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TITLE:
Gender differences in complement-mediated reperfusion injury

PRINCIPAL INVESTIGATOR:
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CONTRACTING ORGANIZATION:
Kansas State University,
Manhattan, KANSAS 66506

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Sherry D Fleming

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Kansas State University,
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Manhattan, KANSAS 66506

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13. SUPPLEMENTARY NOTES

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 is known to include 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.

15. SUBJECT TERMS
sex, female, male, ischemia, hypoxia, complement, inflammation

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	62	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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%
Milestone Achieved: HRPO/ACURO Approval	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)	80%

Subtask 2 Specific immunohistochemistry (IHC) studies will be performed for C3, C1q, C4, Factor B, Membrane attack complex, IgM and β2-glycoprotein I (β2-GPI)	8- 15	Dr. Fleming	90%
Milestone(s) Achieved: Female complement initiation pathways determined and manuscript submitted if applicable	12-15	Dr. Fleming	75%
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)	
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 β2-glycoprotein I (β2-GPI) as appropriate.	11-16	Dr. Fleming	
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	75%
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)	
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 β2-glycoprotein I (β2-GPI) as appropriate.	15-20	Dr. Fleming	

Milestone(s) Achieved: Female complement inhibition pathways determined and manuscript submitted if applicable	20-24	Dr. Fleming	
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 β2-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 β2-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)	10%
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 + β2-GPI.	25-30	Dr. Fleming (total 120 animals)	
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 or on order.

Major task 2: Some of the mice are not breeding as well as necessary for the needed animals, specifically, the MBL-/- mice. The studies using MBL-/- mice will be delayed to acquire the necessary animals. Despite this setback, a manuscript has been written and will be submitted soon (see specifics below and appendix).

Major task 3: The ER-/- mice are on order.

Major task 4: The complement regulator IHC is 75% complete with much of the data being included in the current manuscript (see below).

Major task 6: The time course of the Beta 2 glycoprotein 1 ag is almost complete.

Major task 7: Sera is being collected for antibody purification.

Major task 1 has been accomplished with all animals acquired or on order. This process was somewhat delayed due to unavailability of all the mice. However, all mice for major task 2 have now been acquired and those required for major task 3 are on order.

Major task 2: In addition to a delay in receiving some strains of mice, not all strains are breeding well enough to acquire the needed animals. This has delayed surgeries for the C1q-/- and MBL-/- mice. Despite this setback, a manuscript addressing the male vs female time course of inflammation and injury is in progress and will be submitted soon (see appendix 1 for complete methods and results). Specifically, a post-doc and technician have been trained in the intestinal ischemia/reperfusion (IR) surgery. Using 30 min ischemia followed by 15, 30, 60 or 120 min reperfusion, we performed a time course of injury and inflammation in both sexes of mice. We demonstrated that overall, both sexes of mice sustain IR-induced injury compared to Sham treated animals. However, female mice sustain significantly less intestinal damage than male mice based on the Chui scoring system of histological tissue damage (Fig 1).

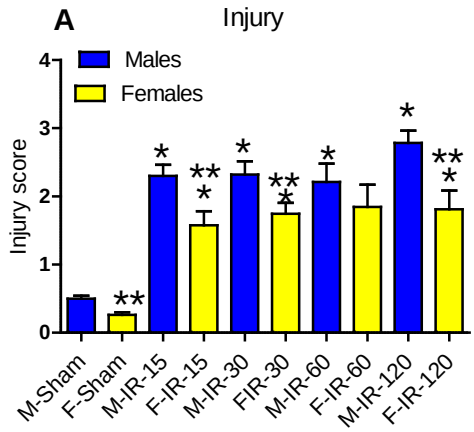


Figure 1. Injury varies by sex over IR time course in male (blue) and female (yellow) mice. C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Mid-jejunal sections were H&E stained and scored for intestinal injury (75-150 villi per animal with 3-10 animals/treatment and each treatment was performed on at least 2 separate days).

Total peroxidase production and myeloperoxidase, a signature of neutrophil infiltration, both matched the histology results with ex vivo intestinal production significantly increased in males compared to females. Additional cytokine analysis indicated that female intestines produced significantly more anti-inflammatory IL-10.

Importantly, the eicosanoid production confirmed our preliminary data in arachidonic acid metabolism. In response to IR, intestines from male mice produced significant Cox-2 and PGE2 with little A5-Lox and LTB4 (Fig 2 A and B). In contrast, intestines from female mice produced significant amounts of A5-Lox and LTB4 with little Cox-2 and PGE2. These opposite results will be examined in all future studies to determine the interactions.

We continued to examine the role of complement initiators and complement regulator differences in male and female mice after intestinal IR surgeries. Using RT-PCR, intestines from male mice produced more classical complement activation in male mice compared to female mice as determined by C1q. In contrast, intestines from female mice produced increased MBL-c compared to intestines from male mice. Although intestines from both sexes produced Factor B, there was no significant difference between sexes (Fig. 3). The RT-PCR results were confirmed by immunohistochemistry (IHC) to determine the amount of each initiation factor (Fig. 4). C1q protein deposition on the intestines after 60 min reperfusion was obvious in male mice whereas intestines from female mice contained significant MBL-C deposits at 30 and 60 mins post reperfusion. These data suggest that male mice preferentially utilize the classical complement activation pathway whereas the lectin pathway is prevalent in female mice. These data will be confirmed using C1q and MBL deficient mice as they become available within the colony. We expect the required studies for Major Task 2 will be completed with at least one manuscript submitted within the next year.

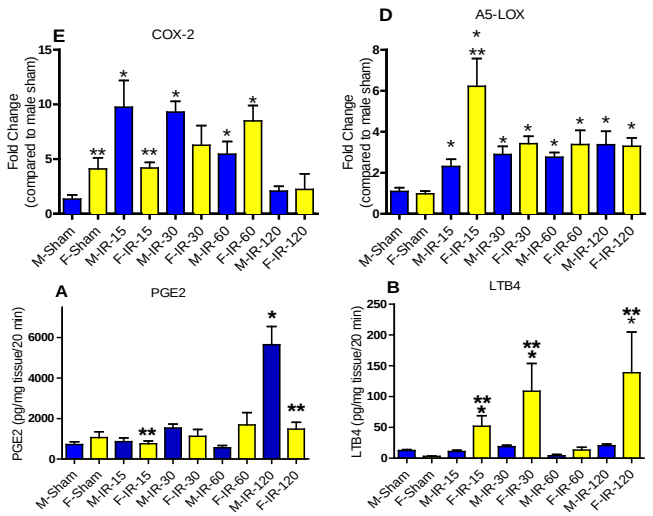


Figure 2. Time course expression of intestinal Cox-2 and A5-Lox RNA gene expression and PGE2 and LTB4 protein expression. Male (blue) and female (yellow) C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were analyzed by RT-PCR for Cox-2 and A5-Lox and ex-vivo for LTB4 and PGE2 by EIA. *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.

