FID-ISPAD Research Grant Progress Report

Year awarded: 2024

Recipient: Dr. Conny Gysemans

Project title: Neutrophils: novel players in the initiation and execution of autoimmune type 1 diabetes?

Project hypotheses and research questions:

As neutrophils can influence cells of both the innate and adaptive immune system, a better characterization of the neutrophil population during type 1 diabetes development, especially at the level of the target organ, is urgently needed and could provide insights into whether neutrophils have divergent developmental branches or different maturation sequences influenced by tissue environmental cues. We want to identify distinct neutrophil populations during type 1 diabetes development in the NOD mouse model using state-of-the-art spatial multi-omics technology. Novel insights can later be verified in the data warehouse from human samples obtained via the European INNODIA consortium.

Project aims:

- Obtain novel information about neutrophils in the pancreatic islets and more distal areas in the pancreas of non-obese diabetic (NOD) mice during different developmental stages of type 1 diabetes using the MILAN method.
- Gather novel insights into how neutrophils within the islet and non-islet microenvironment communicate/interact with other cells of the immune system.

Research Progress:

1) The MILAN method was further optimized and validated to include the following markers.

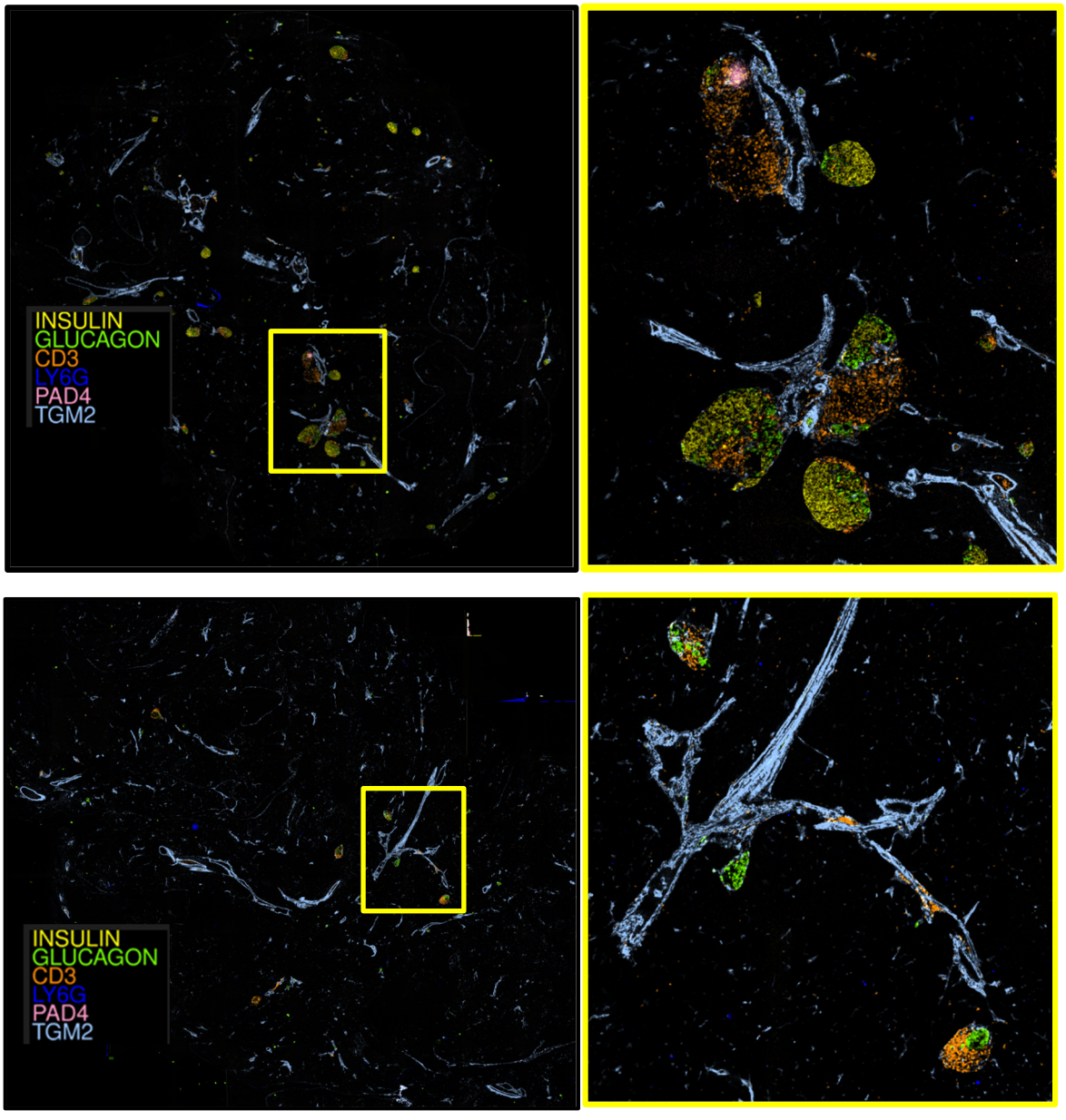
round	FITC	host	TRITC	host	Cy5	host
1	baseline		baseline		baseline	Rb
2	/		f4/80	Rat	CD3	Rb
3	baseline		baseline		baseline	
4	/		CD4	Rat	CD31	Rb
5	/		CD8	Rat	CD19	Rb
6	/		Ly6G	Rat	CD11c	Rb
7	/		/		CD11b	Rb
8	/		/		MPO	Rb
9	baseline		baseline		baseline	
10	/		/		CitH3	Rb
11	/		/		TGM2	Rb
12	/		/		PAD4	Rb
13	/		/		Insulin	Rb
14	/		/		Glucagon	Rb

