

AWARD NUMBER:
W81XWH-18-1-0716

TITLE:
Gender Differences in Complement-mediated Reperfusion Injury

PRINCIPAL INVESTIGATOR:
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CONTRACTING ORGANIZATION:
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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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6. AUTHOR(S) Sherry D Fleming				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
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14. ABSTRACT With increased female soldiers being exposed to combat, the number of women wounded or injured also increases. However, the rate of female combat deaths and extremity or abdominal injuries is significantly higher than within the male population. Thus, understanding mechanistic and treatment differences between men and women is critical. The mechanisms of excessive inflammation in intestinal ischemia/reperfusion injury includes complement activation and neutrophil infiltration in male mice. Due to females experiencing different symptoms, we hypothesized that the mechanisms of inflammation would differ. Our current data demonstrate that female mice use different complement initiators and regulators resulting in distinct kinetics of complement activation as well as inflammatory cell infiltration. Together, these data suggest that sex specific treatments may be required for ischemic events.					
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- 1. INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

With increased female soldiers being exposed to combat, the number of women wounded or injured also increased but the rate of female combat deaths and extremity or abdominal injuries remains significantly higher than within the male population. Thus, understanding mechanistic and treatment differences between men and women is critical. We proposed to examine the mechanisms of inflammation in intestinal ischemia/reperfusion of male and female mice. We will examine intestinal deposition of complement initiators and regulators as well as neutrophilic inflammatory responses in both sexes.

- 2. KEYWORDS:** *Provide a brief list of keywords (limit to 20 words).*

Complement activation, sex, gender, eicosanoid, inflammation, complement regulators, leukotrienes, prostaglandins

- 3. ACCOMPLISHMENTS:** *The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction.*

What were the major goals of the project?

List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project, identify these dates and show actual completion dates or the percentage of completion.

Specific Aim 1 Determine if complement initiation differs between sexes	Timeline	KSU	Percent Complete
Major Task 1 : Establish animal colonies	Months		
Subtask 1: IACUC and ACURO Approvals	1-3	Dr. Fleming	100%
Obtain and breed specific knock out mice	3-6	Dr. Fleming	100%
<i>Milestone Achieved: HRPO/ACURO Approval</i>	6	Dr. Fleming	100%
Major Task 2: Determine specific complement initiation pathway and regulators utilized in females			
Subtask 1 Time course and C1q ^{-/-} and MBL ^{-/-} ischemia/reperfusion (IR) surgeries will be performed and injury and inflammation analyzed. 10 mice per group, with the following groups for both male and female mice: Time course study: wildtype (C57Bl/6 mice) Sham and 30 min ischemia and reperfusion (IR), for 0.5, 1, 2, or 4 hr reperfusion, C1q ^{-/-} Sham, C1q ischemia/reperfusion (IR), MBL (mannose binding lectin) ^{-/-} Sham, MBL ^{-/-} IR. The deficient mice will use the optimal reperfusion time point determined in the time course. Injury will be determined histologically. Inflammatory assays will include complement proteins (C3 and C5) expression determined by PCR and western blot) and activation (plasma C3a/C5a), eicosanoids (leukotrienes and prostaglandins) and cytokines such as IL-1, IL-6, IL-12 and IL-10.	7-12	Dr. Fleming (total 180 animals)	100%

Subtask 2 Specific immunohistochemistry (IHC) studies will be performed for C3, C1q, C4, Factor B, Membrane attack complex, IgM and β 2-glycoprotein I (β 2-GPI)	8- 15	Dr. Fleming	95%
<i>Milestone(s) Achieved: Female complement initiation pathways determined and manuscript submitted if applicable</i>	12-15	Dr. Fleming	100%
Major Task 3: Determine role of estrogen in complement initiation			Percent Complete
Subtask 1: Perform estrogen receptor (ER)-/- IR surgeries and analyze injury; Groups will include ER-/- + Saline Sham, ER-/- + Saline IR, ER-/- + estrogen Sham and ER-/- + estrogen IR, Wildtype Sham and wildtype IR mice.	10-13	Dr. Fleming (total 160 animals)	100%
Subtask 2: Perform inflammatory and IHC studies. Inflammatory assays will include complement proteins (C3 and C5) expression determined by PCR and western blot) and activation (plasma C3a/C5a), eicosanoids (leukotrienes and prostaglandins) and cytokines such as IL-1, IL-6, IL-12 and IL-10. IHC will include C3, C1q, C4, Factor B, Membrane attack complex, IgM and β 2-glycoprotein I (β 2-GPI) as appropriate.	11-16	Dr. Fleming	50%
Milestone(s) Achieved: Role of estrogen receptors in complement initiation determined and manuscript submitted if applicable	14-24	Dr. Fleming	
Specific Aim 2: Determine role of complement regulators in female response to IR			
Major Task 4: Determine role of complement regulators			Percent Complete
Subtask 1: Analyze tissues from time course Aim 1 for complement inhibitors and obtain inhibitors (recombinant Decay accelerating factor (rDAF), (Complement receptor 2- Factor H (CR2-FH), Complement related receptor Y (CRRY), murine Factor H).	8-12	Dr. Fleming	100%
Subtask 2: Perform IR surgeries with complement inhibitors. Male and female C57Bl/6 mice will be randomized and blinded into the following groups: Wildtype C57BL/6 + saline IR and Wildtype C57BL/6 + saline Sham, C57BL/6 + rDAF Sham, C57BL/6 + rDAF IR, C57BL/6 + CR2-FH Sham, C57BL/6 + CR2-FH IR, C57BL/6 + CRRY Sham, C57BL/6 + CRRY IR, C57Bl/6 + FH Sham and C57Bl6 + FH IR.	12-17	Dr. Fleming (total 100 animas)	100%
Subtask 3: Analyze injury and inflammation. Injury will be analyzed histologically. Inflammatory assays will include complement proteins (C3 and C5) expression determined by PCR and western blot) and activation (plasma C3a/C5a), eicosanoids (leukotrienes and prostaglandins) and cytokines such as IL-1, IL- 6, IL-12 and IL-10. IHC will include C3, C1q, C4, Factor B, Membrane attack complex, IgM and β 2-glycoprotein I (β 2-GPI) as appropriate.	15-20	Dr. Fleming	30%

<i>Milestone(s) Achieved: Female complement inhibition pathways determined and manuscript submitted if applicable</i>	20-24	Dr. Fleming	75%
Major Task 5: Determine the role of estrogen on complement regulation			Percent Complete
Subtask 1: Analyze tissues from Aim 2, task 2 for complement regulatory molecules	10-13	Dr. Fleming	
Subtask 2: Perform inflammatory and IHC studies	12-17	Dr. Fleming	
Milestone(s) Achieved: Role of estrogen receptors in complement regulation determined and manuscript submitted if applicable	18-24	Dr. Fleming	
Specific Aim 3: Determine similarities in male and female neoantigens and naturally occurring antibodies			
Major Task 6: Determine female IR-induced neoantigens			Percent Complete
Subtask 1: Determine IR-induced expression of β 2-GPI, NMM, and Annexin IV by IHC and western blot (tissues from time course mice (Aim 1, Task 1))	15-16	Dr. Fleming	20%
Subtask 2: Purify β 2-GPI from mouse serum and analyze from wildtype female serum using ELISA or immunoprecipitation.	20-25	Dr. Fleming (total 50 animals)	
Major Task 7: Determine natural antibody specificities required for IR-injury in females			Percent Complete
Subtask 1: Purify antibodies from mouse sera and deplete specific antibodies	20-25	Dr. Fleming (total 50 animals)	50%
Subtask 2: Perform IR on male and female Rag-1 ^{-/-} mice receiving appropriate antibodies. Sham or IR+ anti-non-muscle myosin, IR + anti-annexin IV, IR + β 2-GPI.	25-30	Dr. Fleming (total 120 animals)	30%
Subtask 3: Analyze injury and inflammation. Eicosanoid and cytokine ELISAs and complement activation products will be determined	30-34	Dr. Fleming	
Milestone(s) Achieved: Differences in natural antibody specificities identified and manuscript submitted if applicable	34-36	Dr. Fleming	

What was accomplished under these goals?

For this reporting period describe: 1) major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative); and/or 4) other achievements. Include a discussion of stated goals not met. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. A succinct description of the methodology used shall be provided. As the project progresses to completion, the emphasis in reporting in this section should shift from reporting activities to reporting accomplishments.

Progress has been made on six of the seven major tasks. The include:

Major task 1 has been accomplished with all animals acquired and breeding well.

Major task 2: A manuscript was **Published in Clinical Immunology (attached)**. In this study we demonstrated that males sustain increased injury, peroxidase and myeloperoxidase (MPO) compared to females. However, the number of PMN and macrophages was not significantly different at later time points. Importantly females produced significant amounts of leukotrienes and males made PGE2. These data were confirmed by treating mice h a Cox-2 inhibitor, NS-398, to block PGE2 production. Injury in male but not female mice was sustained with NS-398 indicating a significant difference between sexes.

A second manuscript will be submitted soon to Molecular Immunology (attached) demonstrating that males use the C1q and MBL pathways, while females primarily use the MBL pathway (Figure 2). As expected, the alternative pathway amplifies the complement cascade in both sexes. Specifically, the time course of complement initiation in wildtype mice demonstrated significantly more RNA of C1q in male mice and MBL-C in female mice, while factor B was similar in each sex (Fig. 1). These data were confirmed with the injury of C1q or MBL or Properdin deficient mice (Fig. 2). In the absence of C1q, male mice sustained limited intestinal damage while female mice sustained damage similar to the wildtype animals. In contrast, in the absence of MBL, damage in both male and female mice was significantly decreased. Properdin deficient (PKO) mice sustained intermediate injury.

Major Task 3: Studies examining the role of ER2 are underway with surgeries completed (Fig. 3). The tissues are now being analyzed. Injury studies indicate significantly more injury in the ESR2-/- female mice compared to wildtype, heterozygote (Het) or male deficient mice. These data suggest that estrogen binding to ESR2 inhibits injury within 60 min post-ischemia.

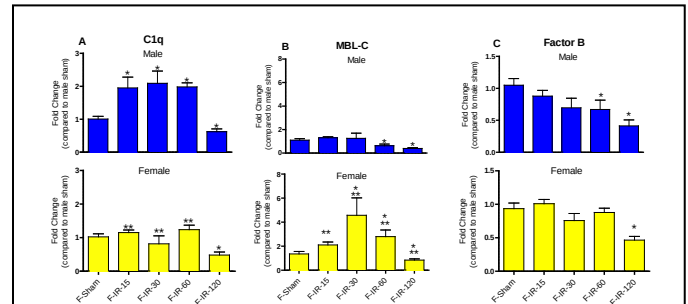


Figure 1. Intestinal C1q, MBL-c and Factor B RNA gene expression time course in male (blue) and female (yellow) mice. Mid-jejunal transcription of (A) C1q, (B) MBL, and (C) FB was determined by qRT-PCR analysis. *indicates significant difference from same sex Sham ($p \leq 0.05$); ** indicates significant difference from opposite sex at same time point ($p \leq 0.05$); n = 4-10 mice per group.

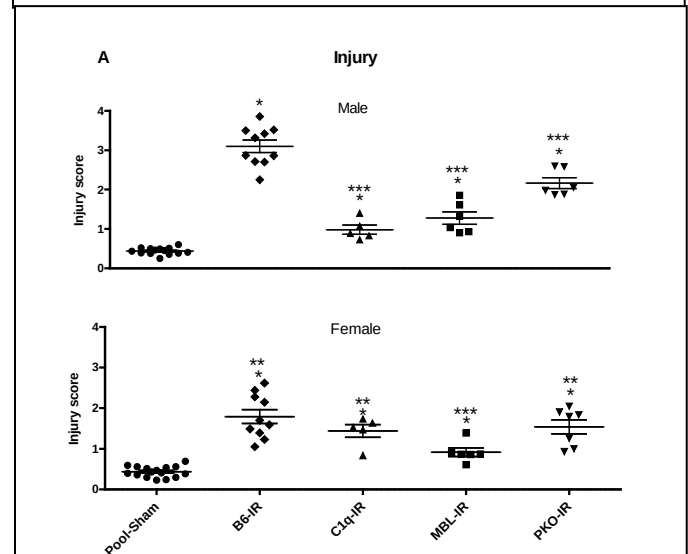


Figure 2. Injury varies by sex complement deficient male and female mice. C57Bl/6 (B6), C1q-/-, MBL-/- or Properdin -/- mice were subjected to Sham or I/R treatment. Mid-jejunal sections were H&E stained and scored for intestinal injury. (75-150 villi per animal). *indicates significant difference from same sex Sham ($p \leq 0.05$); ** indicates significant difference from opposite sex at same time point ($p \leq 0.05$); ***indicates significant differences same sex, compared to its B6 I/R n = 4-10 mice per group.

Major task 4: Significant progress was made on Major task 4 and a manuscript is being assembled. The time course of complement regulatory proteins by RT-PCR (Figure. 4) and IHC is complete. Although CD55 RNA expression changed with additional reperfusion time, there was no significant difference between sexes. In contrast, CRRY RNA significantly increased with reperfusion on intestines from female mice whereas male expression was unchanged. FH RNA was increased within 15 min post ischemia and then decreased over time. However, in female intestines, FH RNA increased towards the end of the time course.

The surgeries are complete on the inhibitory KO mice and wildtype mice treated with the various inhibitors received from the Tomlinson lab. Wildtype C57Bl/6 mice were treated with complement inhibitors (Factor H (FH) and CRRY) which were targeted to areas of C3b deposition (Fig. 5). Interestingly, the additional complement regulators significantly decreased IR-induced intestinal damage in male mice. Together these data indicate that complement inhibitors FH and CRRY play a role in IR-induced damage in male mice. When further analyses are available, an additional manuscript will be submitted as the lab continues to re-open after COVID.

