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14. ABSTRACT

More than 7 in 10 veterans who receive VA care are obese or overweight, putting them at high risk for the development of complications of metabolic syndrome, including type 2 diabetes and non-alcoholic fatty liver disease. Obesity and metabolic syndrome are associated with intestinal dysbiosis and impaired intestinal barrier. These changes in the intestine impact the innate immune system to generate a chronic state of low-grade inflammation, termed metaflammation. We have identified a carbohydrate molecule, hyaluronic acid of 35KD, that has a strong potential to interrupt the deleterious inter-organ cross talk between the gut and innate immune system that drives the progression of obesity/pre-diabetes to diabetes. Given the profound protective effects of HA35 in the gut and anti-inflammatory impact on innate immune cells, we hypothesize that HA35 will have a dually-targeted impact acting to protect gut integrity and reduce metaflammation in a murine model of high fat diet-induced obesity, disruptions that are known to contribute to metabolic syndrome/ pre-diabetes to more severe type 2 diabetes and liver injury.

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W81XWH2010235 - Specific-sized HA35 in Preventing the Transition from Prediabetes to Diabetes
Partnering PI: Carol de la Motte, PhD

STUDY GOALS – Year 1 (09/01/2021- 08/31/2021)

A following table of our goals for year one is extracted from the original Statement of Work designed in July 2019 and initiated September 2020.

Research-Specific Tasks proposed for year 1:

	Months	CC	CC
Major Task 1: Obtain necessary regulatory approvals for working with mice and deidentified human samples			
Subtask 1: Obtain ACURO approval	1-2		Dr. de la Motte
Major Task 4: Intestinal organoid studies (Specific Aim 2)			
Subtask 1: Characterize the effects of lipid, glucose and cytokines on barrier function of intestine in intestinal organoids; determine protective effects of HA35	1-12		Dr. de la Motte
Major Task 6: Generation of cell specific TLR4 and CD44 v7 miceIRF3-/- mice and HFD studies			
Subtask 1: Cross breeding of TLR4fl/fl and CD44v7 fl/fl mice with LysM CRE and villin-CRE mice, breed mice for experiments	2-16		Dr. de la Motte

SPECIFIC AIMS

Major Task 1: Receive ACURO approval for mouse studies
IACUC Approval (Cleveland Clinic) received- Aug. 11, 2020
ACURO- Approval letter received Oct. 9, 2020.

Major task 4: Intestinal organoid studies

Subtask 1: Characterize the effects of lipid, glucose and cytokines on barrier function of intestine in intestinal organoids; determine protective effects of HA35

1.1 Characterize the effect of glucose on barrier function in distal colon (DC) organoids and determine the protective effects of HA35 in hyperglycemic stressed DC organoids

To characterize the effect of glucose on the intestinal barrier function, we evaluated the protein and gene expression of tight junction proteins (ZO1, occludin, and claudin1) in intestinal organoids isolated from genetically wild type (C57BL/6J) murine distal colons (DC). Expanded organoid cultures were treated for 30-48 hours in hyperglycemic (25mM) and normoglycemic (5mM) conditions. Importantly, to determine whether HA35 had protective effects on tight junction proteins, replicate DC organoid cultures were pre-treated with HA35, 24 hours prior to culturing them in hyperglycemia.

We have found that:

A. Intestinal organoids cultured in hyperglycemic medium had lower protein levels of the junctional proteins ZO1, occludin and claudin1 compared to the normoglycemic cultured controls (Fig. 1). HA35 pre-treatment significantly dampened the downregulation of tight junction protein expression of ZO1, occludin, and claudin1 that resulted from culturing DC organoids in high levels of glucose (25mM), yet had little effect in normoglycemic culture levels, especially ZO1 and occludin. Claudin1 may have been upregulated by HA35 alone, either by increased production or slowed degradation. Together these data show HA35 prevents the loss of tight-junctional proteins during hyperglycemia, and in further studies we therefore will continue to conduct time course experiments to see if the three tight junctional proteins are regulated by different mechanisms.

