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Abstract (200 words)
The intestinal mucosa is lined by a single layer of epithelial cells (IECs) that form a selective physical barrier allowing the passage of nutrients while protecting the body against external antigens. Impairment of the intestinal barrier is associated with uncontrolled infiltration of leukocytes into the gut mucosa and represents a hallmark of inflammatory bowel disease (IBD). CAR-Like Membrane Protein (CLMP) belongs to the Cortical Thymocyte marker in Xenopus (CTX) family. CTX members are single transmembrane proteins that have been reported to play a role in epithelial barrier function and leukocyte recruitment to inflammatory sites. Although loss of function mutations of CLMP have been identified in humans with congenital Short Bowel syndrome, its function in the adult intestine is unknown. The aim of this proposal was to characterize the contributions of CLMP in intestinal mucosal homeostasis and inflammatory diseases. During the research period, using IECs that lack or overexpress CLMP and mice with inducible loss of CLMP in intestinal epithelial cells (villin-cre^{ERT2};CLMP$^{fl/fl}$) mice, we have elucidated key roles for CLMP in adult intestinal mucosa including regulation of intestinal epithelial barrier function, cell proliferation and epithelial repair after injury.

15. SUBJECT TERMS
CAR-Like Membrane Protein, intestinal mucosa, cell proliferation, epithelial barrier function, inflammatory bowel disease

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1. Introduction

The intestine is lined by a single layer of epithelial cells that forms a selective-permeable barrier permitting the uptake of luminal nutrients while protecting the body against external noxious substances and pathogens. Intestinal mucosal homeostasis requires coordinated proliferation of epithelial progenitors at the base of the crypt, their migration and differentiation along the crypt-luminal axis, followed by shedding at the luminal surface. During this complex process that lasts 5-7 days, cell-cell cohesion and barrier function are maintained. Importantly, impairment of mucosal homeostasis has been shown to lead to a compromised epithelial permeability which has been linked to the pathogenesis of inflammatory bowel disease (IBD).

At the molecular level, the intestinal barrier is dependent on a collection of proteins that form the apical junctional complex (AJC) that seals the apical-most region of the paracellular space between adjacent epithelial cells. Among the transmembrane proteins of the AJC involved in regulation of intestinal barrier function are members of the Cortical Thymocyte marker for Xenopus (CTX) family including Junctional Adhesion Molecule-A (JAM-A), Coxsackie and Adenovirus Receptor (CAR) and CAR-Like Membrane Protein (CLMP) (1). Unlike JAM-A and CAR, CLMP function has been poorly investigated. Recently, mutations in CLMP have been found in patients with Congenital Short Bowel syndrome, a disease characterized by a shortening of the small intestine and impaired intestinal absorptive function suggesting a role for CLMP in the embryonic development (2-4). However, its function in the adult intestine is largely unknown.

The aim of our project was to characterize the role of CLMP in intestinal mucosal homeostasis and inflammatory bowel disease. Our findings will help to better understand the molecular interactions that control intestinal barrier function and are linked to the pathogenesis of IBD. Furthermore, our results may help to develop new therapeutics to either facilitate the delivery of drugs through the mucosal barrier, or for the treatment of chronic inflammatory gut diseases.

2. Keywords

CAR-Like Membrane Protein, intestinal mucosa, cell proliferation, epithelial barrier function, inflammatory bowel disease.

3. Accomplishments

What were the major goals of the project?

The project was focused on understanding the contributions of CLMP in maintaining intestinal mucosal homeostasis and to identify the roles of CLMP on intestinal barrier compromise upon intestinal inflammation. The study was divided into two specific aims:

Aim 1: To investigate the role of CLMP in regulation of IEC proliferation, cell migration and barrier function.

Aim 2: To probe the role of CLMP in intestinal mucosal homeostasis under inflammatory conditions

What was accomplished under these goals?

- **Task 1.1A (PI: Nusrat): Completed**

  In-vitro studies on CLMP control of intestinal epithelial cells (IECs) homeostasis using assays of proliferation, cell migration and apoptosis with knockdown and overexpressing cell lines.

In order to conduct investigations on the roles of CLMP in the regulation of intestinal epithelial cell proliferation, cell migration, and barrier function in-vitro, we generated stable single clones of intestinal epithelial cell lines (IECs) with overexpression or down-regulation of CLMP protein (figure 1). For the over-expression of CLMP, the coding sequence of human full-length CLMP was cloned into pLEX-MCS lentiviral vector. Human IECs that lack endogenous expression of CLMP (such as SKCO15 and SW480 cells) were transduced with pLEX MCS-CLMP lentivirus or an empty virus as control. For the knock-down of CLMP, Caco-2 cells that express endogenous CLMP were infected with a non-silencing shRNA (control) or shRNA vector against the CLMP gene. In both cases (overexpression or knockdown of CLMP), virus-infected cells were selected with 2mg/mL of puromycin and single clones were obtained by limiting dilution.

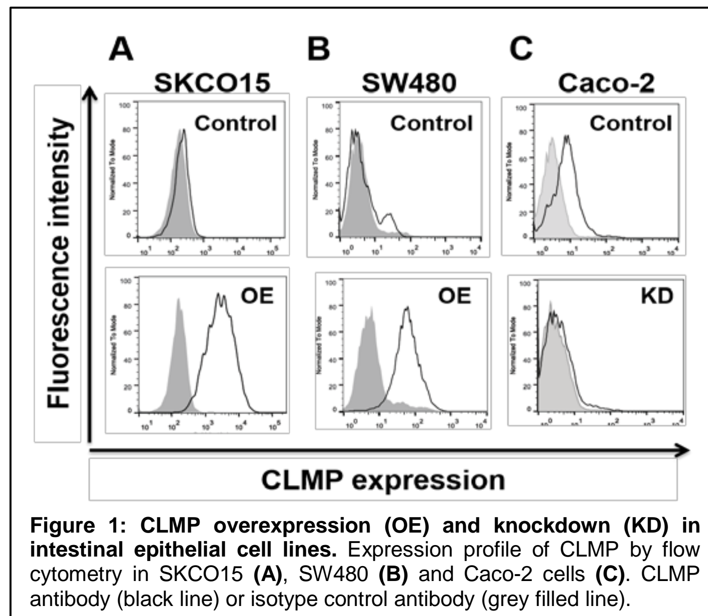

Figure 1: CLMP overexpression (OE) and knockdown (KD) in intestinal epithelial cell lines. Expression profile of CLMP by flow cytometry in SKCO15 **(A)**, SW480 **(B)** and Caco-2 cells **(C)**. CLMP antibody (black line) or isotype control antibody (grey filled line).

To investigate the role of CLMP in epithelial cell proliferation, we evaluated the incorporation of EdU (a modified thymidine analogue) into newly synthesized DNA in cells that overexpress or knockdown for CLMP versus control cells. Cells were seeded at subconfluent density (10^5 cells) on collagen coated glass coverslips. On day 1 following the seeding of the cells, EdU was added in the culture medium for 45 min.

After the incubation time, cells were fixed with formalin and the EdU was detected with fluorescently labeled Alexa Fluor 488 in accordance with the manufacturer's recommendations (Click-it EdU, Invitrogen). Cells were stained with Topro-3 iodide for the detection of total nuclei and the amount of EdU-fluorescent labeling proliferating cells was quantified. As shown in figure 2A, the overexpression of CLMP in SKCO15 and SW480 cells significantly decreased cell proliferation on day 1 following the seeding. In contrast, CLMP knockdown increased Caco-2 cell proliferation (figure 2B). These findings indicate that CLMP regulates epithelial cell proliferation. We further examined whether CLMP expression may affect cell death. For this purpose, we analyzed the expression level of nuclear cleaved Poly (ADP-ribose) polymerase (cleaved PARP) which is a target of caspases and a marker of cells apoptosis. We found that PARP is less subjected to cleavage in cells overexpressing CLMP than control cells suggesting that CLMP overexpression may protect the cells from apoptosis (figure 2C).

