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14. ABSTRACT

Inflammation is known to promote and fuel the progression of CRC. However, the causes that trigger such inflammation and the cells that mediate tumor-promoting inflammation remain unknown. We propose that damage and inflammation is not uncommon in the gut, but that the types of inflammation in the intestine are distinct. Specifically, CRC-promoting inflammation is categorically different, and we propose that inflammation of a non-immune cell known as fibroblast underlies the etiology of CRC. We also posit that a very specific form of cellular damage - the death of intestinal epithelial cell by a molecular program called 'necroptosis' drives inflammation of fibroblasts. Thus, the scientific objective of the research project we propose is to characterize fibroblast inflammation and immune cell inflammation under conditions wherein intestinal epithelial cells die by necroptosis in mouse models of CRC, including new models developed in our laboratory. This will help determine if this specific type of damage and resulting inflammation is preferentially associated with and causes CRC.

15. SUBJECT TERMS

NONE LISTED

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1. **Introduction**
   The research addresses the relationship between inflammation and colorectal cancer (CRC), focusing on the contrasting impact of different types of inflammation on CRC risk. While some inflammatory conditions in the gut, like acute infections, don't increase CRC risk, Inflammatory Bowel Disease (IBD) significantly raises the likelihood of developing CRC. The proposed project aims to understand the inflammatory response associated with a particular cell death mode called necroptosis, hypothesizing it might be a key driver of tumor-promoting inflammation. Two main objectives are set: firstly, to determine if the death of intestinal epithelial cells (IECs) through necroptosis incites an inflammatory reaction in intestinal fibroblasts; and secondly, to examine the impact of inflammation triggered by necroptosis on the evolution of colitis-associated CRC. Using various advanced techniques and mouse models, this project delves into the role of necroptosis in CRC and has the potential to provide novel insights into CRC's etiology and possible therapeutic interventions.

2. **Keywords**
   Inflammation, cell death, necroptosis, fibroblasts, colorectal cancer.

3. **Accomplishments**
   Major goals:
   1. To investigate whether the necroptotic death of IECs triggers an inflammatory response in intestinal fibroblasts.
   2. To investigate the causal effect of necroptosis-induced intestinal fibroblast inflammation in the development of colitis-associated CRC.

   Accomplishments:
   In this first year of funding, we initially: (1) secured IACUC approval and (2) expanded our mouse colony for initiating the experiments proposed. We have additionally advanced in a key proposed area, the single-cell profiling of colonic stromal and immune cells in health and upon necroptotic (NC) injury. In order to assess how the early effector response of immune and stromal populations to NCs compares to a well-established model of colitis (DSS) we performed single-cell RNA-sequencing in Vil1-Cre+ iRipk3Tg/Tg and their Vil1-Cre- iRipk3Tg/Tg counterpart control treated with AP20187 i.p. for three days as well as WT mice left untreated or after DSS treatment (Day 12 and 17). Colons were collected and single cell suspensions were processed using the 10x Genomics Chromium system. Initial clustering analysis based on gene expression with the Seurat package identified 15 distinct populations of cells representing both the immune and stromal compartments (Figure 1). With each of the immune, including phagocytes (Figure 2) and stromal populations defined (Figure 3), we then examined whether necroptotic tissue injury resulted in any significant shifts in the cellular composition. Most significant changes were found in the phagocyte and fibroblasts compartments. In-depth bioinformatic analysis is ongoing, but we have already identified that monocytes and Cd90+ fibroblasts are significantly expanded in response to NC and DSS-induced colitis. We have also detected a subset of proliferating fibroblasts. These initial findings indicate that induction of necroptosis of IECs results in an inflammatory response of macrophages and fibroblasts, resembling aspects of the inflammatory response to DSS.