This panel includes markers for the endocrine pancreas (insulin (beta cells) and glucagon (alpha cells)), the vasculature (endothelial cells (CD31)), and resident or infiltrating immune cells e.g., CD3, CD4, CD8 (T cells), CD19 (B cells), F4/80, CD11b (macrophages), CD11c (dendritic cells), Ly6G (neutrophils), myeloperoxidase (MPO), citrullinated histone 3 (CitH3), Protein-arginine deiminase type-4 (PAD4) and transglutaminase 2 (TGM2). Neutrophils will also be studied functionally. Excessive neutrophil

extracellular traps (NET) formation has been associated with several inflammatory diseases. Therefore, we want to evaluate the expression of NET-associated proteins, including PAD4, CitH3, and MPO.

2) Pancreas specimen from 6-week-old, 12-week-old, and newly diabetic NOD mice (females; n=4) were collected. Formalin-fixed paraffin embedded (FFPE) sections were cut and stained with the panel described above.

3) Despite significant delays faced (Please see '*Bottlenecks*' on page 3) that have impacted on the progress of this project, we have performed the required steps for downstream image analysis using the most common used fluorescent, cyclic methods for multiplexed immunohistochemistry. Images were collected across multiple cycles and were cleaned (quality control), corrected (pre-processing), registered/aligned, auto-fluorescence removed, segmented, and feature extracted. The final steps (phenotypic identification and spatial analyses) will be done in the coming months. Here some representative images can be found from a selected number of markers (insulin, glucagon, CD3, Ly6G, PAD4, and TMG2).



We will detect and measure pancreatic islets and islet cells in fluorescent images with the HALO® software (Indica Labs Inc). The HALO® islet quantification FL module quantifies islets and their total and average area along with measuring nuclear or cytoplasmic positivity and total area for up to 3 dyes. We will get more insight into the biomarker colocalization analysis with the option to output both cell and area markup images. We contacted Indica Labs (HALO software) to implement their HALO® Highplex FL module to analyze the full spectrum of markers in the complete pancreatic area. The module allows to simultaneously analyze an unlimited number of fluorescent markers in any cellar compartment (nucleus, cytoplasm, and/or membrane). We are implementing methods to perform clustering analysis and for visualizing high dimensional HALO® Highplex FL data, such as UMAP and t-SNE.

Bottlenecks:

The following bottlenecks and challenges have contributed to ~3 months of delays in project progress):

- Delayed receipt and access of grant funds (grant was officially awarded in 29th of January 2024 but due to contract negotiations with our institute funds were received on 4th of March 2024)
- Research support staff recruitment issues: the PhD student working on this project went in sick leave September 2023 until recently. She is regaining her PhD training yet only works 20% since July 2024. Moreover, the bioinformatician hired to support the PhD student went in sick leave since July 2024. Her return is still uncertain. Due to these setbacks, we contacted Indica Labs for the image analyses and will use their HALO® Highplex FL module.

Timeline for project completion:

Sept-Oct. 2024: final steps of downstream image analysis
Nov-Dec. 2024: HALO® Highplex FL data analyses
Jan-Feb. 2025: Data interpretation and reporting (peer-reviewed publication and presentation at 2025 ISPAD meeting)