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TITLE: Mitigating the Gastrointestinal Acute Radiation Syndrome by Blocking Calcium/Calmodulin-Dependent Protein Kinase Kinase 2

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CONTRACTING ORGANIZATION: Duke University
DURHAM NC 27708-4677

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14. ABSTRACT

The gastrointestinal acute radiation syndrome (GI-ARS) occurs after high dose total-body or abdominal radiation exposure, which induces extensive damage to crypt stem cells of the small intestines. Severe damage to intestinal stem cells impairs regeneration of the intestinal epithelium, which can result in atrophy of the villi, loss of mucosal barrier, and sepsis. With growing concern over a mass casualty scenario caused by a nuclear attack, it is imperative to develop novel medical countermeasures that mitigate the GI-ARS. Therefore, this project is highly relevant to the health care needs of military service members because currently there is no FDA-approved drug to mitigate the gastrointestinal syndrome induced by accidental exposure to ionizing radiation. Our preliminary data suggested that deletion of calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) markedly protected mice from the GI-ARS induced by partial body irradiation. The overall goal of this study is to define cellular mechanisms by which CaMKK2 regulates the GI-ARS and evaluate the effects of a small molecule CaMKK2 inhibitor STO-609 on mitigating the GI-ARS when administered after radiation exposure. Together, we anticipate that the outcomes from this Discovery Award will provide a foundation that we can build upon to develop a full research program that defines molecular mechanisms by which CaMKK2 functions to regulate the regeneration of the GI epithelium after radiation injury.

15. SUBJECT TERMS

NONE LISTED

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REPORT OUTLINE

1. INTRODUCTION:

The gastrointestinal acute radiation syndrome (GI-ARS) occurs after high dose total-body or abdominal radiation exposure, which induces extensive damage to crypt stem cells of the small intestines. Severe damage to intestinal stem cells impairs regeneration of the intestinal epithelium, which can result in atrophy of the villi, loss of mucosal barrier, and sepsis. With growing concern over a mass casualty scenario caused by a nuclear attack, it is imperative to develop novel medical countermeasures that mitigate the GI-ARS. Therefore, this project is highly relevant to the health care needs of military service members because currently there is no FDA-approved drug to mitigate the gastrointestinal syndrome induced by accidental exposure to ionizing radiation. Our preliminary data suggested that deletion of calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2) markedly protected mice from the GI-ARS induced by partial body irradiation. The overall goal of this study is to define cellular mechanisms by which CaMKK2 regulates the GI-ARS and evaluate the effects of a small molecule CaMKK2 inhibitor STO-609 on mitigating the GI-ARS when administered after radiation exposure. Together, we anticipate that the outcomes from this Discovery Award will provide a foundation that we can build upon to develop a full research program that defines molecular mechanisms by which CaMKK2 functions to regulate the regeneration of the GI epithelium after radiation injury.

2. KEYWORDS:

CaMKK2: calcium/calmodulin-dependent protein kinase kinase 2

GFP: green fluorescent protein

GI-ARS: gastrointestinal acute radiation syndrome

$LD_{50/10}$: lethal dose 50 within 10 days

SBI: sub-total-body irradiation

3. ACCOMPLISHMENTS:

- **What were the major goals of the project?**

There are no significant changes to the major goals of the project as we originally proposed.

Milestone(s) for Specific Aim 1

- Our results will reveal the role of CaMKK2 in regulating the development of the G-ARS, the restoration of the GI mucosal barrier, the regeneration of the GI epithelium as well as cell death and proliferation of the crypt epithelial cells.

Milestone(s) for Specific Aim 2

- Our results will reveal the critical cell type in which CaMKK2 functions to regulate the GI-ARS.

Milestone(s) for Specific Aim 3

- Our results will identify STO-609 as a novel medical countermeasure against the GI-ARS when administered after irradiation

- **What was accomplished under these goals?**

Specific Aim 1: Evaluate the impacts of knocking out CaMKK2 on the regeneration of the GI epithelium.