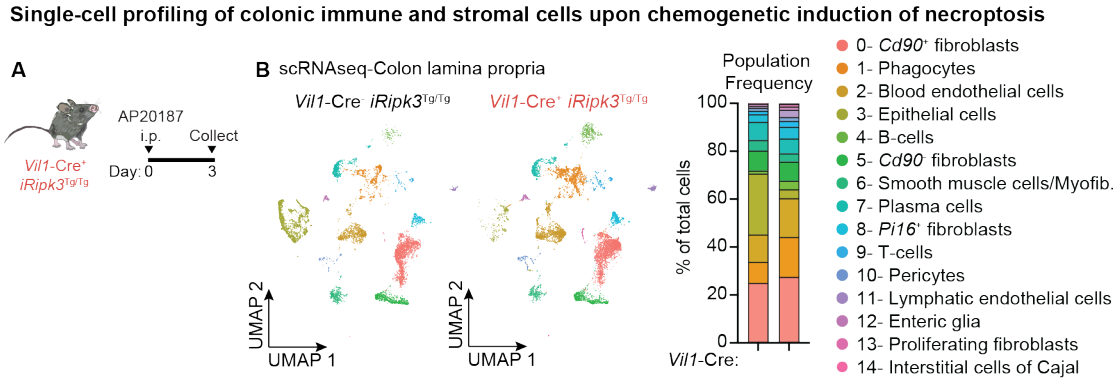

Figure 1. Cellular landscape of colonic immune and stromal cells in necroptotic tissue injury. (A) Scheme of the chemogenetic induction of necroptosis and sample collection. (B) UMAP representation and population frequency of clusters of immune and stromal cells in Vil1-CreiRipk3Tg/Tg and Vil1-Cre+ iRipk3Tg/Tg mice 3 days after AP20187 administration. Single cells are colored by cluster annotation.

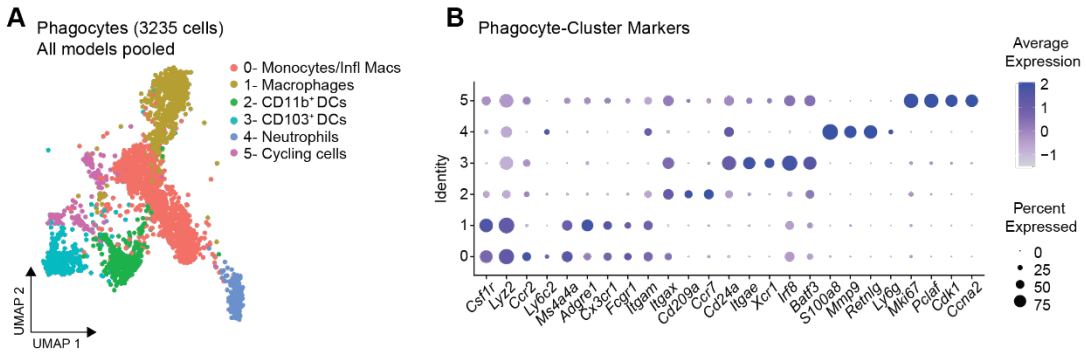

Figure 2. Identification of phagocyte subclusters in the colon by single-cell RNA-sequencing. (A) UMAP representation of the 3,235 cells identified as phagocytes (based on expression of markers Lyz2 and Csf1r). These cells were sub-clustered and identity assigned based on classical marker genes for myeloid and granulocyte populations. Single cells are colored by cluster annotation. (B) Dot plot illustrating expression of cell-type specific genes in each sub-cluster of phagocytes. The size of the dot represents the percent of cells within the sub-cluster that express the given transcript, and the intensity of the color corresponds to the average expression of the transcript.