One major aspect of the intestinal mucosa is its ability to repair after injury in a process called wound healing by collective migration of the epithelial cells. We assessed the putative role of CLMP on wound closure *in-vitro* after a single linear scratch wound on confluent monolayer of SKCO15 or Caco-2 cells. Wound widths were monitored up to day 4 with an optic microscope. As exemplified in figure 2D, overexpression of CLMP in SKCO15 cells increased the restitution of cell monolayers after scratch wound injury while loss of endogenous CLMP in Caco-2 cells resulted in the delay of wound closure. Altogether, these observations strongly suggest that the level of expression of CLMP regulates many aspects of intestinal epithelial homeostasis encompassing intestinal cell proliferation, cell survival and migration after injury.

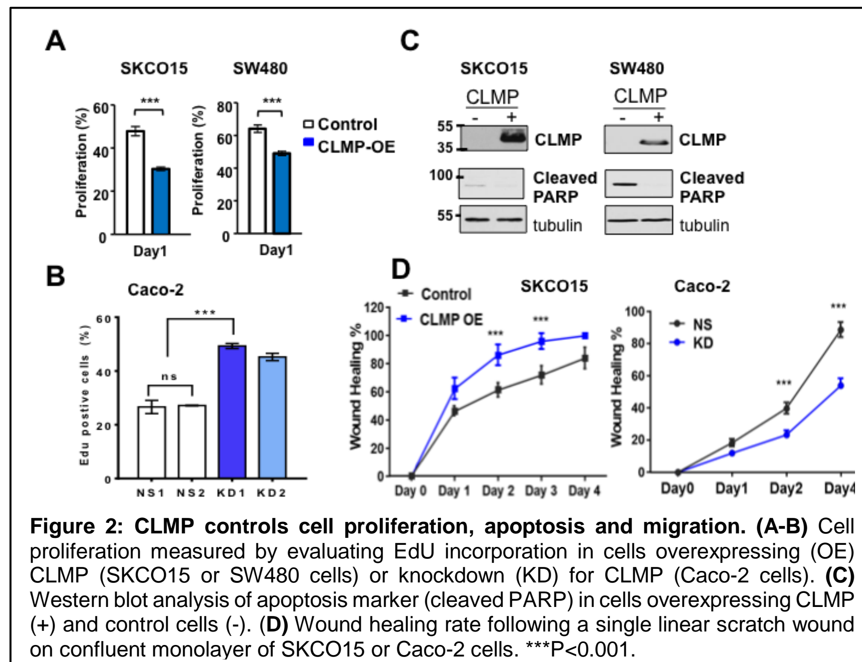

Figure 2: CLMP controls cell proliferation, apoptosis and migration. (A-B) Cell proliferation measured by evaluating EdU incorporation in cells overexpressing (OE) CLMP (SKCO15 or SW480 cells) or knockdown (KD) for CLMP (Caco-2 cells). **(C)** Western blot analysis of apoptosis marker (cleaved PARP) in cells overexpressing CLMP (+) and control cells (-). (**D)** Wound healing rate following a single linear scratch wound on confluent monolayer of SKCO15 or Caco-2 cells. ***P<0.001.

- **<u>Task 1.1B (PI: Parkos):</u> Completed**

  CLMP role in regulation of IECs barrier properties using knockdown and overexpressing cell lines.

CLMP is structurally related to Coxsackie and adenovirus receptor (CAR) that has been reported to be involved in intestinal barrier function. Therefore, we investigated the role of CLMP on the regulation of

epithelial paracellular permeability. For this purpose, cells were plated at confluent density on semi-permeable filters coated with collagen and transepithelial resistance (TER) was measured using an EVOM volt-ohmmeter at different time points up to 5 days after cells seeding. We observed that Caco-2 cell monolayers depleted for CLMP failed to develop high TERs (figure 3A) on day 3. Caco-2 cell monolayers that lack CLMP are leakier than control cells as shown by the increase of paracellular permeability to fluorescein isothiocyanate (FITC)-labeled dextrans (4kDa) when added to the upper chamber. After 2 hours of incubation, the fluorescent content in the basal chamber was measured using a fluorescence plate reader (figure 3B). In contrast, we found that overexpression of CLMP significantly enhanced the TER of SW480 cell monolayers (figure 3C). These results strongly suggest that CLMP participates in the regulation of the intestinal epithelial barrier. We next studied whether CLMP is involved in the formation of epithelial tight junctions. We used a calcium switch assay to study the assembly of intercellular junctions. T84 cells were grown at confluence on semi-permeable filters coated with collagen until they reach a high TER value about 1400 ohm.cm^2. Cells were incubated with 2mM EGTA in Hank's Balanced Salt Solution (HBSS) for 30 min to induce a quick loss of intercellular contacts by chelation of extracellular calcium. Then, complete cell culture media with calcium was added to allow the recovery of intercellular junctions. During the recovery period, anti-CLMP or isotype control antibodies were added. We found that anti-CLMP antibodies delayed the re-assembly of intercellular contacts after calcium switch, suggesting that CLMP supports tight junction formation (figure 3D).

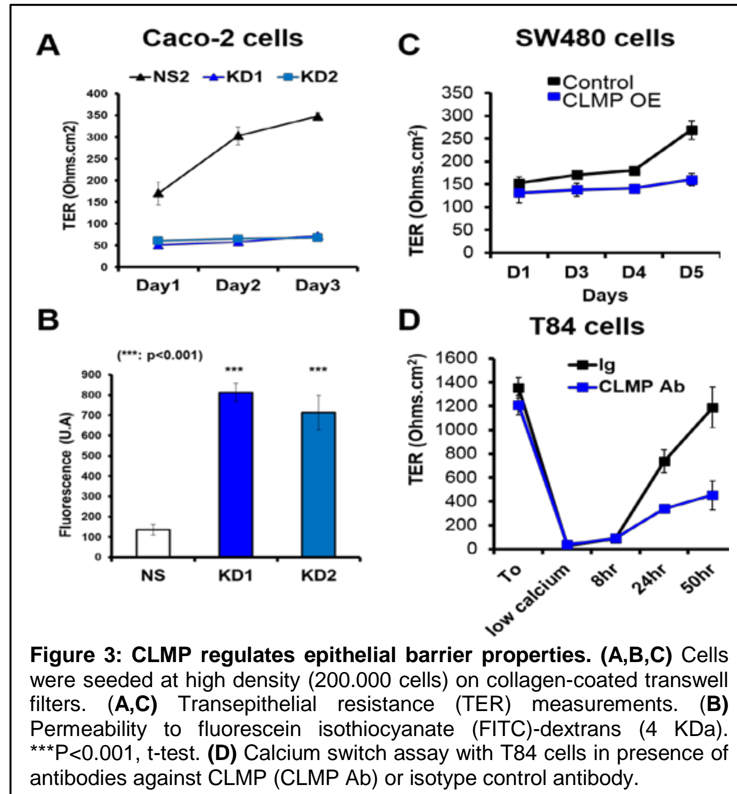

Figure 3: CLMP regulates epithelial barrier properties. (A,B,C) Cells were seeded at high density (200.000 cells) on collagen-coated transwell filters. (**A,C)** Transepithelial resistance (TER) measurements. (**B)** Permeability to fluorescein isothiocyanate (FITC)-dextrans (4 KDa). ***P<0.001, t-test. **(D)** Calcium switch assay with T84 cells in presence of antibodies against CLMP (CLMP Ab) or isotype control antibody.

