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

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Chapter 1

General introduction

The immune system provides protection against pathogens such as bacteria, viruses, parasites and fungi. It comprises a cellular and humoral compartment. The former is a complicated network of immune cells in lymphoid organs, tissues and the circulation and 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, such as macrophages and dendritic cells (DCs), and innate lymphoid cells (ILCs) including cytolytic natural killer (NK) cells and $CD127^+$ 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. Typically, adaptive immune responses will be initiated in the lymph nodes by dendritic cells that have moved into the lymph node upon encounter with and internalization of pathogens in peripheral tissues or fluids, upon which the activated immune cells will leave the lymph node and attack the invading pathogen in the body. Lymphoid organs include primary organs (thymus and bone marrow) and secondary tissues such as spleen and mucosal-associated lymphoid tissue, in which the immune process takes place.

The immune system develops in the fetus during pregnancy. The developing fetus expresses the polymorphic major histocompatibility complex (MHC) molecules, half of which are inherited from the mother and half from the father. This implies that the paternal MHC antigens are potential allogeneic targets for the maternal immune system. Similarly, the non-inherited maternal MHC antigens can potentially be recognized by the fetal immune system. For a successful pregnancy it is thus crucial that both the fetal and maternal immune system remain tolerant to the semi-allogeneic environment, but it is at present unclear how this is achieved. On the other hand, the fetus has to be prepared for the massive colonization of mucosal and epithelial surfaces with commensals and exposure to pathogens directly upon birth. In this respect, it is intriguing that in utero the fetus is thought to be protected from exposure to foreign antigens. In agreement, the vast majority of cord blood T cells exhibit a naive phenotype. Yet, it is unclear how such a naive immune compartment would be able to adequately deal with the rapid microbial colonization upon birth. Thus, the developing immune system should be poised for environmental attack while remaining tolerant to the semi-allogeneic environment, a seemingly conflicting and impossible task.

Hematopoiesis

In human fetus, the first occurrence of hematopoietic stem cells (HSCs) is in the dorsal aorta of the aorta-gonad-mesonephros (AGM) region at week 5 of gestation and before their appearance in the yolk sac[1]. The HSCs then migrate into the fetal liver, which becomes the main hematopoietic site at the 5-6 weeks of gestation. Hematopoiesis in the fetal liver reaches the peak in the second trimester and terminates soon upon birth. In the second trimester, the HSCs are mainly produced by the bone marrow, which remains the primary organ of hematopoiesis throughout whole life. The HSCs from fetal liver are the first to colonize the thymus and spleen[2, 3, 4].

Development of the fetal immune system

T cells

The early prothymocytes can be detected in the fetal liver at week 7 of gestation and are characterized by the expression of CD45, CD7 and cytoplasmic CD3 but lacking expression of membrane CD3, TCR β chain and TdT (terminal deoxynucleotidyl transferase) a DNA polymerase, which can insert random N-nucleotides into the DJ region of the T-cell receptor (TCR), contributing to the diversity of TCR repertoire[5]. T cells first appear in the perithymic mesenchyme at around week 7 of gestation and become mature between week 7 to 12 of gestation as indicated by the expression of P80, a marker for mature thymocytes[6]. These cells begin to migrate to the periphery at approximately week 14 of gestation[7]. The expression of TdT enzyme is not readily detectable before week 19 of gestation in the thymus, however, TdT mRNA can be amplified in the thymus as early as week 8 of gestation at a minimal quantity[8].

