Diabetic Complications Consortium

Application Title: Tissue-derived Factors and Macrophage Polarization in Diabetic Gastroenteropathy

Principal Investigator: Simon James Gibbons PhD

1. Project Accomplishments:

This DiaComp Pilot and Feasibility Program project had one specific aim covering two hypotheses:

Specific Aim: To determine the molecules generated in gastric tissues exposed to diabetes that alter the balance between injurious and protective phenotypes of macrophages in gastric tunica muscularis.

Hypotheses for Aim:

I. Soluble factors generated in the gastric muscularis propria in response to oxidative injury alter the balance of tissue macrophage phenotypes leading to the development of delayed gastric emptying in diabetic mice.

II. Up-regulation of leptin release in the gastric muscularis propria of a subset of diabetic mice induces Stat3-dependent expression of IL6 and TNF in gastric muscularis propria macrophages leading to loss of ICC and delayed gastric emptying.

We have accomplished the following:

i) We identified 4 gene products that were significantly up-regulated in tissues from diabetic mice with delayed gastric emptying. These gene products, encode proteins that regulate the synthesis of medium chain fatty acids and modify plasma membrane lipid content.

ii) We determined that the proteins encoded by these 4 gene products were enriched in myenteric neurons.

iii) We determined by immunohistochemistry that the increased expression of leptin protein in PDGFRα^{+} - fibroblast-like cells was not associated with delayed gastric emptying in diabetic mice.

iv) We applied the novel techniques of mass cytometry and single cell RNA-Sequencing to identify subtypes of myeloid cells in the gastric muscularis propria of diabetic mice to determine the diabetes-induced changes in gene expression between identified subtypes of muscularis propria macrophages.

v) The data resulting from these studies forms the basis for an NIH R01 application that is being prepared and will be submitted for the June 5th, 2020 deadline.

vi) Full analyses of the mass cytometry and single cell RNA-Sequencing experiments are not yet complete, when we have completed these analyses then the data will form the basis for a manuscript that is in preparation. As required by the DiaComp, we will make the full data available when the manuscript is accepted for publication or within 1 year of completion of the project, whichever is sooner.

2. Specific Aims:

Specific Aim 1, Hypothesis I:

i) As outlined in the proposal, our preliminary studies identified leptin as a gene transcript that was more highly expressed in the gastric muscularis propria of diabetic mice with delayed gastric emptying than in diabetic mice with normal gastric emptying. Preliminary, immunohistochemical studies indicated that leptin protein was found in

fibroblast-like cells of the gastric muscularis propria in diabetic mice with delayed gastric emptying. However, studies funded by this DiaComp grant did not support a significant association between leptin protein expression and differences in gastric emptying in the mice. Therefore, we did not pursue the proposed studies on leptin regulation of the phenotypes of muscularis propria macrophages any further.

ii) As a result of the observations regarding leptin expression, we further examined our gene expression data and identified 4 gene products linked to fatty acid metabolism that were also up-regulated in the muscularis propria from diabetic mice with delayed gastric emptying compared to mice with normal gastric emptying (identified in Fig 1). Fatty acid synthase (Fasn), acetyl coA decarboxylase (encoded by Acaca and Acacb) and lipase E (Lipe, aka hormone sensitive lipase) are proteins that contribute to medium chain fatty acid synthesis, and alter the lipid content of the plasma membrane.Therefore we further investigated the distribution of these proteins in the gastric muscularis propria.

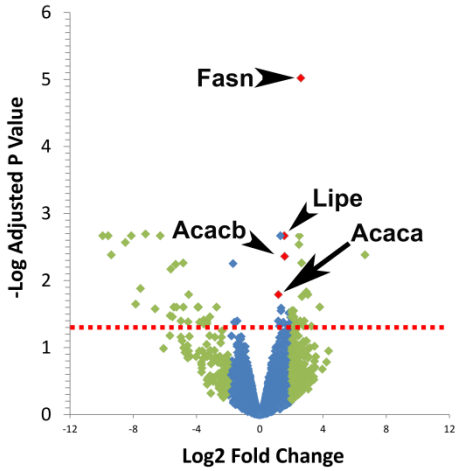
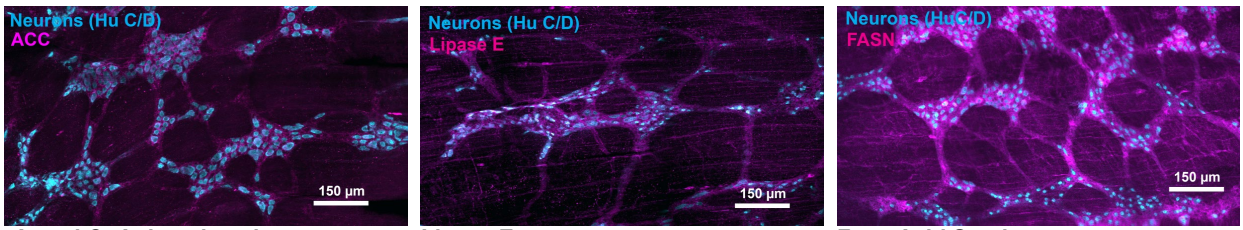


Fig 1. Differential gene expression patterns in the tunica muscularis of mice with normal and delayed gastric emptying.

iii) Dual label immunofluorescence labeling was used to investigate the distribution of fatty acid synthase, acetyl CoA decarboxylase and lipase E in gastric muscularis propria, we found that all 3 proteins were enriched in the myenteric plexus region in whole mount preparations from diabetic mice with delayed gastric emptying and appeared to be most strongly expressed in myenteric neurons (Fig 2)



