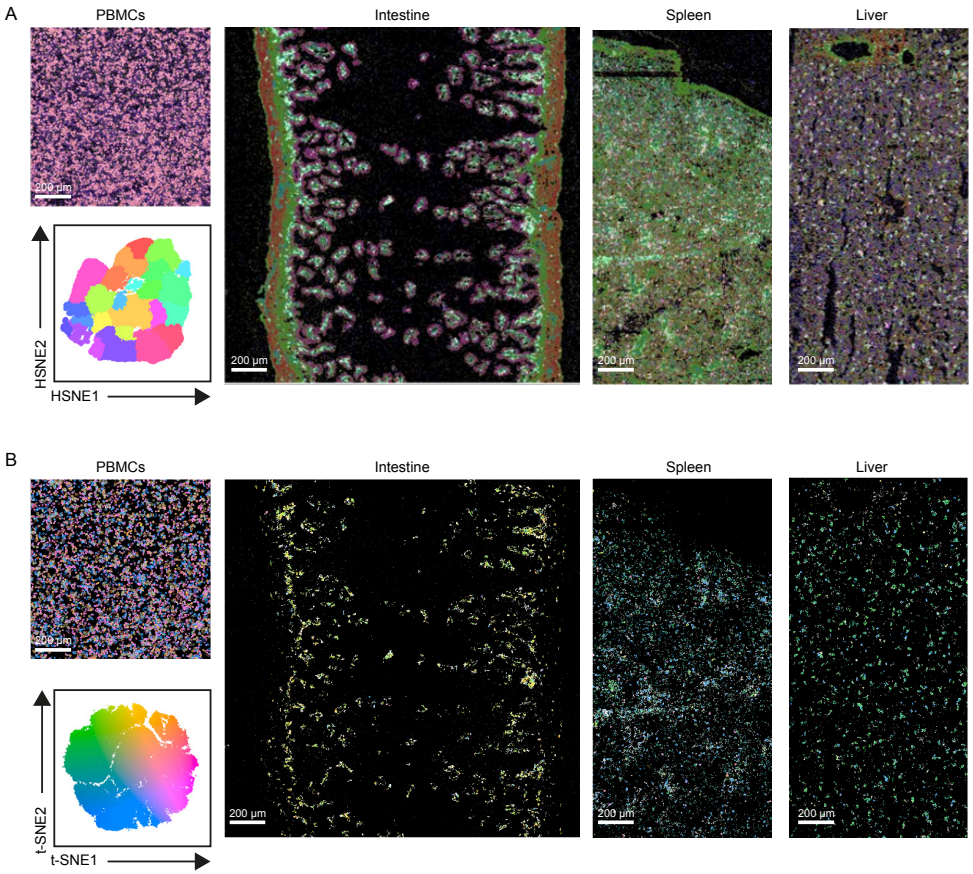

Figure 2 Spatial distribution of the entire immune cells reveals the early immune compartmentalization across tissues *in situ*. (A) Pixels derived from PBMCs (1ROI), fetal intestine (4ROIs), fetal spleen (2ROIs) and fetal livers (2ROIs) based on the expression of E-cadherin, Vimentin, SMA, D2-40, collagen I and CD45 Cytosplore Imaging software (unpublished). This yielded distinct color-coded clusters of pixels that were projected back onto the original images to obtain a global overview of the tissue architecture, reflecting the overall morphological differences between the tissues. Representative mass cytometry images of the PBMCs control, a fetal intestine, spleen and liver, showing the spatial distribution of the immune and stromal clusters. HSNE embedding of all the pixels derived from the PBMCs control, a fetal intestine, spleen and liver. Colors represent the different cell clusters. Scale bar: 200 µm. (B) The similar analysis were performed on the $CD45^+$ pixels based on the 26 immune makers. Representative mass cytometry images of the PBMCs control, a fetal intestine, spleen and liver, showing the spatial distribution of only the immune cell pixels. t-SNE embedding of the $CD45^+$ pixels derived from the PBMCs control, a fetal intestine, spleen and liver. Colors indicate the XY-coordinates of the t-SNE plot. Scale bar: 200 µm.