Compared with a predominant memory T cells ($CD45RO^+$) in the adult tissue, the fetal cord blood T cells mainly display a naive phenotype ($CD45RA^+$) at birth[9]. However, a recent study has identified a minor population of effector memory T cells in cord blood, which expresses the tissue resident and activation marker CD69 and displays both Th1- and Th2-type functional profiles in the absence of known infections during pregnancy[10]. In addition, Byrne et al. have provided evidence for the existence of abundant $CD45RO^+$ memory T cells (including both $CD4^+$ and $CD8^+$ T populations) in the human fetal spleen from 14-22 weeks of gestation, which were able to proliferate in response to IL-2[11]. Between week 16 and 18 of gestation the frequencies of these cells can reach levels comparable to those in the adult spleen but they are dramatically decreased in the neonatal spleen[11]. In

addition, memory T cells have also been identified in the human fetal skin[12] and intestine[13]. Although scarce, T cells are first detectable in the epithelial and lamina propria of the human fetal intestine from week 12 of gestation onwards, and the number of T cells increases along with the gestational age[14]. Bunders et al. demonstrated that in the human fetal mucosa, there is a dominance of $CD45RO^+$ memory T cells with Th1- and Th17-phenotype. These cells express the activation marker HLA-DR and are polyclonal and highly susceptible to HIV infection[13]. The observations of memory T cells in fetal tissues suggest the occurrence of complete T cell maturation in utero but it is unclear how this is induced or regulated. Interestingly, almost 50% of $CD8^+$ T cells in the fetal intestinal lamina propria express CD8aa homodimers, as opposed to CD8αβ heterodimers, commonly found on peripheral $CD8^+$ T cells, which indicates that these CD8aa cells may be thymus-independent and develop in the mucosal tissue niche[15]. As it has been well documented in human studies that from 18 to 24 weeks of gestation, the fetal mesenteric lymph nodes (mLNs) are dominated by $CD45RA^+$ T cells, which can proliferate upon addition of exogenous of IL-2 but not in response to PHA or CD3 specific monoclonal antibodies[16], fetal intestinal memory T cells exhibit unique properties. Finally, mucosal associated invariant T (MAIT) cells have also been identified in the human fetal intestine at the second trimester but their functional properties remain to be further explored[17].

Thus, many issues regarding the nature and development of T cells in the fetal intestine remain unresolved.

Regulatory T cells

Regulatory T cells (Tregs), characterized as $CD3^+CD4^+CD25^{high}FoxP3^+$, play a crucial role in modulating the immune system to maintain immune tolerance and prevent autoimmunity. They are classified into two types: "natural" Tregs, which differentiate from T cell precursors in the thymus[18] and "induced" Tregs, which are generated from the conventional $Foxp3^-CD4^+$ T cells outsize the thymus[19]. It has been reported that Tregs are more abundant in the developing fetus compared with newborns and adult[9, 20] and are present in the human fetal thymus, spleen, lymph nodes (LNs) during the second trimester[20, 21] as well as in cord blood[20]. Moreover, Mold et al. reported that after stimulation with foreign APCs in vitro, human fetal $CD4^+$ T cells derived from fetal LNs and spleens preferentially gave rise to Tregs, which were specific for the non-inherited maternal antigens (NIMAs)[22]. Moreover, HSCs derived from fetal liver and fetal bone marrow were found to generate a significant higher frequency of $Foxp3^+$ Tregs compared to adult derived bone marrow HSCs, indicating that fetal HSCs are prone to Treg differentiation[23].

Finally, the absence of Foxp3$^+$ Tregs in utero has been linked with diseases like type 1 diabetes and dermatitis[24], which further highlights the value to study the Tregs in fetal tissues in detail.