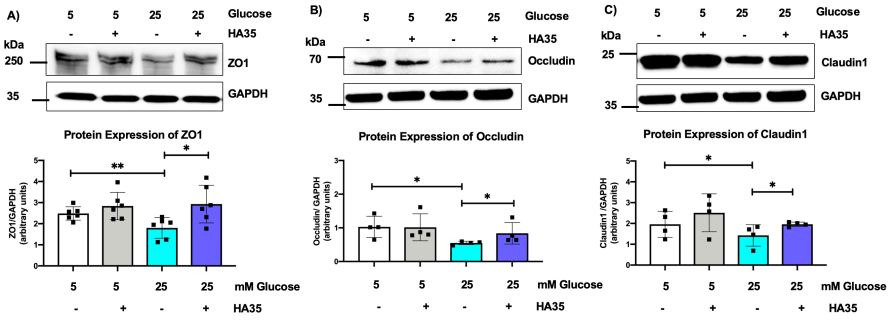

Fig. 1 HA35 protects against the effects of hyperglycemia on tight junction protein expression in distal colon organoids isolated from C57BL/6J wild type mice and cultured *in vitro*. A representative Western Blot shows decreased protein expression of: A) ZO1 after 30h treatment, N=6 different DC organoid isolates; and B) Occludin, N=4; C) Claudin1, N=4; after 48 hours of treatment, in DC organoids cultured in 25 mM glucose compared to 5 mM. HA35 pre-treatment (350 µg/mL, 24 h) of subsequently hyperglycemia stressed DC organoids maintained the levels of ZO1, occludin and claudin1 near basal levels.

B. Interestingly, the relative gene expression of tight junction proteins including ZO1, occludin, and claudin1 is maintained near basal levels and unaffected by the high levels of glucose and/or HA35 as shown in Fig. 2. This suggests that the downregulation of tight junction protein expression in DC organoids under a hyperglycemic stress (Fig. 1) can be the result of protein loss /degradation, which HA35 largely prevents.

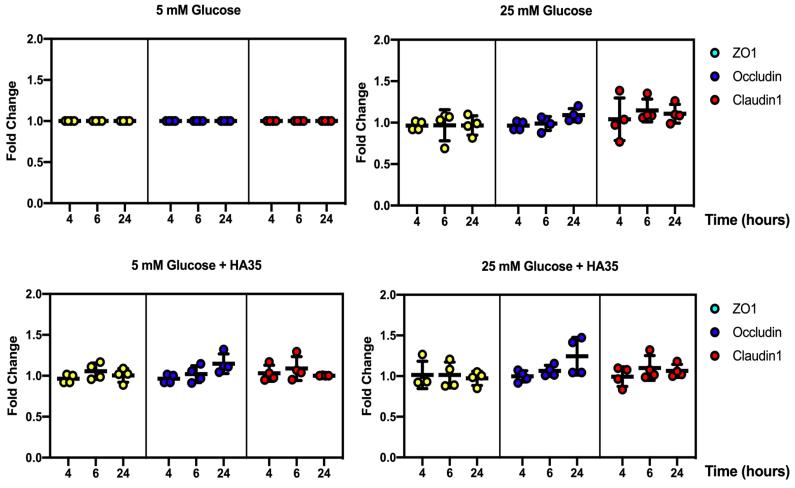

Fig. 2 Relative gene expression of tight junction proteins in distal colon organoids isolated from C57BL/6J wild type mice and cultured *in vitro*. The scatter dot plot graph shows that the relative gene expression of ZO1 (yellow), occludin (blue), claudin1 (red), in 4 separate DC organoid isolates, is maintained near basal level and unaffected by the different treatments at 4, 6 and 24 hours. HA35 pretreatment (350 µg/mL, 24 h) of subsequently hyperglycemia stressed DC, also had no significant effect on gene expression.

C. Unexpectedly we made the observation that, unlike organoids isolated from C57BL/6J WT substrain, high glucose didn't have a significant change on the protein expression of tight junction proteins including ZO1 in DC organoids isolated from wild type C57BL/6N substrain, or the Layilin -/- mice which are on the substrain C57BL/6N background. This necessitated crossing the Layilin -/- mice onto a C57BL/6J background before conducting the in vivo experiments planned, as well as future organoid studies regarding the Layilin receptor. (NB. This is further elaborated on the description of Major Task 6.) Interestingly, the basal protein level of ZO1 in the Layilin -/- DC organoids is higher than that in WT regardless of treatment, suggesting that the junctions are tighter in the Layilin -/- DC organoids (Fig. 3). We plan to evaluate the levels of tight junction proteins in Layilin -/- DC organoids once the backcrossing to substrain C57BL/6J is completed.

