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TITLE: The Role of Complement in Liver Cancer

PRINCIPAL INVESTIGATOR: Joshua Thurman

CONTRACTING ORGANIZATION: University of Colorado Denver, Aurora, CO

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14. ABSTRACT This project explores the role of the complement cascade in the development of hepatocellular carcinoma (HCC). HCC is the second leading cause of cancer-related deaths worldwide, and the incidence may be up to five times higher among U.S. veterans than in the general population. Chronic inflammatory diseases of the liver, such as hepatitis and nonalcoholic steatohepatitis (NASH), are strongly associated with an increased risk of HCC, and a large body of literature shows that the complement system is activated in these diseases (1-5). The association of inflammatory liver diseases with HCC suggests that prolonged inflammation directly contributes to tumor formation. The underlying mechanisms of this are incompletely understood, however, limiting our ability to prevent HCC in at-risk patients. Based on our recent work, we believe that complement inhibitory drugs may be effective for reducing liver inflammation and preventing and/or treating HCC.					
15. SUBJECT TERMS None listed.					
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1. INTRODUCTION:

This project is examining the role of the complement cascade in the development of hepatocellular carcinoma (HCC). HCC is the second leading cause of cancer-related deaths worldwide, and the incidence may be up to five times higher among U.S. veterans than in the general population. Chronic inflammatory diseases of the liver, such as hepatitis and nonalcoholic steatohepatitis (NASH), are strongly associated with an increased risk of HCC, and a large body of literature shows that the complement system is activated in these diseases. The association of inflammatory liver diseases with HCC suggests that prolonged inflammation directly contributes to tumor formation. The underlying mechanisms of this are incompletely understood, however, limiting our ability to prevent HCC in at-risk patients. Based on our previous work, we believe that complement inhibitory drugs may be effective for reducing liver inflammation and preventing and/or treating HCC. We will examine the mechanisms linking complement activation with the development of HCC, and explore whether complement inhibition will reduce the risk of disease.

2. KEYWORDS:

Liver, hepatocellular carcinoma, complement, inflammation, therapy

3. ACCOMPLISHMENTS:

What were the major goals of the project?

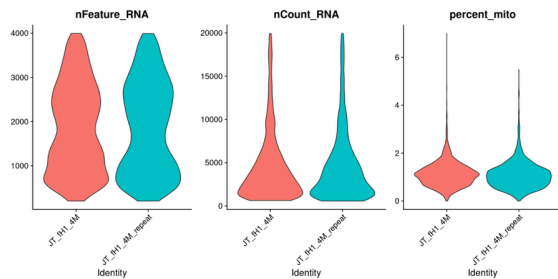
What was accomplished under these goals?

Specific Aim 1: Identify the mechanisms by which uncontrolled complement activation causes HCC.

Major Task 1: *Identify the effects of complement activation on the different cell types in the liver.*

Subtask 1: *Identify the effects of complement activation on the transcriptional profiles of individual cell types within the liver. (24 mos)*

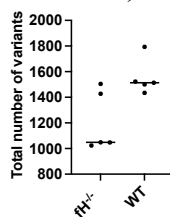
Accomplishments. The goal of this Subtask is to use transcriptional analysis to study the effects of complement activation in the liver (as is seen in $fH^{-/-}$ mice) on different cell types. To accomplish this task, we have bred $fH^{-/-}$ and control mice and begun analyzing the livers by single cell RNAseq. We have tested methods of generating single cell suspensions from the livers in order to analyze them by single cell RNA sequencing. Cell fixation methods may prevent the dropout of some cell types, such as hepatocytes, so we have compared the effects of cell fixation on quality of the single cell suspensions. Using these cell isolation methods, we analyzed the cells from a $fH^{-/-}$ mouse liver by single cell RNA sequencing. We have performed preliminary analysis of these results, and the quality of the data appears to be good. Below are violin plots of a repeated sample demonstrating: the number of features per cell (nFeature_RNA), the number of molecules per cell (nCount_RNA), and the percent of mitochondrial genes per cell (percent_mito).



More detailed analysis of the results by our bioinformatician (Jihye Kim, PhD) is ongoing. We will await this analysis, including a summary of the cell types identified, before repeating this experiment in additional mice.