Major Task 5:
Analysis of ER2-/- mice have begun and will continue in the next year as the lab re-opening continues.

Major task 6: The time course of the Beta 2 glycoprotein 1 ag is complete. Additional IHC of other neoantigens will be continued upon complete re-opening of the lab

Major task 7: Sera is collected and awaiting antibody purification.

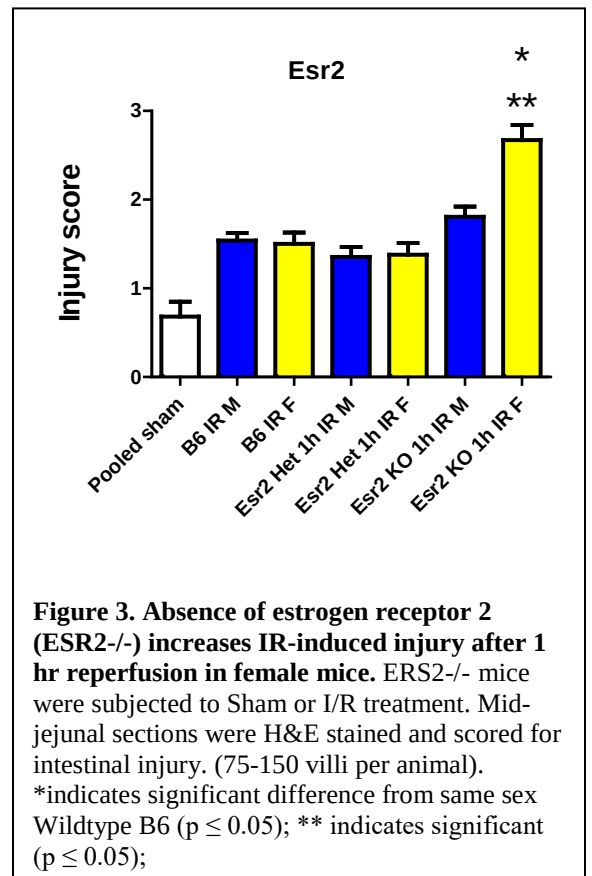


Figure 3. Absence of estrogen receptor 2 (ESR2-/-) increases IR-induced injury after 1 hr reperfusion in female mice. ESR2-/- mice were subjected to Sham or I/R treatment. Mid-jejunal sections were H&E stained and scored for intestinal injury. (75-150 villi per animal). *indicates significant difference from same sex Wildtype B6 ($p \leq 0.05$); ** indicates significant ($p \leq 0.05$);

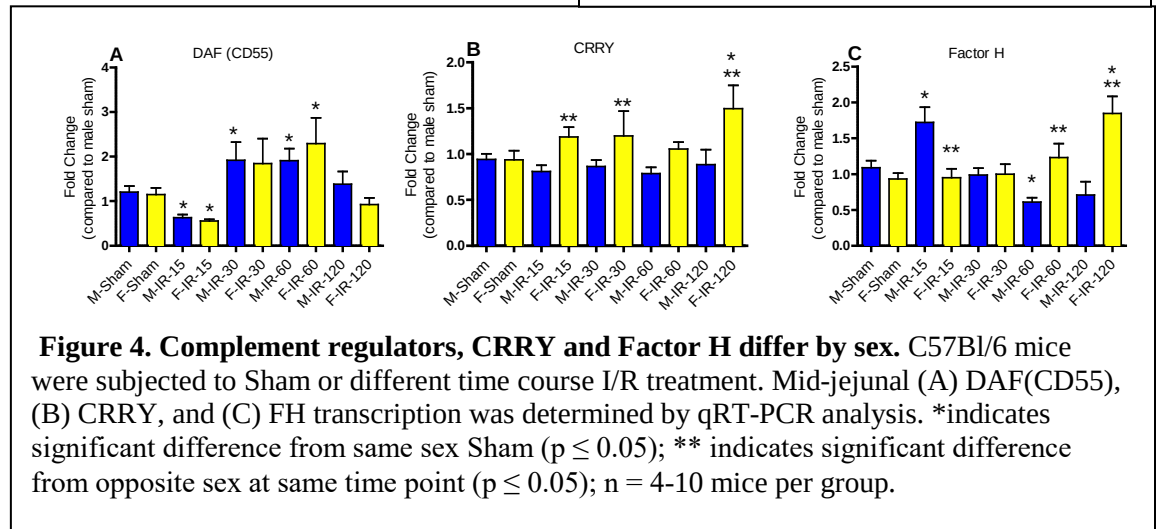


Figure 4. Complement regulators, CRRY and Factor H differ by sex. C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Mid-jejunal (A) DAF(CD55), (B) CRRY, and (C) FH transcription was determined by qRT-PCR analysis. *indicates significant difference from same sex Sham ($p \leq 0.05$); ** indicates significant difference from opposite sex at same time point ($p \leq 0.05$); $n = 4-10$ mice per group.

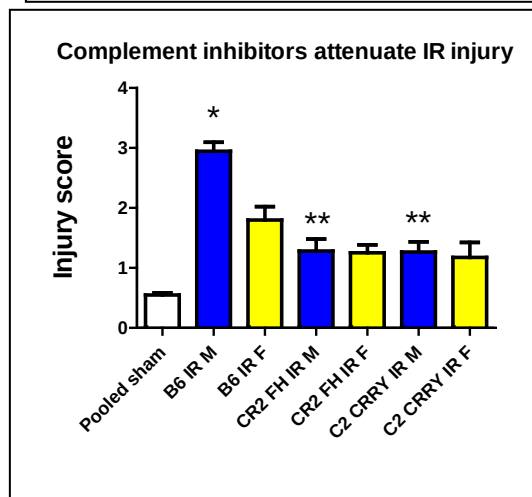


Figure 5. C57Bl/6 mice treated with targeted Factor H (FH) or Crry inhibits IR-induced injury in both sexes of mice. C57Bl/6 mice were subjected to Sham or I/R treatment. Mid-jejunal sections were H&E stained and scored for intestinal injury. (75-150 villi per animal). *indicates significant difference from same sex ($p \leq 0.05$); ** indicates significant difference from opposite sex at same time point ($p \leq 0.05$);

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

If the project was not intended to provide training and professional development opportunities or there is nothing significant to report during this reporting period, state “Nothing to Report.”

Describe opportunities for training and professional development provided to anyone who worked on the project or anyone who was involved in the activities supported by the project. “Training” activities are those in which individuals with advanced professional skills and experience assist others in attaining greater proficiency. Training activities may include, for example, courses or one-on-one work with a mentor. “Professional development” activities result in increased knowledge or skill in one’s area of expertise and may include workshops, conferences, seminars, study groups, and individual study. Include participation in conferences, workshops, and seminars not listed under major activities.

The professional development of the post-doc resulted in her successfully obtained a faculty position. All lab members participate the Immunity journal club. Ms. Rowe, technician, obtained authorship and is building her CV.

How were the results disseminated to communities of interest?

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

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities.

Publication of a manuscript in Clinical Immunology. Nov. 2, 2020, I will present to the American Legion as an outreach.

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

If this is the final report, state “Nothing to Report.”

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

The remaining surgeries will be completed and data analysis with IHC, RT-PCR and Luminex studies continued. Publications will be written and submitted.

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?

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

Describe how findings, results, techniques that were developed or extended, or other products from the project made an impact or are likely to make an impact on the base of knowledge, theory, and research in the principal disciplinary field(s) of the project. Summarize using language that an intelligent lay audience can understand (Scientific American style).

Identifying mechanistic sex differences may provide for more appropriate treating of each sex in the future.

What was the impact on other disciplines?

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

Describe how the findings, results, or techniques that were developed or improved, or other products from the project made an impact or are likely to make an impact on other disciplines.

Nothing to Report

What was the impact on technology transfer?

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

Describe ways in which the project made an impact, or is likely to make an impact, on commercial technology or public use, including:

- *transfer of results to entities in government or industry;*
- *instances where the research has led to the initiation of a start-up company; or*
- *adoption of new practices.*

Nothing to Report

What was the impact on society beyond science and technology?

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

Describe how results from the project made an impact, or are likely to make an impact, beyond the bounds of science, engineering, and the academic world on areas such as:

- *improving public knowledge, attitudes, skills, and abilities;*
- *changing behavior, practices, decision making, policies (including regulatory policies), or social actions; or*
- *improving social, economic, civic, or environmental conditions.*

Nothing to Report

5. **CHANGES/PROBLEMS:** *The PD/PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency grants official whenever there are significant changes in the project or its direction. If not previously reported in writing, provide the following additional information or state, “Nothing to Report,” if applicable:*

Changes in approach and reasons for change

Describe any changes in approach during the reporting period and reasons for these changes. Remember that significant changes in objectives and scope require prior approval of the agency.

Due to the availability of the DAF KO, and Factor H mutant mice, our initial data that only Factor H and CRRY are critical were confirmed with the mutant mice.

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

Describe problems or delays encountered during the reporting period and actions or plans to resolve them.

Covid-19 has delayed our progress. We had limited lab function from Mar-June. However, all personnel were paid during this time. Data was analyzed and manuscripts were written and submitted but actual lab work was extremely limited.

Changes that had a significant impact on expenditures

Describe changes during the reporting period that may have had a significant impact on expenditures, for example, delays in hiring staff or favorable developments that enable meeting objectives at less cost than anticipated.

Dr. Miaomiao Wu, post-doc, acquired a faculty position in Sept. At this time, we do not have a post-doc and may end up hiring a technician. This will save money but will also delay the project as anyone hired will need to learn the surgeries and the literature prior to writing a manuscript.

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

Describe significant deviations, unexpected outcomes, or changes in approved protocols for the use or care of human subjects, vertebrate animals, biohazards, and/or select agents during the reporting period. If required,

were these changes approved by the applicable institution committee (or equivalent) and reported to the agency? Also specify the applicable Institutional Review Board/Institutional Animal Care and Use Committee approval dates.

Significant changes in use or care of human subjects

Nothing to Report

Significant changes in use or care of vertebrate animals

Nothing to Report

Significant changes in use of biohazards and/or select agents

Nothing to Report

6. PRODUCTS: *List any products resulting from the project during the reporting period. If there is nothing to report under a particular item, state "Nothing to Report."*

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

Report only the major publication(s) resulting from the work under this award.

Journal publications. *List peer-reviewed articles or papers appearing in scientific, technical, or professional journals. Identify for each publication: Author(s); title; journal; volume: year; page numbers; status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Journal publications:

Wu M, Rowe JM, Fleming SD. Eicosanoid production varies by sex in mesenteric ischemia reperfusion injury. Clin Immunol. 2020 Nov;220:108596. doi: 10.1016/j.clim.2020.108596. Epub 2020 Sep 19. PMID: 32961332. Published Online. Acknowledgement of federal support: Yes.

Books or other non-periodical, one-time publications. *Report any book, monograph, dissertation, abstract, or the like published as or in a separate publication, rather than a periodical or series. Include any significant publication in the proceedings of a one-time conference or in the report of a one-time study, commission, or the like. Identify for each one-time publication: author(s); title; editor; title of collection, if applicable; bibliographic information; year; type of publication (e.g., book, thesis or dissertation); status of publication (published; accepted, awaiting publication; submitted, under review; other); acknowledgement of federal support (yes/no).*

Nothing to Report

Other publications, conference papers and presentations. *Identify any other publications, conference papers and/or presentations not reported above. Specify the status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.*

Abstracts were submitted but meetings were all cancelled. So, nothing to report

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

List the URL for any Internet site(s) that disseminates the results of the research activities. A short description of each site should be provided. It is not necessary to include the publications already specified above in this section.

Nothing to Report

- **Technologies or techniques**

Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

Nothing to Report

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

Identify inventions, patent applications with date, and/or licenses that have resulted from the research. Submission of this information as part of an interim research performance progress report is not a substitute for any other invention reporting required under the terms and conditions of an award.

Nothing to Report

- **Other Products**

Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that

makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:

- *data or databases;*
- *physical collections;*
- *audio or video products;*
- *software;*
- *models;*
- *educational aids or curricula;*
- *instruments or equipment;*
- *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
- *clinical interventions;*
- *new business creation; and*
- *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Name: Sherry Fleming

Project Role: PI

Researcher Identifier <https://orcid.org>

Nearest person month worked: 1 mo

Contribution to Project: Dr. Fleming has coordinated the approval of the regulatory animal protocols and ordering of animals and the appropriate animal surgeries.

Name: Miaomiao Wu

Project Role: Post-doc

Researcher Identifier;

Nearest person month worked: 9 mo

Contribution to Project:.

Name: Jennifer Rowe

Project Role: Animal Technician

Researcher Identifier; <https://orcid.org>

Nearest person month worked: 9 mo

Contribution to Project: Ms Rowe has coordinated the ordering and breeding of the specific mice. In addition, she has performed the animal surgeries.

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.”

If the active support has changed for the PD/PI(s) or senior/key personnel, then describe what the change has been. Changes may occur, for example, if a previously active grant has closed and/or if a previously pending grant is now active. Annotate this information so it is clear what has changed from the previous submission. Submission of other support information is not necessary for pending changes or for changes in the level of effort for active support reported previously. The awarding agency may require prior written approval if a change in active other support significantly impacts the effort on the project that is the subject of the project report.