- <u>**Task 1.1C (PI: Nusrat):**</u> **Completed**

  CLMP role in growth and polarity using knockdown and overexpressing cell lines.

As mentioned above, we found that the overexpression of CLMP decreased cell proliferation in contrast to its down-regulation that promoted cell proliferation indicating that CLMP expression regulates cell growth. In order to investigate the polarity of CLMP in intestinal epithelial cells, we have performed immunofluorescence staining followed by microscopy analysis of endogenous CLMP in Caco-2 cells (figure

4A) and in SKCO15 cells transfected with a vector expressing CLMP (figure 4B). As seen in Caco-2 cells, endogenous CLMP is detected along the basolateral membrane and it also co-localized with the tight junction (TJ) protein ZO-1. Using of SKCO15 cells, we found that ectopic expression of CLMP did not alter the localization of ZO-1 that is still concentrated at the plasma membrane and in cell junctional complexes (figure 4B). Furthermore, we did not observe any change in expression of TJ and AJ proteins including ZO-1, CAR, JAM-A and E-Cadherin (not shown). Altogether, these observations suggest that the level of expression of CLMP modulate cell growth without impairment of cell polarity.

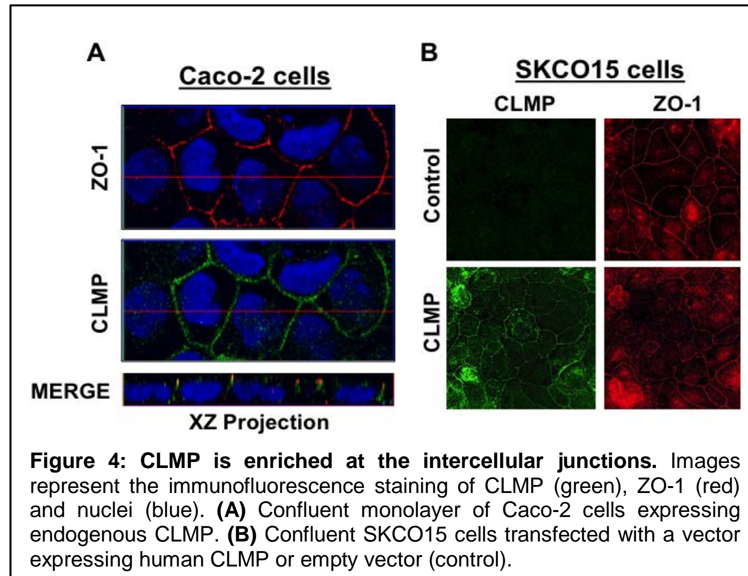

Figure 4: CLMP is enriched at the intercellular junctions. Images represent the immunofluorescence staining of CLMP (green), ZO-1 (red) and nuclei (blue). **(A)** Confluent monolayer of Caco-2 cells expressing endogenous CLMP. **(B)** Confluent SKCO15 cells transfected with a vector expressing human CLMP or empty vector (control).

- **<u>Task 1.1D (PI: Nusrat):</u> Completed**
  CLMP role in epithelial growth in Xenografts in-vivo.

To analyze the putative role of CLMP in proliferative capacity of epithelial cells and tumor growth in-vivo, we performed xenograft experiments with Rag 1-deficient (-/-) mice as recipients. We injected subcutaneously 10^6 SKCO15 cells overexpressing CLMP or control cells into twelve-week-old Rag $1^{-/-}$ mice (7 mice per condition). Tumor growth was monitored every day by palpation and mice were sacrificed when tumors reached ≈ 2 cm in size. As shown in figure 5A, the overexpression of CLMP prevented tumor growth, since tumors developed in 100 percent of mice injected with control cells lacking CLMP, while only 1 mouse that was injected with cells overexpressing CLMP produced a small tumor (33.5 mm^3). In contrast, the depletion of CLMP in Caco-2 cells (KD) increased the size of tumor growth in-vivo (figure 5B). Altogether, our results provide evidence that CLMP expression controls epithelial cells' proliferation in-vitro and in-vivo.

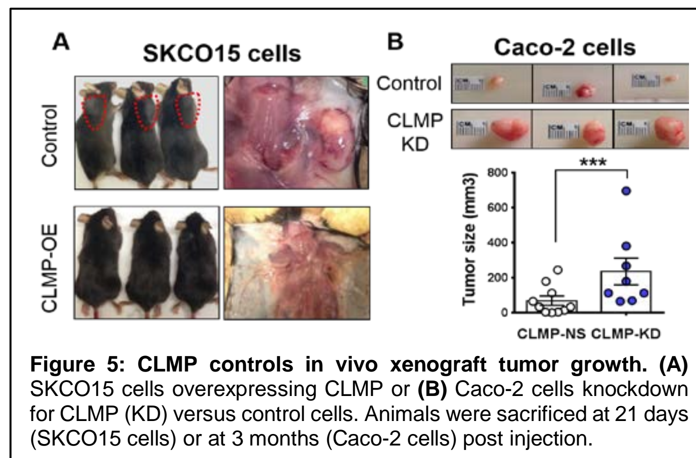

Figure 5: CLMP controls in vivo xenograft tumor growth. (A) SKCO15 cells overexpressing CLMP or **(B)** Caco-2 cells knockdown for CLMP (KD) versus control cells. Animals were sacrificed at 21 days (SKCO15 cells) or at 3 months (Caco-2 cells) post injection.

- **Task 1.2A (PI: Parkos): Completed**

  CLMP co-association with tight junction (TJ) proteins by immunoprecipitation, immunofluorescence labeling and confocal microscopy.

CLMP is structurally related to Junctional adhesion molecule -A and CAR which have been reported to be localized at TJs and interact with TJ-associated proteins such as ZO-1 (5). As mentioned above, we found endogenous CLMP co-distributed at the plasma membrane with ZO-1 in Caco-2 and SKCO15 cells (figure 4). Because TJ complexes have been reported to be associated with lipid-enriched microdomains, we investigated whether CLMP is present in these plasma membrane microdomains. We performed a sucrose gradient ultracentrifugation to separate lipid rafts by flotation as described previously (6). Caco-2 cells were lysed with 1.5% Triton X-100 lysis buffer, then lysates were adjusted to a final concentration of 40% sucrose (w/w) and separated in a linear 5%-30% sucrose gradient by ultracentrifugation. 0,5 ml fractions were collected and proteins were resolved on SDS-PAGE gel. Lipid raft-associated proteins such as flotilin-1 and desmoglein-2 were used as markers for western blotting (figure 6A). The majority of CLMP proteins was not found in lipid rafts, however at higher exposure we detected CLMP in flotilin-1 and desmoglein-2 rich fractions. We conclude that CLMP is localized in two pools in the lateral cell membrane and is enriched in lipid microdomains. We further studied the interaction between CLMP and the TJ-associated protein ZO-1 using proximity ligation assays. This technique is a sensitive method for detecting protein-protein interactions (closer than 40 nm) visualized as individual dots by fluorescent microscopy. We detected intensive PLA signals between endogenous CLMP and ZO-1 in Caco-2 cells at intercellular contacts when cells were incubated with specific antibodies against both CLMP and ZO-1 (figure 6B). In contrast, no fluorescent dots were detected with the antibody combination containing anti-CLMP and isotype control. We then confirmed the physical interaction between CLMP and ZO-1 by co-immunoprecipitation assay using Caco-2 cell lysates in RIPA buffer (figure 6C). Furthermore, by immunoprecipitation assay, we also identified the TJ-associated protein Afadin/AF-6 and AJ-associated protein β-catenin as co-precipitate binding partners of CLMP.