Acetyl CoA decarboxylase **Lipase E** **Fatty Acid Synthase**

Fig 2. Immunofluorescence labeling in gastric myenteric plexus of diabetic C57Bl6 mice with delayed gastric emtpying

Specific Aim 1, Hypothesis II:

Since the results of the studies directed at SA1, Hypothesis I did not support a clear association of leptin with patterns of cellular changes linked to diabetic gastroparesis, we used two novel approaches to determine other potential upstream regulators of muscularis propria macrophage phenotype and interstitial cell of Cajal loss.

i) We determined the diversity of myeloid cells in the gastric muscularis propria of non-diabetic mice and diabetic mice with delayed and normal gastric emptying, by isolating and labeling all cells from dissociated gastric muscularis propria and profiling the immunophenotype of the cells using the Fluidigm Helios cytometry by time of flight (CyTOF) mass spectrometry system. Using this approach, we identified

3 major clusters of cells in the dissociated tissue that correspond to tissue macrophages based on expression of MHCII, F480, CX3CR1 and CD11B (Fig 3). We are currently repeating these studies to determine how the representation of the cell populations differs between the 3 groups of mice. The cell surface markers identified by CyTOF will be used to isolate the sub-sets of cells identified in this study for further characterization of the gene expression patterns and investigation of the signaling pathways activated by tissue factors in the gastric muscularis propria.

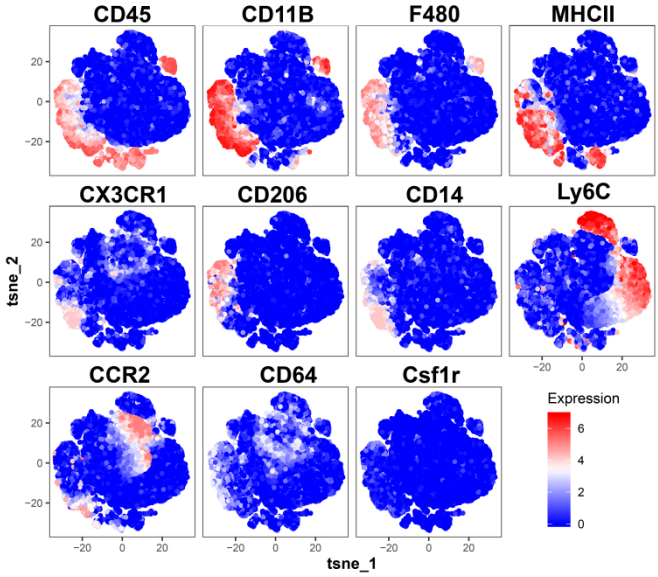


Fig 3. "Marker Expression Level" plots showing expression of myeloid cell markers in gastric muscularis clusters from CYTOF

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ii) In studies on a separate group of mice, we isolated CD45-positive cells from dissociated gastric muscularis propria of non-diabetic mice and diabetic mice with delayed and normal gastric emptying by single cell RNA sequencing. Dissociated cells were labeled with Alexa Fluor450-conjugated anti-mouse CD45 antibodies, sorted on a FACS Aria cytometer (BD Biosciences) and live cells were resuspended in 0.5% bovine serum albumin in phosphate buffered saline for single cell sorting on the 10X Chromium platform. We obtained data from 350-400 cells per sample that were separated into discrete compact clusters of cells (Fig 4) on a tSNE plot. Two clear clusters of macrophages were detected by single cell RNA-Sequencing, (Clusters 0 and 2 in Fig 4) expressing MHCII genes, Itgam (CD11B), and Cx3cr1 RNAs. These cells also expressed Csf1r and Cd14 indicating monocyte origin. We are currently repeating these studies to determine how the abundance of the subsets of myeloid cells change with onset of diabetes and delayed gastric emptying, with the goal of identifying signaling pathways activated in subsets of macrophages and the upstream activators of those pathways.

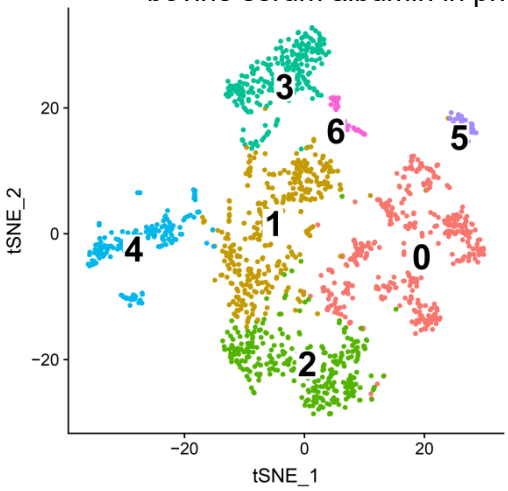

Fig 4. Clustering of myeloid cells in gastric muscularis based on SC RNA-Seq

In conclusion, we have carefully tested the hypotheses proposed in this application and while we have not found data to support our original hypothesis regarding the role of leptin in determining the activation phenotype of gastric muscularis propria macrophages in diabetes, we have identified a completely new hypothesis regarding the importance of tissue-derived lipids in diabetic gastroparesis. As intended, we have generated significant amounts of preliminary data to support new hypotheses regarding the mechanisms behind development of diabetic complications in general and more specifically diabetic gastroparesis.

3. Publications:

No publications have resulted from this project so far. However the data have formed the basis for a new R01 application that will be submitted for the June 5th, 2020 deadline. We anticipate that at least one major publication will result from these studies after the completion of our grant proposal.