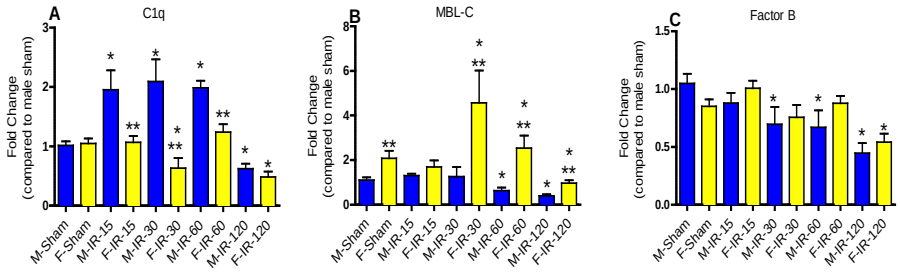


Figure 3. C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Mid-jejunal transcription of (A) C1q, (B) MBL, and (C) FB was determined by qRT-PCR analysis. *indicates significant difference from same sex Sham ($p \leq 0.05$); ** indicates significant difference from opposite sex at same time point ($p \leq 0.05$); n = 4-10 mice per group.

Complement regulation occurs by production of complement regulators as well as complement inititations. Thus, significant progress has been made on Major Task 4 as well. The time course of IR-induced injury also examined complement regulators, CD55 (DAF), Factor H (FH) and CRRY, the rodent equivalent to CD46 in humans. Similar to the complement initiators, we examined message for each regulator using RT-PCR (Fig. 5) and protein expression on cell surfaces by IHC (Fig. 6). 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.

As protein is required for actual complement inhibition, additional IHC studies were performed on tissues from the entire time course (Fig 6). Again a sexual dichotomy was observed with female intestines expressing significant CRRY on the intestinal villi and submucosa by 60 min post reperfusion, while little to no CRRY was expressed on male tissues at any time point. In contrast, FH was deposited on male tissue by 60 min and little to no deposition occurred on the female tissue. Similar to Major task 2, these results will be confirmed and extended using immunodeficient mice and/or immunopharmacological agents.

Due to the delay in breeding of mice, we have begun the analysis of neoantigen expression on tissues from the IR-induced time course. Thus, approximately 20% of Major Task 6 is completed. These preliminary data suggest that there may be differences in the timing and specificity of neoantigen expression. These studies will be completed within the next year.

Finally, sera has been collected for antibody purification in Major Task 7. The antibody purification will continue as time allows in the next year.

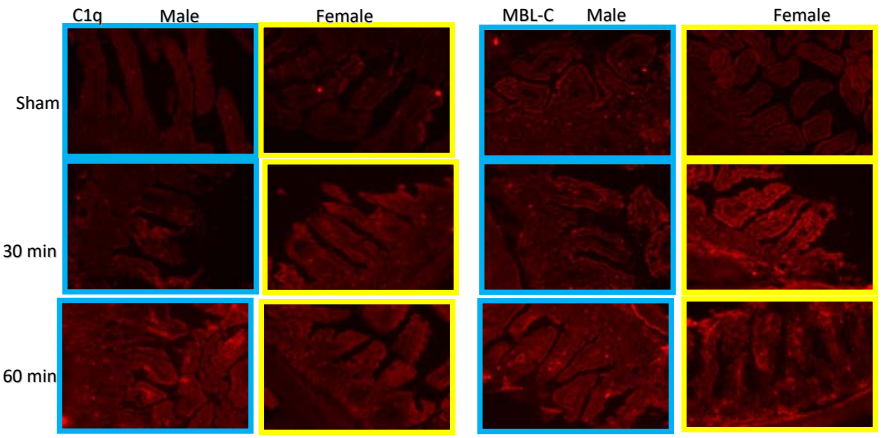


Figure 4. IR-induced injury induces C1q deposition on male intestines and MBL-C deposition on female mice. C57Bl/6 mice were subjected to Sham or time course I/R treatment. Intestinal sections were stained for C1q (Left) and MBL (Right) by immunohistochemistry. Microphotographs (200x) are representative of 3-4 animals stained in at least 3 independent experiments.

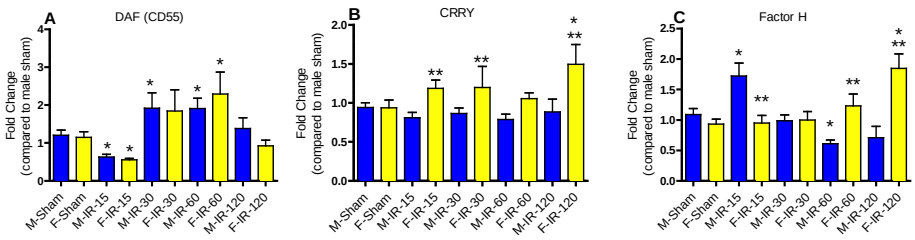


Figure 5. Complement regulators, CRRY and Factor H differ by sex. C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Mid-jejunal (A) DAF(CD55), (B) CRRY, and (C) FH transcription was determined by qRT-PCR analysis. *indicates significant difference from same sex Sham ($p \leq 0.05$); ** indicates significant difference from opposite sex at same time point ($p \leq 0.05$); n = 4-10 mice per group.

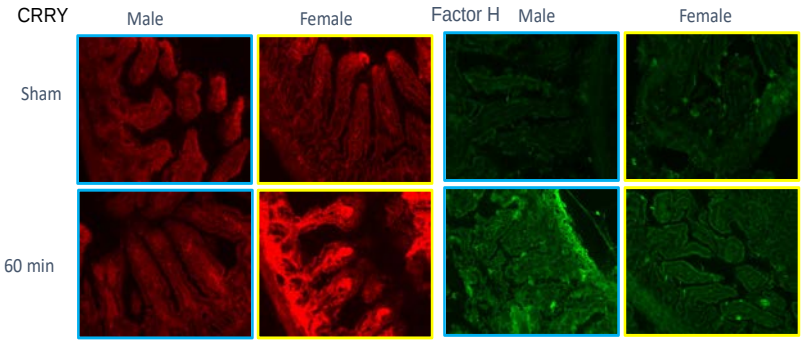


Figure 6. In response to IR, intestines from female mice express CRRY but male mice express Factor H. C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were stained for CRRY (left), and FH (right) by immunohistochemistry. Microphotographs (200x) are representative of 3-4 animals stained in at least 3 independent experiments.

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 post-doc was hired and trained in performing the surgeries and the necessary microscopic analyses. She has also expanded the labs abilities in molecular analysis. All lab members participate the Immunity journal club.

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.

Nothing to Report

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 manuscript will be submitted and published. The C1q and MBL -/- mice as well as DAF and FP KO mice and FH mut mice will be subjected to IR and analyses performed.
The neoantigen IHC will be continued.

4. **IMPACT:** D*escribe 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, Properidan KO and Factor H mutant mice, our initial data that only Factor H and CRRY are critical will be confirmed with the mutant mice prior to use of the inhibitors.

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.

Some mice have not bred as expected, this has delayed the surgeries for the C1q and MBL deficient mice. However, in the mean time, we have begun staining tissues for neoantigens (Main task 6) and collecting sera for antibody purification (Main task 7). These tasks will continue while the mice produce enough animals for the surgeries. All tasks are expected to be completed but the order will be somewhat altered.

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.

By confirming the preliminary data with deficient mice, it will decrease the number and therefore, expensive costs of the inhibitors.

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).*

Nothing to Report (manuscript in progress is attached)

  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.*

Sherry Fleming, Jennifer Rowe, Jonathan Ferm Jennifer Amrein, and Miaomiao Wu, Complement activation delay causes decreased intestinal ischemia-reperfusion injury in female mice, Poster presentation, AAI national meeting, May 2019
Sherry Fleming, Jonathan Ferm and Jennifer Amrein, Complement Regulators and Mechanisms Vary by Sex in Intestinal Ischema-Reperfusion Injury, Poster presentation, Complement International meeting, Sept 2018

- **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/0000-0001-5385-7233
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: Jennifer Rowe
Project Role: Animal Technician
Researcher Identifier; https://orcid.org/0000-0001-6812-4315
Nearest person month worked: 6 mo
Contribution to Project: Ms Rowe has coordinated the ordering and breeding of the specific mice. In addition she has performed the animal surgeries.