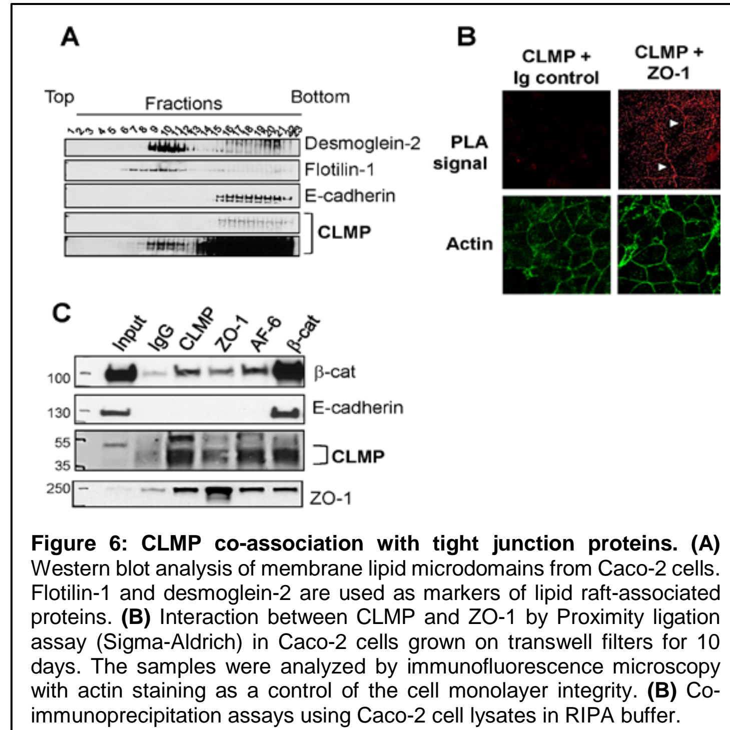

Figure 6: CLMP co-association with tight junction proteins. (A) Western blot analysis of membrane lipid microdomains from Caco-2 cells. Flotilin-1 and desmoglein-2 are used as markers of lipid raft-associated proteins. **(B)** Interaction between CLMP and ZO-1 by Proximity ligation assay (Sigma-Aldrich) in Caco-2 cells grown on transwell filters for 10 days. The samples were analyzed by immunofluorescence microscopy with actin staining as a control of the cell monolayer integrity. **(B)** Co-immunoprecipitation assays using Caco-2 cell lysates in RIPA buffer.

- **Task 1.2B (PI: Parkos): Partially completed; technical challenges encountered.**
  Mass spectrometry of CLMP immunoprecipitates to identify associated proteins and verification by immunofluorescence and western blot of immunoprecipitates.

As mentioned above, we succeeded in performing co-immunoprecipitation assays to study the physical interaction between CLMP and partners by using commercially available antibodies against CLMP and specific candidate proteins such as ZO-1 or Afadin/AF-6. However, using these conditions we were unable to detect bands by Coomassie blue of stained proteins co-precipitated with CLMP and separated by SDS-PAGE. Despite our efforts to enrich a huge amount of CLMP and the use of mild detergents in the cell lysate buffer (for example Brj97 instead of triton X-100), we found CLMP slightly enriched by immunoprecipitation and did not distinguish clear protein bands for putative partners that we could extract for mass spectrometry analysis and for identification of putative partners (figure 7).

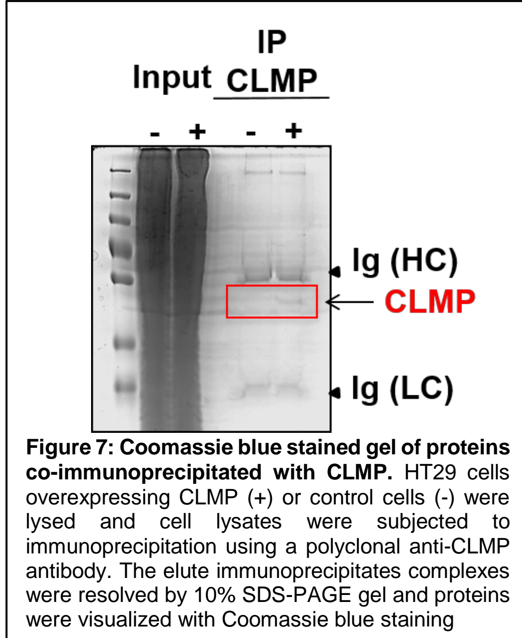

Figure 7: Coomassie blue stained gel of proteins co-immunoprecipitated with CLMP. HT29 cells overexpressing CLMP (+) or control cells (-) were lysed and cell lysates were subjected to immunoprecipitation using a polyclonal anti-CLMP antibody. The elute immunoprecipitates complexes were resolved by 10% SDS-PAGE gel and proteins were visualized with Coomassie blue staining

- **Task 1.3 (PI: Parkos): Partially completed; extracellular domain studied, intracellular domain studies incomplete.**
  CLMP Structure-function studies that include identification of CLMP domain responsible for functional effects.

We have produced soluble recombinant proteins of CLMP (sCLMP) consisting of the two extracellular immunoglobulin (Ig)-like domains at the C-terminus: a His-tag or a Fc-tag (figure 8A). Since CLMP possesses a putative dimerization motif (analogous to CAR), we performed an ELISA-based assay to investigate whether the sCLMP proteins homodimerize and kept a native configuration. We found that immobilized sCLMP proteins bind specifically to sCLMP in solution. This indicates that sCLMP present the same features as CLMP when expressed in

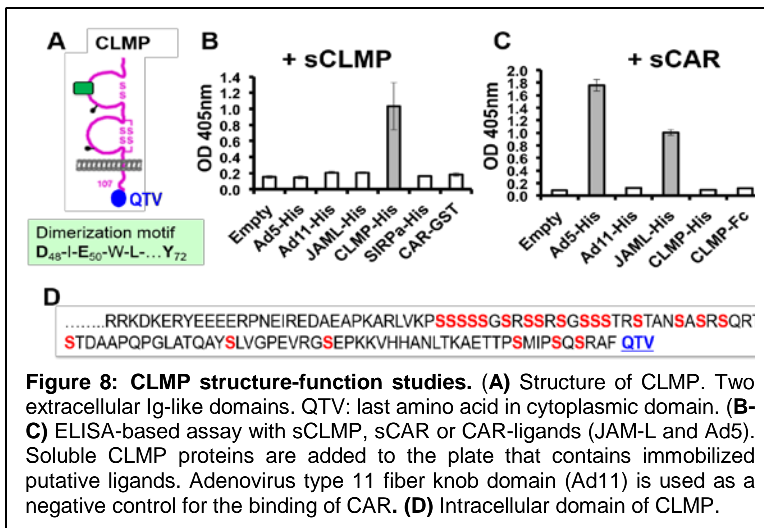

Figure 8: CLMP structure-function studies. (**A)** Structure of CLMP. Two extracellular Ig-like domains. QTV: last amino acid in cytoplasmic domain. (**B-C)** ELISA-based assay with sCLMP, sCAR or CAR-ligands (JAM-L and Ad5). Soluble CLMP proteins are added to the plate that contains immobilized putative ligands. Adenovirus type 11 fiber knob domain (Ad11) is used as a negative control for the binding of CAR**. (D)** Intracellular domain of CLMP.

cells (figure 8B). Then, we tested whether sCLMP proteins can bind to the closely related protein Coxsackie and Adenovirus Receptor (CAR) or CAR ligands (such as Junctional Adhesion Molecule-Like/JAM-L and adenovirus type 5 fiber knob domain/Ad5). No binding interactions were detected with the extracellular domains of CAR or CAR ligands (figure 8B). As a control experiment, figure 8C shows that soluble recombinant CAR proteins bind to its immobilized ligands (Ad5 and JAM-L) but not to CLMP. Altogether our finding indicates that CLMP forms dimers through its extracellular domain and may not substitute CAR to its ligands. We have also generated sCLMP with a deletion of the distal Ig loop (CLMP-D1) or of the proximal Ig loop (CLMP-D2). Furthermore, as shown in figure 8D, the intracellular domain of CLMP presents several Serine amino acids that might act as putative sites for phosphorylation or dephosphorylation by kinases or phosphates, respectively. The role of the truncated soluble forms of the extracellular domain of CLMP as well as its intracellular domain requires further investigations.

