Diabetic Complications Consortium

Application Title: Role of Macrophage Mechanobiology in Diabetic Gastroenteropathy
Principal Investigator: Gianluca Cipriani, PhD

1. Project Accomplishments:

This DiaComp Pilot and Feasibility Program project had one central hypothesis that was tested by 2 AIMS.

Hypothesis: In diabetic gastroparesis, pro-inflammatory Piezo1 muscularis macrophages release IL6 and TNFα in response to mechanical force, induce loss of ICC and promote gastric dysfunction.
Aims:
i) Determine how MMs respond to mechanical force through Piezo1.
ii) Determine the location of Piezo1 MMs and the effect of their depletion on gastric function in diabetic mice.

We have accomplished the following points that are instrumental in completing this award and will be supportive and central for a new R01 application planned for the fall of 2023.
a) We developed novel cultures of sorted GI muscularis macrophages. This novel primary culture allows to test the effect of different stiffness grades on muscularis macrophages' phenotype.
b) Muscularis macrophages responded to different subtrates with increased inflammatory response.
c) To test changes to intracellular calcium after applying different forces, we generated a LysM^{Gcamp} mouse model.
d) We successfully developed a mouse model that allows conditional depletion of Piezo1 from the myeloid cell population. This mouse will serve to study the second aim of the grant about the possible involvement of this population in the development of delayed gastric emptying.

In the following sections, we will describe in detail the data achieved during the length of the project.

2. Specific Aims:

Aim1

Determine how MMs respond to mechanical force through Piezo1.

1. As outlined in the proposal, we developed a novel primary culture of muscularis macrophages to study the effect of mechanical forces on MMs phenotype. First, we isolated muscularis macrophages from the gut muscularis propria of CSF1r^{GFP} mice (**Fig.1**) using a protocol previously done in our laboratory. We then kept the cells in culture for up to 7 days and found that the number of muscularis macrophages was not changed over time, suggesting that stiffness is not affecting the overall macrophage population. In addition, we see that the canonical markers for macrophages were not changed after seven days in cultures, suggesting that the overall cell phenotype was maintained after seven days.

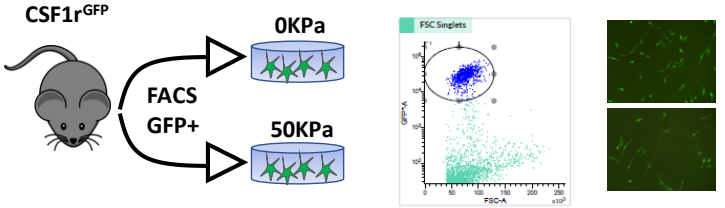

Figure1. Experiment plan used to generate muscularis macrophage primary culture.

2. We next used our novel primary cell culture model to investigate whether cultured muscularis macrophages change their phenotype in the presence of substrates of different stiffness. RNA from

muscularis macrophages exposed to 0KDa and 50 KDa was isolated, and the expression of pro- and anti-inflammatory macrophages' canonical markers was quantified by RT-qPCR. We found that muscularis macrophages express higher level of IL1β, a pro-inflammatory cytokine, after exposure to a stiffer substrate (50KDa) (**Fig.2A**). This is in line with the grant's overall hypothesis about stiffness driving muscularis macrophage activation to a pro-inflammatory phenotype.

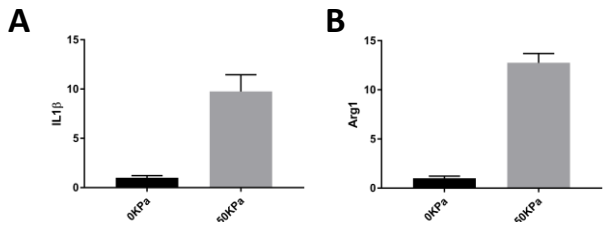

Figure2. Muscularis macrophages exposed to stiffer substrate express both pro- and anti-inflammatory markers.

However, we unexpectedly found higher expression of a canonical anti-inflammatory marker, such as Arg1, after exposure to a stiffer substrate (**Fig.2B**). This result can be explained by the high heterogeneity shared between tissue-resident muscularis macrophages, which have a prominent anti-inflammatory phenotype at steady state. Notably, Piezo1 expression levels were not changed on muscularis macrophages after exposure to stiffer substrates (data not shown).