Name: Miaomiao Wu
Project Role: Post-doctoral fellow
Researcher Identifier;
Nearest person month worked: 6 mo
Contribution to Project: Ms.Wu has completed her online training, assisted with tissue processing and performing all qRT-PCR. She will learn the surgeries once she is approved by ACURO.

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, eskurich@d.umn.edu, 218- 726- 8104
Performance Period:	8/1/2015 - 7/31/2019 with no cost extension
Level of Funding:	$69,581
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 (ET_A) 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.*

Gender differences in complement-mediated reperfusion injury
BA170055
W81XWH1810716

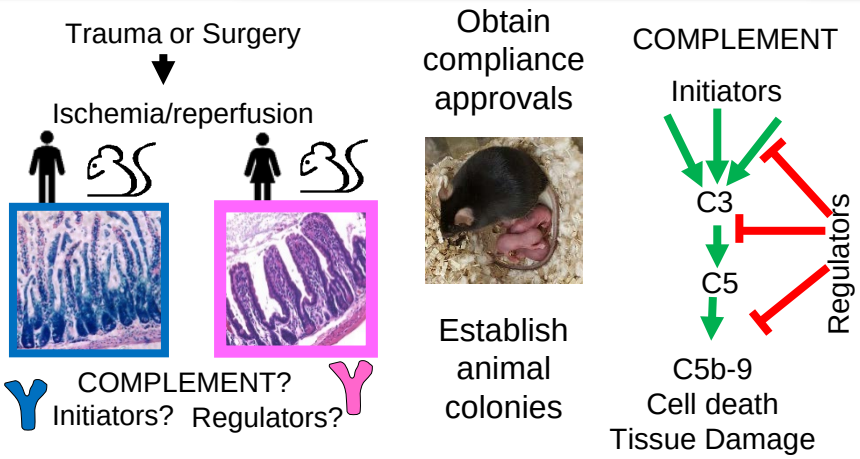
PI: Fleming, Sherry D **Org:** Kansas State University **Award Amount:$** 657,217.00

Study/Product Aim(s)

- **Aim 1**: Determine if complement initiation differs between sexes
- **Aim 2:** Determine role of complement regulators in female response to IR
- **Aim 3**: Determine similarities in male and female neoantigens and naturally occurring antibodies

Approach

Each Aim's activities will span parts of 3 (out of 4) calendar years. This proposal will determine if gender alters complement initiation pathways during intestinal ischemia/reperfusion using complement deficient or estrogen receptor deficient mice. The role of complement regulators will be examined by treating both sexes of mice with complement regulators. Finally, the neoantigen expression between male and female mice will be analyzed and antibodies collected from male and female mice will attempt to restore injury in antibody deficient mice.



Accomplishments: Compliance approvals and mice have been obtained. Surgical training is completed and reperfusion time course surgeries are completed. Evaluation of complement initiators and regulators, injury and eicosanoids are in progress.

Timeline and Cost

Activities CY	18	19	20	21
Aim 1: Determine female complement initiation				
Aim 2: Determine female complement regulators				
Aim 3: Determine female neoantigens and natural antibodies				
Estimated Budget ($K)	**$10K**	**$220K**	**$211K**	**$225K**

Updated: (Oct 2019)

Goals/Milestones

CY18 Goal - Obtain compliance approval and establish colonies

- ☑ IACUC and ACURO approvals
- ☑ Establish animal colonies
- ☑ **CY19 Goals** –Determine female complement initiators and regulators
- ☑ Time course surgeries/data collection completed in both sexes
- ☐Determine role of complement regulators (in progress)
- ☐Determine female complement initiation pathway (in progress)

CY20 Goals – Determine similarities in male and female neoantigens

- ☐ Determine female IR-induced neoantigens (in progress)
- ☐ Determine role of estrogen in complement initiation
- ☐ Determine the role of estrogen on complement regulation
- ☐ Determine female natural antibody specificities (in progress)

CY21 Goals – Determine female naturally occurring antibodies

Budget Expenditure to Date

Projected Expenditure:$200,500 Actual Expenditure: $197,614

Manuscript in Progress

Complement regulators and mechanisms vary by sex in intestinal ischemia reperfusion injury

ABSTRACT

Complement activation plays a pivotal role in intestinal ischemia reperfusion (I/R)-induced injury, which is an inevitable inflammatory response leading to significant morbidity and mortality. Intestinal I/R was established primarily using a male model to identify intestinal I/R related diseases in both sexes and female patients are provided with similar treatment to males. However, sexual dimorphism has been shown in intestinal I/R related diseases. Complement components show sex differences in animal models of kidney and heart ischemia. In addition, I/R injury in female mice is attenuated or delayed. Thus, we hypothesized that complement regulation may differ by sex during intestinal I/R and mechanism of I/R-induced injury in females may differ in timing, quantity or required molecules. Male and female C57Bl/6 mice were subjected to Sham treatment or 30 min intestinal ischemia followed by 15 min, 30 min, 60 min, and 120 min reperfusion. We demonstrate that compared to male mice, females maintain lower intestinal I/R-induced tissue damage. In response to I/R, males produce PGE2 regulated by COX-2, while females produce LTB4 by A5-LOX. Although both sexes activate both classical and MBL complement pathways during I/R, males tend to use classical pathway more than females and females tend to use MBL pathway more than males. Together these data suggest that local control of complement activation and regulation, eicosanoid production and neutrophil infiltration in response to intestinal I/R varies between sexes suggesting that distinct therapeutic intervention may be needed in clinical ischemic diseases.

INTRODUCTION

Ischemia-reperfusion (I/R) injury is an important medical condition that poses a serious clinical therapeutic problem for multiple conditions including surgery, myocardial infarction and stroke, gastrointestinal dysfunction, cerebral and hepatic diseases, systemic inflammatory response syndrome, and organ transplantation. Among the ischemia-reperfusion in other internal organs, the intestine appears to be

the most sensitive to ischemia reperfusion injury {Sasaki, 2007 #5}. During an ischemic event, tissue injury occurs. However, reperfusion of a temporarily ischemic tissue significantly magnifies the tissue damage. Mesenteric ischemia/reperfusion (IR) results in local intestinal inflammation and damage, and may progress to systemic inflammatory responses in remote organs including the lungs. The exact mechanism of this enhanced injury is unknown, although during the reperfusion phase, multiple inflammatory mediators including complement, cytokines, and adhesion molecules are generated both locally and systemically in the blood. [Austen, 1999 #77][Eror, 1999 #78][Williams, 1999 #79]. In mice, intestinal ischemia induces neutrophil infiltration, cytokine and eicosanoid production, and complement activation leading local mucosal injury {Otamiri, 1989 #31;Austen, 2003 #32;Hernandez, 1987 #11;Rehrig, 2001 #12;Stahl, 2003 #13;Simpson, 1993 #14;Fleming, 2002 #1}.