Previously active grant which has closed:	
Title:	Complement Activation in Pregnancy and Hypertension
Effort:	8.33%
Supporting Agency:	US Department of Health and Human Services, D004708901 through Regents of the University of Minnesota
Grants Officer:	Elliot Skurich, 409 Darland Administration Building, Duluth, MN 55812
Performance Period:	8/1/2015 - 7/31/2019 with no cost extension
Level of Funding:	
Project Goals:	Determine therapeutic utility of manipulating complement system to mitigate maternal and fetal consequences of preeclampsia
Specific Aims:	1. Identify the mechanism of increased complement activation after placental ischemia. 2. Identify endothelin A receptor (ETA) as important in placental ischemia-induced endothelial dysfunction and hypertension following complement activation.
Overlap with Proposed:	No overlap

What other organizations were involved as partners?

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

Describe partner organizations – academic institutions, other nonprofits, industrial or commercial firms, state or local governments, schools or school systems, or other organizations (foreign or domestic) – that were involved with the project. Partner organizations may have provided financial or in-kind support, supplied facilities or equipment, collaborated in the research, exchanged personnel, or otherwise contributed.

Provide the following information for each partnership:

Organization Name:

Location of Organization: (if foreign location list country)

Partner’s contribution to the project (identify one or more)

- Financial support;
- In-kind support (e.g., partner makes software, computers, equipment, etc., available to project staff);
- Facilities (e.g., project staff use the partner’s facilities for project activities);
- Collaboration (e.g., partner’s staff work with project staff on the project);
- Personnel exchanges (e.g., project staff and/or partner’s staff use each other’s facilities, work at each other’s site); and
- Other.

Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: *For collaborative awards, independent reports are required from BOTH the Initiating Principal Investigator (PI) and the Collaborating/Partnering PI. A duplicative report is acceptable; however, tasks shall be clearly marked with the responsible PI and research site. A report shall be submitted to <https://ers.amedd.army.mil> for each unique award.*

QUAD CHARTS: *If applicable, the Quad Chart (available on <https://www.usamraa.army.mil>) should be updated and submitted with attachments.*

9. **APPENDICES:** *Attach all appendices that contain information that supplements, clarifies or supports the text. Examples include original copies of journal articles, reprints of manuscripts and abstracts, a curriculum vitae, patent applications, study questionnaires, and surveys, etc.*



Eicosanoid production varies by sex in mesenteric ischemia reperfusion injury

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ABSTRACT

Intestinal ischemia/reperfusion (I/R)-induced injury is an inflammatory response with significant morbidity and mortality. The early inflammatory response includes neutrophil infiltration. However, the majority of rodent studies utilize male mice despite a sexual dimorphism in intestinal I/R-related diseases. We hypothesized that sex may alter inflammation by changing neutrophil infiltration and eicosanoid production. To test this hypothesis, male and female C57Bl/6 mice were subjected to sham treatment or 30 min intestinal ischemia followed by a time course of reperfusion. We demonstrate that compared to male mice, females sustain significantly less intestinal I/R-induced tissue damage and produced significant LTB₄ concentrations. Male mice release PGE₂. Finally, treatment with a COX-2 specific inhibitor, NS-398, attenuated I/R-induced injury, total peroxidase level, and PGE₂ production in males, but not in similarly treated female mice. Thus, I/R-induced eicosanoid production and neutrophil infiltration varies between sexes suggesting that distinct therapeutic intervention may be needed in clinical ischemic diseases.

1. Introduction

Ischemia-reperfusion (I/R) injury poses a serious clinical therapeutic problem during multiple conditions including surgery, myocardial infarction and stroke, gastrointestinal dysfunction, cerebral and hepatic diseases, systemic inflammatory response syndrome, and organ transplantation. Compared to other internal organs, the intestine appears exquisitely sensitive to I/R injury [1]. Ischemia induces local tissue injury, which is significantly magnified during reperfusion. The local intestinal inflammatory response may progress to a systemic inflammatory response in remote organs including the lungs. Reperfusion-induced inflammation includes complement, cytokines, and cellular influx by increased adhesion molecules generated both locally and systemically in the blood [2–4]. In mice, intestinal ischemia also induces neutrophil infiltration and secretion of cytokines and eicosanoids leading local mucosal injury [5–11].

Numerous clinical studies of I/R-related conditions support the importance of sex-associated differences [12–14]. After myocardial infarction, women generally have a higher in-hospital mortality than men and tend to develop heart failure [15]. The operative mortality of women after coronary artery bypass grafting surgery is 2–3 times higher than men [16]. Although the prevalence of ischemic heart

disease is 1.8% higher in men than in women [17], the hospital discharges for cardiovascular diseases of men were higher than women during the same time period in the U.S [18]. Together, these data suggest that sex may be an independent factor and affect the clinical treatment. Quantitative trait locus expression analysis of patients with ischemia also indicated that genomic sex differences affect myocardial gene expression and cell type enrichment analysis demonstrated sex-bias in different proportions of specific cell types [12]. Finally, females present distinct symptomology compared to males in response to myocardial I/R, also suggesting sex as an independent risk factor for I/R [19].

Despite the systematic differences between women and men in the I/R injuries, women remain underrepresented in clinical research studies [20–22]. In addition, the mouse model of intestinal I/R was established primarily using male mice, as female mice show decreased injury compared to male mice in multiple forms of I/R including, hemorrhage [23], myocardial [24], kidney [25], and intestine [26], and sepsis [25]. Although I/R injury in female mice is attenuated or delayed, the specific protective mechanisms were not defined [27–29]. Recent studies demonstrated that female sex hormones could reduce pro-inflammatory cytokines production in inflammatory bowel disease [30], reduce inflammation in heart and kidney I/R injuries [31,32] and

Abbreviations: I/R, ischemia reperfusion; LTB₄, leukotriene B₄; PGE₂, prostaglandins E₂; COX-2, cyclooxygenase-2; 5-LOX, 5-lipoxygenase; PGs, prostaglandins; HIF-1α, hypoxia-inducible factor-1α; MPO, myeloperoxidase; SMI, mucosal injury score; TMB, 3,3',5,5'-tetramethylbenzidine.

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preserve endothelial integrity [33].

Arachidonic acid, cleaved from membrane phospholipids by activated phospholipase A2 during ischemia, is metabolized into prostaglandins E_2 (PGE_2) by cyclooxygenase-2 (COX-2) [34–37]. COX-2 is expressed at low levels constitutively and is strongly expressed induced by multiple stimuli [38]. In addition, COX-2 plays a predominant role in prostaglandins (PGs) production in the pathophysiological processes, such as ischemic injury [39–41]. A5-LOX also metabolizes arachidonic acid into leukotriene B_4 (LTB_4). Additional literature indicates that LTB_4 recruits neutrophils in response to I/R by chemotaxis [11,42].

Understanding the molecular mechanisms of female attenuated or delayed I/R injury will provide insight into the sexual dimorphism of intestinal I/R and may allow the development of sex-specific therapies to reduce the injuries. To test the hypothesis that male and female I/R-induced damage is mechanistically different, we examined the kinetics of intestinal I/R-induced mediators and pathology in C57BL/6 female and male mice subjected to 30 min ischemia followed by 15 min, 30 min, 60 min, and 120 min reperfusion. Our data indicate that sex differences alter the mechanisms of intestinal I/R injury. Specifically, males produce PGE_2 via the COX-2 pathways in response to I/R injury, while under similar conditions, female mice use A5-LOX to produce LTB_4 .

2. Materials and methods

2.1. Mice

C57BL/6 J mice were purchased from Jackson Laboratory and bred in the Division of Biology at Kansas State University, Manhattan, KS. All mice were allowed free access to food and water, and maintained under 12 h light/dark cycles in specific pathogen free conditions (*Helicobacter* species, mouse hepatitis virus, minute virus of mice, mouse parvovirus, Sendai virus, murine norovirus, *Mycoplasma pulmonis*, Theiler's murine encephalomyelitis virus, and *endo*- and *ecto*-parasites). All studies were approved by the Institutional Animal Care and Use Committee and conducted in compliance with the Animal Welfare Act and other federal statutes and regulations concerning animals and experiments involving animals.

2.2. Ischemia and reperfusion procedure

Animals were subjected to I/R, as previously described [39]. Briefly, isoflurane (2–3%) anesthetized mice were subjected to a midline laparotomy and covered with a moistened surgical gauze to prevent desiccation. After a 30 min equilibration period, the superior mesenteric artery was identified, isolated, and a small vascular clamp (Roboz Surgical Instruments) applied to induce ischemia. Ischemia was confirmed by the blanching of the intestine and the absence of pulsations distal to the clamp. After 30 min of ischemia, the clamp was removed and the intestines were reperused for 15, 30, 60, or 120 min. Reperfusion was confirmed by the return of pinkish color to the intestine. All mice received buprenorphine (0.075 mg/kg) for pain. Sham treated animals underwent the same procedure with manipulation of the intestines but without clamping. After reperfusion, the mice were deeply anesthetized, bled for serum collection and euthanized. Then beginning approximately 10 cm distal to the gastroduodenal junction, five sections of the mid-jejunum (2 cm each) were collected for histological and other analyses. All procedures were performed with all animals breathing spontaneously and anesthetized mice were kept on a 37 °C water-circulating heating pad to maintain body temperature. In some studies, 17 h prior to surgery mice were injected intraperitoneally with NS-398 (0.25 mg/mouse), a cyclooxygenase-2-specific inhibitor (Sigma-Aldrich) prepared as indicated in the company's instructions. A second dose of NS-398 was injected intravenously 3 h prior to I/R surgery.

2.3. Histology and immunohistochemistry

Immediately after removal, a 2 cm mid-jejunum tissue section was fixed in 10% buffered formalin phosphate, embedded in paraffin, cut transversely (8 μ m), and H&E stained. The mucosal injury score (SMI) was graded based on a six-tiered scale modified from Chiu et al. [43,44]. Briefly, the average intestinal mucosal injury was calculated by the average of two well trained, blinded observers. Each observer graded 90–150 villi from 0 to 6. Normal villi were assigned a score of zero; a score of 1 was assigned when the villi tip was distorted; villi with Guggenheims' spaces were scored 2; a score of 3 was assigned to villi with small regions of disruption in the epithelial cells; villi with large regions of exposed but intact lamina propria with epithelial sloughing were scored 4; villi with extruding lamina propria were assigned to a score of 5; a score of 6 was assigned when the villi displayed hemorrhage or were denuded. Photomicrographs were obtained from H&E stained slides by utilizing a 40 $\times$, 0.5 Plan Fluor objective on Nikon 80i microscope. Images were acquired by using a Nikon DS-5 M camera with the DS-L2 software at room temperature (Nikon).

Additional 2 cm intestinal sections were immediately snap frozen in optimal cutting temperature (O.C.T) freezing medium and serial 8 μ m transverse sections were placed on the slides for future immunohistochemistry, as described previously [45]. Briefly, slides were fixed using acetone, and followed by incubating with 10% sera in phosphate buffered saline (PBS) for 30 min to block nonspecific binding. After PBS washes, the intestinal sections were incubated with primary antibodies in the dark for 1 h at room temperature or ON at 4 °C. The F4/80 and myeloperoxidase (MPO) deposition on the tissue was detected by staining with a rat-anti-mouse anti-F4/80 (Biolegend) Alexa Fluor 594 or purified rabbit-anti-MPO (Spring Bioscience) followed by an AlexaFluor 594 conjugated donkey-anti-rabbit IgG secondary antibody (Jackson ImmunoResearch). All experiments contained serial sections stained with appropriate isotype control antibodies. Each slide was mounted with ProLong Gold (Invitrogen). Four to eight images per section were acquired by a blinded observer at room temperature by utilizing a Nikon eclipse 80i microscope equipped with a Infinity 3S camera (Lumenera) and analyzed by using Infinity Analyzer software (Lumenera). Densitometry used ImageJ (<https://imagej.nih.gov/ij/>).

2.4. Ex vivo eicosanoid, cytokine, and peroxidase determination

The ex vivo generation of eicosanoids and cytokines in the small intestine tissue section was detected using a modified method previously described [46]. Immediately after removal, a 2 cm intestinal section was minced, and washed in cold Tyrode buffer and resuspended in 37 °C freshly oxygenated Tyrode buffer (Sigma-Aldrich). After incubation at 37 °C for 20 min, the supernatants were collected. Levels of LTB_4 and PGE_2 were determined using enzyme immunoassay kits (Cayman Chemical). Analysis of cytokines present in the same intestinal supernatants was determined by using a Milliplex MAP immunoassay kit (Millipore) that was analyzed on a Milliplex Analyzer (Millipore). Supernatants generated for eicosanoids and cytokines were also used to determine peroxidase activity by measuring oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) as described previously [11]. Protein content was determined using the bicinchoninic acid (Pierce) assay adapted for use with microtiter plates. All eicosanoid and cytokines concentrations were standardized per mg protein per 20 min.

2.5. MPO activity and C5a level determination by ELISA

Blood was collected from mice after intestinal I/R, allowed to clot on ice at least 30 min and whole serum collected by centrifugation for 20 min at approximately 1000 $\times$ g. Serum MPO activity and serum C5a level were determined based on the sandwich ELISA principle. The mouse MPO and C5a capture antibody, detection antibody, standard,

Table 1
Real time PCR primer sequences.

Gene	Tm (°C) ^a	Sequence
HIF-1 α	51	FWD: ATGAGAGAAATGCTTACACAC REV: TGAGGTGGTTACTGTTGG
COX-2	54	FWD: ATCCTGCCAGCTCCACCG REV: TGGTCAAATCCTGTGCTCATACAT
A5-LOX	52	FWD: ATCTGAGCGAGTCAAGAACC REV: TGAAAATGTTCCCTTCCTGT
Ribosomal 18s ^b	56	FWD: GGTGATCCTGCCAGTAGC REV: GCGACCAAAGGAACCATAAC

^a Annealing temperature.