Subtask 2: Whole exome sequencing of livers from wild-type, $fH^{-/-}$, and $fH^{-/-}fB^{-/-}$ mice. (15 mos)

Accomplishments. $fH^{-/-}$ mice develop hepatocellular carcinoma at a higher rate than control mice. We hypothesized that this is because complement-mediated inflammation in the livers of these mice causes somatic mutations in liver cells, thereby increasing the rate of carcinogenesis. To test this hypothesis, we have performed whole exome sequencing of livers from $fH^{-/-}$ and control mice harvested at various ages. The sequencing has been performed, and our bioinformatician (Jihye Kim, PhD) has begun analyzing the results. To compare the mutation burden in livers of $fH^{-/-}$ and control mice, Dr. Kim quantified the number of: 1) frameshift deletions, 2) frameshift insertions, 3) nonframeshift deletions, 4) nonframeshift insertions, 5) nonsynonymous single nucleotide polymorphisms (SNPs), 6) startloss, 7) stopgain, 8) stoploss, and 9) splice variants. The goal of this Although the analysis is ongoing, preliminary analysis of the total number of genetic variants (e.g., sum of 1-9) was lower in aged $fH^{-/-}$ mice had fewer mutations than age-matched wild-type controls.



Specific Aim 2: Determine whether complement activation in the liver suppresses anti-tumor immunity.

Major task 1: Test whether myeloid cells in the livers of $fH^{-/-}$ mice suppress T cell function.

Subtask 1: Myeloid cells isolated from livers wild-type, $fH^{-/-}$, and $fH^{-/-}fB^{-/-}$ mice. Functional suppression by these cells tested in vitro. (24 mos)

Accomplishments. Not yet started.

Subtask 2: *Determine whether C3a and C5a attract immunosuppressive myeloid cells to the liver.*

Accomplishments. $fH^{-/-}$ mice develop hepatocellular carcinoma at a higher rate than control mice. We hypothesized that complement activation in the livers of these mice attracts immunosuppressive myeloid cells, impairing the body's ability to eliminate tumors. To test this hypothesis we are using mice in which the myeloid cells cannot respond to complement fragments, because the receptors for these fragments have been deleted. We have performed bone marrow transplantation in which bone marrow was harvested from mice with targeted deletion of C3aR/C5aR in myeloid cells was transplanted into $fH^{-/-}$ mice. These mice will be harvested at planned timepoints, and cells in the liver will be analyzed.

Major Task 2: Examine whether complement activation affects the anti-tumor function of CD4 and CD8 T cells.

Subtask 1: Deplete CD4 and CD8 cells in $C3aR^{fl/fl}C5aR^{fl/fl}LysM-Cre$ mice to determine whether it reverses the effects of C3aR and C5aR deficiency on tumor growth. (24 months).

Accomplishments. Not yet started.

Specific Aim 3: Test whether complement inhibition is effective for preventing and treating HCC.

Major Task 1: Test whether complement inhibition is effective for treatment of spontaneous HCC in $fH^{-/-}$ mice.

Subtask 1: Assess whether treatment of $fH^{-/-}$ mice with 3d8b-Crry prevents or slows the development of liver tumors. (36 months).

Accomplishments. Not yet started.

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

Nothing to report.

How were the results disseminated to communities of interest?

Preliminary results from these experiments have been presented at a "Tumor Immunology" research seminar, at the University of Colorado Anschutz Medical Campus.

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

Specific Aim 1: Identify the mechanisms by which uncontrolled complement activation causes HCC.

Major Task 1: *Identify the effects of complement activation on the different cell types in the liver.*

Subtask 1: *Identify the effects of complement activation on the transcriptional profiles of individual cell types within the liver.* (24 mos)

Planned activities. After confirming that our current protocol for this experiment yields results of sufficient quality, we will continue to harvest livers of $fH^{-/-}$ and control mice. We will make single cell suspensions from the livers and analyze them by single cell RNAseq. The results will then be analyzed to compare the transcriptional responses in cells from $fH^{-/-}$ mice at various ages with those of age-matched control mice.

Subtask 2: Whole exome sequencing of livers from wild-type, $fH^{-/-}$, and $fH^{-/-}fB^{-/-}$ mice. (15 mos)

Planned activities. Whole exome sequencing has been completed, and we will finish analyzing the data.

Specific Aim 2: *Determine whether complement activation in the liver suppresses anti-tumor immunity.*

Major task 1: *Test whether myeloid cells in the livers of $fH^{-/-}$ mice suppress T cell function.*

Subtask 1: Myeloid cells isolated from livers wild-type, $fH^{-/-}$, and $fH^{-/-}fB^{-/-}$ mice. Functional suppression by these cells tested in vitro. (24 mos)

Planned activities. We will begin the process of isolating myeloid cells from the livers of $fH^{-/-}$ and control mice.