Examine the effects of deleting CaMKK2 on the GI-ARS

To investigate the role of Camkk2 in regulating the gastrointestinal acute radiation syndrome (GI-ARS), we used mice in which Camkk2 is deletion in the germline ($Camkk2^{-/-}$). To minimize the effect of genetic background, we performed GI-ARS experiments by comparing $Camkk2^{-/-}$ mice to their $Camkk2^{+/-}$ littermates. Based on the results from our dose-escalation experiments (data not shown), we determined to study the GI-ARS using 15.5 Gy sub-total-body irradiation (SBI) because this radiation dose is closed to a $LD_{50/10}$ dose for mice that retain wild-type Camkk2 (data not shown). We then exposed both male and female $Camkk2^{-/-}$ mice and $Camkk2^{+/-}$ littermates to 15.5 Gy SBI. These mice were followed for the development of GI-ARS up to 30 days after irradiation. Our results showed that male $Camkk2^{-/-}$ mice developed the GI-ARS at a similar percentage as their $Camkk2^{+/-}$ littermates. However, female $Camkk2^{-/-}$ mice appeared to be more resistant to the GI-ARS compared to their $Camkk2^{+/-}$ littermates (p=0.051) (**Figure 1**). These data suggest that Camkk2 may regulate the GI-ARS in a sex-specific manner.

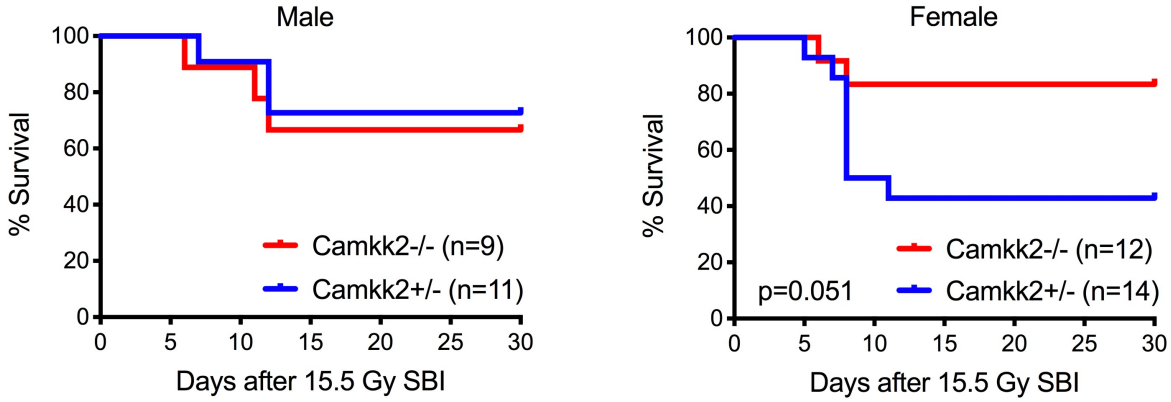

Figure 1. Deletion of Camkk2 may protect female mice from the GI-ARS. Kaplan-Meier survival analysis of 8 to 10-week-old $CaMKK2^{-/-}$ and $Camkk2^{+/-}$ littermates after 15.5 Gy SBI. *P* value was calculated by log-rank test.

Examine the effects of CaMKK2 knockout on the regeneration of intestinal crypt epithelial cells after irradiation

To examine the role of Camkk2 in regulating the regeneration of intestinal crypts after irradiation, we counted the number of $BrdU^+$ proliferating crypts 96 hours after 12 Gy SBI. For each mouse, 5 to 10 correctly orientated circumferences that do not contain Peyer's patches were scored. Our preliminary results did not show a difference in the number of $BrdU^+$ crypts after 12 Gy SBI between $Camkk2^{+/+}$, $Camkk2^{+/-}$, and $Camkk2^{-/-}$ mice (**Figure 2**). However, given that these data were generated from a mixed of male and female mice, we will increase the number of mice for this experiment to have enough power to evaluate the potential effect of sex.

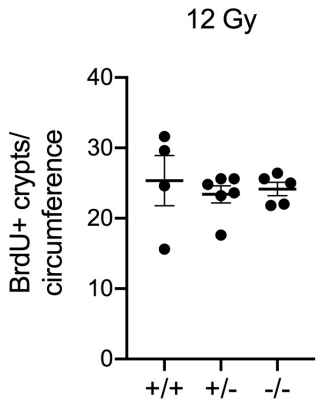

Figure 2. Quantification of surviving crypts in $Camkk2^{+/+}$, $Camkk2^{+/-}$, and $Camkk2^{-/-}$ mice 96 hours after 12 Gy SBI. Mice were injected with 100 mg/kg BrdU 2 hours before euthanization to label proliferating cells. A surviving crypt is defined as one that has 10 or more tightly packed BrdU positive cells (excluding Paneth cells). Each dot represents one mouse.