^b House-Keeping gene to which genes of interest were normalized.

and Streptavidin-HRP were purchased from R&D SYSTEMS.

2.6. Quantitative real-time PCR

One 2 cm jejunal section was snap frozen in liquid nitrogen and stored at -80°C until used for RNA analysis. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. RNA quantity and quality was determined by using the Nano drop (Thermo Fisher Scientific). Total RNA (1 μg) was reverse transcribed into cDNA by using aScript first strand cDNA synthesis kit (Quanta Biosciences). Quantitative real-time PCR analysis was performed in 25 μl total volume using an Applied Biosciences StepOnePlus thermocyclers (Thermo Fisher) with Perfecta SYBR Green Fastmix reagent (Quanta Biosciences) using the following protocol: 3 min at 95°C ; 50 cycles of 10 s at 95°C , 20 s at Tm (Table 1), 10 s at 72°C ; melt curve start at 60°C , increasing by 0.5°C up to 95°C . After amplification, relative gene expression was normalized to 18 s rRNA values, and $\Delta\Delta\text{Ct}$ fold change relative to sham-treated mice was determined.

2.7. Statistical analysis

Data are presented as mean $\pm$ the standard error of the mean (SEM) and were compared by unpaired *t*-test with Mann-Whitney post hoc analysis (Graph Pad/Instant Software Inc). Differences ($P \leq 0.05$) between groups were considered significant.

3. Results

3.1. Higher intestinal I/R-induced tissue damage and inflammation in male mice

Previous studies indicated that the prevalence of ischemic heart disease is higher in men than in women with 8.3% of cardiovascular deaths in men vs. 6.1% in women in 2008 [47], and intestinal I/R-induced injury in female mice is attenuated or delayed in multiple forms of I/R and hemorrhage [28,29,48]. It is likely the time course of the I/R damage differs between sexes. To test this hypothesis, we subjected both male and female wild-type, C57Bl/6 mice to 30 min ischemia, followed by 15 min, 30 min, 60 min, and 120 min reperfusion, and evaluated intestinal epithelial damage. Sham treatment was not significantly different at various time points of the same gender of mice. Thus, the scores of damage and subsequent analyses were pooled by gender for sham treatment only. As expected, after I/R, male mice sustained significant tissue damage (Fig. 1A). Although female mice also sustained significant intestinal damage, the damage was significantly lower than that sustained by male mice at a similar time points (Fig. 1A). Thus, these data indicate that intestinal I/R-induced significant tissue injury in both sexes, but male mice sustained significantly higher mucosal tissue damage than females throughout the time course of intestinal I/R.

Intestinal I/R-induced tissue damage is accompanied by

inflammation with significantly elevated levels of total intestinal peroxidase [11], myeloperoxidase [49] and cytokines [39]. Myeloperoxidase (MPO), one of the most abundant enzymes released by activated neutrophils, is an inflammatory molecule common ischemic tissue [50]. We hypothesized that total tissue peroxidase and specifically myeloperoxidase would be increased after intestinal I/R, but the dynamic change may differ between male and female mice. As expected in a time course of I/R, total peroxidase in males was significantly increased compared to sham treated animals (Fig. 1B). In contrast, the total tissue peroxidase was not significantly increased at any time point examined in female mice (Fig. 1B). After I/R, both males and females had significantly elevated serum MPO level compared with their corresponding shams (Fig. 1C). In addition, serum MPO level in males was slightly higher than it was in females during all I/R time course, with significance at 15 min, 30 min, and 120 min (Fig. 1C). As complement plays a significant role in intestinal I/R tissue damage [51], we examined neutrophil chemotactic C5a as well. After I/R at 30 and 60 min, males had significantly decreased serum C5a level compared with their corresponding shams (Fig. 1D). In contrast, in female mice the serum C5a level did not increase during I/R compared to its corresponding sham treatment (Fig. 1D). Importantly, between males and females within the same treatment, males had significantly higher level of C5a. Together, the intestinal injury, total peroxidase and MPO and C5a indicate that compared to females, males have higher levels of tissue damage and inflammation during intestinal I/R, which begins earlier. These data suggest the inflammatory response to intestinal I/R differs between males and females.

3.2. Male mice recruit more neutrophils and macrophages during intestinal I/R

MPO and F4/80 identify infiltration of neutrophil and macrophage, respectively. To examine differences between neutrophil and macrophage infiltration in males and females after intestinal I/R, we performed immunohistochemistry to examine MPO and F4/80 expression. In addition, MPO and F4/80 staining was significantly increased in I/R treated intestines compared to sham treatment. Importantly, in response to I/R, MPO and F4/80 staining was significantly higher in male intestines compared to female intestines (Fig. 2A, B). Taken together, intestinal I/R induces neutrophil and macrophage infiltration in both sexes but I/R induces more neutrophil and macrophage infiltrates in males than females. Together these data indicate the inflammatory infiltrate differs between sexes.

As the cellular influx induces significant cytokine secretion, which further contributes to the inflammatory response to I/R, we examined ex vivo cytokine production (Fig. 3A-F). Intestines from both sexes secreted similar levels of IL-12p40 and p70, CXCL1 (KC) and TNF- α . However, female intestinal IL-6 and IL-10 secretion were significantly higher at 15 and 30 min post ischemia, respectively. The increased IL-10 early during reperfusion suggests that female intestines maintain an anti-inflammatory response longer compared to male intestines.

3.3. Female mice produce LTB₄ whereas male mice produce PGE₂

We previously demonstrated pivotal roles in intestinal I/R for the arachidonic acid metabolites, PGE₂ and LTB₄ [39]. LTB₄ induces neutrophil chemotaxis [52,53] while COX-2 production of PGE₂ regulates hypoxia-inducible factor-1 α (HIF-1 α), a master regulator of oxygen homeostasis [54]. Thus, we performed RT-PCR to determine if expression of HIF-1 α , COX-2 and A5-LOX in the intestinal tissue from male and female mice differed (Fig. 4A-C). As indicated in Fig. 4A, sham-treated intestines from females expressed increased HIF-1 α compared to those from males. In response to I/R, male intestines expressed significantly more HIF-1 α at 30 min I/R (Fig. 4A), while HIF-1 α expression changed little when I/R was compared to sham treatment in female intestines. These data suggest that female intestines may be more

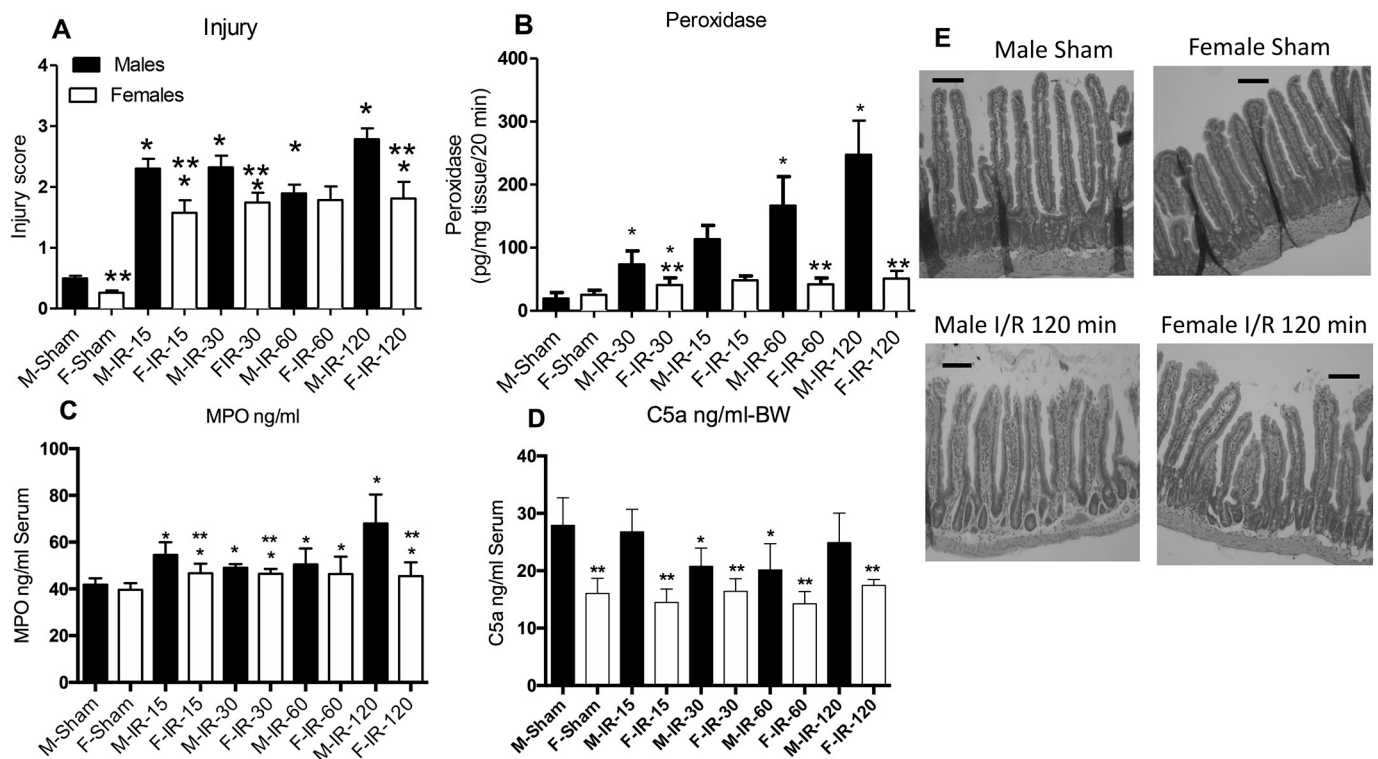


Fig. 1. Intestinal I/R-induced tissue damage and inflammation differs between male and female mice. (A) C57Bl/6 J mice were subjected to sham or 15, 30, 60, or 120 min intestinal I/R treatment and mid-jejunal sections H&E stained and scored for mucosal injury (75–150 villi per section); (B) Total peroxidase levels were determined by measuring oxidation of 3,3',5,5'-tetramethylbenzidine (TMB); (C, D) Serum myeloperoxidase (MPO) and C5a were measured with sandwich ELISA. (E) Representative microphotographs of H&E stained sections. Bar = 40 μm. *, Significant difference between I/R treatment and sham within the same sex ($P \leq 0.05$), as determined by unpaired *t*-test; **, Significant difference between sexes with the same treatment ($P \leq 0.05$). Each bar is representative of 6–10 animals per group.

sensitive to handling than male intestines. Similar to HIF-1 α expression, males expressed significant levels of COX-2 at early time points (15 and 30 min) I/R (Fig. 4B), while female expression of COX-2 increased at 60 min (Fig. 4B). Surprisingly, intestines from female mice expressed significantly increased levels of A5-LOX from 15 min to 120 min I/R and the level of A5-LOX was significantly higher than males at 15 min I/R (Fig. 4C).

As demonstrated previously, intestinal I/R also increases the chemotactic eicosanoids, LTB₄ and PGE₂ in wild-type mice [11,46]. To determine alteration of LTB₄ and PGE₂ production in all time points in male and female mice, we detected ex vivo generation of LTB₄ and PGE₂. Similar to previous studies, ex vivo intestinal sections from I/R-treated male mice released significantly more PGE₂ at 120 min I/R. PGE₂ production by female intestines after I/R was not elevated compared to female sham mice (Fig. 5A). In contrast, intestines from female mice consistently produced increased LTB₄ from 15 min I/R to 120 min I/R, while male mice maintained the same level of LTB₄ compared to male sham mice (Fig. 5B). Taken together, after intestinal I/R, arachidonic acid is converted to LTB₄ in female intestines whereas male intestines produce PGE₂.

3.4. Male but not female mice are protected with COX-2 inhibitor, NS-398

The dichotomous effect of mesenteric I/R-induced production of LTB₄ and PGE₂ raised the question of whether COX-2 plays different roles females and males in I/R-induced tissue damage and eicosanoids production. To explore the possibility that the inhibition of COX-2 would produce distinct protective effects in males and females during intestinal I/R, we treated both sexes of mice with a COX-2 specific inhibitor, NS-398, and evaluated injury, peroxidase and PGE₂ and LTB₄ production. As expected, treatment with NS-398 significantly reduced

ischemic intestinal injury in males compared with non-treated males (Fig. 6A). As peroxidase is a critical catalytic step of COX-2 [55], treatment with NS-398 also decreased total peroxidase concentrations significantly in males after I/R compared to untreated I/R male mice (Fig. 6B). Peroxidase levels in females increased significantly after NS-398 treatment without changing the injury level (Fig. 6B). Importantly, after I/R NS-398 treatment decreased PGE₂ production in males, but did not alter production in females (Fig. 6C). NS-398 treatment did not affect LTB₄ production in either sexes after I/R (Fig. 6D). Taken together, NS-398 inhibits tissue damage, total peroxidase level, and PGE₂ production in male intestines after I/R, but not in intestines from females. These data demonstrate a sexual dimorphism in I/R-induced arachidonic acid metabolism.

4. Discussion

Differences in sex play a critical role in the outcome of an I/R event in both experimental and clinical studies. Males appear to sustain increased tissue damage but females sustain greater mortality and longer hospital stays after an ischemic insult [19,56–58]. However, our understanding of sex-related time course and mechanisms of I/R-induced injury was unclear, largely due to the lack of experimental modeling. While males are larger and appear to sustain more damage in response to kidney, intestine and heart IR [25,26,59,60], we demonstrate that sex also impacts intestinal I/R-induced tissue injury by changing neutrophil recruitment, and eicosanoid production.