Initial complement studies attenuated injury by blocking activation of C3 or C5 activation {Fleming, 2002 #35;Austen, 1999 #39;Wada, 2001 #38;Rehrig, 2001 #37;Williams, 1999 #36}. Subsequent studies in male mice indicate that I/R activates multiple complement pathways to induce tissue damage {Stahl, 2003 #41;Austen, 2004 #40;Williams, 1999 #36}. Decreased I/R-induced intestinal injury found in Rag-1-/- or C4-/- mice and IgM restored damage to wildtype levels in Rag-1-/- mice {Williams, 1999 #36}. These studies suggested that intestinal I/R induces neo-antigen(s) recognized by IgM to activate classic complement pathway. Similar studies demonstrated that the lectin and alternative pathways are also involved in local IR-induced injury {Austen, 2004 #40;Stahl, 2003 #41}. Together, these studies indicate that the effector phase of intestinal I/R includes direct complement activation by all three pathways in male mice.

Numerous studies support the importance of sex-associated differences in I/R related conditions {Nemeth, 2012 #6;Mester, 2018 #7;Stone, 2019 #8}. After myocardial infarction, women generally have a higher in-hospital mortality and tend to develop heart failure {Vaccarino, 2002 #9}. The operative mortality of women after coronary artery bypass grafting surgery is 2-3 times higher than men {Stramba-Badiale, 2010 #10}. Although the prevalence of ischemic heart disease is 1.8% higher in men than in women {Roger, 2012 #43}, the hospital discharges for

cardiovascular diseases of men were higher than women during the same time period in the U.S {Benjamin, 2019 #44}. 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 deconvolution analysis of cell types demonstrated differing proportions of specific cell types {Stone, 2019 #19}. Finally, females present distinct symptomology compared to males also suggesting sex as an independent risk factor for I/R. (REF)

Despite the systematic differences between women and men in the I/R injuries, women remain underrepresented in clinical research studies. 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 {Kahlke, 2000 #20}, myocardial {Li, 2015 #21}, kidney {Kawasaki, 2012 #22}, and intestine {Ba, 2005 #23}), and sepsis {Kawasaki, 2012 #22}. Researchers have found that I/R injury in female mice is attenuated or delayed, but the specific protective mechanisms were not defined {Doucet, 2010 #18;Breithaupt-Faloppa, 2014 #17;Breithaupt-Faloppa, 2013 #16}. Recent studies demonstrated that female sex hormones could reduce pro-inflammatory cytokines production in inflammatory bowel disease {van der Giessen, 2019 #27}, reduce inflammation in heart and kidney I/R injuries {Perez-Alvarez, 2016 #25;Kang, 2014 #26} and preserve endothelial integrity {Gardner, 1999 #24}. Clinical studies show an association between the use of oral contraceptives and I/R injury as estrogen changes the vessel diameter while testosterone blockade reduces nitric oxide production {Xue, 2015 #30;Egger, 1974 #29;Brennan, 1968 #28}. Complement components also show sex differences in other animal models including heart {Li, 2015 #34} and kidney {Hutchens, 2008 #33} ischemia. However, it is unknown if complement initiation or complement regulation by natural inhibitors differ in female mice. Thus, mechanism of I/R injury in females may be different in timing, quantity or required molecules.

Understanding the molecular mechanism of female attenuated or delayed I/R injury will provide insight for the corresponding treatment of males. To determine the mechanistic sex differences in intestinal I/R will provide insight into the sexual

dimorphism of intestinal I/R and may allow development of sex-specific therapies to reduce the injuries. Therefore, we examined the kinetics of intestinal I/R induced mediators in relation to intestinal pathologies 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 in females alters the mechanisms of intestinal I/R injury compared with males.

MATERIALS AND METHODS

Mice

C57Bl/6 mice were purchased from Jackson Laboratory, and maintained under 12 h light/dark cycles in the Division of Biology at Kansas State University, Manhattan, KS. All mice were allowed free access to food and water, and maintained under 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.

Ischemia and Reperfusion Procedure

Animals were subjected to I/R, as previously described {Fleming, 2002 #1}. Briefly, isofluorane (2-3%) anesthetized mice were subjected to a midline laparotomy, and covered with a moistened surgical gauze to prevent desiccation. After 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 observing the blanching of the intestine and visualize the absence of pulsations distal to the clamp. After 30 min of ischemia, the clamp was removed and the intestines reperfused for 15, 30n, 60, or 120 min. Reperfusion was confirmed by observing the pinkish color change of the intestine and visualize the pulsatile flow to the superior mesenteric artery. All mice received buprenorphine

(0.075mg/kg) for pain. Sham treated animals underwent the same procedure but without clamping. After reperfusion, the mice were 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 mice were put on a 37°C water-circulating heating pad to keep their body temperature.

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 (8um), and H&E stained. The mucosal injury score (SMI) was graded based on a six-tiered scale modified from Chiu et al {Chiu, 1970 #2}. Briefly, the average intestinal mucosal injury was calculated by the average of two well trained, blinded observers. Each observer graded 90-150 villi from 0-6. 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 exposure but intact lamina propria with epithelial sloughing were score 4; when the lamina propria was extruding, the villi was 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 20X, 0.5 Plan Fluor objective on Nikon 80i microscope. Images were acquired by using a Nikon DS-5M camera with the DS-L2 software at room temperature (Nikon, Melville, NY).

Additional 2 cm intestinal sections were immediately snap frozen in optimal cutting temperature (O.C.T) freezing medium and 8 um transverse sections were placed on the slides for future immunohistochemistry, similarly as described previously {Pope, 2015 #3}. 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 1h at room temperature or ON at 4°C. The C3, IgM, C1q, C4, Factor B

(FB), Membrane attack complex (MAC) expression, or b2-glycoprotein I (b2-GPI) deposition on the tissue were detected by staining with antibodies identified in Table 1. All experiments contained serial sections stained with appropriate isotype control antibodies. Each slide was mounted with ProLong Gold (Invitrogen). Images were acquired by a blinded observer at room temperature by utilizing a Nikon eclipse 80i microscope equipped with a CoolSnap CF camera (Photometrics) and analyzed by using Metavue software (Molecular Devices).

Ex vivo Eicosanoid, and Cytokine Determination

The ex vivo generation of eicosanoids and cytokines in small intestine tissue section was detected using a modified method previously described {Sjogren, 1994 #4}. One 2 cm intestinal section was minced and washed in cold Tyrode buffer and resuspended in 37°C oxygenated Tyrode buffer (Sigma-Aldrich, St. Louis, MO). After incubation at 37°C in a heat block for 20 min, the supernatants were collected. Levels of leukotriene B_4 (LTB_4) and prostaglandins E_2 (PGE_2) were determined using enzyme immunoassay kits (Cayman Chemical, Ann Arbor, MI). Analysis of cytokines present in the same intestinal supernatants was determined by using a Milliplex MAP immunoassay kit (Millipore) and data was read on a Milliplex Analyzer (Millipore). Protein content was determined using the bicinchoninic acid (Pierce, Rockford, IL) assay adapted for use with microtiter plates. All eicosanoid generation and cytokines concentrations were expressed per mg protein per 20 min.

Quantitative Real-time PCR

One 2 cm jejunal section was snap froze in liquid nitrogen and stored at -80 oC until used. Total RNA was isolated using TRIzol reagent (Invitrogen) according to the instructions of the manufacturer. RNA integrity and genomic DNA (gDNA) contamination was assessed by using an Agilent 2100 Bioanalyzer (Agilent) and RNA quantity and purity was determined by using the Nanodrop (Thermo Fisher Scientific). Samples with RIN values greater than 7.0 and no gDNA contamination were used for the following cDNA synthesis. Total RNA (1 ug) was reverse transcribed into cDNA by using aScript first strand cDNA synthesis kit (Quanta

Biosciences). Quantitative real-time PCR analysis was performed in 25 ul total volume using an Applied Biosciences StepOnePlus thermocyclers (Thermo Fisher) with Perfecta SYBR Green Fastmix reagent (Quanta Biosciences). The following protocol was used: 3 min at 95°C; 50 cycles of 10 sec at 95°C, 20 sec at Tm, 10 sec at 72°C; melt curve start at 60°C, increasing by 0.5°C up to 95°C. After amplication, relative gene expression was normalized to 18s rRNA values, and ΔΔCt fold change relative to Sham-treated mice was determined.