subcellular level. Currently, we are integrating the analysis at the pixel level with that at the cellular level in the tissue context. In the future, we would like to integrate the imaging-mass cytometry data with mass cytometry and RNA-seq

data. Eventually, a 3D image of the fetal tissues could also be reconstructed by using a series of tissue sections, in which the entire network between immune cells and surrounding stromal cells could be visualized in the tissue microenvironment.

Although there are many advantages to imaging-mass cytometry, a fundamental limitation is the speed of acquisition: 1 mm^2 in 2 hours. This hampers the identification of rare cell populations such as the int-ILCs and $CD117^+$ T cells in the tissue samples. To overcome this limitation, we are currently developing 7-color Vectra antibody panels to detect int-ILCs in sections to allow preselection of regions of interest (ROIs) in a serial section for analysis with imaging-mass cytometry. Such an approach can also be applied to tissue samples from patients where there is substantial heterogeneity in the cell distribution patterns, such as in tumors or inflamed intestine. Finally, imaging-mass cytometry can also be used as an explorative tool to identify disease-specific clusters in the tissue context. This can form the basis for the development of a targeted Vectra or immunochemistry antibody panel to detect such disease-specific features for diagnostic and/or prognostic purposes.

System immunology

System immunology is a new approach, where the immune system is investigated by integrating the results of a variety of approaches including single-cell mass cytometry, (single-cell) transcriptomics, TCR and BCR repertoire sequencing, metabolomics, proteomics, multiplexed imaging and epidemiology[44]. In **chapter 3**, we used this approach by combining results obtained from single-cell mass cytometry, with single-cell RNA sequencing, TCR sequencing, flow cytometry and imaging-mass cytometry, to investigate the $CD4^+$ T cell compartment in the human fetal intestine. Here, all techniques used reinforced the conclusion that memory-like $CD4^+$ T cells are generated in the human fetal intestines in utero. Such a multidisciplinary systems immunology approach is particularly useful for studies on the human immune system as there are very limited possibilities for experiments to determine causal correlations in vivo. Importantly, for such a cooperative approach, a multi-disciplinary team of clinicians, technicians, bioinformaticians and immunologists is crucial to the success. Continuous integration of novel technologies will further enhance the power of this approach. For example, at the time we were conducting our studies, Cite-seq to quantify the expression of proteins and transcripts simultaneously at the single-cell level was not yet available so we had to rely on indirect approaches to distinguish between $CD45RA^+$ and $CD45RO^+$ T cells. Thus, with the development of more advanced techniques such as the third generation RNA sequencing, imaging-mass cytometry

and the scalable, interactive and user-friendly computational tools, the system-immunology approach will provide us an unique opportunity to determine how the complex immune system operates in the tissue niche in health and disease.

A prenatal window of opportunity?