Our data demonstrate that both male and female mice have significantly increased injury after 30 min ischemia followed by as little as 15 min reperfusion and was sustained through 120 min reperfusion. Previous studies indicate that females are more resistant to oxygen deprivation than males [61,62]. In addition, increased tolerance of

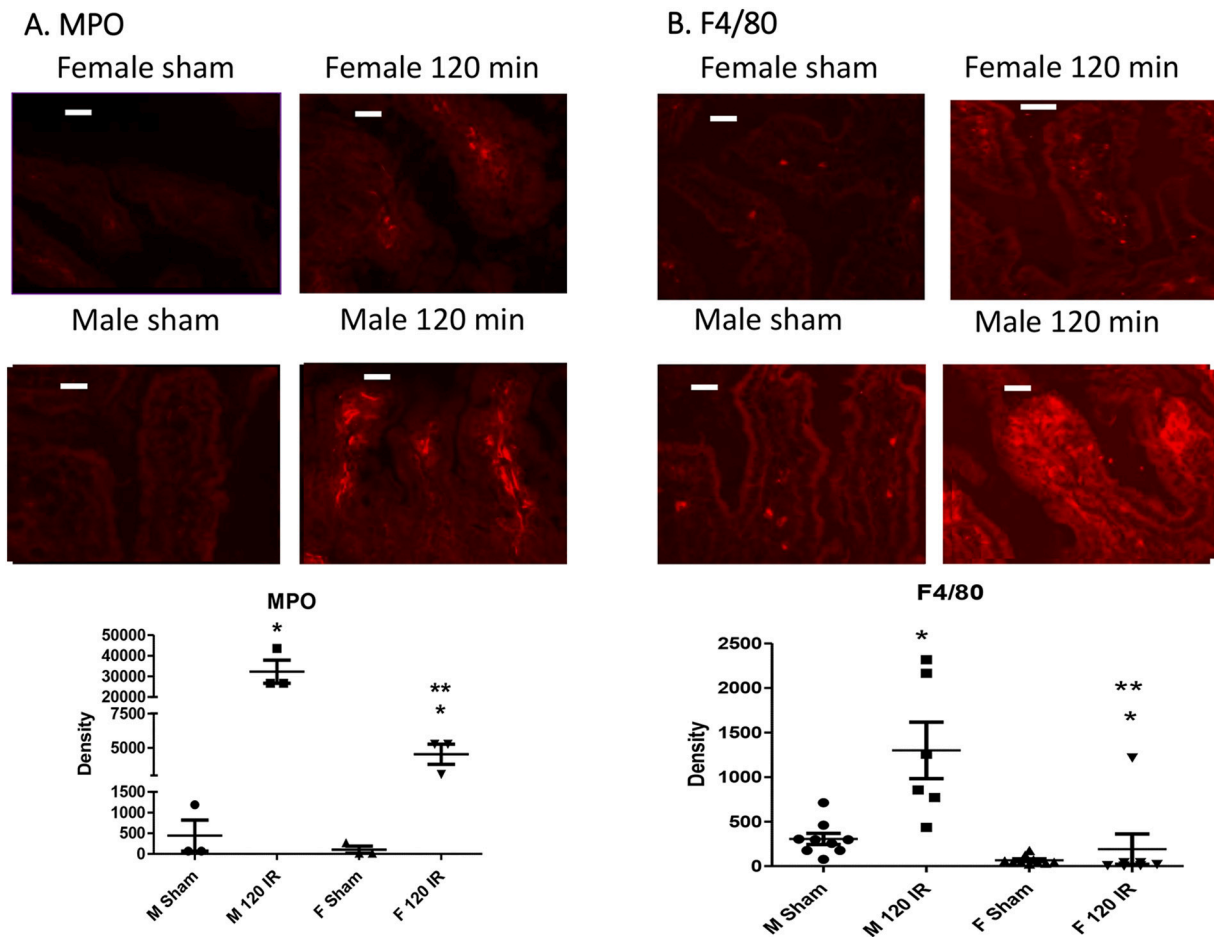


Fig. 2. Intestine tissue myeloperoxidase (MPO) and F4/80 staining differs between sexes.

C57Bl/6 J mice were subjected to sham and I/R treatment with 120 min reperfusion. Intestinal sections were stained for (A) MPO and (B) F4/80 by immunohistochemistry. Bar = 40 μ m. Densitometry was performed for each animal using ImageJ. Microphotographs (400 $\times$) are representative of at least 3–4 animals stained in at least 3 independent experiments.

females to I/R damage was observed in rabbits, rats, mice, and dogs [59,63–65]. In agreement with the above studies, males sustained increased histological damage compared to females throughout the reperfusion period.

Following I/R, intestines from female mice produced a marked increase of LTB₄ with little increase in PGE₂. In contrast, intestines from male mice produced little LTB₄ and significant amounts of PGE₂. This observation is consistent with prior studies showing *in vivo* sex differences in leukotriene biosynthesis with males producing more PGE₂ [66] and females producing more LTB₄ [67,68]. Furthermore, previous studies found significant HIF-1 differences between male and female cardiac responses to chronic hypoxia [69] similar to the current data in response to intestinal I/R (Fig. 4A). Specifically, similar hypoxic conditions induced significantly higher HIF-1 α expression in male mouse intestines compared to intestines from female mice (Fig. 4A). Our data also indicate that during intestinal I/R, female intestines use A5-LOX to upregulate LTB₄ production, rather than inducing COX-2 and PGE₂ production. This suggests that attenuating inflammation during I/R in females may require a LTB₄ inhibitor rather than PGE₂ inhibitor. In a trauma model using male mice, the COX-2 inhibiting drug, NS-398, decreased PGE₂ production to approximate levels found in females [66]. Another study found the A5-LOX product (5-lipoxygenase) is critical to increasing cutaneous wound healing of female mice [70]. It is possible that LTB₄ has a similar role in intestinal I/R. In the trauma study, PGE₂ production remained unchanged in females treated with or without NS-398 [66]. Importantly, our current studies correlate with

previous studies that demonstrated that NS-398 attenuated PGE₂ production and intestinal injury in males in response to I/R [39,71]. However, our current studies also show that peroxidase levels in female intestines increased significantly after NS-398 treatment suggesting a compensatory effect may occur. Although females do not upregulate the COX-2 pathway during I/R, the inhibition of COX-2 appears to increase other pathways such as neutrophil infiltration in females. Taken together, our data indicate that in response to I/R, male mice sustain significant injury which appears to require COX-2-mediated PGE₂ production while females appear to use the A5-LOX to produce LTB₄.

Myeloperoxidase, an abundant enzyme released by tissue-infiltrating neutrophils correlates with tissue injury in both acute and chronic inflammation and plays a critical role in organ damage after renal, myocardial, hepatic and intestinal I/R injury as well as other forms of tissue injury including end stage renal disease [72–75] [76,77]. C5a is generated via complement pathway activation, and acts as a potent chemoattractant for recruiting neutrophils to the sites of injury or inflammation [78]. Our data demonstrate that females produce significantly less myeloperoxidase and C5a than males at both the early and late stages of I/R. However, additional studies are needed. While MPO is a potent neutrophilic mechanism of tissue damage, other studies indicate that TLR9 mediated neutrophil extracellular traps (NETs) create significant damage in hepatic I/R of male mice [76]. However, there is no sex dimorphism in NETs during end stage renal disease. Thus, future studies should compare mechanistic differences between sexes as therapeutic targets. Mouse studies demonstrate that

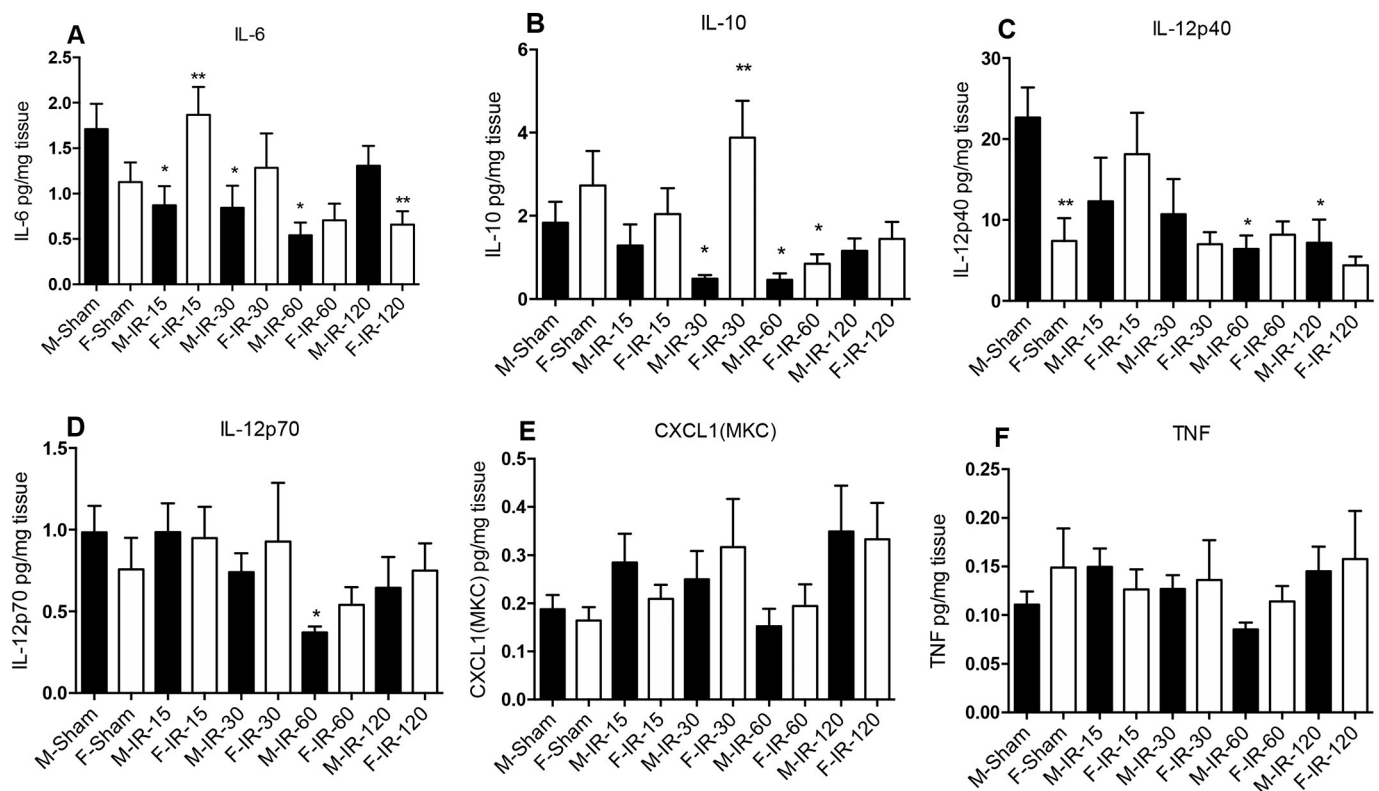


Fig. 3. Cytokine production in different sexes after intestinal I/R.

C57Bl/6 J mice were subjected to sham and I/R with 15, 30, 60 or 120 mins of reperfusion. Intestinal sections were analyzed ex vivo for (A) IL-6, (B) IL-10, (C) IL-12p40, (D) IL-12p70, (E) CXCL1(MKC), and (F) TNF production and normalized to a per mg tissue protein; *, Significant difference between I/R treatment and sham within the same sex ($P \leq 0.05$), as determined by unpaired t-test; **, Significant difference between sexes with the same treatment ($P \leq 0.05$). Each bar is representative of 5–10 animals per group.

TLRs including TLR4 [45] and TLR9 [76] are critical to induce PGE2 or NETs. Thus, TLRs may provide therapeutic targets for I/R damage but female mice should be examined as well [45,76]. Importantly, therapeutics should normalize both male and female differences as cetirizine in allergic inflammation [84].

F4/80+ macrophages, also participate in tissue injury after hepatic, myocardial, cerebral, and intestinal I/R [79–83]. In addition, intestines from male mice express higher levels of F4/80+ macrophages after I/R compared to intestines from female mice. Male neutrophils showed greater pro-inflammatory phenotype polarization, while female

neutrophils displayed lower inflammatory markers and were polarized to the anti-inflammatory phenotype [85]. This suggests a longer and more sustained I/R-induced inflammatory cell infiltration in intestines from male than female mice. Similarly, a previous study indicated that during myocardial infarction, the leukocyte ratios and wound healing kinetics benefit females by preventing the excessive myocardial infarction-induced inflammation [86]. In agreement with these studies, a recent publication reported that during myocardial infarctions, males have a higher neutrophil abundance than females [85]. Importantly, clinical gender differences exist in the immune cell changes in a

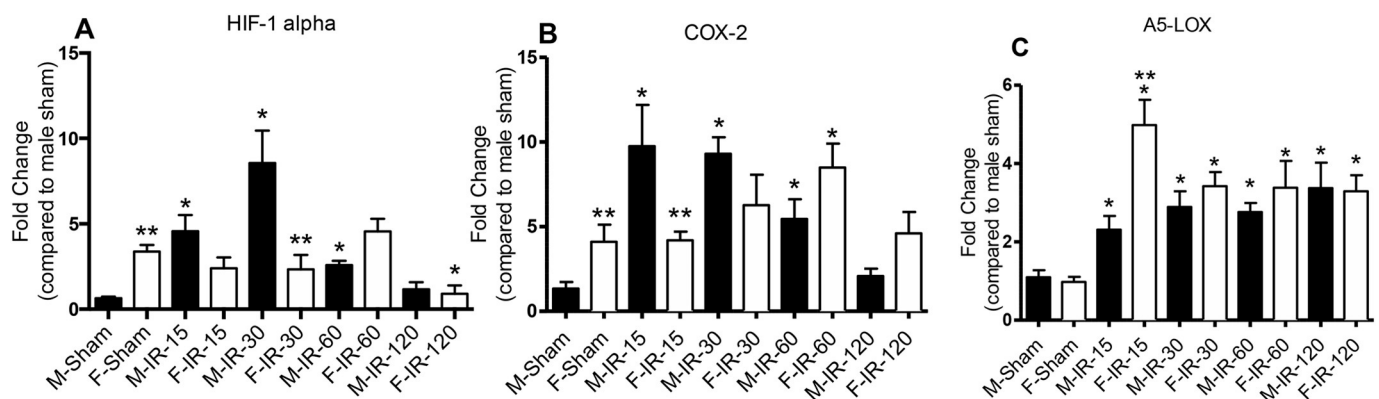


Fig. 4. Intestinal I/R-induced HIF-1α, COX-2, and A5-LOX transcription differs between sexes.