Innate lymphoid cells

Innate lymphoid cells (ILCs) lack the expression of antigen-specific receptors and the major immune lineage markers but otherwise they are the innate counterparts of T cells. NK cells, group 1 ILCs (ILC1s), group 2 ILCs (ILC2s) and group 3 ILCs (ILC3s) mirror CD8$^+$ cytotoxic T cells, CD4$^+$ Th1, Th2 and Th17 cells, respectively, with respect to their function and development[25]. It has been reported that fetal ILCs are found in the human liver, lymphoid tissue, lung and intestine[26, 27, 28]. In the fetal liver, NKp44$^-$ ILC3s are abundant and appear as early as week 6 of gestation whereas the other subsets are detectable only after week 15[26]. Compared to fetal liver, NKp44$^+$ ILC3s are enriched in the human fetal intestine, where there are hardly any ILC1s, suggesting that the differentiation of ILC1s may be related to microbiota colonization[29]. Lymphoid tissue inducer (LTi) cells, a subtype of ILC3s expressing Neuropilin-1[27], play a crucial role in second lymphoid organs formation during the fetal development, and have been identified in the fetal LNs and spleen in the first and second trimester[27, 30, 31]. In addition, Mjösberg et al. has provided evidence for the existence of ILC2s in the mucosal tissue such as fetal intestine and lung during the second trimester[32]. Thus, tissue specific localization of particular subsets is already evident in the developing fetus. In recent years, substantial plasticity and heterogeneity within ILC compartment have been described in adults[25, 33] but this is still unexplored in the human fetus.

The knowledge on ILC development is mainly based on studies in mice, where the common lymphoid progenitors (CLPs) give rise to the common innate lymphoid progenitors (CILPs), which further differentiate into NK cell precursors (NKP) and common helper innate lymphoid progenitors (CHILPs). CHILPs give rise to lymphoid tissue inducer progenitors and innate lymphoid cell precursors (ILCPs), which generate LTi and ILC1s, ILC2s, and ILC3s, respectively[25]. In humans, multipotent CLP-like CD34$^+$ progenitor and CD34$^+$CD117$^+$ CILPs have been identified in human lymphoid tissues, such as tonsil[34, 35]. Additionally, a subset of CD34$^-$CD127$^+$CD117$^+$ ILCPs has been described in different tissues, including fetal liver[36]. Recently, Chen et al. demonstrated that human CD56$^+$ ILCPs can give rise to NK cells and ILC3s whereas CD56$^-$ ILCPs generate ILC2s, pointing towards differences in the developmental pathways of ILC in human and mice[37]. Finally, as only the NKP and ILC3 precursor have been identified in humans[38], several other precursors and intermediates of the ILC developmental pathways still need to be

identified.

B cells and dendritic cells

B cells appear in the fetal liver as early as week 8 of gestation[39] and in the spleen by week 13[40] and B cells with a diverse B cell receptor repertoire are detectable in cord blood already at week 12 of gestation[41]. The presence of DCs has been demonstrated in different fetal tissues, such as spleen, skin and intestine, where the DCs suppress the T-cell immunity by promoting the induction of Tregs and inhibiting the production of tumor-necrosis factor (TNF)[42]. In addition, follicular DCs co-localized with B cells in the fetal spleen, indicating antigen presentation to B cells[43]. Although different types of subsets in both innate and adaptive immunity have been identified in utero, most of the knowledge is based on studies with cord blood collected at birth due to scarcity of human fetal tissues. In addition, the recent discoveries of effector T cells and mature DCs in the developing fetus suggest that the fetal immune system is more complex than previously appreciated. Therefore, further investigations into the development of early-life immunity with a global and detailed view across fetal tissues such as the intestine are needed.

Intestinal mucosal immune system

The intestine harbors the largest immune compartment in our body and is a main interface for direct interaction between the immune system and a large array of environmental antigens. It is continuously exposed to both antigens derived from harmless dietary constituents and potential harmful (commensal) bacteria, separated from each other by only a single layer of epithelial cells. For this, an elaborate intestinal immune system has evolved to distinguish the harmful from beneficial antigens. T cells are scattered throughout the epithelium and the underlying lamina propria harbors both adaptive and innate immune cells as well as a variety of antigen presenting cells. In addition, there are gut-associated lymphoid tissues, such as mesenteric lymph nodes and Peyer's patches[44], the latter of which are first detectable in the fetal intestine between week 16-19 of gestation[45]. While there is extensive literature on the organization and composition of the immune system in the intestine in children and adults, relatively little is known about this issue in the developing fetal intestine. 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.