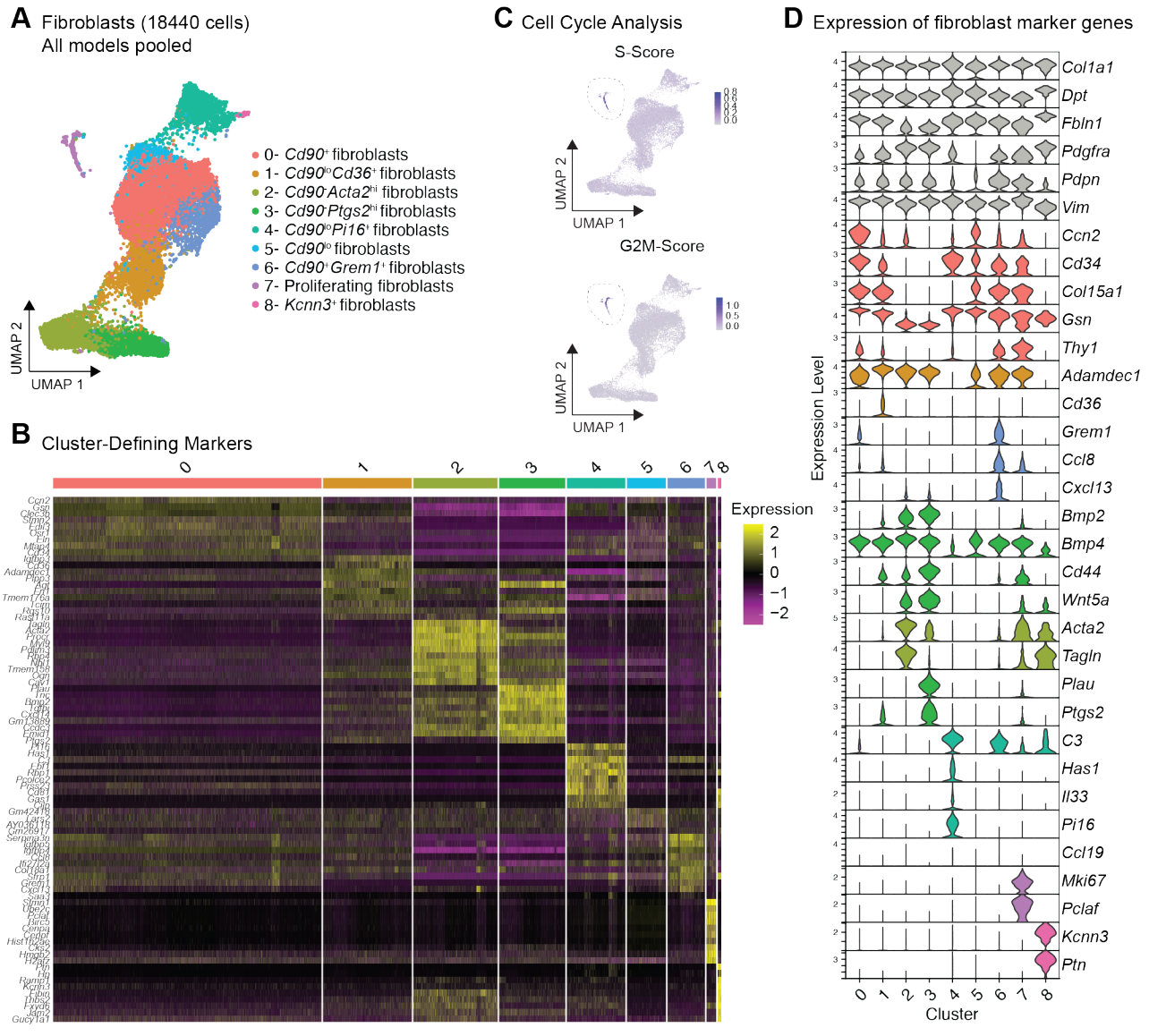

Figure 3. Characterization of fibroblast subclusters in the colon by single-cell RNA-sequencing. (A) UMAP representation of the 18,440 cells identified as fibroblasts. These cells were sub-clustered and identity assigned based on expression of known marker genes for fibroblast subsets (Cd90, Col15a1, Pi16, Adamdec1, Ptgs2) and cluster-defining markers from (B). Single cells are colored by cluster annotation. (B) Expression heatmap showing the top 10 cluster-defining marker genes for each fibroblast sub-cluster across all sub-clusters. (C) Scores for cell cycle were

generated based on expression of genes associated with S-phase or G2/M-phase (Tirosh et al., 2016). Feature plot showing expression of these suites of genes in fibroblast subclusters. Dotted line highlights the sub-cluster of cells identified to be actively proliferating, by this method. (D) Violin plot showing expression of pan-fibroblast marker genes (gray) and sub-cluster-specific fibroblast marker genes (colored to match sub-clusters in (A)) across all sub-clusters.

Opportunities for training and professional development:
As part of their professional development, Dr. Nevin and Ms. Gray presented their work in weekly lab meetings and discussed current scientific literature in weekly journal clubs. We have also met every two weeks with them to guide them in the planning of their experimental approaches and discuss the fundamental biological basis of their project.
All postdoctoral trainees at Yale University, including Dr. Nevin, are required to create an individual development plan and to provide annual progress reports for review by, and discussion with, the faculty mentor (Dr. Rothlin and Dr. Ghosh). Progress reports are then submitted to the Yale Office for Postdoctoral Affairs as a condition of the trainee's reappointment by this Office.

Results dissemination:
Results from this research project were presented by Dr. Rothlin at the following forums.

2023 IBioBA, Buenos Aires, Argentina
2023 Academia Nacional de Medicina, Buenos Aires, Argentina
2023 Ingebi, Buenos Aires, Argentina
2023 FASEB Autoimmunity Conference, Southbridge, Massachusetts, USA
2023 Cell Symposia on Myeloid Cells, Shanghai, China
2023 Institute of Translational Medicine (ITM), Zhejiang University, China
2023 IMCB, Singapore, Singapore
2023 Department of Zoology, Kolkata University, Kolkata, India
2023 Indian Institute of Chemical Biology, Kolkata, India
2023 InStem, Bengaluru, India
2023 Indian Institute of Science, Bengaluru, India
2023 National Institute of Immunology, Delhi, India
2023 Department of Microbiology, Immunology & Molecular Genetics, UT Health San Antonio, Texas, USA
2023 CNIC, Madrid, Spain

Plan for next report period:
For the next report period, we will aim to finalize the analysis of the immune and stromal response to necroptosis in comparison to DSS-induced colitis. This will include not only scRNAseq analysis, but also validation by flow cytometry, qPCR, immunofluorescence, Luminex, etc. Additionally, we will advance the proposed studies on longitudinal assessment of this novel disease model. We will extend the studies to include histological assessment of disease progression. With this foundation, we will complement our studies in the RIPK3 model with AOM administration to test whether necroptosis induced inflammation confers increased susceptibility to AOM induced colon cancer. We will compare the susceptibility of these models to that in which apoptosis, but not necroptosis, is induced in intestinal epithelial cells.

