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Imaging the (un)imaginable of the Barrier Immune system

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Appendices

Summary in English

Imaging mass cytometry

Imaging mass cytometry (IMC) is an extension of CyTOF technology with a laser ablation system, which can be applied on snap-frozen tissue slides and archived paraffin slides. Like in single cell mass cytometry, the technique makes use of metal-labelled antibodies to allow the incorporation of a high number of antibodies simultaneously.

In **Chapter 2**, the optimal staining procedures for IMC on snap-frozen tissue slides from fetal and adult intestinal samples were developed to visualize the tissue architecture and the spatial distribution of the stromal and immune cells. Moreover, in **Chapter 3**, we built up the *Cytosplore Imaging* platform to analyze dense immune infiltrates identified within the IMC images. This allowed for the identification and detailed characterization of lymphoid follicles in the fetal intestine that are composed of B cells, T cells, $CD3^{-}CD7^{+}$ ILCs and myeloid cells and found just below the epithelium in the developing intestine. Moreover, there was abundant co-expression of CD69 and CD161 in lymphoid follicles of the human fetal intestine.

In **Chapter 4**, an IMC dataset on normal skin samples and skin samples from patients with mycosis fungoides were acquired and analyzed to obtain a global overview of the distribution of $CD4^{+}$ T cells, Tregs, $CD8^{+}$ T cells, malignant T cells, and various myeloid cell subsets. Here we observed distinct clusters of $CD4^{+}$ T cells and multiple types of dendritic cells identified through differential expression of CD11c, CD1a and CD1c in the dermis of MF skin.

In **Chapter 5**, IMC was used to compare major immune compartments in non-lesional and lesinal skin from psoriasis patients. The analysis based on pixels in *Cytosplore Imaging* quantified the number of pixels per immune cell subset (the area of per immune cell subset) and demonstrated a significant increase of $CD4^{+}$ and $CD8^{+}$ T cells, macrophages and mast cells in lesional skin, while no significant differences were observed for the dendritic cell subsets. Moreover, the analysis of the IMC dataset at the pixel level revealed the presence of pixels harboring expression of both T and myeloid cell markers, so-called double feature pixels, most likely representing close interactions between these cell subsets.

Development of the human fetal intestinal immune system

In **Chapter 3**, we have used a combination of spectral flow cytometry, imaging mass cytometry and RNAscope to obtain detailed insight into the composition and spatial organization of the developing fetal intestinal immune system throughout the second trimester of gestation.

The spectral flow cytometry analysis readily identified all major immune subsets and heterogeneity therein in the developing human fetal intestines in time. Moreover, this identified stable clusters of Ki-67 expressing cells within all identified immune subsets that remained present in time. Also, imaging mass cytometry identified the formation of lymphoid follicles just below the epithelium in the developing intestine from week 16 onwards, harboring B cells, T cells, $CD3^{-}CD7^{+}$ ILCs and myeloid cells and confirmed the presence of Ki-67^{+} cells in the various immune subsets *in situ*. This also identified the presence of $CD1c^{+}$ B cells and $ROR\gamma t^{+}CD117^{+}CD127^{+}$ LTi- like cells, compatible with the formation of lymphoid follicles the human fetal intestine. Moreover, the presence of CXCL13 mRNA transcripts *in situ* provided further evidence for the formation of such follicles in the developing human fetal intestine.

Finally, we observed that a $CD69^{+}CD117^{+}CD161^{+}CCR6^{+}CD127^{+}$ phenotype was shared by subsets of fetal intestinal $CD3^{-}CD7^{+}$ ILCs and T cells and that these cells preferentially reside in the LFs, and harbor Ki-67^{+} cells, indicating a role in the development of these follicles. *In vitro*, Ki-67^{+} cells in these major immune subsets were capable of spontaneous proliferation, and IL-7 stimulation enhanced the proliferation of these cells. Together with the demonstration of the presence of IL-7 mRNA transcripts in both the lamina propria and epithelium of the human fetal intestine this supports the hypothesis that IL-7 plays an important role in the development of the human fetal intestinal immune system.

These observations indicate the presence of immune subset-committed cells capable of local proliferation, contributing to the development and growth of organized immune structures throughout the 2^{nd} trimester in the human fetal intestine, presumably preparing the infant for the microbial colonization right after birth.

The immune system in skin diseases

Mycosis Fungoides

In **Chapter 4**, we aimed to gain a better understanding of the immune microenvironment in early stage mycosis fungoides (MF). We applied both single-cell and imaging mass cytometry to characterize the immune composition in individual patients. We observed shared $CD4^+$ T cell and myeloid cell subsets in all patients while unique subsets to particular patients were identified as well imaging mass cytometry revealed dense aggregates of T cells with various dendritic cell types in the dermis, likely implied in the disease pathogenesis and representing a potential target for treatment. These results might be helpful for improved diagnosis and personalized treatment of patients with mycosis fungoides.

Psoriasis

In **Chapter 5,** we applied imaging mass cytometry to investigate immune infiltration in patients with mild to moderate psoriasis. The *Cytosplore Imaging* pixel analysis of the IMC dataset identified distinct composition and organization of the immune compartment lesional skin biopsies as compared to non-lesional skin biopsies. In addition, we identified several clusters of "double feature pixels", indicative of close interactions between T cells and various myeloid/DC cell subsets, and likely linked to disease pathogenesis. Finally, preliminary evidence indicates that the quantity of the "double feature pixels" in a given sample may correlate with the severity of psoriasis, an issue that should be explored in a larger number of patient samples.

Overall, the work described in this thesis demonstrates show that the novel high-dimensional technologies provide unprecedented insight into the composition and spatial organization the barrier immune system in health and diseases.