Statistical Analysis

Data are presented as mean ± the standard error of the mean (SEM) and were compared by one-way analysis of variance with *post hoc* analysis using Newman-Kuels test (Graph Pad/Instant Software Inc. Philadelphia, PA). Differences ($P < 0.05$) between groups were considered significant.

RESULTS

Higher intestinal I/R induced tissue damage 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 {Roger, 2012 #42}, and intestinal I/R induced injury in female mice is attenuated or delayed in multiple forms of I/R and hemorrhage {Doucet, 2010 #18;Breithaupt-Faloppa, 2014 #17;Breithaupt-Faloppa, 2013 #16}. It is likely that the time course of the I/R damage differs between sexes. To test this hypothesis, we subjected wild-type both male and female C57Bl/6 mice to 30 min ischemia, followed by 15 min, 30 min, 60 min, and 120 min reperfusion, and evaluated intestinal epithelial damage. As Sham treatment was not significantly different between time points of the same gender of mice, the scores of damage and subsequent analyses were pooled by gender. As expected, after I/R, male mice sustained significant tissue damage (Fig. 1A). Female mice also sustained significant intestinal damage but the damage was significantly lower than that sustained by male mice at a similar time point. (Fig. 1A). Thus, these data indicate that intestinal I/R induced significant

tissue injury in both sexes, but male mice sustained significantly higher tissue damage than female throughout the time course of intestinal I/R.

As intestinal I/R-induced tissue damage is accompanied by inflammation with significantly elevated levels of intestinal peroxidase {Fleming, 2002 #35} and LTB4 and PGE2 {Stojadinovic, 1995 #45}, we also detected the total tissue peroxidase and ex vivo generation of LTB4 and PGE2.
We hypothesized that total tissue peroxidase would be increased after intestinal I/R, but the dynamic change may differ between male and female mice. As expected in response to time course I/R, total peroxidase in mice of both sexes was increased constitutively, except for female mice in 120 min reperfusion (Fig. 1B). There was a significant increase in male mice in the total tissue peroxidase level in the ischemia followed by 120 min reperfusion, whereas the peroxidase level was significantly decreased in female mice (Fig. 1B).

RESULTS

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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 {Roger, 2012 #42}, and intestinal I/R induced injury in female mice is attenuated or delayed in multiple forms of I/R and hemorrhage {Doucet, 2010 #18;Breithaupt-Faloppa, 2014 #17;Breithaupt-Faloppa, 2013 #16}. It is likely that the time course of the I/R damage differs between sexes. To test this hypothesis, we subjected wild-type both male and female C57Bl/6 mice to 30 min ischemia, followed by 15 min, 30 min, 60 min, and 120 min reperfusion, and evaluated intestinal epithelial damage. As Sham treatment was not significantly different between time points of the same gender of mice, the scores of damage and subsequent analyses were pooled by gender. As expected, after I/R, male mice sustained significant tissue damage (Fig. 1A). Female mice also sustained significant intestinal damage but the damage was significantly lower than that sustained by male mice at a similar time point. (Fig. 1A). Thus, these data indicate that intestinal I/R induced significant tissue injury in both sexes, but male mice sustained significantly higher tissue damage than female throughout the time course of intestinal I/R.As intestinal I/R-

induced tissue damage is accompanied by inflammation with significantly elevated levels of total intestinal peroxidase {Fleming, 2002 #35}, myeloperoxidase (REF) and cytokines (fleming paper), we also detected the total tissue peroxidase and ex vivo generation of LTB4 and PGE2. We hypothesized that total tissue peroxidase may increase after intestinal I/R, but the dynamic change might be different between male and female mice. As expected in a time course of I/R, total peroxidase in mice of both gender was significantly increased compared to sham treated animals (Fig. 1B). However, there total tissue peroxidase at all time points was significantly increased in male mice compared to female mice (Fig. 1B).

Myeloperoxidase (MPO), one of the most abundant enzymes released by activated neutrophils, is linked with continuous activation and recruitment of leukocytes to infarcted tissue [Askari, 2003 #80]. Thus, MPO is characterized by powerful pro-inflammatory properties. To further determine whether MPO is involved in adverse outcome after intestinal I/R and the difference between females and males, we also determined serum MPO level in the time course. After I/R, both males and females had significantly elevated serum MPO level compared with their corresponding shams (Fig 1C). Besides, serum MPO level in males were higher than it was in females during all I/R time course, males had significantly higher MPO level at 15 min, 30 min, and 120 min (Fig 1C). We also determined cytokine levels in the intestine tissues (Fig 2A-2F). Compared with males, the majority of intestinal cytokine production by females was similar. However, female intestinal IL-6 and IL-10 production was significantly higher at 15 and 30 min post ischemia, respectively. IThe increased IL-10 early during reperfusion suggests that female intestines may sustain earlier protection compared to male intestines.

Together with the intestinal injury and total peroxidase, these data indicate the males not only have higher level of inflammation during intestinal I/R and tissue damage, but the inflammation and damage starts earlier in males compared with females, which suggest males and females respond differently in inflammation and tissue damage after intestinal I/R.

Female mice produce LTB4 whereas male mice produce PGE2

As demonstrated previously, intestinal I/R also increases the chemotactic eicosanoid, LTB4 and PGE2 in wild-type mice {Stojadinovic, 1995 #45;Fleming, 2002 #35}. Similar to previous studies, intestinal sections from I/R treated male mice

released significantly increased PGE2 at 120 min I/R. However, PGE2 production by female intestines after I/R was not elevated compared to female Sham mice (Fig. 3A). 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. 3B). / Importantly, there was significantly more LTB4 production in females after I/R, while PGE2 production was much higher in male mice (Fig. 3A, 3B). In addition, there was minimal change in the LTB4 production in male mice and PGE2 production in female mice compared to the corresponding Shams (Fig. 3A, 3B). Taken together, after the intestinal I/R, arachidonic acid is converted to LTB4 in female intestines whereas male intestines produce PGE2.

Oxygen is one of the most essential nutrients changed significantly during I/R. Hypoxia-inducible factor-1 alpha (HIF-1a) is a master regulator of oxygen homeostasis, and the expression of HIF-1a is regulated by oxygen levels [Majmundar, 2010 #81]. HIF-1a has been observed to be regulated by the COX-2 product PGE2 [Liu, 2002 #82]. LTB4, one of metabolites of arachidonic acid through 5-lipoxygenase (5-LOX), plays an important role in leukocyte recruitment [Martel-Pelletier, 2004 #84;Werz, 2002 #83]. To further determine whether males and females uses different pathways to induce LTB4 and PGE2 production after I/R, we performed RT-PCR to detect the expression of HIF-1a, COX-2 and A5-LOX in the intestinal tissue. As indicated in Figure 3C-3E, males express significantly more HIF-1a at 30 min I/R (Fig 3C), while HIF-1a expression changed little after I/R in female intestines. Males also expressed and significant levels of COX-2 at early time points (15 and 30 min) I/R (Fig 3D), while females expression of COX-2 increased at 60 min (Fig 3D). , In agreement with LTB4 production, females significantly increased level of A5-LOX from 15min to 120 min I/R and the level of A5-LOX was significantly higher than males by 15 min I/R (Fig 3E). Together with the PGE2 and LTB4 production, these data indicate that in response to I/R, male mouse intestines produce PGE2 through COX-2 regulation, while females produce LTB4 through 5-LOX regulation.