Mass cytometry

Mass cytometry or cytometry by time-of-flight (CyTOF) 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[46, 47]. Theoretically, with the availability of additional metal reporters, over 100 markers can be measured simultaneously in the future. The schematic overview of the mass cytometry workflow is described in **Figure 1**. Therefore, mass cytometry now provides the opportunity to study the diversity and heterogeneity of the immune system with an unprecedent high-resolution. Although mass cytometry has many advantages over traditional flow cytometry, there are still some challenges with mass cytometry[46]. First, the sensitivity of the metal reporters used in mass cytometry is lower than the brightest fluorophores in flow cytometry. Second, the acquisition speed can reach thousands of events per second with around 95% recovery in flow cytometry, compared to only 300 events per second with around 60% recovery (Helios) in mass cytometry. Third, as the cells are destroyed during the data acquisition, mass cytometry cannot be used to sort cells for further functional analysis. Finally, in mass cytometry the classical forward and size scatter parameters are lacking.

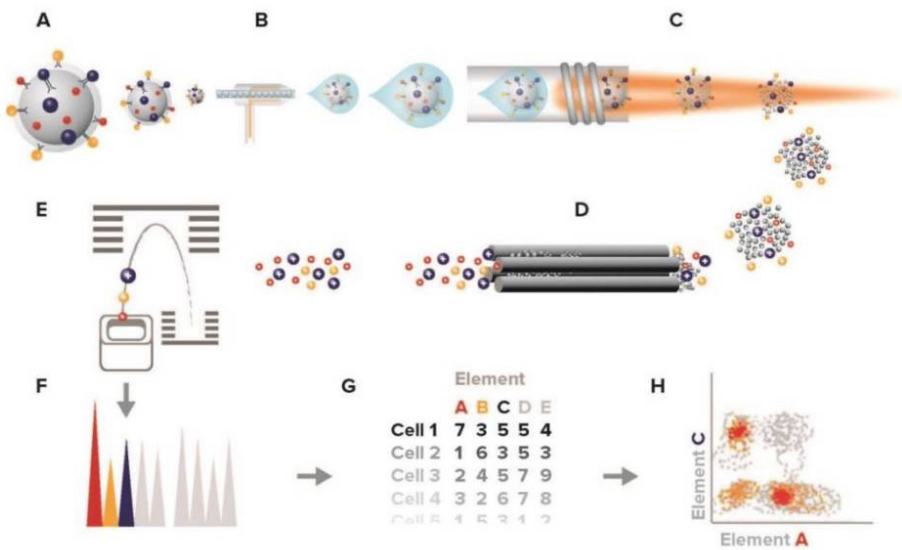

Figure 1.Workflow of CyTOF. A single-cell suspension is labeled with metal-conjugated antibodies (A) and injected into the nebulizer (B). After aerosolizing into a single-cell droplet, it is directed into the inductively coupled plasma (ICP) torch, in which it is vaporized, atomized and ionized (C). The low mass ions are removed by the high pass optic (D), resulting in an ion cloud enriched with heavy metal isotypes, which then enters the time of flight (TOF) chamber (E). The ions are then separated based on the their mass as they accelerate to the detector, where the intensity of each metal for each individual cells will be measured (F). Finally, data is generated in the FSC format (G) and analyzed using third-party software (H). Adapted from Fluidigm.

Data analysis in mass cytometry

In flow cytometry, cell populations are manually identified by using sequential gating based on bimodal expression patterns within one- or two-dimensional plots. With the substantial increase in the number of parameters in mass cytometry, the complexity of the data interpretation has challenged this manual "gating strategy" and inspired the development of new computational tools. t-distributed Stochastic Neighbor Embedding (t-SNE) is a non-linear dimensionality reduction algorithm and currently widely used for the analysis of mass cytometry data. t-SNE takes all the marker expression patterns into account simultaneously and projects the high dimensional data onto two dimensional plots[48]. Nevertheless, conventional t-SNE has several limitations like a restriction of the size of the dataset and long computing time. Hierarchical Stochastic Neighbor Embedding (HSNE), an in-house developed software, removes the scalability limitation of the conventional t-SNE analysis and allows the rapid exploration of millions of cells simultaneously. Furthermore, the implementation of HSNE in Cytosplore[49], named Cytosplore^{+HSNE} [50], allows the user to analyze the entire dataset at several levels of hierarchy, going from a global overview of the data structure to full single cell resolution.