- <u>**Task 1.4 (PI: Nusrat):**</u> **Completed**
  CLMP signaling pathways.

We have found that CLMP overexpression negatively regulates cell proliferation (figure 2A). Therefore, we have initiated experiments to explore how CLMP may modulate cell proliferation. We analyzed by western blot the expression of well-accepted markers of cell proliferation such as AKT and members of the cyclin family that control the cell cycle. Among the molecules tested, we found that the overexpression of CLMP significantly decreased the level of proteins of p-AKT (S473) as well as the cyclin D family such as cyclin D1 and D2 using two distinct human IECs (SKCO15 and SW480 cells) (figure 9A). These results suggest that CLMP delayed cell proliferation by decreasing the expression level of cyclin D proteins that drive the G1/S phase transition. Given that Cyclin D family members have been reported to be downstream targets of β-catenin transcription activity, we studied whether CLMP controls cyclin D levels through β-catenin. Using a TOP/FOP luciferase reporter assay that is well-accepted for studying β-catenin transcriptional activity (7), we found that over-expression of CLMP in SKCO15 cells resulted in reduce transcriptional activity of β-catenin. In contrast, knockdown of CLMP enhanced β-catenin activity (figure 9B). In conclusion, we have identified a CLMP-dependent signaling pathway that involves β-catenin activity to regulate cell proliferation.

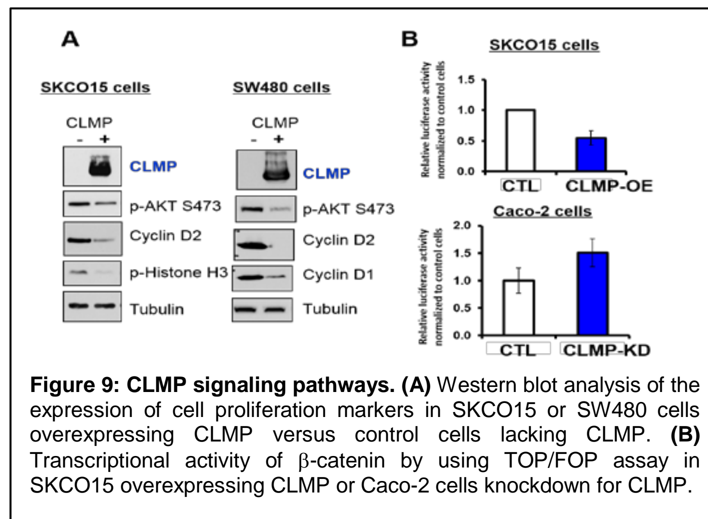

Figure 9: CLMP signaling pathways. (A) Western blot analysis of the expression of cell proliferation markers in SKCO15 or SW480 cells overexpressing CLMP versus control cells lacking CLMP. **(B)** Transcriptional activity of β-catenin by using TOP/FOP assay in SKCO15 overexpressing CLMP or Caco-2 cells knockdown for CLMP.

- **Task 1.5A (PI: Parkos): Completed**

  Generation of mice with inducible targeted deletion of CLMP in IECs.

Since CLMP has been reported to be critical in embryonic gut development, the global CLMP gene loss in mice would be problematic to study the role of CLMP in adult intestine. To circumvent this issue, we generated transgenic mice in which the CLMP gene (exon 3) is flanked by loxP sites that will allow conditional tissue-specific deletion of CLMP when such animals were mated with tamoxifen tissue-specific promoter, gene-Cre^{+} animals (Figure 10A). For the study of CLMP in intestinal mucosa, these animals were crossed with tamoxifen-inducible villin-Cre (Villin-Cre^{ERT2}) animals to generate CLMP knockdown specifically in intestinal epithelial cells. As seen in figures 10 B-C, CLMP is depleted in IECs, which form ileum at the level of messenger RNA and protein.

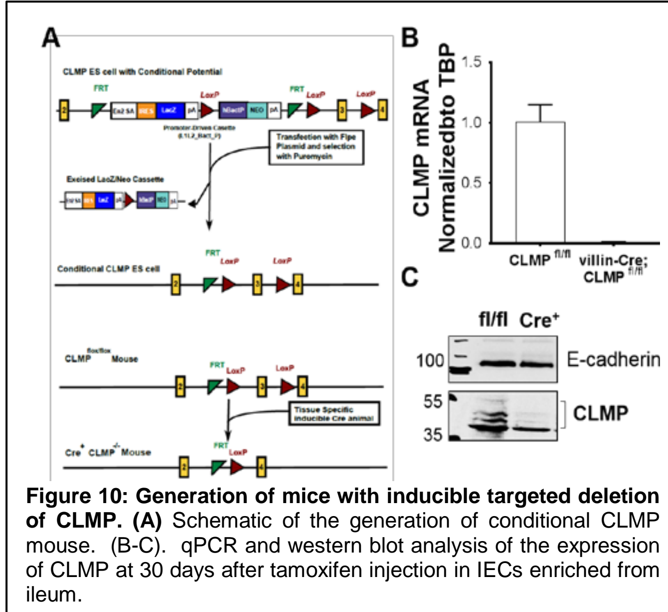

Figure 10: Generation of mice with inducible targeted deletion of CLMP. (A) Schematic of the generation of conditional CLMP mouse. (B-C). qPCR and western blot analysis of the expression of CLMP at 30 days after tamoxifen injection in IECs enriched from ileum.

- **Task 1.5B (PI: Nusrat): Completed**

  In-vivo analysis of CLMP function. Effect of inducible targeted deletion of CLMP effects on IEC homeostasis and barrier function.

Using tamoxifen-inducible CLMP deficient mice, the acute depletion of CLMP did not result in an alteration of the gross architecture of the colon and ileum. As shown in figure 11 A, H&E coloration showed no difference in the overall organization and size of the crypts and villi in the ileum of CLMP $^{fl/fl}$ and villin-Cre^{ERT2}; CLMPfl$^{/fl}$ mice. In addition, loss of CLMP in the ileum did not impair the polarized localization of the tight junction protein claudin-4 at the tip of the villi, as detected by in situ hybridization analyses (figure 11 A). Furthermore, in basal condition, we studied the effect of the depletion of CLMP in IECs on mucosal permeability to FITC-dextrans (4kDa) in-vivo by using a well-established method in our laboratory of ileal