Complement initiation in response to intestinal I/R differs between sexes

Complement initiation occurs via three distinct primary pathways {Merle, 2015 #47;Ricklin, 2010 #46}: the classical pathway begins with antibody-antigen-mediated

activation through C1q recognition; carbohydrate binding to mannose binding protein (MBP) triggers the lectin complement pathway; and the alternative pathway involves cleavage of Factor B (FB) and stabilization. Previous studies indicate intestinal ischemia followed by reperfusion causes local inflammation characterized by complement activation and deposition with mucosal injury {Austen, 1999 #39;Fleming, 2002 #35;Fleming, 2002 #1}. Compared with male mice, the total complement activation in females decreased despite similar amounts of C3 activation {Kotimaa, 2016 #48}, indicating sex plays an important role in I/R induced complement activation. We hypothesized that the initiation of complement components from different pathways may be different in different sexes in different time course. Thus, we performed RT-PCR to examine the mRNA level expression of C1q, MBL-c, and Factor B. The expression of C1q was low and changed little during the four point time course of I/R in females, while it increased in males significantly at 15 min and remained at high level from 15 min to 60 min I/R (Fig. 4A). In addition, C1q levels in males were significantly higher at 15- 60 min I/R, compared to females (Fig. 4A). In contrast, MBL-c expression was altered little in males after I/R, and actually decreased significantly from 60-120 min compared to sham-treated males (Fig. 4B). However, the expression of MBL-c in females increased significantly from 30-60 min before decreasing at 120 min I/R (Fig. 4B). Compared to males, females express significantly more MBL in shams, and in I/R from 30-120 min (Fig. 4B). Males and females had a similar level of FB with no significant change in FB expression in females from 15-60 min I/R, while the expression of FB in males continuously decreased after I/R and was significantly decreasedfrom 30-120 min (Fig. 4C).

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, C4, and C3. In response to I/R, IgM was upregulated in females by 30- 120 min, while it was regulated in males by 60-120 min, meanwhile, females express more IgM than males (Fig 5A). C1q changed little in females, but was upregulated in males from 30-60 min, and overall males expessed more C1q expressed compared to females at later timepoints (Fig 5B). In contrast, females express MBL-c on intestinal endothelium by 30 min but males expressed little MBL-c until 60 min post-ischemia (Fig 5C). Importantly, much more

MBL-c was deposited on female intestines than on male intestines (Fig 5C). C4 was upregulated both in males and females, and starts between 15-30min (Fig 5D). Both females and males have C4 and C3 upregulated after I/R (Fig 5D and 5E, respectively), but females express more C3 earlier (at 30min), while males express more C3 later (at 60-120 min) (Fig 5E).

Our data indicate that all three complement pathways are activated in both sexes after intestinal I/R. However, males tend to use the classic pathway more than lectin pathway, while females tend to use lectin pathway more than classical pathway. Together with tissue injury and eicosanoid production, these studies suggest that sex alters the mode of inflammation and subsequent tissue injury.

Complement inhibitors vary by sex

Complement regulators help control and adjust complement induced injuries. In the alternative pathway, C3 activation is primarily controlled by FH. Decay accelerating factor (DAF/CD55) and complement receptor 1-related gene/protein y (CRRY), a rodent-specific complement regulator, are exposed in most cells to regulate C3 and C5 activation {Miwa, 2007 #49}. In rodents, CRRY is a key complement regulator with similar activities to human DAF and membrane cofactor protein (MCP). . Previous studies showed all complement regulators have critical roles during I/R {Miao, 2014 #50;Wang, 2010 #51}. Thus, to test the hypothesis that different sexes exhibit a varied profile of regulator expression, we detected both RNA and protein expression levels of DAF, FH, and CRRY in both sexes after intestinal I/R. As expected, male and female expressed very similar levels of all three complement regulators in intestines of Sham-treated mice (Fig 6A, B, C). , In response to I/R, RT-PCR indicated a signficnant increase in DAF expression in male intestines after 30 and 60 min reperfusion and decreased to background levels by 120 min reperfusion (Fig. 6A). Females also had increased expression after 30 min and 60 min reperfusion, but was significant only after 60 min (Fig. 6A). Similar to males, the level of DAF decreased to its Sham levels after 120 min reperfusion (Fig. 6A). The expression of DAF is similar in females and males both in sham and I/R groups (Fig. 6A). In contrast, CRRY only increased significantly in females after 120 min I/R, while CRRY remained at background levels throughout 120 min reperfusion in males (Fig. 6B). Importantly, female intestines expressed significantly more CRRY at 30,

60, and 120 min I/R compared to male intestines (Fig. 6B).The FH expression increased significantly increased after 15 min reperfusion in males, prior to decreasing to Sham levels (Fig. 6C). In contrast, expression of FH in females remained low until 120 min I/R (Fig. 6B). Males expressed significant higher level of FH after 15 min, then the FH expression level are higher in female after 60 min and 120 min I/R (Fig. 6B). Thus, our data suggest that complement inhibitors also vary by males and females.

To further confirm the expression of complement inhibitors, we conducted IHC to detect the expression levels of DAF, CRRY, and FH. In response to I/R, DAF was upregulated both in males and females from 15-60 min (Fig 7A). Females expressed more CRRY from 60-120 min than males (Fig 7B). FH was upregulated in females from 60-120 min, while it was upregulated in males by 15-60 min (Fig 7C). There was more FH expressed in males compared to females (Fig 7C).

DISCUSSION

Differences in sex play a critical role in the final outcome of an ischemic event in both experimental and clinical studies. Males appear to sustain increased tissue damage but females sustain greater mortality and longer hospital stay after an ischemic insult. [Ostadal, 2009 #56][Kaidonis, 2019 #54]. [Park, 2016 #57][Choleris, 2018 #55]. However, our understanding of sex-related mechanistic and time course 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, a complete time course of mechanisms of I/R-induced in each sex had been examined[Robert, 2011 #52;Ba, 2005 #23;Kawasaki, 2012 #22;Siegel, 2010 #53]. In the present study, we demonstrated that sex impacts intestinal ischemia reperfusion (I/R)-induced tissue injury, eicosanoids production, complement initiating pathways, and complement inhibitor expression.

Previous studies demonstrated that intestinal, renal, and myocardial I/R induced tissue damage is largely dependent on complement activation [Morrison, 2011 #59;Fleming, 2003 #58]. In addition, I/R induced injury is attenuated or delayed in female mice {Doucet, 2010 #18; Breithaupt-Faloppa, 2013 #16}. In other animal models, complement also show sex differences such as heart {Li, 2015 #34} and kidney {Hutchens, 2008 #33} ischemia. Thus, these studies support the hypothesis

that complement initiation or complement regulation by natural inhibitors may differ in female mice, and mechanism of I/R injury in females may be different in timing, quantity or required molecules.