Imaging-mass cytometry

Recently, imaging-mass cytometry has been developed as a new technology to gain spatial information in situ, 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[51]. A schematic overview of the imaging-mass cytometry workflow is described in **Figure 2**. In addition, the traditional imaging analysis can be performed at the cell level by applying cell segmentation, cell neighborhood analysis and distance measurement. Up till now, imaging-mass cytometry has been mainly applied for biomarker identification in pharmaceutical research[52] and analysis of the tumor environment in cancer research[51, 53, 54]. However, imaging-mass cytometry can be of great value in many other studies as well as it offers an opportunity to gain unprecedented insight into the organization of the immune system in situ.

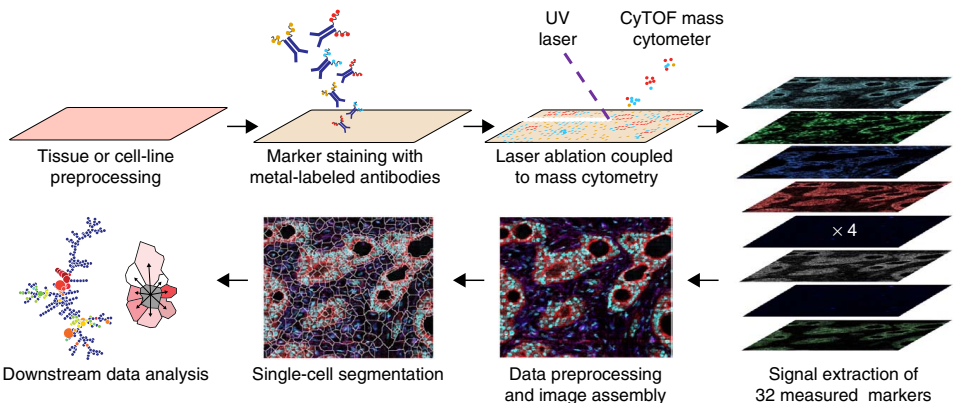

Figure 2. Work flow of imaging-mass cytometry. Adapted from Giesen et al. (2014)

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. In recent years, a varieties of single-cell RNA-sequencing techniques have been developed such as microfluidic-, plate- and droplet-based approaches[55]. As each of them has its own advantages and drawbacks[56], the users can choose the best approach based on their research goals. As such, single-cell RNA-sequencing provides an opportunity to determine the cell transcriptomic heterogeneity, allows the discovery of rare cell populations, can be used to determine cellular differentiation trajectories and to reveal regulatory networks among cells at the gene level[57].

Outline of the thesis

In recent years, several studies have highlighted the uniqueness of the human immune system in early life. Due to the scarceness of human fetal tissues and technical limitations, a system-wide and detailed phenotypical characterization of the composition and development of the human fetal immune system was lacking. In this thesis, I describe my work to gain further insight in the composition and development of the human fetal immune system using an array of advanced high-throughput technologies.

The immune system comprises both the innate and adaptive immune compartments. With respect to the innate compartment, I was the first to apply mass cytometry to delineate the innate lymphoid cells (ILCs) in the human fetal intestine (**Chapter 2**). This revealed extensive heterogeneity of ILCs. Beside

the known ILC subsets, a previously unrecognized subset was identified, named int-ILCs, with a cell surface phenotype that combined CD127$^+$ ILC and NK cell properties. In line with this, visualization of the t-SNE computation demonstrated that this cell positioned itself in between the CD127$^+$ ILC and NK clusters, giving rise to the hypothesis that these cells might be precursors that could give rise to both NK cells and CD127$^+$ ILCs. In functional in vitro studies I demonstrated that this was indeed the case. This study was one of the first to generate a data-driven hypothesis by exploring the heterogeneity of the immune system using mass cytometry, followed by functional validation.