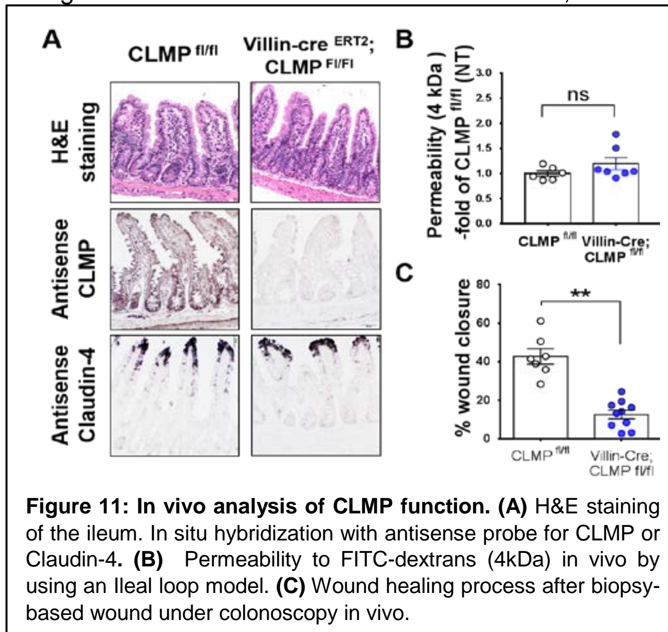

Figure 11: In vivo analysis of CLMP function. (A) H&E staining of the ileum. In situ hybridization with antisense probe for CLMP or Claudin-4**. (B)** Permeability to FITC-dextrans (4kDa) in vivo by using an Ileal loop model. **(C)** Wound healing process after biopsy-based wound under colonoscopy in vivo.

loop model (8, 9). We found no difference in FITC-dextrans paracellular permeability between CLMP deficient and control animals, indicating that loss of CLMP in-vivo did not affect intestinal barrier function in basal condition. Given our in-vitro findings described above (figure 3), these results suggest that compensatory mechanisms might be involved in-vivo to prevent a leaky barrier secondary of CLMP loss. Interestingly, following colonoscopy-based wound injuries, the absence of CLMP resulted in reduced wound healing in-vivo compared to control mice. These findings strongly suggest that while CLMP does not play a major role in barrier function under homeostasis, CLMP is required for an efficient epithelial restitution after wound injury.

- **<u>Task 2.1A (PI: Nusrat):</u> Completed**
  In-vitro assays of IFNγ and TNFα effects on CLMP expression and subcellular localization in model intestinal epithelial cell lines.

To study the expression of CLMP under inflammatory conditions in-vitro, we treated human model IECs (Caco-2 and T84 cells) cultured on semi-permeable filters coated with collagen with pro-inflammatory cytokines for 24 hours or 48 hours: IFNγ or TNFα or a combination of IFNγ + TNFα (100ug/ml each). Interestingly, TNFα alone did not affect the expression of CLMP in both cell lines (Caco-2 and T84), while IFNγ, or a treatment IFNγ + TNFα resulted in increased expression of CLMP mRNA (figure 12). We were not able to detect an increased expression of CLMP protein in IECs treated with pro-inflammatory cytokines either by western blotting nor changes in CLMP subcellular localization by immunofluorescence microscopy.

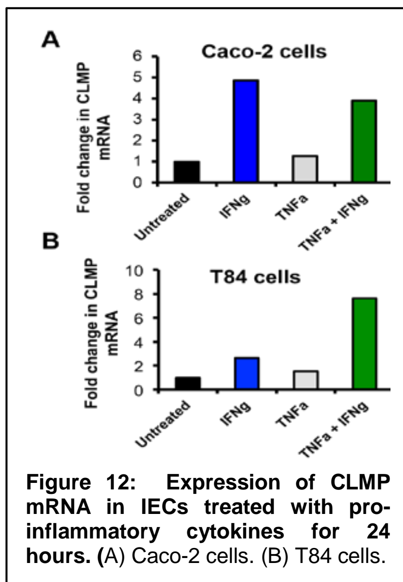

Figure 12: Expression of CLMP mRNA in IECs treated with pro-inflammatory cytokines for 24 hours. (A) Caco-2 cells. (B) T84 cells.

- **<u>Task 2.1B (PI: Nusrat):</u> Completed**
  In-vivo analysis of CLMP expression in colitis and in colonic mucosa from individuals with inflammatory bowel disease (IBD).

To determine whether the expression of CLMP is altered under inflammatory conditions in-vivo, wild-type C57BL6 mice were subjected to dextran sulfate sodium (DSS)-induced colitis by chemical mucosal injury. DSS (2.5%) were administrated in drinking water daily for up to 6 days. Since DSS mainly affects the distal colon, pieces of distal colon were processed to extract total RNA at 2, 4, and 6 days of treatment with DSS. CLMP mRNA was quantified by qPCR. We found increased expression of CLMP mRNA in mice treated

with DSS, indicating that CLMP is up-regulated in the intestinal mucosa under inflammation in-vivo (figure 13A). The increased expression of CLMP protein was also detected in tissue from individuals with IBD compared to control tissue (non-inflamed) by immunofluorescence staining and western blot analysis (figure 13B). Altogether, these observations indicate that CLMP expression is enhanced under inflammatory conditions in-vivo in human and mouse colon.

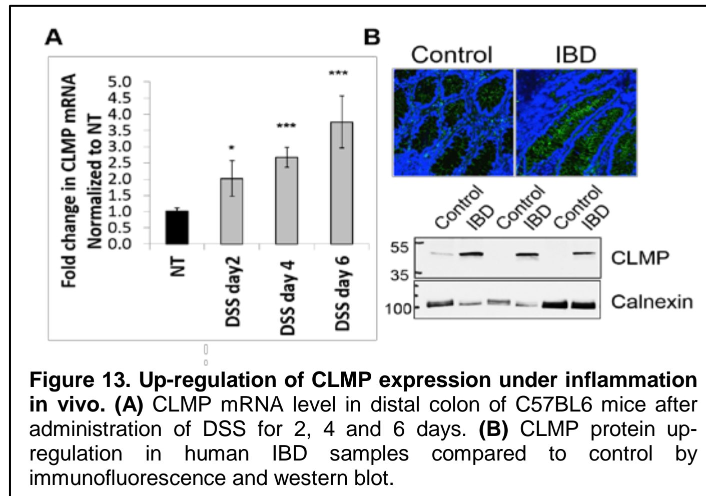

Figure 13. Up-regulation of CLMP expression under inflammation in vivo. (A) CLMP mRNA level in distal colon of C57BL6 mice after administration of DSS for 2, 4 and 6 days. **(B)** CLMP protein up-regulation in human IBD samples compared to control by immunofluorescence and western blot.

- **<u>Task 2.2 (PI: Nusrat):</u> Completed**

  Effects of increased CLMP expression on IEC function under conditions of inflammation in-vitro.

As indicated above (Task 2.1A), we were not able to detect an increase of CLMP at protein level in IECs treated with pro-inflammatory cytokines (TNFα and IFNγ) in-vitro, while CLMP mRNA is found up-regulated (figure 12). Therefore, we did not investigate the functional role of CLMP under inflammation in-vitro, since we had no evidence of an alteration of the level of expression of CLMP protein in these conditions.

- **<u>Task 2.3 (PI: Parkos):</u> Completed**

  Role of CLMP in PMN transepithelial (TEpM) migration in-vitro and in-vivo.