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 throught 120 min reperfusion. . Previous studies indicate that females are more resistant to oxygen deprivation than males [Mirza, 2015 #65][Ostadal, 1984 #60]. In addition, increased tolerance of females to I/R damage was observed in rabbits, rats, mice, and dogs [Robert, 2011 #64][Ross, 2012 #63;Murphy, 2007 #62;Johnson, 2006 #61]. In agreement with the literature, males sustained increased histological damage compared to females throughout the reperfusion period. . 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 the induction of organ damage after renal, myocardial and intestinal I/R injury [Matthijsen, 2007 #70;Ali, 2016 #71;Kocael, 2016 #72;Roman, 2008 #66]. . Our data also demonstrate that females produce significantly lower myeloperoxidase than males both at the early and late stage of I/R, indicating a sex-related alteration. Similarly, previous study indicates that leukocyte ratios during myocardial infarction inflammation and would healing kinetics benefit females by preventing the excessive myocardial infarction-induced inflammation [Cavasin, 2004 #67]. In agreement with these studies, a recent publication reported that males show a higher neutrophil abundance [DeLeon-Pennell, 2018 #68]. In this study, females had an overall lower mortality rate and an accelerated IL-6 plasma resolution than males [DeLeon-Pennell, 2018 #68]. 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 [Kamimura, 2003 #99]. IL-6 has also been reported to mediate anti-inflammatory effects to protect intestine from I/R injury [Kimizuka, 2004 #100]. No sex-differences in TNF-a production were reported [Fang, 2007 #69][DeLeon-Pennell, 2018 #68]. Our results are consistent with prior work that showed females secrete higher IL-6 after I/R, while there is no difference on TNF-a production between different sexes or over the time course for each sex. Male neutrophils showed greater pro-inflammatory phenotype polarization, while female neutrophils displayed lower inflammatory markers and were rather polarized to the anti-

inflammatory phenotype [DeLeon-Pennell, 2018 #68]. In addition, our data also demonstrate that females produce increased IL-10 after 30 min reperfusion. As noted in this paper, I/R is considered one of the inflammatory diseases which induces pro-inflammatory cytokines and chemokines produced from monocytes or damaged intestine by I/R. IL-10, an anti-inflammatory cytokine, was reported to inhibit the production of pro-inflammatory cytokines and has been demonstrated to reduce inflammation-mediated tissue damage in both non-infectious [Gloor, 1998 #74;Schneider, 2004 #73] and infectious [Gazzinelli, 1996 #76;Sewnath, 2001 #75] causes of inflammatory responses. One explanation is that our data show females produced increased IL-6 and IL-10 to balance or inhibit the extra pro-inflammatory cytokine production to protect the immune system from extra inflammation or the tissue from getting damaged.

Following the I/R, a marked production of LTB4 was observed in females, but not males. In the meanwhile, males produce significant PGE2, but females do not. This observation is consistent with prior studies showing in vivo sex differences in leukotriene biosynthesis that males produce more PGE2 [Kahlke, 2004 #87], females produce more LTB4 [Rossi, 2014 #86;Rossi, 2019 #85]. Furthermore, previous studies have found that there are significant differences between males and females in adaptive responses to hypoxia [Bohuslavova, 2010 #88], and our date reveals significant differences between males and females in intestinal adaptive responses to hypoxic transcriptional factor and related regulator expression in I/R. Males are more sensitive to oxygen reduction as it express significantly higher HIF-1a after I/R (Fig 3C), while females are more adaptive to oxygen depletion (Fig 3C). It has been shown that in males, the drug NS-398, which is a COX-2 inhibitor, decreases COX-2 induced PGE2 production towards levels found in females [Kahlke, 2004 #87]. However, in the same study PGE2 production remained unchanges in females treated with or without NS-398. Importantly, NS-398 attenuates PGE2 production and intestinal injury in males in response to I/R as well [Sato, 2005 #90;Moses, 2009 #89]. Although these studies have not been extended to females, our data suggest that during intestinal I/R, females 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.

Complement activation and regulation is critical to intestinal, myocardial, and renal I/R-induced damage, though 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 [Morrison, 2011 #59]. We observed increased expression of IgM, C3, and C4 in both sexes, increased expression of C1q in males, and increased expression of MBL in females, indicating that intestinal I/R-induced complement activation, especially classical and MBL pathways, which is in accord with previous studies [Collard, 2000 #93;Collard, 1997 #92]. Our data demonstrate that males have increased level of IgM from 30-120 min I/R (Fig 5A), increased level of C1q from 30-60 min I/R (Fig 5B), increased level of MBL from 60-120 min (Fig 5C), increased level of C4 from 30-120 min (Fig 5D), increased level of C3 from 30-120 min (Fig 5E). One recent study shows that IgM forms complement-activating complexes with C1q on cell membranes highly similar to IgG1-C1 to mediate complement activation [Sharp, 2019 #96]. Together, our results indicate both classical and MBL pathways are involved in intestinal I/R-induced injury in males, as the classical pathway can be initiated by the binding of antibody-antigen complexes to the C1q protein and MBL pathway is triggered by MBL recognition of certain patterns of carbohydrate structures [Gadjeva, 2004 #95;Roos, 2003 #94]. However, females have increased level of IgM from 30-120 min I/R (Fig 5A), little change on C1q (Fig 5B), increased level of MBL from 30-120 min (Fig 5C), increased level of C4 from 30-120 min (Fig 5D), increased level of C3 from 30-120 min (Fig 5E). Furthermore, males have more C1q from 30-60 min I/R, females have more MBL-c from 30-120 min than males. Interestingly, MBL has been shown to bind to IgM to initiate complement activation due to its high specificity for mannose which is found to be rich in the carbohydrate regions of IgM [Arnold, 2005 #98;McMullen, 2006 #97]. Besides, the classical pathway appears to interact with the natural IgM-ischemic antigen complex in an intestinal I/R injury study [Lee, 2010 #91]. Thus, correlating with our data here, it is likely that MBL pathway activation is more important and might be the dominant complement pathway for females to induce intestinal injury during I/R.

Taken together, these data indicate that males appear to preferentially utilize the classical pathway in intestinal injury, while females tend to use the MBL pathway.

Previous studies show that in intestinal I/R-induced injury, IgM is important, MBL and classical complement pathways are critical, while alternative complement pathway is not involved [Lee, 2010 #91]. Also, our study shows that early component of the alternative complement pathway probably was not involved in either sexes for I/R injury (Fig 4C), which is in accordance with previous studies [Lee, 2010 #91].

For complement inhibitors, DAF was upregulated both in males and females from 15-60min, CRRY increased from 60-120 min, while FH was upregulated from 60-120min in females, and from 15-60 min in males. Meanwhile, males express more DAF and FH, females express more CRRY. This indicates that the regulation of complement inhibitors is different in males and females.

This study has provided in vivo evidence that males and females respond differently in intestinal I/R-induced injury in inflammation, neutrophil recruitment, and complement activation and regulation. Thus, this study indicates specific ischemia studies examining gender differences require greater attention in order to better understand the different mechanisms, develop appropriate medicines or medical treatment selections.

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Figure Legends

Figure 1. Female mice sustain less intestinal I/R-induced tissue injury and inflammation than male mice.

C57Bl/6 mice were subjected to Sham or different time course I/R treatment. (A) Mid-jejunal sections were H&E stained and scored for intestinal injury (75-150 villi per animal with 3-10 animals/treatment and each treatment was performed on at least 2 separate days). (B) Intestinal sections were analyzed ex-vivo for total peroxidase production. (C) Serum were analyzed by Elisa for myeloperoxidase (MPO). *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.