Subtask 2: *Determine whether C3a and C5a attract immunosuppressive myeloid cells to the liver.*

Planned activities. We have generated the C3aR/C5aR deficient mice. At the appropriate ages the mice will be harvested for tissue and cell analysis.

Major Task 2: *Examine whether complement activation affects the anti-tumor function of CD4 and CD8 T cells.*

Subtask 1: Deplete CD4 and CD8 cells in $C3aR^{fl/fl}C5aR^{fl/fl}LysM-Cre$ mice to determine whether it reverses the effects of C3aR and C5aR deficiency on tumor growth. (24 months).

Planned activities. This experiment will be started once we have confirmed whether C3aR/C5aR affect tumor growth.

Specific Aim 3: Test whether complement inhibition is effective for preventing and treating HCC.

Major Task 1: Test whether complement inhibition is effective for treatment of spontaneous HCC in $fH^{-/-}$ mice.

Subtask 1: Assess whether treatment of $fH^{-/-}$ mice with 3d8b-Crry prevents or slows the development of liver tumors. (36 months).

Planned activities. We are generating the complement inhibitory drug for this experiment, and we are breeding $fH^{-/-}$ mice. We will also test whether tumor growth can be monitored by radiologic imaging.

4. IMPACT:

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

We believe that complement activation is likely to promote tumor growth in the liver by two possible mechanisms: 1) increased somatic mutation rate, or 2) creation of an immunosuppressed environment in the liver. Thus far our data suggest that it is not due to an effect on the rate of somatic mutations.

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?

Nothing to report.

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

Nothing to report.

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

Significant changes in use or care of human subjects

Not applicable.

Significant changes in use or care of vertebrate animals

Nothing to report.

Institutional Animal Care and Use Committee approval: 1/10/23

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications. Nothing to report.

Books or other non-periodical, one-time publications. Nothing to report.

Other publications, conference papers and presentations.

Preliminary results from these experiments have been presented locally at a “Tumor Immunology” research seminar, held on the University of Colorado Anschutz Medical Campus.

- **Website(s) or other Internet site(s)**

Nothing to report.

- **Technologies or techniques**

Nothing to report.

- **Inventions, patent applications, and/or licenses**

Nothing to report.

- **Other Products**

Nothing to report.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: Joshua M. Thurman

Project Role: Principal Investigator

Research Identifier: 0000-0002-3476-9956

Nearest person month worked: 1.2 calendar months

Contribution to project: Dr. Thurman oversees all aspects of this project, including animal models of hepatocellular carcinoma, recombinant protein production and characterization, and interpretation of the data.

Funding support: National Institutes of Health and Department of Defense.

Other grants from which Dr. Thurman received support during this funding period

- R01 DK125823, no overlap
- DOD W81XWH-19-1-0717, no overlap
- R01 DK130255, no overlap
- R01 DK076690, no overlap
- R01 CA236222, no overlap
- P01 HL152961, no overlap

Name: Jihye Kim

Project Role: Co-Investigator

Research Identifier:

Nearest person month worked: 1.2 calendar months

Contribution to project: Dr. Kim is a bioinformatician. She will oversee the whole exome sequencing and mutational burden analysis

Funding support: National Institutes of Health

Other grants from which Dr. Kim received support during this funding period.

- R21 NS123546, no overlap
- R01 CA219896, no overlap
- R35 NS097303, no overlap

Name: Rebecca McCullough

Project Role: Co-Investigator

Research Identifier:

Nearest person month worked: 1.2 calendar months

Contribution to project: Dr. McCullough is overseeing the histologic assessment of liver inflammation, and assisting with interpretation of assays examining leukocyte subsets in the livers of experimental animals.

Funding support: National Institutes of Health

Other grants from which Dr. McCullough received support during this funding period.

- R21 ES032529, no overlap
- R01 DK125595, no overlap
- R00 AA025386, no overlap

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

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Nothing to report.

What other organizations were involved as partners?

If there is nothing significant to report during this reporting period, state “Nothing to Report.”

Provide the following information for each partnership:

Organization Name: Cleveland Clinic Foundation

Location of Organization: Cleveland, OH

Partner’s contribution to the project:

- Collaboration (e.g., partner’s staff work with project staff on the project)

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report.

9. APPENDICES:

Nothing to report.