We used a transwell-based system in-vitro (as described previously (10)) to assess the role of CLMP in PMN TEpM. Human neutrophils freshly isolated were added on the upper compartment of the transwells at the basal side of a polarized monolayer of T84 cells. Bacterial formyl peptide fMLF was added in the lower chamber of the transwell to attract the PMN. After one hour, PMN that have transmigrated into the lower compartment of the transwell were quantified by the measure of MPO activity. First we confirmed that CLMP mRNA is expressed in human PMN (figure 14A). Then, we used two distinct

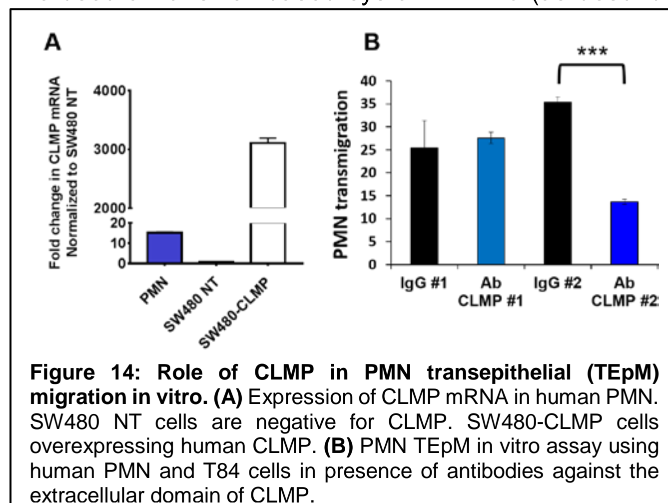

Figure 14: Role of CLMP in PMN transepithelial (TEpM) migration in vitro. (A) Expression of CLMP mRNA in human PMN. SW480 NT cells are negative for CLMP. SW480-CLMP cells overexpressing human CLMP. **(B)** PMN TEpM in vitro assay using human PMN and T84 cells in presence of antibodies against the extracellular domain of CLMP.

antibodies (commercially available) against the extracellular domain of CLMP and found reduced TEpM indicating that neutralization of CLMP has prevented PMN TEpM in-vitro (figure 14B). Since CLMP is expressed on neutrophils and IECs, it is likely that CLMP-CLMP homophilic interactions might be engaged in this process. Interestingly, using the ileal loop model for TEpM assay (as described previously, (9)) with CLMP deficient mice in intestinal epithelium (villin-Cre^{ERT2};CLMP$^{fl/fl}$ mice), we found no difference in PMN TEpM (not shown). These observations suggest that in-vivo, other molecules/factors may compensate the loss of CLMP. This observation is similar with the absence of CLMP effect on barrier function in-vivo while we detected a defect using in-vitro assays as mentioned above (figures 3 and 11B).

- **<u>Task 2.4 (PI: Parkos):</u> Completed**
  The effect of targeted deletion of CLMP on colitis induction and recovery using DSS model of colitis.

We performed DSS-induced colitis model in-vivo using Villin-Cre^{ERT2}; CLMPfl$^{/fl}$ and CLMP$^{fl/fl}$ mice. 2.5% DSS was administrated ad libitum in the drinking water for 9 days to mimic acute mucosal injury or by cycles of 5 days of DSS followed by 10 days of water only to induce injury/ recovery cycles as seen during chronic inflammation. Disease activity index (DAI) was determined by measure of the weight loss, presence of blood in stool and stool consistency. The maximal value of DAI of 4 correlate with high levels of inflammation and clinical symptoms. We observed that loss of CLMP in IECs did not affect the severity of disease in both acute and chronic colitis (figure 15). These findings indicate that CLMP does not play a role in development and progression of colitis in-vivo.

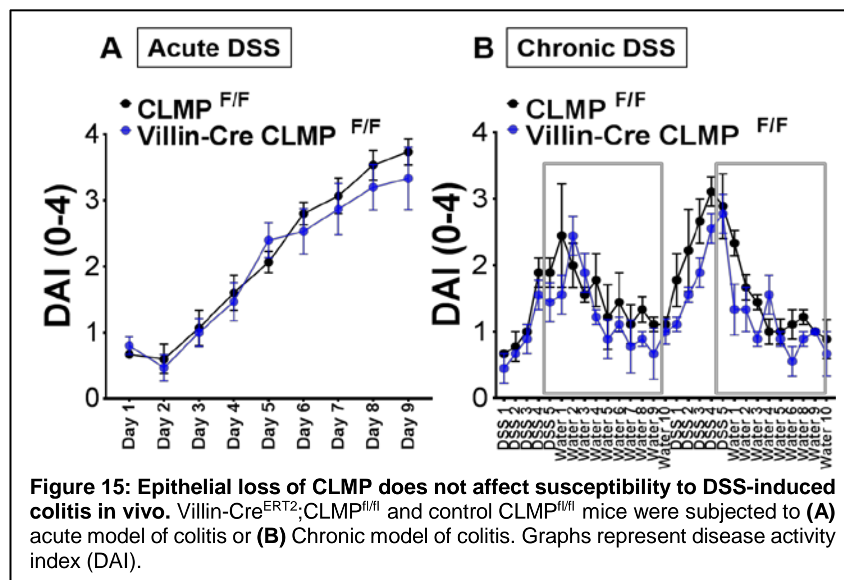

Figure 15: Epithelial loss of CLMP does not affect susceptibility to DSS-induced colitis in vivo. Villin-Cre^{ERT2};CLMP$^{fl/fl}$ and control CLMP$^{fl/fl}$ mice were subjected to **(A)** acute model of colitis or **(B)** Chronic model of colitis. Graphs represent disease activity index (DAI).

What opportunities for training and professional development has the project provided?

- Anny-Claude Luissint (PhD) did a presentation at the **Annual Meeting of the American Society for Investigative Pathology (ASIP)** in conjunction with Experimental Biology 2014 at San Diego (April 26-30). Title: "*CAR-like membrane protein negatively regulates the intestinal barrier and promotes neutrophil transepithelial migration",* published in ***The FASEB Journal. April 2014. Abstract Number:60.6.***

- Anny-Claude Luissint (PhD) did a presentation at the **Annual Meeting of ASIP** in conjunction with Experimental Biology 2015 at Boston (April 28-May1st). Title: "*CLMP Expression is Increased in the Intestinal Epithelium Under Inflammatory Conditions and Regulates Intercellular Adhesion, Proliferation and Migration".* ***The FASEB Journal. April 2015. Abstract Number: 282.9.***

- Anny-Claude Luissint (PhD) did a presentation at ***Military Health System Research Symposium (MHSRS)*** at Fort Lauderdale, FL (August 19th, 2015). Title: "*Increased expression of CAR-Like Membrane Protein (CLMP) in inflammatory bowel disease positively regulates intestinal barrier function and wound healing".*

- Anny-Claude Luissint (PhD) presented a poster at the ***PISA conference*** entitled Pathways to Translational Medicine: Recent Advances in Cell injury, Inflammation, and Neoplasia in Baltimore, MD, October 8-10, 2015. Title: "*Upregulation of CAR-Like Membrane Protein (CLMP) expression in the inflamed intestinal mucosa promotes cell migration and repair"*.

- Anny-Claude Luissint (PhD) did a presentation at Gastrointestinal Function and Development "***Gut" Group meeting April 11th, 2018 at University of Michigan.*** Ann Arbor, MI. Title: "*CAR-Like Membrane Protein (CLMP), a member of the CTX family that regulates intestinal mucosal homeostasis and inflammation".*

How were the results disseminated to communities of interest?

Research results were presented at the following national and local scientific meetings

Annual Meeting of the American Society for Investigative Pathology (ASIP) in conjunction with Experimental Biology 2014 at San Diego (April 26-30). Title: "*CAR-like membrane protein negatively regulates the intestinal barrier and promotes neutrophil transepithelial migration",* published in ***The FASEB Journal. April 2014. Abstract Number:60.6.***

Annual Meeting of ASIP in conjunction with Experimental Biology 2015 at Boston (April 28-May1st). Title: "*CLMP Expression is Increased in the Intestinal Epithelium Under Inflammatory Conditions and Regulates*

*Intercellular Adhesion, Proliferation and Migration". **The FASEB Journal. April 2015. Abstract Number: 282.9.***

Military Health System Research Symposium (MHSRS) at Fort Lauderdale, FL (August 19th, 2015). Title: "*Increased expression of CAR-Like Membrane Protein (CLMP) in inflammatory bowel disease positively regulates intestinal barrier function and wound healing".*

Pathobiology for students, investigators and academicians (PISA) conference entitled Pathways to Translational Medicine: Recent Advances in Cell injury, Inflammation, and Neoplasia in Baltimore, MD, October 8-10, 2015. Title: "*Upregulation of CAR-Like Membrane Protein (CLMP) expression in the inflamed intestinal mucosa promotes cell migration and repair"*.