The early postnatal period is considered a period in which environmental factors strongly affect the development of the immune system, which is likely linked to protection or susceptibility to a variety of immune-mediated diseases later in life[45]. In several studies, the impact of environmental factors on the fetal immune system have been investigated. Van de Pavert et al. reported that there was a significantly reduction of the lymph size and a decrease of the immune response efficiency in offspring that was fed a vitamin A-deficient diet[46]. Furthermore, an elegant study has shown that the maternal microbiome reprogramed the intestinal transcriptional expression profiles and shaped the immune composition of the offspring[47]. Also, allergen-specific T cell reactivity has been found in cord blood as early as week 23 of gestation[26, 27] and, as mentioned above, antigen-specific T cells have been identified in fetuses whose mothers were suffering from human cytomegalovirus (HCMV)[25, 48, 49], HIV[28], hepatitis C virus[29], malaria[30], Cruzi[50] and toxoplasmosis[51] infection during pregnancy. Consistent with these findings, in the present study, tissue-resident memory-like $CD4^+$ T cells have been identified in the fetal intestine whose mothers were with the normal pregnancy as early as the second trimester (**Chapter 3**). Moreover, DCs were identified in the human fetal intestine (**Chapter 3 and 4**), which may mediate T cell priming[17]. Also, intestinal NK cells readily produce granzyme B and perforin, suggesting functional maturation of NK cells in the developing fetus as well (**Chapter 2**). The formation of memory $CD4^+$ T cell responses is accompanied by the appearance of $Foxp3^+$ Tregs in several fetal tissues, the fetal intestine in particular (**Chapter 3 and 4**). In addition, it has been shown that fetal DCs strongly induced the production of Tregs[17]. Finally, several B cell clusters were $CD20^+CD27^{int}CD38^+$, indicative of a regulatory B cell phenotype, in the fetal tissues. As such it is likely that the fetal immune system can be "trained" by the maternal-derived environmental factors before birth, which may provide an opportunity for the development of preventive strategies for the development of immune-mediated diseases such as celiac disease, inflammatory bowel diseases and allergy by influencing the balance between the development of memory $CD4^+$ T cells and $Foxp3^+$ Tregs in utero.

Conclusions and future perspectives

Our studies provide a global, comprehensive and detailed description of the fetal

immune system during healthy pregnancy by integrating an array of advanced high-parameter single-cell techniques. We further determined the function of several identified cell clusters and identified a novel intestinal ILC subset that can give rise to NK cells and ILC3s in vitro (**Figure 3**), which adds another layer of understanding of ILC differentiation and plasticity. The full differentiation potential of this new subset needs to be explored in the future. What's more, we revealed the generation of memory-like $CD4^+$ T cells in the developing human fetal intestine (**Figure 4**), indicating the exposure to antigens in utero. Determination of the nature of the antigens, as well as the function of these memory-like T cells would be crucial for understanding the transition from the relatively "clean" womb to the relatively "dirty" external world. Additionally, the observation of site-specific immunity highlighted the importance to investigate the immune system in the tissue niche. The crosstalk between immune cells and surrounding stromal cells can now be determined using imaging-mass cytometry, which can be integrated with other omics data as well. Next to the immune landscape of the fetal spleen, liver and intestine that we have described, it will be relevant to investigate the composition of the developing immune system in other fetal lymphoid and non-lymphoid tissues. In particular, comparisons of the fetal immune system at several time points in gestation and the neonatal period would be worthwhile as this will give a global picture of the development of human immune system and fill the gaps between prenatal and neonatal immunity. Overall, the results presented in the current thesis deepens our understanding of prenatal immunity and may ultimately be useful for the development of "early" intervention strategies to prevent the development of immune mediated diseases later in life.

