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

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English summary

Fetal immune system

The immune system provides protection against pathogens such as bacteria, viruses, parasites and fungi. It comprises a cellular and humoral compartment. The former is classically divided into the innate and adaptive immune compartment. Innate immunity provides a crucial first line of defense mediated by a swift, general and non-specific response to the invader. The innate compartment consists of various types of phagocytic granulocytes, myeloid cells and innate lymphoid cells (ILCs). Simultaneously, the adaptive immune system will mount a more tailored and specific response through antigen-specific receptors-expressing B and T lymphocytes, the latter including helper $CD4^+$ T cells and cytotoxic $CD8^+$ T cells. Ultimately, the combined innate and adaptive will lead to suppression or elimination of the invading pathogen and the generation of immunological memory that provides long-lasting immunity to the pathogen.

The fetal immune system develops during pregnancy and it has to remain tolerant to the semi-allogeneic environment while being prepared for the immense exposure to foreign antigens directly upon birth. As the largest immune compartment in our body, the intestine is continuously exposed to antigens derived from both harmless dietary constituents and potential harmful (commensal) bacteria. For this, an elaborate intestinal immune system has evolved to distinguish the harmful from beneficial antigens. Although a few studies have reported the identification of several immune cell subsets in the fetal tissues such as memory T cells in spleen and intestine, due to the scarcity of the fetal tissues and technique limitations, most investigation of the fetal immune system are based on cord blood collected at birth. Therefore, a system-wide and detailed phenotypical characterization of the composition and development of the human fetal immune system, especially the intestine was lacking. In the current study, we have approached this issue by making use of recently introduced single cell technologies, including (imaging-) mass cytometry and single-cell RNA-sequencing.

(Imaging-) Mass cytometry

Mass cytometry is a new platform that couples flow cytometry with mass spectrometry. This technique uses rare earth metals as reporters instead of fluorophores, which removes the limitation on the number of antibodies that can simultaneously be used due to the spectral overlap of the fluorochromes, and eliminates the problem of autofluorescence. Through mass cytometry up

to 42 markers can now be used simultaneously at single-cell resolution with minimal overlap between channels. Therefore, mass cytometry now provides the opportunity to study the diversity and heterogeneity of the immune system with an unprecedent high resolution.

To gain spatial information *in situ*, imaging-mass cytometry has been developed recently as a new technology, which couples a laser ablation system with a mass cytometer. In this way, it enables the visualization of dozens of markers on the same tissue section simultaneously with a sub-cellular resolution of 1 μm. Therefore, imaging-mass cytometry further offers an opportunity to gain unprecedented insight into the organization of the immune system *in situ*.

Single-cell RNA-sequencing

As a complementary approach to mass cytometry, single-cell RNA sequencing dissects the gene expression profiles on tens of thousands of individual cells at the single-cell level. As such, it provides an opportunity to determine the cell transcriptomic heterogeneity and the discovery of rare cell populations. Moreover, it facilitates the identification of potential cellular differentiation trajectories and regulatory networks among cells at the gene level.

Heterogeneity and differentiation of innate lymphoid cells

In **Chapter 2** we explored the heterogeneity and development of the innate lymphoid cell (ILC) compartment in the fetal intestine using mass cytometry with a 35-antibody panel. 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. Moreover, by visualizing the dynamics of the t-SNE computation, we identified smooth phenotypic transitions from cells within the $Lin^-CD7^+CD127^-CD45RO^+CD56^+$ (int-ILCs) cluster to both NK cells and $CD127^+$ ILCs, revealing potential differentiation trajectories. Finally, in functional assays, we validated that the int-ILCs can indeed give rise to both NK cells and ILC3s.

Memory formation in the human fetal intestine

In **Chapter 3** we combined mass cytometry with single-cell RNA-sequencing, TCR-sequencing, imaging-mass cytometry and flow cytometry to dissect the $CD4^+$ T cell compartment in the human fetal intestine. Here, mass cytometry identified 22 phenotypically distinct clusters, including naive-like, memory-like and regulatory-

like subpopulations, which were confirmed and further characterized at the transcriptional level. Moreover, we observed that the memory-like $CD4^+$ T cells readily produced the cytokines TNF, IFN-γ and IL-2. Furthermore, the integrated single-cell analysis revealed a robust memory differentiation trajectory. Finally, clonal expansion and co-localization of memory-like $CD4^+$ T cells with APCs further supported the concept of the generation of memory-like $CD4^+$ T cells in the human fetal intestine, suggesting exposure to foreign antigens *in utero*.

Site-specific immune signatures across and within tissues

To further extend the understanding of the fetal immune system, in **Chapter 4** we applied mass cytometry to profile the innate and adaptive immune compartment in the fetal intestine, spleen and liver. Here, we identified 177 phenotypically distinct clusters including both previously identified and novel cell clusters. PCA analysis indicated substantial differences between the composition of the immune system in the different tissues. Moreover, imaging-mass cytometry further underpinned the distinctness of the immune system in the tissues *in situ.* Thus, our results provide evidence for early-life compartmentalization of the immune system in fetal spleen, liver and intestine.

In **Chapter 5**, we applied the mass cytometry to analyze the composition of the mucosal immune system of patients with inflammatory intestinal diseases and controls. Here, we revealed previously unrecognized heterogeneity in the mucosal immune system and tissue- and disease-specific immune subsets.

Conclusion

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*, which adds another layer of understanding of ILC differentiation and plasticity. Moreover, we revealed the generation of memory-like $CD4^+$ T cells in the developing human fetal intestine, indicating the exposure to antigens *in utero*. Additionally, our results provided evidence for the early-life immune compartmentalization across tissues as early as second trimester, which highlights the importance to investigate the immune system in the tissue niche. Together, our studies deepens our understanding of prenatal immunity and may provide a prenatal window of opportunity for the development of preventive strategies for the immune-mediated diseases later in life.