C57Bl/6 J mice were subjected to sham and I/R treatment with 15, 30, 60 or 120 min of reperfusion. Mid-jejunal expression of (A) HIF-1α, (B) COX-2 and (C) A5-LOX, RNA was determined by RT-PCR analysis. Samples were normalized to 18S followed by fold change compared to Sham treatment. *, Significant difference between I/R treatment and sham within the same sex ($P \leq 0.05$), as determined by unpaired t-test; **, Significant difference between sexes with the same treatment ($P \leq 0.05$). Each bar is representative of 5–10 animals per group.

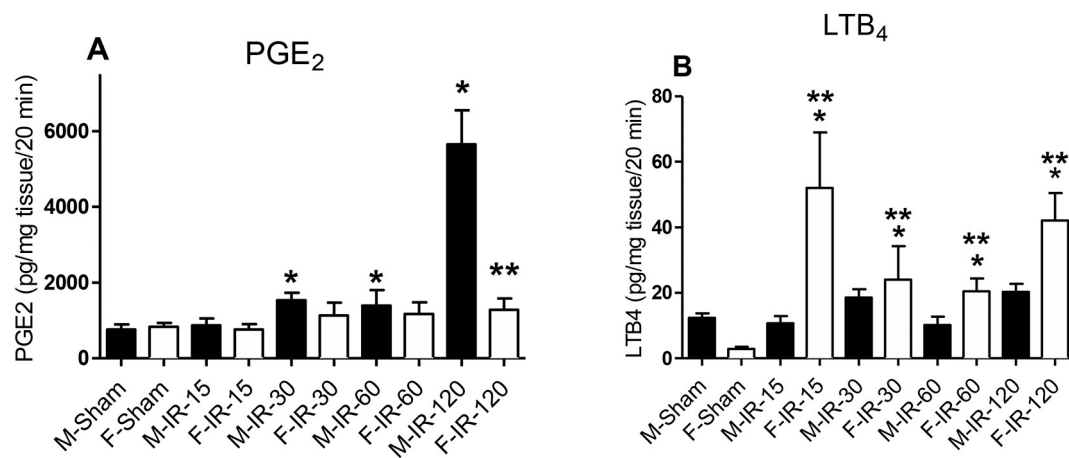


Fig. 5. Ex vivo production of PGE₂ and LTB₄ differs between sexes over reperfusion times.

C57Bl/6 J mice were subjected to sham and I/R treatment with distinct 15, 30, 60, or 120 min reperfusion. Intestinal sections were analyzed ex vivo for (A) PGE₂ and (B) LTB₄ production and standardized to mg total tissue protein. *, Significant difference between I/R treatment and sham within the same sex ($P \leq 0.05$), as determined by unpaired t-test; **, Significant difference between sexes with the same treatment ($P \leq 0.05$). Each bar is representative of 5–10 animals per group.

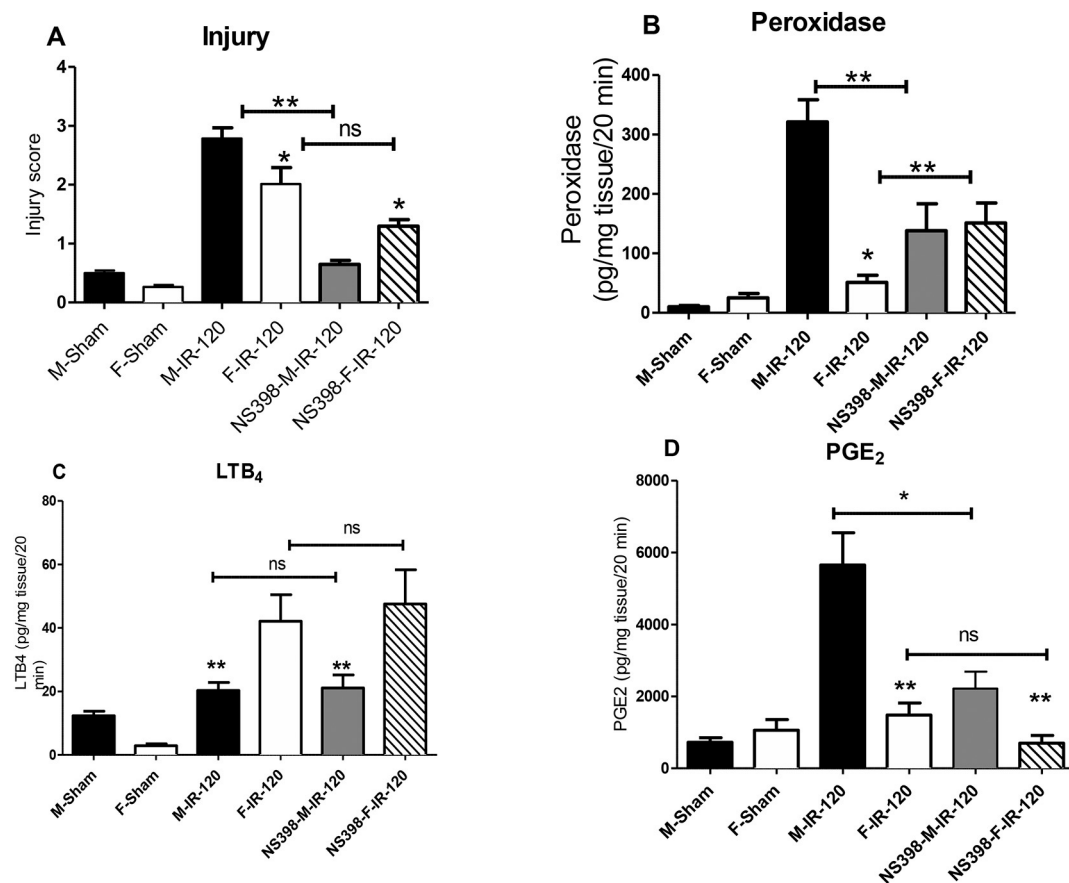


Fig. 6. COX-2 inhibition attenuates intestinal I/R-induced tissue damage in male but not female mice.

(A) C57Bl/6 J mice were subjected to sham or 120 min intestinal I/R treatment with or without administration of COX-2 inhibitor, NS-398. After 120 min reperfusion, mid-jejunal sections were H&E stained and scored for mucosal injury (75–150 villi per section); (B) Total peroxidase levels were determined by measuring oxidation of 3,3',5,5'-tetramethylbenzidine (TMB); Intestinal sections were also used to analyze ex vivo (C) LTB₄ and (D) PGE₂ production. Peroxidase, LTB₄ and PGE₂ were normalized to mg protein within the tissue. *, Significant difference between sexes within the same treatment ($P \leq 0.05$), as determined by unpaired t-test; **, Significant difference between untreated I/R and NS-398 treated mice within the same sex ($P \leq 0.05$). Each bar is representative of 5–10 animals per group.

multitude of diseases including allergic rhinitis where females rapidly induced a monocytic response compared to a significant eosinophilic decrease in males [84]. A complete profile of the cellular responses between males and females remains to be accomplished.

I/R is considered an inflammatory disease which induces pro-

inflammatory cytokines and chemokines produced from monocytes or damaged intestine by I/R [87]. Pro-inflammatory TNF is produced in response to myocardial ischemia and lung ischemia/reperfusion [87,88]. Our results demonstrate little TNF- α production in response to I/R and importantly, no differences between sexes suggesting that the

pro-inflammatory cytokine was not instrumental in the sexual dimorphism. IL-6 is a unique pleiotropic cytokine displaying both pro-inflammatory and anti-inflammatory properties depending on the type of injury and the target cell type [89]. Importantly, females with an overall lower mortality rate in myocardial infarctions also resolved increased plasma IL-6 faster than males [85]. These data correlate with the current data demonstrating that females secrete more IL-6 after I/R and reports that IL-6 mediates anti-inflammatory effects to protect intestine from I/R injury [90]. IL-10, an anti-inflammatory cytokine inhibits the production of pro-inflammatory cytokines and reduces inflammation-mediated tissue damage in both non-infectious [91,92] and infectious [93,94] causes of inflammatory responses. Our data also demonstrate that females produce increased IL-10, an anti-inflammatory cytokine, after 30 min reperfusion. One explanation for this dichotomy of cytokines (IL-6 and IL-10) in females is to balance or inhibit excessive pro-inflammatory cytokine production to protect the intestine and surrounding tissue from damage.

5. Conclusion

This study provides *in vivo* evidence that males and females respond differently to intestinal I/R-induced injury with distinct inflammatory differences. Specifically, compared to male mice, females secrete significantly more LTB₄, less PGE₂ and more anti-inflammatory cytokines. Thus, this study demonstrates specific sex differences and suggests that understanding the different mechanisms will be critical for development of appropriate medications or medical treatments.

Author contribution

SDF provided funding. SDF and MMW participated in study conception and design, data analysis, and interpretation of the data. MMW and JMR acquired data. All authors revised and approved the final manuscript.

Data availability statement

The data sets used and/or analyzed during the current research are available from the corresponding author on reasonable request.

Declaration of Competing Interest

None of the authors has any potential financial conflict of interest related to this manuscript.

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Running title: Complement activation induced in intestinal IR is sex-dependent

Complement initiation varies by sex in intestinal ischemia reperfusion injury

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Abbreviations used in this article:

ischemia reperfusion, IR; leukotriene B₄, LTB₄; prostaglandins E₂, PGE₂;
mucosal injury score, SMI; 3,3',5,5'-tetramethylbenzidine, TMB.

Key words: Prostaglandins, leukotrienes, C1q, MBL, properdin, male, female,
inflammation

ABSTRACT

Intestinal ischemia reperfusion (IR)-induced tissue injury represents an acute inflammatory response with significant morbidity and mortality. The mechanism of IR-induced injury is not fully elucidated, but recent studies suggest a critical role for complement activation and for differences between sexes. To test the hypothesis that complement initiation differs by sex in intestinal IR, we performed surgical intestinal IR on male and female WT C57BL/6, C1q^{-/-}, MBL-C^{-/-}, properdin^{-/-} mice. Initial studies demonstrated a difference in complement activation in male and female WT mice. Based on mRNA and protein expression, we demonstrated that males utilize all three pathways whereas females utilized only classical pathway was initiated in males, while females used lectin and alternative pathways.. Furthermore, WT, C1q^{-/-} and MBL-C^{-/-} male mice attenuated tissue damage, decreased ex vivo generation of eicosanoids, and reduced serum levels of C5a. In contrast only MBL-C deficiency had a similar effect in females and **decreased serum C5a level in PKO**. Our findings suggested that complement activation induced in intestinal IR in a sex-dependent manner.

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INTRODUCTION

Ischemia reperfusion (IR) generally stems from the interruption of organ blood flow and its subsequent restoration and contributes to pathology in a wide range of clinical conditions, ranging from hemorrhagic shock, stroke, myocardial infarction, and early organ transplant rejection, (1-5). These diseases are major causes of disability and even mortality. While not as common, acute mesenteric ischemia represents a potentially fatal vascular problem with an overall mortality rate of 70% to 90% (7, 8). It is becoming increasingly appreciated that gender differences exist in several models of IR, such as kidney (23), myocardial (24), hemorrhage (25), and intestine (26). After cardiac or brain ischemic events, females sustain less tissue injury, but remain hospitalized longer with higher in-hospital mortality (33-36). Importantly, after heart attacks or strokes, more females are permanently disabled than males (32). Furthermore, whole genome RNA-seq analysis also showed sexual dimorphism in transcriptional response to myocardial ischemia (37).

Similar to clinical studies, female mice sustain reduced and delayed intestinal damage in multiple forms of IR and hemorrhage (29-31). Previous studies suggested that the damaging inflammatory response consists of both neutrophil and complement activation (40) and POPE/MOSES AND COMPLEMENT papers. Many studies demonstrated that arachidonic acid metabolism to eicosanoid production is critical for IR-induced intestinal damage. In male mice prostaglandins E2 was required (POPE/MOSES). However, a recent study demonstrates that intestines from female mice secrete potent chemoattractants, the eicosanoid LTB4 and C5a from the complement cascade (PAPER 1). While previous studies suggested a sexual dimorphism in eicosanoid production, the IR-induced difference between sexes in complement activation are not clear.

The complement system consists of over 30 soluble and membrane bound proteins and protects from invading pathogens. (9). Recent studies have demonstrated that the complement system has a central role in orchestrating inflammatory responses and IR-induced tissue injury (11) of multiple organs, including the intestine (8), kidneys (12), brain (13), and heart (14), of humans and animals (15). Previous studies characterize the reperfusion inflammatory response after intestinal ischemia

by deposition of IgM and C3 in ischemic tissues (1, 16, 17)(16, 47). Mice deficient in multiple complement components sustain reduced renal and/or cerebral IR induced injury to varying degrees (18)(13, 19). Importantly, IgM initiates intestinal, myocardial, and cerebral IR injury (17, 21, 22).

