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

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Chapter 7

Appendices

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.

Nederlandse samenvatting

Het foetale afweersysteem

Het afweersysteem beschermt ons tegen pathogenen zoals bacteriën, virussen en schimmels. De cellulaire component van het afweersysteem bevat twee compartimenten, het innate en het adaptieve compartiment. Het innate afweersyteem bevat diverse phagocyterende cellen, antigeen presenterende cellen en lymphocyten die zorgen voor een snelle en aspecifieke reactie tegen binnendringende pathogenen. Tegelijkertijd reageert ook het adaptieve afweersysteem dat een veel specifiekere reactie opbouwt waarbij het gebruik maakt van antigeen-specifieke receptoren op B- en T-lymphocyten. Deze gecombineerde reactie zal uiteindelijk tot onderdrukking of eliminatie van het pathogeen leiden waarbij tegelijk geheugen wordt opgebouwd zodat hetzelfde pathogeen bij een volgende infectie sneller onschadelijk kan worden gemaakt.

Het foetale afweersysteem ontwikkelt zich tijdens de zwangerschap waarin het tolerant moet zijn tegen de deels allogene omgeving waarin de foetus zich ontwikkelt terwijl het ook moet worden voorbereid op de ontwikkeling van de bacteriële microbiota in de darm direct na de geboorte. Het afweersysteem in de darm is het grootste in ons lichaam en is continue blootgesteld aan zowel onschuldige voedselcomponenten als potentieel schadelijke bacteriën die dit afweersysteem van elkaar moet kunnen onderscheiden. Hoewel enkele studies al hebben aangetoond dat het foetale afweersysteem diverse componenten bevat, is vanwege de beperkte beschikbaarheid van foetaal weefsel de meeste kennis over het foetale afweersysteem gebaseerd op studies met navelstrengbloed. Een gedegen en brede studie naar de compositie van het foetale afweersysteem, met name in de darm, ontbrak tot op heden. In het huidige proefschrift heb ik dit onderzocht waarbij ik gebruik gemaakt heb van de nieuwste ontwikkelingen op het gebied van cellulaire analyse, waaronder massa cytometrie en RNA-sequentie bepalingen.

Massa cytometrie

Massa cytometrie is een nieuwe methode waar flow cytometrie is gekoppeld aan massa spectrometrie. In massa cytometrie worden metalen als reporters gebruikt waardoor de beperkingen van het traditionele gebruik van fluorochomen opgeheven wordt en tot 42 markers gelijktijdig geanalyseerd kunnen worden. Door deze revolutionaire innovatie kan de diversiteit en compositie van het afweersysteem tot in detail worden bestudeerd.

Het is ook mogelijk om deze techniek op weefselcoupes toe te passen, imaging massa cytometrie genaamd, waarmee het mogelijk is om de diversiteit en organisatie van het afweersysteem in de context van het weefsel te bestuderen.

Bepalen van genexpressie op cel niveau

Complementair aan massa cytometrie kan de expressie van genen van tienduizenden cellen op single-cel niveau worden bepaald waarmee de heterogeniteit van cellen en de ontdekking van zeldzame celtypes mogelijk wordt. Ook kan deze aanpak leiden tot de identificatie van cel ontwikkelingstrajecten en regulatoire netwerken binnen het afweersysteem.

Heterogeniteit en differentiatie van innate lymphocyten

In **hoofdstuk 2** is de heterogeniteit en ontwikkeling van het innate lymphoide cel compartiment in de foetale darm bestudeerd m.b.v. massa cytometrie. Hierbij werden 34 fenotypisch verschillende innate cel clusters gevonden, waaronder reeds eerder geïdentificeerde cel clusters zoals NK cellen en $CD127^+$ ILC subsets, maar ook een aantal niet eerder beschreven cel clusters. Gebruikmakend van de eigenschappen van de computationele analyse methoden konden mogelijke ontwikkelingstrajecten binnen het innate lymphoide compartiment worden geïdentificeerd die erop duiden dat de NK cellen en ILC3 kunnen ontstaan uit $Lin^-CD7^+CD127^-CD45RO^+CD56^+$ (int-ILCs) cellen. In functionele assays werd deze hypothese verder onderbouwd.

De opbouw van geheugen in de foetale darm

in **hoofdstuk 3** Is massa cytometrie gecombineerd met single-cel RNA-sequencen, T cel receptor sequencen, imaging massa cytometrie en flow cytometrie om het CD4 positieve T cel compartiment in de humane foetale darm te ontrafelen. Met massa cytometrie konden 22 CD4 T cel subsets worden onderscheiden waaronder naïeve, geheugen en regulatoire subsets. Middels RNA-sequencen werd dit verder onderbouwd. Opmerkelijk genoeg produceerden de geheugen cellen ontstekings mediatoren zoals TNF-alpha en Interferon-γ. Ook legde een gedetailleerde en geïntegreerde analyse van de data een differentiatie traject bloot dat de vorming van geheugen ondersteunde. Tenslotte wezen zowel de co-lokalisatie van de geheugen T cellen met antigeen presenterende cellen in het darmweefsel zelf, en aanwijzingen voor klonale cel expansie in de darm erop dat de geheugenvorming in de foetale darm het gevolg was van blootstelling van de foetus aan omgevingsfactoren in de baarmoeder.