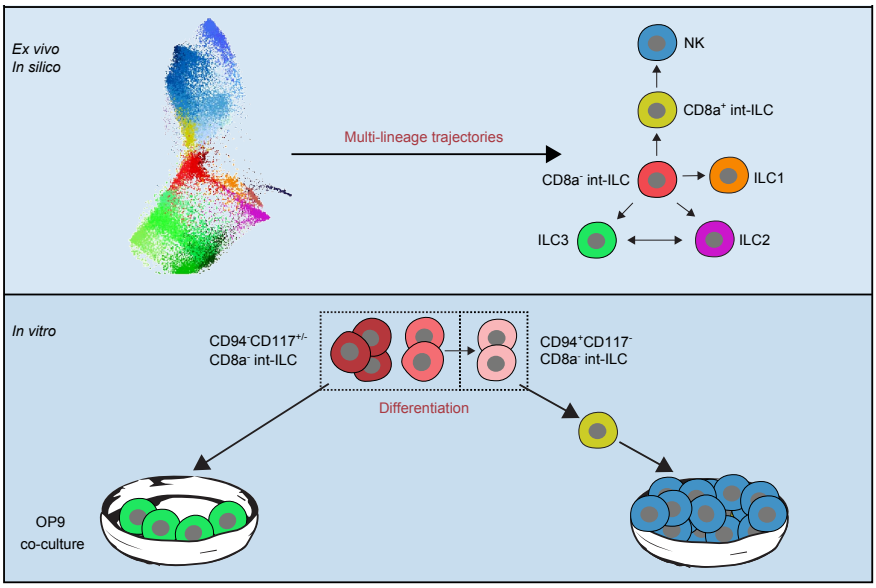

Figure 3. Differentiation of int-ILCs. Monitoring t-SNE computation dynamics predicts the potential differential trajectories *in silico* (upper panel). Upon co-culturing with OP-DL1, the int-ILCs can give rise to NK cells (blue) and ILC3s (green) (bottom panel).

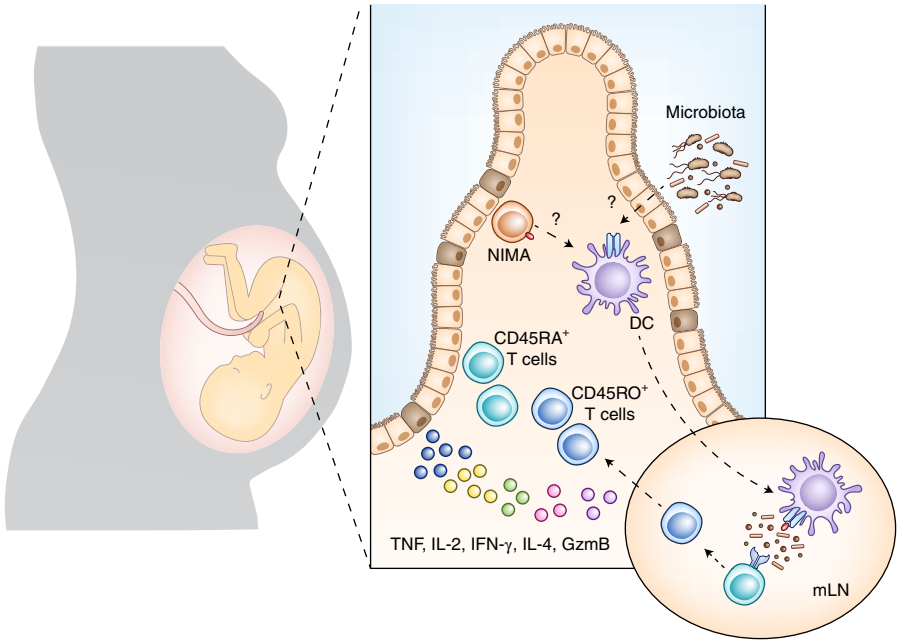

Figure 4. Memory generation of CD4⁺ T cells in the human fetal intestine. Phenotypically distinct naive (CD45RA⁺) and memory-like (CD45RO⁺) CD4⁺ T cells reside in the human fetal intestine at the second trimester during healthy pregnancy. The naive CD4⁺ T cells can produce TNF and IL-2, whereas memory-like T cells can produce not only TNF and IL-2, but also IFN-γ, IL-4 and granzyme B (GzmB), indicating functionality of the CD4⁺ T cell compartment. The identification of resident DCs and migratory DCs in the intestinal lamina propria and mesenteric lymph nodes (mLNs) by the second trimester prompted us to speculate their role in the generation of functional fetal memory-like CD4⁺ T cells. Although commensal microbe-derived antigens or NIMAs have been thought to be important in the memory generation of fetal intestinal T cells, the antigen specificity of these fetal memory-like CD4⁺ T cells remains unidentified. Adapted from Lim et al. (2019).

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