Mesenteric IR damage was alleviated in C2, C3, C4, MBL deficient mice (3, 8, 20). Three distinct primary pathways initiate complement activation: the antibody-antigen-binding activates complement through C1q; mannose binding protein (MBP) binding to carbohydrate triggers the lectin complement pathway; and the alternative pathway involves cleavage of Factor B (FB) and stabilization by the plasma glycoprotein, properdin(9): Compared with male mice, total complement activation in females decreased despite similar accounts of C3 activation (39), indicating sex plays an important role in IR induced complement activation. Sexual dimorphism in the complement system poses interesting questions for immune biology response. Interestingly, accumulated research supports the idea that sex impact the immune system significantly (38) and complement activity may differ by gender (39). We hypothesized that the initiation of complement pathways may differ between males and females.

MATERIALS AND METHODS

Mice

WT C57BL/6, C1q-knockout, and MBL-C-knockout, (C1q^{-/-}, MBL-C^{-/-}, -) mice were purchased from The Jackson Laboratory and Properdin deficient mice were obtained from Dr. Wenchou Song, Univ. Penn. All mice were bred and maintained in the Division of Biology at Kansas State University, Manhattan, KS with a 12-h light/12-h dark cycle with a standard diet. Sex and age-matched (8 to 10 week old) mice were selected for experiments. All studies were approved by the Institutional Animal Care and Use Committee at Kansas State University and conducted according to the Animal Welfare Act and other federal statutes and regulations concerning animals and experiments involving animals.

Treatment of intestinal IR

The surgical protocol for intestinal IR was performed as previously described (42). Briefly, isoflurane (2-3%) anesthetized mice were subjected to a midline laparotomy and administered buprenorphine (0.06 mg/kg) for pain. After a 30 min equilibration period, the superior mesenteric artery was identified and ischemia was induced by application of a small nontraumatic vascular clamp (Roboz Surgical Instruments) and visually affirmed by blanching of the mid-jejunum. After a 30 min ischemic phase, the clamp was removed and the intestine was allowed to reperfuse for 15, 30, 60, or 120 min in WT mice, or 60 (data not shown) or 120 min in deficient mice. Reperfusion was affirmed by observing the return of pinkish color to the intestine. Sham mice were subjected to the same surgical intervention but without occlusion. All procedures were performed with the mice breathing spontaneously and anesthetized mice were kept on a 37°C water-circulating heating pad to maintain body temperature. Additional isoflurane was administered immediately prior to sacrifice. After sacrifice, serum and four sections of the mid-jejunum (2 cm each), beginning approximately 10 cm distal to the gastroduodenal junction, were harvested for histological evaluation, immunohistochemistry, RT-PCR, as well as eicosanoid and **cytokine** determination.

Histology and Immunohistochemistry

After euthanasia, 2 cm mid-jejunum specimens were immediately fixed in 10% neutral buffered formalin phosphate, embedded in paraffin, cut transversely in 8 µm sections, transferred to positively charged slides, and stained with hematoxylin and eosin (H&E). The score of mucosal injury (SMI) for each slide was assessed using a six-tiered scale adapted from Chiu et al (43), as described previously (44). Briefly, the average mucosal damage score of the mid-jejunum intestinal section was calculated by two well trained observers unaware of the treatment. Each observer graded 90-150 villi on a scale of 0-6 with the following categories: Normal villi were assigned a score of zero; a score of 1 were assigned to villi with tip distortion; score 2 was assigned when Guggenheims' spaces were present; a score of 3 was assigned to villi with slight disruption of the epithelial cells; villi in which the lamina propria was exposed but intact were scored as 4; villi with exuding lamina propria were scored as 5; and score 6 was assigned when the villi displayed hemorrhage or

denuded. Photomicrographs were acquired from H&E stained slides by using a 20X, 0.5 Plan Fluor objective on Nikon 80i microscope. Images were obtained by utilizing a Nikon DS-5 M camera with the DS-L2 software at room temperature (Nikon). Immediately after removal, additional 2 cm mid-jejunum sections were snap-frozen in optimal cutting temperature (O.C.T.) freezing medium. Specimen were cut transversely in 8 μ m sections and placed on the slides for future immunohistochemistry, as described previously (45). Briefly, pre-chilled acetone fixed slides were incubated with 10% normal donkey sera in phosphate buffered saline (PBS) for 30 min at 37°C to block nonspecific binding. Following PBS washes, the intestinal sections were incubated with primary antibodies in the dark for 1 h at room temperature (RT) or overnight (O/N) at 4°C. The C3b, C1q, MBL-c, Factor B, and IgM deposition on the tissue sections was determined by staining with a purified rat-anti-mouse anti-C3b antibody (Hycult Biotechnologies), or anti-C1q antibody (Cedarlane), or anti-MBL-c antibody (Cedarlane), or rabbit-anti-mouse anti-Factor B antibody (Abcam) followed by a Texas-red conjugated donkey-anti-rat IgG secondary antibody (Jackson ImmunoResearch) or FITC-green conjugated donkey-anti-rabbit IgG secondary (Jackson ImmunoResearch). The detection of IgM was performed similarly with the exception of using FITC AffiniPure Fragment goat-anti-mouse IgG antibody (Jackson ImmunoResearch), and no secondary antibody was needed. All experiments contained serial sections stained with the appropriate isotype control antibodies. The slides were then mounted with ProLong Gold (Invitrogen). Images (4-8 images per section) were acquired by a well-trained blinded observer at RT by using a Nikon eclipse 80i microscope equipped with a Infinity 3S camera (Lumenera) and analyzed by utilizing Infinity Analysis software (Lumenera). All microphotographs were examined by Image J software (National Institutes of Health) using the fluorescent area fraction after setting threshold for each experiment. The average of the isotype control was subtracted from each image. The average of 5 images from 3-4 animals per treatment group is analyzed and reported.

Ex vivo Cytokine and Eicosanoid Determination

Mid-jejunal tissue ex vivo generation of eicosanoids and cytokines was determined by using a adapted procedure previously described (46). Briefly, immediately after collection, 2 cm mid-jejunum sections were minced, washed in pre-chilled Tyrode

buffer (Sigma-Aldrich), and resuspended in 37°C freshly oxygenated Tyrode buffer. After 20 min incubation at 37°C, the supernatants and tissues were collected and stored at -80°C until assayed. The tissue protein content was detected by utilizing the bicinchoninic acid assay (Pierce) modified for use with microtiter plates. Total peroxidase activity was determined by measuring oxidation of 3,3',5,5'-tetramethylbenzidine (TMB) and the reaction was stopped with 0.18 M sulfuric acid as described previously (47). Horseradish peroxidase (HRP) (Sigma) was used as a standard. The same intestinal tissue supernatants were used to determine concentrations of leukotriene B₄ (LTB₄) and prostaglandins E₂ (PGE₂) utilizing enzyme immunoassay kits (Cayman Chemical). Supernatants generated for total peroxidase and eicosanoids were also used to detect cytokine levels by using a Milliplex MAP immunoassay kit (Millipore) that was analyzed on a Milliplex Analyzer (Millipore). The concentrations of peroxidase, eicosanoid, and cytokines were standardized per mg intestinal tissue protein per 20 min.

C5a level determination by ELISA

Blood was collected from mice after intestinal IR, allowed to clot on ice at least 30 min and serum collected by centrifugation for 20 min at approximately 1000 × g. Serum C5a level was determined based on the sandwich ELISA principle. The mouse C5a capture antibody, detection antibody, standard, and Streptavidin-HRP were purchased from R&D SYSTEMS.

Quantitative Real-time PCR

After euthanasia, one 2 cm mid-jejunal tissue approximately 10 cm distal to gastroduodenal junction was immediately snap froze in liquid nitrogen and stored at -80°C until used for RNA analysis. Total RNA was isolated from intestine tissues using TRIzol reagent (Invitrogen) and then treated with DNase I (Invitrogen) according to manufacturer's instructions. RNA quantity and quality was evaluated and measured by utilizing the Nano drop (Thermo Fisher Scientific). The reverse transcription was conducted at 25°C for 5 min, 42°C for 30 min, and 85°C for 5 min to get cDNA with 1 µg total RNA by using qScript first strand cDNA synthesis kit (Quanta Biosciences). Primers (Table 1) were designed with Primer 5.0 according to the mouse gene sequence. Quantitative real-time PCR was conducted in a 25 µl total volume using an Applied Biosciences StepOnePlus thermocyclers (Thermo

Fisher) with Perfecta SYBR Green Fastmix reagent (Quanta Biosciences). The PCR protocol is as follows: (i) pre-denaturation for 3 min at 95°C; (ii) an amplification and quantification program including 50 cycles of 10 sec at 95°C, 20 sec at T_m (Table 1), 10 sec at 72°C; (iii) melt curve start at 60°C, increasing by 0.5°C up to 95°C. After amplification, 18s rRNA was used as an internal control to normalize relative gene transcript levels, $\Delta\Delta C_t$ fold change relative to sham-treated wildtype mice was determined.

Statistical Analysis

Data are presented as mean $\pm$ the standard error of the mean (SEM), and were statistically analyzed by unpaired t-test with Mann-Whitney *post hoc* analysis (Graph Pad/Instant Software Inc). Differences between groups were considered significant when P value ≤ 0.05 .

RESULTS

Complement initiation in response to intestinal IR differs between sexes

Complement activation is initiated by multiple pathways which may lead to distinct complement activation in male vs female mice during intestinal IR. Initial studies examined wildtype male and female mice complement initiation mRNA expression in wildtype male and females by performing RT-PCR of C1q, MBL-c, and Factor B. The expression of C1q increased in males significantly at 15 min and remained at high level from 15 min to 60 min IR (Fig. 1A top), while in females C1q remained low during the four time points of IR in females (Fig. 1A bottom). In addition, male C1q expression was significantly higher at 15- 60 min IR, compared to females (Fig. 1A). C1q transcription level decreased at 120 min IR in both male and female (Fig. 1A). In contrast, MBL-c expression was altered little in males after IR, and actually decreased significantly from 60-120 min compared to sham-treated males (Fig. 1B top). However, the expression of MBL-c in females increased significantly from 30-60 min before decreasing at 120 min IR (Fig. 1B bottom). Compared to males, females express significantly more MBL-c RNA in shams, and in IR from 30-120 min (Fig. 1B). From 15-60 min after IR, the expression of FB in males continuously decreased and the decrease became significant from 30-60 min (Fig. 1C top), while it was not altered in females at 30-60 mins (Fig. 1C bottom). Both male and female had a

significantly decreased level of FB at 120 min IR (Fig. 1C). There were no significant differences between males and females, during the IR time course (Fig. 1C).

To confirm the expression of complement initiators and major players in the complement pathways, we conducted immunohistochemistry (IHC) to detect the intestinal deposition of IgM, C1q, MBL, FB, and C3b. In response to IR, IgM was deposited in both males and females, although females expressed more IgM than males after 120 min reperfusion (Fig. 2). Similar to mRNA expression levels, C1q tissue deposition was increased in males at 60 min (data not shown) and was similar to its shams at 120 min after ischemia (Fig. 2). Besides, C1q deposition was also changed little in females at either 60 min (data now shown) or 120 min of reperfusion (Fig. 2). Overall males deposited more C1q compared to females (Fig. 2). In contrast, both males and females deposited increased levels of MBL-c after IR by 60 min (data now shown) and 120 min of reperfusion (Fig. 2). Importantly, much more MBL-c accumulated on female intestines than on male intestines by 120 mins post ischemia (Fig. 2). FB was expressed consistently in both sham and IR in both in sexes (Fig. 2). Tissue deposition of C3b was enhanced in males by both 60 min (data now shown) and 120 min (Fig. 2), and it was also increased in females. Intestines from both sexes deposited increased C3b at both time points.

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Taken together, our data indicate that all three complement pathways play a role in both sexes after intestinal IR in wildtype mice. However, males tend to use both lectin pathway and classical pathway, while females tend to depend on lectin pathway. Males tend to use the classic pathway for a short period during early IR. Amplification pathway is important for both sexes in wildtype mice.

Complement deficiency protects mice from intestinal IR injury in a sex dependent manner

To understand the specific role of each initiation pathway intestinal IR-induced tissue damage, we subjected C1q^{-/-}, MBL-C^{-/-}, and FP^{-/-} male and female mice to sham or 120 min mesenteric IR. As there is no significance among sham treatment at different time points of the same sex of either wildtype (WT) or knockout (KO) mice, injury scores and subsequent analyses were pooled by sex for sham treatment only. Compared to sham mice, intestinal damage was increased significantly in both sexes

in both WT mice after 120 min of reperfusion (Fig. 3). Although IR induced significant injury in both sexes of C1q^{-/-}, MBL^{-/-}, and FP^{-/-} mice (Fig. 3), a significant difference between sexes also existed. In males, compared to WT after IR, intestinal injury was reduced significantly in all deficient mice (Fig. 3). In contrast, compared to corresponding WT after IR, intestinal injury in females was decreased significantly in only MBL^{-/-} (Fig. 3). Between males and females, WT and deletion of C1q or properdin resulted in significantly reduced intestinal damage in females, compared to its corresponding male mice after IR (Fig. 3).

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These data suggest that males are protected with C1q, MBL, or properdin deficiency, while females are only protected from MBL-deficiency. Together with current data in C1q, MBL, and FB expression in IHC experiments, these results indicate that all three complement pathways are required for inducing intestinal IR injury in males, while the lectin pathway is particularly critical for inducing tissue damage in females. Among the three pathways, IR-induced tissue damage requires C1q in males more than females, MBL is critical for both sexes, and alternative pathway appears to amplify the injury during intestinal IR.