Gastrointestinal Function and Development "***Gut" Group meeting April 11th, 2018 at University of Michigan.*** Ann Arbor, MI. Title: "*CAR-Like Membrane Protein (CLMP), a member of the CTX family that regulates intestinal mucosal homeostasis and inflammation".*

What do you plan to do during the next reporting period to accomplish the goals?

This is the final report and plan to submit a manuscript for publication in a peer reviewed journal that highlights the results in this report during the next year.

4. Impact

What was the impact on the development of the principal discipline(s) of the project?

Given the high impact of IBD (ulcerative colitis and Crohn's disease) on health and medical management of both military recruits and veterans, studies directed at understanding the pathogenesis and treatment of IBD are warranted.

At the beginning of the study, CLMP was previously reported to play a role in intestinal development since loss of function mutations result in congenital short bowel syndrome in human neonates; however, its contributions in adult intestine were unknown. The aim of our study was to identify the roles of CLMP in the adult intestine and open directions for further studies as well as pharmacological approaches for the treatment of individuals with IBD. During the funding period, in order to study the role of CLMP in intestinal epithelial mucosal homeostasis and inflammation in-vitro and in-vivo, we successfully produced human intestinal epithelial cell lines (knock-down or overexpressing CLMP), and generated tamoxifen-inducible knock-out mice of CLMP in intestinal epithelial cells (Villin-Cre^{ERT2}; CLMP $^{fl/fl}$), respectively. Overall, our results indicate that CLMP is a key regulator of intestinal mucosal homeostasis encompassing epithelial barrier function, cell proliferation, and cell migration: (1) we found that over-expression of CLMP prevented

xenograft tumor growth in mouse while its loss of expression promotes tumor growth, indicating that CLMP controls cell proliferation and might act as a tumor suppressor. (2) We have identified a CLMP-dependent signaling pathway that involve molecules that are well-described to modulate epithelial cell proliferation (such as β-catenin and cyclin D); however, further investigations are required to deeply characterize the mechanisms by which CLMP prevents intestinal epithelial cell proliferation. Furthermore, (3) we have observed that CLMP promoted barrier function to fluorescein dextrans (4kDa) as well as epithelial restitution after wound injury in-vitro and in-vivo. Interestingly, since the expression of CLMP is upregulated during inflammation in the intestine from individuals with IBD as well as in colon from mice under dextran sulfate sodium (DSS)-induced colitis, altogether these results strongly indicate that CLMP plays protective roles in the intestine during inflammation.

In conclusion, our findings are making an impact on a better understanding of the molecular mechanisms that control intestinal epithelial barrier function during homeostasis, inflammation and mucosal repair after injury. A long term benefit is that such studies may yield new therapeutic targets to help with medical management of military recruits and veterans with IBD.

What was the impact on other disciplines?

Nothing to Report

What was the impact on technology transfer?

Nothing to Report

What was the impact on society beyond science and technology?

A long term benefit is that such studies may yield new therapeutic targets to help with medical management of military recruits and veterans with IBD.

5. Changes/Problems

Changes in approach and reasons for change:

Nothing to Report

Actual or anticipated problems or delays and actions or plans to resolve them:

Nothing to Report

Changes that had a significant impact on expenditures

During the reporting period (on March 2015), Drs. Parkos and Nusrat moved their laboratory from Emory University (Atlanta, GA) to the University of Michigan (Ann Arbor, MI). The DOD funds were discontinued

from 02/28/2015 to 01/15/2016. This loss of resources has unfavorably impacted the development of the project, notably in terms of delays in hiring individuals to work on the project, no funds to buy reagents and materials required for experiments and to maintain the CLMP animal colonies.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report

6. Products

Publications (in preparation):

"Loss of CAR-Like Membrane Protein (CLMP) expression promotes cell proliferation and tumor growth". Anny-Claude Luissint, Liu Xiaoming, Ashley Bennett, Roland Hilgarth, Hikaru Nishio, Charles Parkos and **Asma Nusrat.**

"*CAR-Like Membrane Protein (CLMP) is up-regulated in the inflamed intestinal mucosa promoting epithelial barrier function and repair after injury*". Anny-Claude Luissint, Sven Flemming, Vicky García-Hernández, Roland Hilgarth, Michele Reed, Ashley Bennett, Asma Nusrat and **Charles Parkos.**

Review articles:

"JAM related proteins in mucosal homeostasis and inflammation". Luissint AC, Nusrat A, **Parkos CA**. Semin Immunopathol. 2014; 36(2): 211–226.

"Inflammation and the Intestinal Barrier: Leukocyte-Epithelial Cell Interactions, Cell Junction Remodeling, and Mucosal Repair". Luissint AC, Parkos CA, **Nusrat A**. Gastroenterology. 2016;151(4):616-32

Presentations:

- Anny-Claude Luissint (PhD) did a presentation at the **Annual Meeting of the American Society for Investigative Pathology (ASIP)** in conjunction with Experimental Biology 2014 at San Diego (April 26-30). Title: "*CAR-like membrane protein negatively regulates the intestinal barrier and promotes neutrophil transepithelial migration",* published in ***The FASEB Journal. April 2014. Abstract Number:60.6.***

- Anny-Claude Luissint (PhD) did a presentation at the **Annual Meeting of ASIP** in conjunction with Experimental Biology 2015 at Boston (April 28-May1st). Title: "*CLMP Expression is Increased in the Intestinal Epithelium Under Inflammatory Conditions and Regulates Intercellular Adhesion, Proliferation and Migration".* ***The FASEB Journal. April 2015. Abstract Number: 282.9.***

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Journal publications. Books or other non-periodical, one-time publications. Other publications, conference papers, and presentations. Website(s) or other Internet site(s). Technologies or techniques. Inventions, patent applications, and/or licenses. Other Products.

Nothing to Report

7. Participants & Other Collaborating Organizations

What individuals have worked on the project?

Name:	***Dr. Charles Parkos***
Project Role:	*PI (Initiating PI)*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No Change
Contribution to Project:	No Change
Funding Support:	No Change

Name:	***Dr. Asma Nusrat***
Project Role:	*PI (Partnering PI)*
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No Change
Contribution to Project:	No Change
Funding Support:	No Change

Name:	***Dr. Anny-Claude Luissint***
Project Role:	Postdoctoral fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	No Change
Contribution to Project:	No Change
Funding Support:	*No change*

Name:	***Ashley Bennett***
Project Role:	Research specialist
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*100% effort through Feb 28th 2015*
Contribution to Project:	Assisting in performing experiments, assisting in maintaining inducible tissue targeted CLMP knockout mice.
Funding Support:	*DoD*

Name:	***Shuling Fan***
Project Role:	Research Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	*100% effort from 12-1-16 to 3-31-18*
Contribution to Project:	Cell line generation and basic molecular and cellular biology
Funding Support:	*DoD*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to Report.

What other organizations were involved as partners?

Nothing to Report.

8. Special Reporting Requirements

A Duplicative report will be submitted for both, Dr. Charles Parkos (Initiating PI) and Dr. Asma Nusrat (Partnering PI).

9. Appendices

None

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