With respect to the adaptive immune compartment, I used an array of advanced single-cell techniques, including high-throughput mass cytometry and imaging-mass cytometry, single-cell RNA-sequencing and TCR sequencing, to characterize the CD4$^+$ T cell compartment in the human fetal intestine (**Chapter 3**). By combining the acquired datasets with advanced computational analysis tools and functional analysis this revealed that memory-like CD4$^+$ T cells with pro-inflammatory properties were already generated in the developing human fetal intestinal immune system, indicative of in utero exposure to foreign antigens.

To further extend the understating of the fetal immune system, I applied mass cytometry and imaging-mass cytometry to both the innate and adaptive immune compartments in the fetal intestine, spleen and liver (**Chapter 4**). This revealed an early-life immune compartmentalization in these different fetal tissues.

Also, I was involved in a study in which we analyzed the composition of the mucosal immune system of patients with inflammatory intestinal diseases and controls using mass cytometry (**Chapter 5**). This first in its kind study revealed previously unrecognized heterogeneity in the mucosal immune system and tissue- and disease-specific immune subsets.

Finally, in **chapter 6**, I discuss the major findings described in **Chapter 2-5** and their implications for shaping the prenatal immune system. In addition, potential directions for future research are discussed.

References

1. Ivanovs, A. et al. Highly potent human hematopoietic stem cells first emerge in the intraembryonic aorta-gonad-mesonephros region. J Exp Med 208, 2417-2427 (2011).
2. Christensen, R.D. Hematopoiesis in the fetus and neonate. Pediatr Res 26, 531-535 (1989).
3. Migliaccio, G. et al. Human embryonic hemopoiesis. Kinetics of progenitors and precursors underlying the yolk sac----liver transition. J Clin Invest 78, 51-60 (1986).
4. Zhang, X., Zhivaki, D. & Lo-Man, R. Unique aspects of the perinatal immune system. Nat Rev Immunol 17, 495-507 (2017).
5. Haynes, B.F., Denning, S.M., Singer, K.H. & Kurtzberg, J. Ontogeny of T-cell precursors: a model for the initial stages of human T-cell development. Immunol Today 10, 87-91 (1989).
6. Lobach, D.F., Hensley, L.L., Ho, W. & Haynes, B.F. Human T-Cell Antigen Expression during the Early Stages of Fetal Thymic Maturation. Journal of Immunology 135, 1752-1759 (1985).
7. Haynes, B.F. The Human Thymic Microenvironment. Advances in Immunology 36, 87-142 (1984).
8. George, J.F., Jr. & Schroeder, H.W., Jr. Developmental regulation of D beta reading frame and junctional diversity in T cell receptor-beta transcripts from human thymus. J Immunol 148, 1230-1239 (1992).
9. Thome, J.J. et al. Early-life compartmentalization of human T cell differentiation and regulatory function in mucosal and lymphoid tissues. Nat Med 22, 72-77 (2016).
10. Zhang, X. et al. CD4 T cells with effector memory phenotype and function develop in the sterile environment of the fetus. Sci Transl Med 6, 238ra272 (2014).
11. Byrne, J.A., Stankovic, A.K. & Cooper, M.D. A novel subpopulation of primed T cells in the human fetus. J Immunol 152, 3098-3106 (1994).
12. Schuster, C. et al. Phenotypic characterization of leukocytes in prenatal human dermis. J Invest Dermatol 132, 2581-2592 (2012).
13. Bunders, M.J. et al. Memory CD4(+)CCR5(+) T cells are abundantly present in the gut of newborn infants to facilitate mother-to-child transmission of HIV-1. Blood 120, 4383-4390 (2012).
14. Spencer, J., MacDonald, T.T., Finn, T. & Isaacson, P.G. The development of gut associated lymphoid tissue in the terminal ileum of fetal human intestine. Clin Exp Immunol 64, 536-543 (1986).
15. Latthe, M., Terry, L. & MacDonald, T.T. High frequency of CD8 alpha alpha homodimer-bearing T cells in human fetal intestine. Eur J Immunol 24, 1703-1705 (1994).
16. von Hoegen, P., Sarin, S. & Krowka, J.F. Deficiency in T cell responses of human fetal lymph node cells: a lack of accessory cells. Immunol Cell Biol 73, 353-361 (1995).
17. Leeansyah, E., Loh, L., Nixon, D.F. & Sandberg, J.K. Acquisition of innate-like microbial reactivity in mucosal tissues during human fetal MAIT-cell development. Nat Commun 5, 3143 (2014).
18. Geiger, T.L. & Tauro, S. Nature and nurture in Foxp3(+) regulatory T cell development, stability, and function. Hum Immunol 73, 232-239 (2012).
19. Chen, W. et al. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med 198, 1875-1886 (2003).
20. Michaelsson, J., Mold, J.E., McCune, J.M. & Nixon, D.F. Regulation of T cell responses in the developing human fetus. J Immunol 176, 5741-5748 (2006).
21. Cupedo, T., Nagasawa, M., Weijer, K., Blom, B. & Spits, H. Development and activation of regulatory T cells in the human fetus. Eur J Immunol 35, 383-390 (2005).
22. Mold, J.E. et al. Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero. Science 322, 1562-1565 (2008).
23. Mold, J.E. et al. Fetal and adult hematopoietic stem cells give rise to distinct T cell lineages in humans. Science 330, 1695-1699 (2010).
24. Bennett, C.L. et al. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nature Genetics 27, 20-21 (2001).
25. Vivier, E. et al. Innate Lymphoid Cells: 10 Years On. Cell 174, 1054-1066 (2018).