Specific Aim 2: Define the critical cell type in which CaMKK2 functions to regulate the GI-ARS

Examine cells that express CaMKK2 in the small intestine before and after irradiation

To determine cells in the small intestine that express *Camkk2*, we first used CaMKK2-GFP^{Tg} reporter mice to examine GFP^+ cells by immunohistochemistry (IHC) staining. As shown in **Figure 3**, We observed that GFP was expressed by intestinal crypt cells (red arrows) as well as stroma cells in the lamina propria (green arrows).

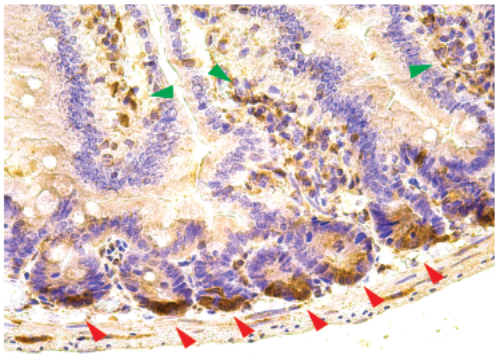

Figure 3. Examination of GFP^+ cells in the small intestine of CaMKK2-GFP^{Tg} reporter mice. Immunohistochemistry staining shows that the expression of GFP in the small intestine of Camkk2-GFP reporter mice is present in intestinal crypt cells (red arrows) and stromal cells in the lamina propria (green arrows). Image represents data from 9 mice.

To better profile the expression of *Camkk2* in intestinal epithelial cells, we analyzed a single-cell RNAseq (scRNAseq) database of mouse small intestines under homeostasis (Haber et al. 2017). ScRNAseq data indicate that although the overall expression of *Camkk2* in mouse intestines is low, *Camkk2*-expressing cells are highly enriched in a specific type of intestinal epithelial cells called tuft cells (**Figure 4, A and B**). This finding was validated in an independent dataset that compares the transcriptome of sorted intestinal tuft cells to non-tuft epithelial cells (Miller et al. 2018) (**Figure 4C**). Collectively, gene expression data of two independent studies reveal that

within intestinal epithelial cells *Camkk2* is preferentially expressed by tuft cells under homeostasis.