4. Impact

Impact on the development of the principal discipline(s) of the project

Impact on other disciplines
Nothing to report.

Impact on technology transfer
Nothing to report.

Impact on society beyond science and technology
Currently there are no disease-modifying therapies for Inflammatory Bowel Disease (IBD) or colitis-associated CRC. Immunotherapy is indicated only for metastatic MSI-H/MMR-D CRC. Other types of CRC, including colitis-associated CRC, are refractory to immunotherapy. Our project proposes an entirely new idea for the mechanism of CRC development. We posit that while certain types of inflammation in the gut can resolve, once fibroblasts in the gut become inflammatory this represents the inflection point to non-resolving inflammation that drives colitis-associated CRC. We also hypothesize that fibroblast inflammatory response is driven by necroptotic death of intestinal epithelial cells. Our preliminary findings indicate that indeed,

necroptosis of intestinal epithelial cells induces an inflammatory response of fibroblasts similar to the one detected in mouse models of colitis. Currently, the spotlight is only on immune cells and for the most part, fibroblasts are ignored. Therefore, the impact of our project can be a paradigm shift in examining the etiology of colitis-associated CRC. The pathway to drug discovery starts with comprehensive understanding of the etiology and mechanisms of diseases. We are hopeful that short-term, the impact of our studies would be to open a new line of CRC research focused on target identification.

5. Changes/Problems

Nothing to report.

6. Products

Nothing to report.

7. Participants & Other Collaborating Organizations

Individuals that worked on the project

Name:	**Dr. Carla Rothlin**
Project Role:	**PI**
Researcher Identifier (e.g. ORCID ID):	***0000-0002-5693-5572***
Nearest person month worked:	**0.6 calendar months**
Contribution to Project:	**Dr. Rothlin directed the project**

Name:	**Dr. Sourav Ghosh**
Project Role:	**Co-I**
Researcher Identifier (e.g. ORCID ID):	**0000-0001-5990-8708**
Nearest person month worked:	**0.6 calendar months**
Contribution to Project:	**Dr. Ghosh co-directed the project with Dr. Rothlin**

Name:	**Dr. James Nevin**
Project Role:	**Postdoctoral Associate**
Researcher Identifier (e.g. ORCID ID):	**0000-0001-6286-5622**
Nearest person month worked:	**6 calendar months**
Contribution to Project:	**Dr. Nevin perform research**

Name:	**Ms Gabrielle Gray**
Project Role:	**Research Assistant**
Researcher Identifier (e.g. ORCID ID):	**0009-0005-0220-6912**
Nearest person month worked:	**11.4 calendar months**

Contribution to Project:	Ms Gray assisted with animal colony management and experiments

Changes in active support of the PI
The PI have obtained the following new awards. There is no overlap between any of the new awards and this award.
Agency: NIH/NIA, 1RF1AG082190
Title: Augmenting AXL and MERTK function to restrain cognitive decline and improve health span in mouse models of Alzheimer's Disease
PI: Rothlin (contact), Ghosh, Grutzendler, Horvath
Project period: 04/01/2023 - 03/31/2028

Agency: NIH/NEI, R01EY034003
Title: Inflammation in MERTK-dependent Retinitis Pigmentosa
PI: Rothlin (contact), Ghosh, Finnemann
Project period: 09/01/2023 - 01/31/2028

Agency: NIH/NIAID, R21AI174387
Title: Naïve T cell archetypes and anti-tumor immunity
PI: Rothlin (contact), Ghosh
Project period: 07/01/2023 - 06/31/2028

Agency: Bright Focus
Title: Functional understanding of AXL effector role(s) in microglia towards developing disease modifying therapies in Alzheimer's Disease
PI: Rothlin (contact), Ghosh
Project period: 07/01/2023 - 06/30/2026

Agency: Fighting Blindness
Title: A novel, rationally designed pharmacological approach to countering vision loss in a preclinical model of MERTK-associated Retinitis Pigmentosa
PI: Finnemann (contact), Rothlin, Ghosh
Project period: 09/01/2023 – 08/31/2028

Other organizations involved as partners
Nothing to report.

8. Special Reporting Requirements
Nothing to report.

9. Appendices
Not applicable.