Complement deficiency decreases intestinal eicosanoid production in a sex dependent manner

Previous studies showed that eicosanoids, LTB₄ and PGE₂ is rapidly elevated in response to intestinal IR (48, 49). To confirm the role of C1q, MBL, and properdin in mesenteric IR-induced tissue damage, we examined ex vivo generation of both eicosanoids in intestine tissues after IR in all deficient mice. Similar to previous studies, intestines from IR treated WT female mice generated elevated LTB₄ at 120 min, while WT male mice did not increase LTB₄ compared to male sham treated mice (Fig. 4A). After IR, female intestines produced significantly more LTB₄ than males (Fig. 4A). Deletion of C1q, MBL, or properdin in male mice did not alter the production of LTB₄ (Fig. 4A). In contrast, deletion of C1q or properdin resulted in a decrease of LTB₄ production in females, and deletion of MBL or properdin resulted in a significant decrease of LTB₄ production, compared to its corresponding female WT mice after IR (Fig. 4A).

In contrast, intestines from IR treated WT male mice generated significantly elevated PGE₂ at 120 min post-ischemia, while WT female mice maintained PGE₂ production similar to sham treated mice (Fig. 4B). As expected, after IR, female intestines

produced significantly lower PGE2 than males (Fig. 4B). Deletion of C1q, MBL, or properdin in male mice significantly reduced production of PGE2 (Fig. 4B). Whereas, there was minimal change in the PGE2 production in female MBL^{-/-} or FP^{-/-} mice compared to the corresponding WT mice (Fig. 4B). However, deletion of C1q resulted in an increase of PGE2 production in females (Fig. 4B).

These results suggest that MBL is critical for inducing intestinal tissue damage during IR for both sexes. C1q and properdin are critical for IR injury induced PGE2 production in males. These provide evidence for the crucial role of all three complementary pathways in IR induced damage in males. Together with current data in FB expression in IHC experiment, and injury scores, these results suggest that alternative pathway is required for complementary induced injury during intestinal IR, but it is not enough to induce significant tissue injury.

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Depletion of MBL or properdin decreases serum C5a levels in both sexes following intestinal IR

Complement plays a significant role in intestinal I/R tissue damage (11).

Complement activation leads to series of proteolytic amplifying events, including the cleavage of C3 and C5 which are common to all three initiation pathways. Cleavage of each component results in deposition of C3b or C5b respectively. Measurement of C3b deposition on intestinal tissue in each of the deficient mice demonstrated...

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Similarly, C5 cleavage generates the anaphylatoxin C5a. C5a is critical for initiation of neutrophil recruitment and activation, thus we examined neutrophil chemotactic C5a levels in the blood. Serum levels of C5a were markedly elevated in WT and C1q^{-/-} after IR in both sexes, compared to their corresponding shams (Fig. 5). In contrast, C5a levels were significantly decreased in MBL^{-/-} and FP^{-/-} after IR in both sexes (Fig. 5). Within the same sex, compared to WT after IR, males produced significantly lower levels of C5a in the serum of MBL^{-/-} and FP^{-/-} mice, females also produced significantly lower levels of C5a in the serum of MBL^{-/-} and FP^{-/-} mice. However, it was different in C1q^{-/-} between sexes compared to the corresponding shams, serum level of C5a in males was not altered, but it was significantly increased in females. Between males and females, females had significantly lower serum level of C5a in WT, C1q^{-/-}, and P^{-/-}. Taken together, our data suggest that deletion of MBL or properdin decreases serum C5a levels in both sexes after IR,

deletion of C1q does not change it in males, while deletion of C1q increases it in females.

DISCUSSION

Complement activation is critical in the mesenteric IR and other forms of IR induced injury and inflammation (14, 50, 51). Previous studies showed that intestinal, renal, and myocardial IR induced tissue damage are largely dependent on complement activation (52, 53). Interestingly, renal I/R-induced damage uses more alternative pathway of complement activation whereas intestinal and myocardial I/R-induced injury is more dependent on classical and MBL pathways rather than the alternative pathway (52). Thus, the involvement of the complement in IR induced damage is well established. However the mechanistic differences between the sexes are not well understood. In humans and in other models of complement activation, complement activations appears to differ by sex,. Most studies used male mice as females are smaller and sustain reduced damage. These studies support the hypothesis that complement initiation may differ between sexes after IR we hypothesized that females may initiate complement by a different pathway than males. The sex dimorphism in intestinal IR induced complement activation differed in wildtype mice as well as in C1q^{-/-}, and MBL^{-/-} mice. Our data demonstrate that although both sexes activate complement females appear to depend on MBL initiation while males also use C1q to initiate complement.

In the current study, we present evidence that both male and female WT mice have significantly increased intestinal damage after IR. We observed significantly increased transcription of C1q gene and MBL-C gene in male and female mice, respectively (Fig. 1A and 1B) after IR. The RNA levels of C1q and MBL-C gene are significantly different between males and females after IR (Fig. 1A and 1B). In addition, FB gene maintained a similar transcription level during IR in both sexes, and there is no significant difference between sexes (Fig. 1C). Here, we conclude that complement activation is critical during intestinal IR, which is in accordance with previous studies (8, 54, 55). In addition, a sex bias seemingly correlated with IR induced complement initiator genes expression differences between sexes.

The classical pathway can be initiated by the binding of antibody-antigen complexes to the C1q protein and MBL pathway is triggered by MBL recognizing carbohydrate patterns (57, 58). A recent study shows that IgM forms complement-activating complexes with C1q on cell membranes highly similar to IgG1-C1 to mediate complement activation (56). Additional studies demonstrate that MBL may also recognize carbohydrate structures on the IgM (Ming Zhao and Stahl). To address whether C1q and MBL play different role in IR induced injury in different sexes, WT mice and mice genetically deficient in early components of either C1q-dependent classical or MBL pathways were characterized in intestinal model of IR damage. Male mice showed significantly lower tissue injury in absence of C1q or MBL, while females showed significantly lower damage in absence of MBL, but not C1q, at least under the conditions of our model.

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As suggested by others using intestinal IR model (17, 47, 59), if the C1q-dependent classical pathway is involved in IR induced damage by binding to natural antibodies, mice deficient of C1q should be protected from IR damage. However, previous studies show disagreement on whether mice deficient in C1q protected in IR models. The Classical pathway was robustly activated during heart and kidney ischemia (14, 51) and C1q deficiency protected animals from cerebral IR injury (60, 61). However, C1q deficiency did not protect in intestinal (3, 8) or myocardial injuries (62). These studies demonstrated that C1q-deficient male mice were not protected when subjected to 20-40 min ischemia and followed by 3 h reperfusion in both intestinal and myocardial IR models. Our data was the first study to show female mice are not protected from C1q deficiency. In addition, male mice deficient in C1q were protected at 120 min reperfusion indicated from both injury scores (Fig. 3A) and PGE2 production (Fig. 3D). This may be explained that males maintained a higher tissue injury from early to late stage compared to females, which could be a complicated combination of injuries from all directions. They started to have many inflammatory related genes translated, but during the response to IR, selected genes were finally translated and expressed into active proteins to induce inflammation and injury, which may be correlated with sex related factors according to our results. Therefore, it is likely that C1q accounts for male mice tissue injury induced by intestinal IR, possibly just for a short phase.

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We also found that in the absence of MBL both male and female mice were protected from mesenteric IR induced injury. Previously, studies have documented that MBL-deficient male mice were protected in renal (12), intestinal (3, 8), and myocardial (62) IR injuries. It is important to note that only male mice were used throughout these experiments. Here, we also found significantly reduced tissue damage in male mice with the absence of MBL, which was consistent with them. Importantly, our data was the first to report that female mice were also protected from the absence of MBL.

It is also important to note that C3b and FB were consistently expressed during mesenteric IR for both sexes, indicating that alternative pathway was not essential or not enough to induce local injury. This may be explained that as C3b serves as the binding partner for FB during IR induced amplification of the complement cascade through the alternative pathway. They could also have another role such as amplification of remote injury.

The establishment of the crucial roles of complementary pathways in intestinal IR injury in different sexes does not necessarily exclude the involvement of other innate immune components. It is likely that various components of innate immunity participate in different stages of injury in different sexes.

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In conclusion, we demonstrated that C1q-deficient male mice, but not females, were protected, at least for a short period, from intestinal IR injury. Whereas MBL-deficiency protect both male and female mice in the mesenteric model. This study has provided in vivo evidence that males and females response differently to intestinal IR in the complementary initiator aspect. Therefore, sex should be considered as an important factor to ischemic researches, and future research is required to focus on developing treatments that are effective in women.

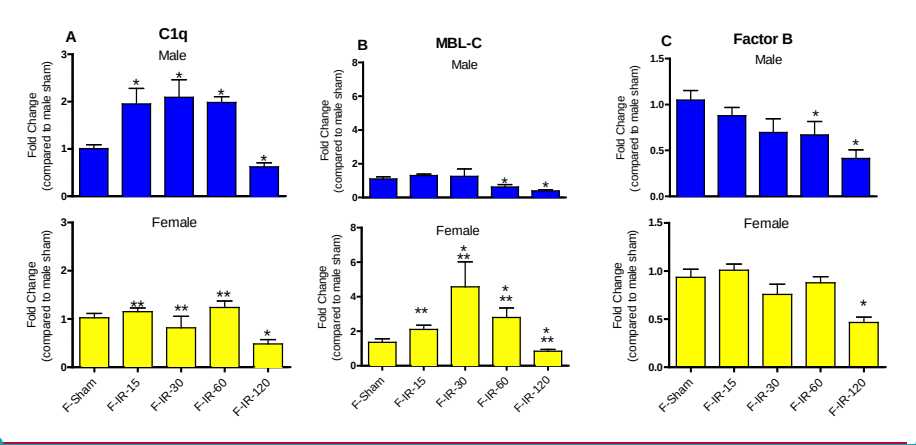
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Figure Legends

Figure 1:



Field Code Changed

Figure 2:[IHC](#)

Figure 3:

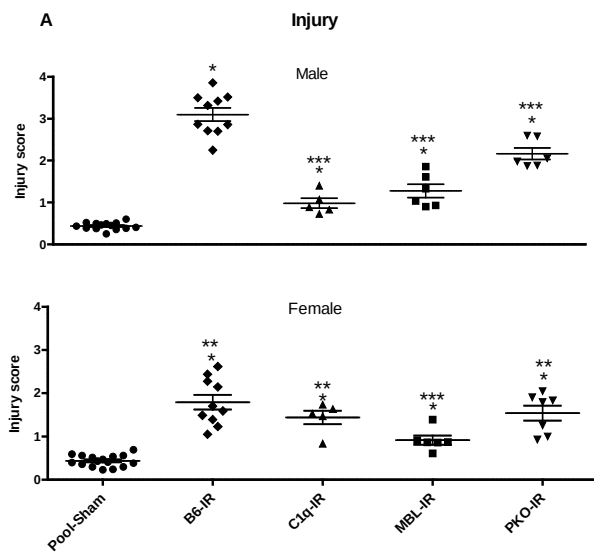


Figure 4:

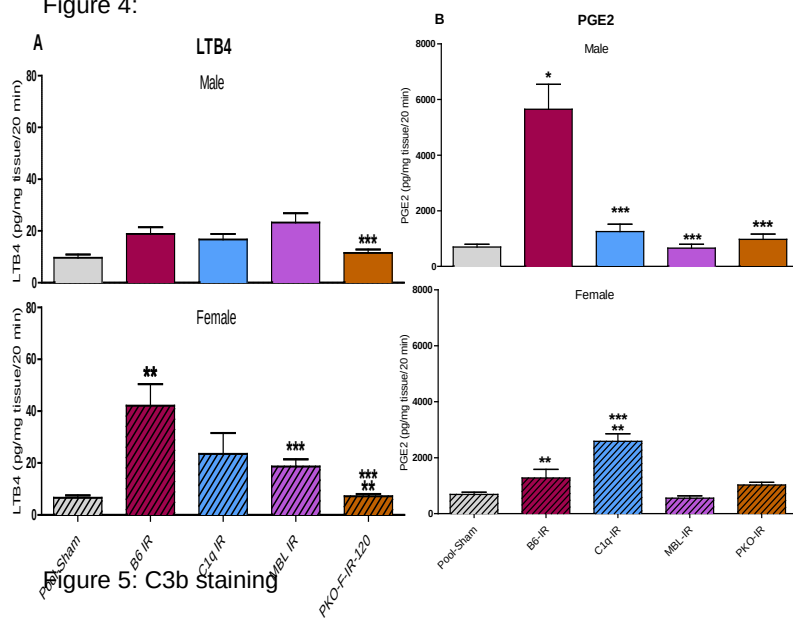


Figure 5: C3b Staining

Table 1 Real Time PCR Primer Sequences

Gene	Tm (°C) ^a	Sequence
C1q	58	FWD: CAC CAA CCA GGA GAG TCC AT
		REV: ACC TGA AAG AGC CCC TTG TT
MBL	56	FWD: ATGCCTACTTGGGCATCAC
		REV: ATCGTTCCACTTGCCATTTC
Factor B	58	FWD: CCA GCA TTT GGG TTT CAG TT
		REV: CAC ACC TCC AGA GGA GAA GC
Ribosomal 18S ^b	56	FWD: GGTGATCCTGCCAGTAGC
		REV: GCGACCAAAGGAACCATAAC

^aAnnealing temperature

^bHouse-Keeping gene to which genes of interest were normalized

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