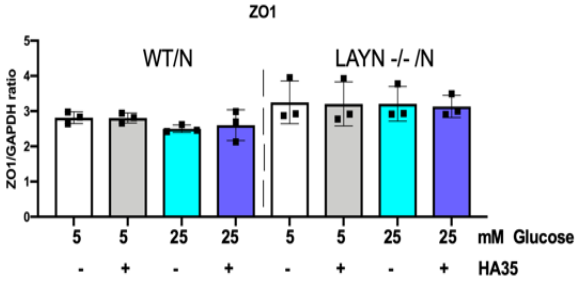

Fig. 3 Basal level of tight junction protein, ZO1, is higher in Layilin -/- distal colon organoids than that in WT. The bar graph shows the protein expression of ZO1 is higher in distal colon organoids isolated from WT compared to Layilin -/- mice of sub-strain C57BL/6N, N=3 different organoid isolates. High glucose did not cause a significant change on the protein expression of tight junction component ZO1, nor for occludin and claudin1 (data not shown).

1.2 Determine the protective effects of HA35 on DC organoids treated with cytokines.

Type 2 diabetes is a metabolic disorder characterized by chronic hyperglycemia and increased levels of circulating cytokines, including TNF-α and IL-6. To determine whether HA35 protects against the effects of TNF-α and IL-6 on the intestinal barrier function, we evaluated the protein and gene expression of tight junction proteins (ZO1, occludin, and claudin1) in DC organoids pre-treated without or with HA35 for 24 hours prior to cytokine treatment.

We have found that:

A. TNF-α treatment significantly reduces the protein levels of ZO1 and occludin (Fig. 4) but not claudin1 (not shown), in wild type (C57BL/6J substrain) DC organoids. HA35 pre-treatment prevents the downregulation of tight junction proteins ZO1 and occludin in this system. Relative gene expression of tight junction proteins ZO1, occludin, and claudin1 was maintained near basal levels and unaffected by stimulating DC organoids with TNF-α with or without HA35 (not shown).

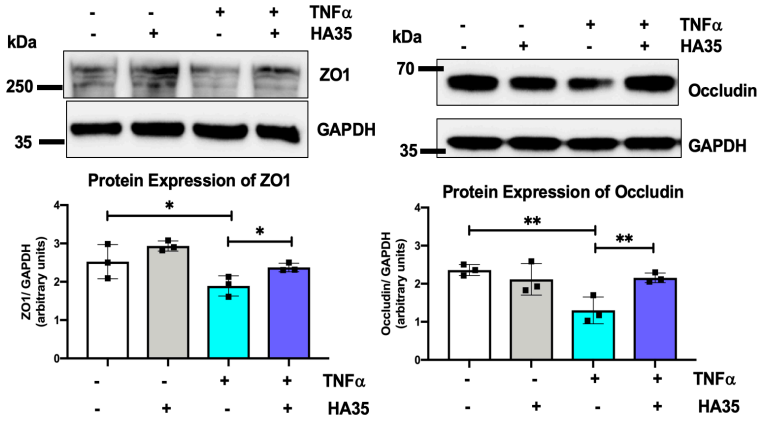

Fig. 4 HA35 protects against the effects of TNF-α on tight junction protein expression in mouse DC organoids. A representative Western Blot shows the decreased protein expression of ZO1 and occludin after treatment with TNF-α (10 ng/mL, 48 hour) (N=3, separate isolates of DC organoids from C57BL/6J WT mice). HA35 pretreatment (350 μg/mL, 24h) maintained ZO1 protein near basal levels in the TNF-α treatment group.