26. Forkel, M. et al. Composition and functionality of the intrahepatic innate lymphoid cell-compartment in human nonfibrotic and fibrotic livers. Eur J Immunol 47, 1280-1294 (2017).

27. Shikhagaie, M.M. et al. Neuropilin-1 Is Expressed on Lymphoid Tissue Residing LTi-like Group 3 Innate Lymphoid Cells and Associated with Ectopic Lymphoid Aggregates. Cell Reports 18, 1761-1773 (2017).

28. Miller, D., Motomura, K., Garcia-Flores, V., Romero, R. & Gomez-Lopez, N. Innate Lymphoid Cells in the Maternal and Fetal Compartments. Front Immunol 9, 2396 (2018).

29. Hazenberg, M.D. & Spits, H. Human innate lymphoid cells. Blood 124, 700-709 (2014).

30. Hoorweg, K. et al. A Stromal Cell Niche for Human and Mouse Type 3 Innate Lymphoid Cells. J Immunol 195, 4257-4263 (2015).

31. Cupedo, T. et al. Human fetal lymphoid tissue-inducer cells are interleukin 17-producing precursors to RORC+ CD127+ natural killer-like cells. Nat Immunol 10, 66-74 (2009).

32. Mjosberg, J.M. et al. Human IL-25- and IL-33-responsive type 2 innate lymphoid cells are defined by expression of CRTH2 and CD161. Nat Immunol 12, 1055-1062 (2011).

33. Bjorklund, A.K. et al. The heterogeneity of human CD127(+) innate lymphoid cells revealed by single-cell RNA sequencing. Nat Immunol 17, 451-460 (2016).

34. Freud, A.G. et al. Evidence for discrete stages of human natural killer cell differentiation in vivo. J Exp Med 203, 1033-1043 (2006).

35. Scoville, S.D. et al. A Progenitor Cell Expressing Transcription Factor RORgammat Generates All Human Innate Lymphoid Cell Subsets. Immunity 44, 1140-1150 (2016).