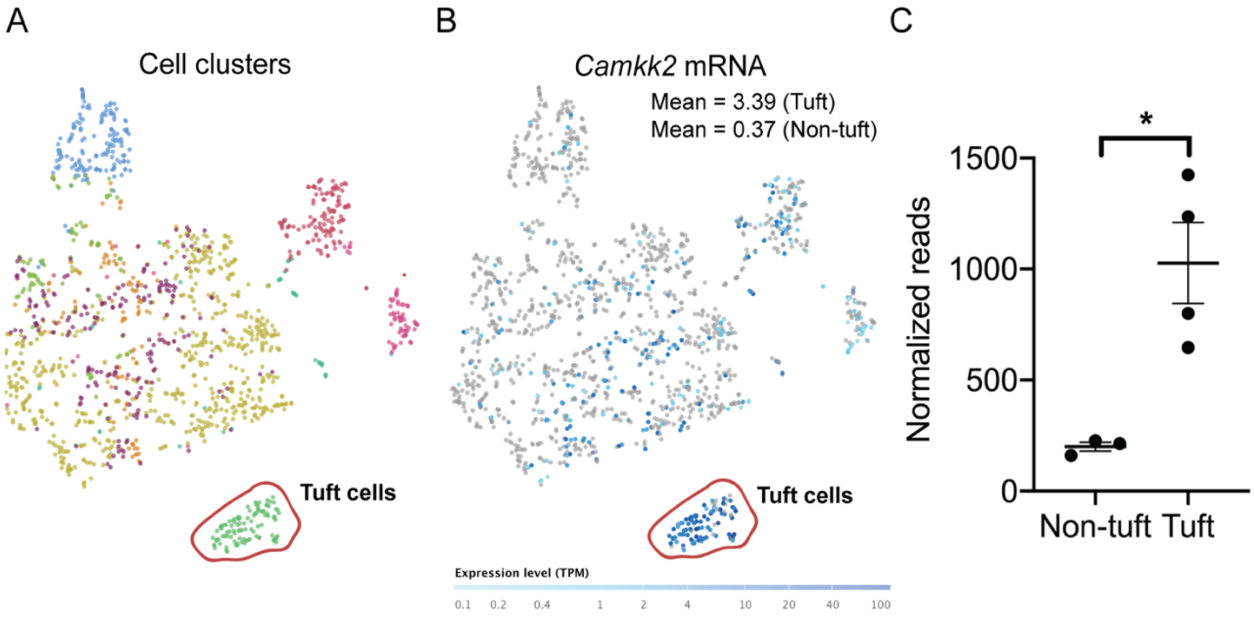

Figure 4. The expression of *Camkk2* in the intestinal epithelium is enriched in tuft cells. (**A and B**) ScRNAseq data showing that *Camkk2* is preferentially expressed by tuft cells. The mean expression intensity of *Camkk2* is 3.39 and 0.37 in tuft cells and non-tuft epithelial cells, respectively. (**C**) The transcriptome of intestinal tuft cells, which were sorted based on an IL-25 reporter, was compared to non-tuft epithelial cells. The expression of *Camkk2* in tuft cells is significantly higher than which in non-tuft cells. Each dot represents on mouse. **P*<0.05 by two-tailed t-test.

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

- Nothing to Report

- **How were the results disseminated to communities of interest?**
  - Nothing to Report.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Aim 1:
    - Increase the sample size of both male and female mice for the GI-ARS and crypt regeneration experiments.
    - Perform FITC-dextran assays to examine the permeability of the GI epithelium after irradiation
  - Aim 2:
    - Study GI-ARS using mice in which Camkk2 is deleted specifically in the GI epithelium or stromal cells.

- Aim 3:
    - Investigate the effect of the Camkk2 inhibitor STO-609 on the development of the GI-ARS.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*
    - **What was the impact on the development of the principal discipline(s) of the project?**
        - Our findings reveal that Camkk2 potentially regulates the GI-ARS in a sex-specific manner. If this finding is reproducible, it would warrant further studies to dissect underlying mechanisms.
    - **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**
        - The research operation of my lab was postponed between March to June 2020 because of COVID-19 pandemic. During this shutdown period, we were asked to stop ongoing experiments and decrease the number of mouse cages. Therefore, this incidence caused a significant delay in the experiments we planned to do for this DoD grant. Nevertheless, since my lab resumed lab operation in June, we have been gradually increasing the number of breeders to perform additional experiments we proposed in Aim 1 and Aim 2. In addition, we will order mice to start the drug studies we proposed in Aim 3.
    - **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**

- Nothing to Report.

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

6. **PRODUCTS:**
   - **Publications, conference papers, and presentations**
     - **Journal publications.**

       Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**
   - **What individuals have worked on the project?**

Name:	Lee, Chang-Lung
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	0000-0002-0673-633X
Nearest person month worked:	3.24
Contribution to Project:	Dr. Lee is responsible for designing experiments, overseeing projects and analyzing data in the proposed study.
Funding Support:	N/A

Name:	Racioppi, Luigi
Project Role:	Co-investigator
Researcher Identifier (e.g. ORCID ID):	N/A
Nearest person month worked:	1.2
Contribution to Project:	Dr. Racioppi provided genetic models required to develop the project. He also contributes to experimental design and data analysis.

Funding Support:	*N/A*

Name:	*Hasapis, Stephanie*
Project Role:	Lab Research Analyst I
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4.8
Contribution to Project:	Ms. Hasapis manages mouse colonies as well as performs *in vivo* and *in vitro* experiments to study GI-ARS for this project.
Funding Support:	*N/A*

- **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**
   - **COLLABORATIVE AWARDS:**
   - N/A
   - **QUAD CHARTS:**
   - N/A
9. **APPENDICES:** N/A