B. IL-6 treatment significantly reduces the protein level of ZO1 (Fig. 5) but not that of claudin1 nor occludin (not shown), in wild type (C57BL/6J substrain) DC organoids. HA35 pre-treatment prevents the downregulation of tight junction proteins ZO1 in this system. Similar to TNF-α and high levels of glucose, IL-6 treatment did not have a significant effect on the relative gene expression of tight junction proteins ZO1, occluding and claudin1 (not shown).

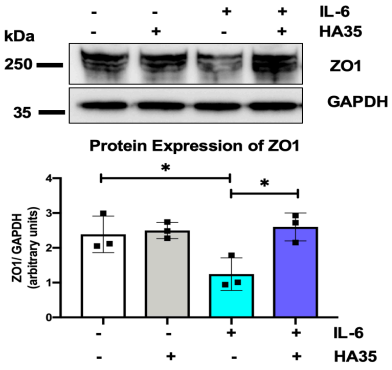

Fig. 5 HA35 protects against the effects of IL-6 on ZO1 protein expression in mouse DC organoids.
A representative Western Blot shows the decreased protein expression of ZO1 after treatment with IL-6 (100 ng/mL, 48 hour) (N=3, separate isolates of DC organoids from C57BL/6J WT mice). HA35 pretreatment (350 μg/mL, 24h) maintained ZO1 protein near basal levels in the IL−6 treatment group.

Major Task 6: Generation of cell specific TLR4 and CD44 v7 mice and HFD studies (Specific Aim 1)

Several variant isoforms of CD44 (CD44v) are expressed by alternative splicing of variant exons encoding extracellular regions. Particularly isoforms containing CD44v7 are expressed on T cells and macrophages in T-helper-1 (Th1)-mediated chronic inflammation in the gut. However, the first five (non-variable) exons of CD44 encode an amino-terminal globular protein domain, which is known as the 'link' domain, enables CD44 to bind to hyaluronan. For this reason, the best way to determine whether CD44 functions as a receptor for HA35 is by cell-specific conditional inactivation of all CD44 isoforms. Therefore, we obtained CD44 flox/flox mice from Dr. Ellen Puré at the University of Pennsylvania. When combined with a Cre, this will eliminate an essential constant exon, and thereby all CD44 isoforms.

Since our data supports that C57BL/6**J** genetic background is a more consistent model to study the prophylactic effects of HA35 against a high glucose and fat diet, we started backcrossing our transgenic mice to a C57BL/6**J** genetic background. Based on the single nucleotide polymorphism (SNP) analysis, used by Jackson laboratories to characterize sub-strain background and distinguish between C57BL/6**J** and C57BL/6**N**, we were able to achieve a 60.42% C57BL/6**J** genetic background in CD44 flox/flox, 97.55% in TLR4 flox/flox, 99.66% in Villin Cre, 99.31 % LysM Cre, and 100% in Layilin -/- mice. As soon as we accomplish a 100% C57BL/6**J** genetic background in all of our transgenic mice, we will start cross breeding them with the LysM Cre and Villin Cre for our experiments.

Key Accomplishments and Outcomes:

Experiments performed in the first year largely confirmed our hypothesis that HA35 would protect against barrier loss in the face of challenge with high glucose and pro-inflammatory conditions, and provided new insights the mode of action:

1) Demonstrated that hyperglycemic conditions and inflammatory cytokines compromise the tight junction protein composition in the primary cultures of mouse gut epithelium grown as distal colon organoids. The protein downregulation of ZO1, occludin and claudin1 is indicative of leaky barrier functions associated with bacterial and metabolite translocation and is considered a precursor to inflammation associated with many aspects of metabolic disease.
2) Demonstrated that HA35 pretreatment maintains the levels of tight junction in colon epithelial cell organoids treated with hyperglycemic conditions or inflammatory cytokines.

3) Since gene expression levels of the tight junction proteins in epithelium of colon organoids are not changed significantly by HA35, we now hypothesize that it acts mainly in pathways that prevent the loss of the junctional proteins, rather than by replacing them.

4) Technically, the complex crossing of genetically specific mice is well underway. Importantly, having identified an unexpected complication in substrains of mice (C57BL/6**J** vs C57BL/6**N**) early has averted potential complications in data interpretation in multiple experiments proposed for years 2 and 3 of this proposal, while only minimally slowing progress.

Publications: none to date
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