36. Lim, A.I. et al. Systemic Human ILC Precursors Provide a Substrate for Tissue ILC Differentiation. Cell 168, 1086-1100 e1010 (2017).

37. Chen, L. et al. CD56 Expression Marks Human Group 2 Innate Lymphoid Cell Divergence from a Shared NK Cell and Group 3 Innate Lymphoid Cell Developmental Pathway. Immunity 49, 464-476 e464 (2018).

38. Juelke, K. & Romagnani, C. Differentiation of human innate lymphoid cells (ILCs). Curr Opin Immunol 38, 75-85 (2016).

39. Thilaganathan, B., Nicolaides, K.H., Mansur, C.A., Levinsky, R.J. & Morgan, G. Fetal B lymphocyte subpopulations in normal pregnancies. Fetal Diagn Ther 8, 15-21 (1993).

40. Namikawa, R. et al. Ontogenic development of T and B cells and non-lymphoid cells in the white pulp of human spleen. Immunology 57, 61-69 (1986).

41. Rechavi, E. et al. Timely and spatially regulated maturation of B and T cell repertoire during human fetal development. Sci Transl Med 7, 276ra225 (2015).

42. McGovern, N. et al. Human fetal dendritic cells promote prenatal T-cell immune suppression through arginase-2. Nature 546, 662-+ (2017).

43. Timens, W., Rozeboom, T. & Poppema, S. Fetal and neonatal development of human spleen: an immunohistological study. Immunology 60, 603-609 (1987).

44. Mowat, A.M. & Agace, W.W. Regional specialization within the intestinal immune system. Nat Rev Immunol 14, 667-685 (2014).

45. Howie, D. et al. Extrathymic T cell differentiation in the human intestine early in life. J Immunol 161, 5862-5872 (1998).

46. Stern, L. et al. Mass Cytometry for the Assessment of Immune Reconstitution After Hematopoietic Stem Cell Transplantation. Front Immunol 9, 1672 (2018).

47. Bendall, S.C., Nolan, G.P., Roederer, M. & Chattopadhyay, P.K. A deep profiler's guide to cytometry. Trends Immunol 33, 323-332 (2012).

48. van der Maaten, L. & Hinton, G. Visualizing Data using t-SNE. Journal of Machine Learning Research 9, 2579-2605 (2008).

49. Hollt, T. et al. Cytosplore: Interactive Immune Cell Phenotyping for Large Single-Cell Datasets. Computer Graphics Forum 35, 171-180 (2016).

50. van Unen, V. et al. Visual analysis of mass cytometry data by hierarchical stochastic neighbour embedding reveals rare cell types. Nat Commun 8, 1740 (2017).

51. Giesen, C. et al. Highly multiplexed imaging of tumor tissues with subcellular resolution by

mass cytometry. Nature Methods 11, 417-+ (2014).

52. Chang, Q. et al. Biodistribution of cisplatin revealed by imaging mass cytometry identifies extensive collagen binding in tumor and normal tissues. Sci Rep 6, 36641 (2016).

53. Singh, M. et al. Highly Multiplexed Imaging Mass Cytometry Allows Visualization of Tumor and Immune Cell Interactions of the Tumor Microenvironment in FFPE Tissue Sections. Blood 130 (2017).

54. Schulz, D. et al. Simultaneous Multiplexed Imaging of mRNA and Proteins with Subcellular Resolution in Breast Cancer Tissue Samples by Mass Cytometry. Cell Syst 6, 25-36 e25 (2018).

55. Zheng, G.X. et al. Massively parallel digital transcriptional profiling of single cells. Nat Commun 8, 14049 (2017).

56. Chen, G., Ning, B. & Shi, T. Single-Cell RNA-Seq Technologies and Related Computational Data Analysis. Front Genet 10, 317 (2019).

57. Hwang, B., Lee, J.H. & Bang, D. Single-cell RNA sequencing technologies and bioinformatics pipelines. Exp Mol Med 50, 96 (2018).