To test the contribution of Piezo1 to stiffness-associated effect on muscularis macrophage phenotype, we treated the cells to induce siRNA mediated ko of Piezo1 in both conditions by inducing 75% reduction of Piezo1 expression compared to vehicle treated cells (**Fig.3A**). Interestingly, Piezo1 ko muscularis macrophages after exposure to 50KPa substrate do not show any change in pro-inflammatory markers (**Fig.3B**), however, Arg1 (**Fig.3C**) expression was still upregulated compared to the appropriate vehicle treated control. All together, these data suggest the primary role of Piezo1 in driving stiffness-associated activation of muscularis macrophages to a pro-inflammatory phenotype.

4. Our data suggest that Piezo1 induces pro-inflammatory muscularis macrophage activation. In addition to changes to the phenotype, we tested whether changes to stiffness levels affect intracellular calcium in muscularis macrophages. To test this, we have generated a mouse model to look at internal calcium changes upon application of different forces. We generated a LysM^{Gcamp} mouse model that will allow us to visualize calcium oscillation in muscularis macrophages in both *vivo* and *vitro*. We will take advantage of our collaborative environment within the Enteric Neuroscience Program, where other group members have successfully done this type of experiment (Dr. Beyder, Dr. Linden) in different cell types, such as enteric neurons and ICC.

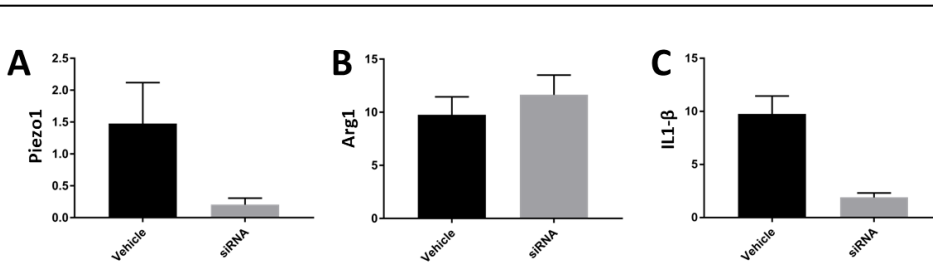

Figure3. Piezo1 regulates stiffness associated muscularis macrophage activation to pro-inflammatory phenotype.

Aim2:

Determine the location of Piezo1 MMs and the effect of their depletion on gastric function in diabetic mice.

***1*.**To study this aim, we generated a mouse model in which we conditionally depleted Piezo1 from MMs by breeding LysM^{cre} with Piezo1$^{flox/flox}$ mice. The resulting LysM^{piezo1} mouse model is viable and showed no significant changes in size and gut anatomy compared to CTRL. We used 10 LysM^{piezo1} and the appropriate control mice for a longitudinal study. These mice were treated with STZ to induce diabetes as previously done, and gastric emptying will be monitored for up to 8 weeks after the onset of diabetes. After onset of diabetes, we did not see any significant changes to glucose levels between LysM^{piezo1} and CTRL mice. Interestingly smooth muscle layers taken from LysM^{piezo1} mice showed reduced expression levels of pro-inflammatory cytokines compared to controls. This result aligns with

the overall hypothesis of the grant about Piezo1 driving pro-inflammatory muscularis macrophages activation. As proposed, we will monitor solid gastric emptying for these mice for 10 weeks after the onset of diabetes.

In general, we have done all the experiments proposed in the original application and produced all the mice models we planned. As anticipated in the proposal, our goal is to use the data generated from this application to develop a new Ro1 proposal that we would like to submit by the end of next year (October 2023). At this point, we are expanding this proposal's original aim and hypotheses to new and exciting hypotheses that will be part of the future application.

3. Publications & Book chapter:

a) Ji S, Traini C, Mischopoulou M, Gibbons SJ, Ligresti G, Faussone-Pellegrini MS, Sha L, Farrugia G, Vannucchi MG, Cipriani G. Muscularis macrophages establish cell-to-cell contacts with telocytes/PDGFRα-positive cells and smooth muscle cells in the human and mouse gastrointestinal tract. Neurogastroenterol Motil. 2021 Mar;33(3):e13993.

b) Mischopoulou M, D'Ambrosio M, Bigagli E, Luceri C, Farrugia G, Cipriani G. Role of Macrophages and Mast Cells as Key Players in the Maintenance of Gastrointestinal Smooth Muscle Homeostasis and Disease. Cell Mol Gastroenterol Hepatol. 2022;13(6):1849-1862.

c) Cipriani, G., Pullapantula, S., 2022, 'Macrophages in the Smooth Muscle Layers of the Gastrointestinal Tract', in V. Kumar (ed.), Macrophages - Celebrating 140 Years of Discovery [Working Title], IntechOpen, London. 10.5772/intechopen.102530.