Weefselspecifieke samenstelling van het afweersysteem

Om verder inzicht in het foetale afweersysteem te verkrijgen wordt in **hoofdstuk 4** beschreven hoe massa cytometrie is gebruikt om het innate and adaptieve afweersysteem in de foetale darm, milt en lever met elkaar te vergelijken. Deze analyse leidde tot de identificatie van 117 fenotypisch verschillende cel clusters waaronder zowel eerder beschreven alsmede nieuw ontdekte clusters. PCA analyse liet zien dat er grote verschillen zijn in de samenstelling van het afweersysteem in de drie geanalyseerde organen. Met imaging massa cytometrie werd deze weefsel-specifieke samenstelling bevestigd. Deze resultaten laten zien dat heel vroeg in het leven weefsel-specifieke compartimentalisatie van het afweersysteem ontstaat.

In **hoofdstuk 5** is massa cytometrie gebruikt om het afweersysteem te analyseren in darmbiopten van patiënten die leiden aan chronische darmontsteking en dat te vergelijken met dat in gezonde controles. Deze analyse liet zien dat zowel weefsel- als ziekte-specifieke immuun subsets konden worden gedetecteerd.

Conclusies

Het in dit proefschrift beschreven werk geeft een breed en gedetailleerd inzicht in de samenstelling van het foetale afweersysteem gedurende de zwangerschap. Hiervoor is gebruik gemaakt van een serie geavanceerde multiparameter technieken om cellen op single-cel niveau te bestuderen. Daarnaast is de functie van een aantal nieuw geïdentificeerde cel subsets bepaald. Hierbij is een nieuwe ILC subset gevonden die *in vitro* kan differentiëren tot zowel NK cellen als ILC3's. Hiermee is nieuw inzicht verkregen in de plasticiteit van het ILC compartiment. Verder werd gevonden dat gedurende de zwangerschap geheugen cellen worden gevormd in de foetale darm, wat er op wijst dat de foetus in de baarmoeder is blootgesteld aan omgevingsfactoren. Tenslotte is gevonden dat reeds heel vroeg in het leven het afweersysteem in verschillende weefsels een eigen signatuur heeft en het afweersysteem zich dus aanpast aan het weefsel waarin het moet functioneren. Om de functie van het afweersysteem goed te doorgronden is het dus noodzakelijk om het afweersysteem in de weefselcontext te bestuderen. Al met al biedt dit werk nieuw inzicht in de samenstelling en functie van het foetale afweersysteem, kennis die mogelijk gebruikt kan gaan worden om de ontwikkeling van ziekten die door ontregelde reacties van het afweersysteem worden veroorzaakt te voorkomen.

Curriculum Vitae

Na Li was born on May 28th 1987 in Tangshan, Hebei, China, where she grew up and attended primary, middle and high school. In 2007, she continued her studies on Veterinary Medicine at Human Agriculture University and obtained her bachelor degree in 2011 with the thesis titled "Compound of melamine and cyanuric acid on liver damage in mice". This thesis was honored as an 'Outstanding undergraduate dissertation'. During her master, she started to study on microbiology and immunology in Veterinary Medicine at Jilin University under the supervision of Prof. Liancheng Lei. In 2014, she obtained her master degree with the research project on "Screening of virulence genes contributing to *Streptococcus Suis Type 2* translate across the blood brain barrier and the mechanism of meningitis pathogenesis". During her bachelor's and master's degree from 2008 to 2013, she obtained National scholarship for Encouragement or first-grade scholarship. After obtaining funding for a four-year PhD abroad from the Chinese Scholarship, she started her PhD trajectory in October 2014 at the department of Immunohematology and Blood Transfusion (IHB) at Leiden University Medical Center (LUMC) under the supervision of Prof. Frits Koning. During her PhD, she investigated the development of the human fetal immune system using high-parameter advanced techniques, which have been described in this thesis. She was one of the pioneers using the mass cytometry and imaging-mass cytometry. This also resulted in covers for Journal of Experimental Medicine (Vol 215, No 5, May 2018) and Nature Immunology (Vol 20, No 3, March 2019) and a prize "DIRK BARTZ PRIZE FOR VISUAL COMPUTING IN MEDICINE" at Eurographics 2019. After her PhD graduation in 2019, she will move back to China and continue her research in the field of immunology.

List of publications

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