1 Injury and inflammation (including PMN)
1A Injury score
B total peroxidase
C Myeloperoxidase （MPO）

Figure 2. Female mice produce more anti-inflammatory cytokines than male mice after intestinal I/R.

C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were analyzed ex-vivo for (A) IL-10, (B) IL-12p40, (C) IL-12p70, (D)CXCL1 (KC), and (E) TNF production. *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.

Fig 2A Cytokines IL-6
B Cytokines IL-10
C Cytokines IL-12p40
D Cytokines IL-12p70
E Cytokines CXCL1(KC)
F Cytokines TNF

Figure 3. Females produce LTB4 whereas males produce PGE2 in response to intestinal I/R, and they use different hypoxia pathways for different eicosanoid production.

C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were analyzed ex-vivo for (A) LTB4 and (B) PGE2. Mid-jejunal (C) HIF-1a, (D) A5-LOX, and (E) COX-2 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.

3 ECOIS and different pathway for F and M
3A LTB4
B PGE2
C RT-PCR HIF-1a
D RT-PCR A5-lox
E RT-PCR COX-2

Figure 4. Complement initiation in response to intestinal I/R differs between sexes.

C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Mid-jejunal (A) C1q, (B) MBL, and (C) FB 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.

4 Initiators and major players in complement
A RT-PCR C1q
B RT-PCR MBL-c
C RT-PCR FB

Figure 5. Complement components and IgM deposition in response to intestinal I/R differs between sexes.
C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were stained for (A) IgM, (B) C1q, (C) MBL, (D) C4, and (E) C3 by immunohistochemistry. Microphotographs (200x) are representative of 3-4 animals stained in at least 3 independent experiments.
Fig 5 A IHC IgM
B IHC C1q
C IHC MBL
D IHC FB (we will do FB for IHC)
D IHC C4 (sherry said we may not put it in paper)
E IHC C3 (do C3b)

Figure 6. Complement inhibitors in response to intestinal I/R differs between sexes.
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.
6 Inhibitors in complement
6A RT-PCR DAF(CD55)
B RT-PCR CRRY
C RT-PCR FH

Figure 7. Complement inhibitor deposition in response to intestinal I/R differs between sexes.
C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were stained for (A) DAF(CD55), (B) CRRY, and (C) FH by immunohistochemistry. Microphotographs (200x) are representative of 3-4 animals stained in at least 3 independent experiments.
Fig 7 A IHC DAF(CD55)
B IHC CRRY
C IHC FH

Antibody Table 1

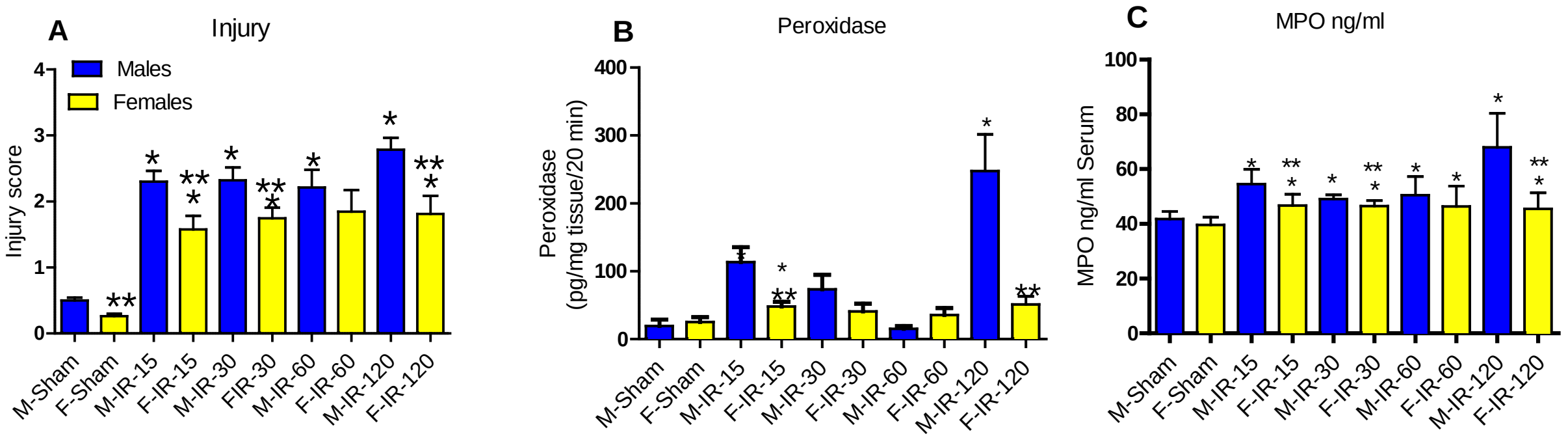
Primary antibody	**Product Information**
IgM	Fluorescein (FITC) AffiniPure F(ab')$_2$ Fragment Goat Anti-Mouse IgM, μ chain specific (Jackson ImmunoResearch Lab, 115096075, 1:50)
C1q	Anti-Mouse Complement Component C1q (Cedarlane, CL7501AP, 1:50)
MBL-c	Anti-Mouse MBL-C (Cedarlane, CL7303AP, 1:50)
C4	Anti-Mouse Complement Component C4, (Cedarlane, CL7504AP, 1:100)
C3	C3, Mouse, mAb 11H9 (HycultBiotech, HM1045-5714M18, 1:100)
DAF(CD55)	CD55 Antibody (Invitrogen, MA5-29678, 1:25)
CRRY	Anti-Crry antibody [8AE6] (Abcam, ab180638, 1:50)
FH	Anti-Factor H antibody (Abcam, ab8842, 1:25)

ISO	**Product Information**
Rat IgG2a	Purified Rat IgG2a, κ Isotype Ctrl Antibody (Biolegend, #400502)
Goat Gamma Globulin	Goat Gamma Globulin (Jackson ImmunoResearch Lab, 005000002)
Rabbit Gamma Globulin	Rabbit Gamma Globulin (Jackson ImmunoReserach Lab, 011000002)
Sheep Gamma Globulin	Sheep Gamma Globulin (Jackson ImmunoResearch Lab, 013 000 002)
Secondary Antibody	**Product Information**
Rat Alexa	Alexa Fluor 594 AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rat IgG (H+L) (Jackson ImmunoResearch Lab, 711096152, 1:1000)
Rabbit FITC	Fluorescein (FITC) AffiniPure F(ab')$_2$ Fragment Donkey Anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Lab, 711096152, 1:1000)
Sheep FITC	Fluorescein (FITC) AffiniPure F(ab')$_2$ Fragment Donkey Anti-Sheep IgG (H+L) (Jackson ImmunoResearch Lab, 713096147, 1:1000)

	Male (M)	Female (F)	Between sexes
Injury	Up at 15 min, kept high from 15-120 min.	Slightly up at 15 min, kept similar level from 15-120 min.	M sustain higher injury level than F at all time courses.
Total peroxidase	Up at 15 min and peak at 120 min.	Similar at all time courses.	M produce more peroxidase than F at all time courses.
MPO (Elisa)	Up at 15 min, kept high at 30 and 60 min, and peak at 120 min.	Slightly up at 15 min, kept similar level from 15-120 min.	M produce more MPO than F at all time courses.
IL-6 (Luminix)	Down at 15 min, kept low from 15-120 min.	Up at 15 min, but drop to similar level to Sham at other time points.	F produce more IL-6 than M at 15 min.
IL-10 (Luminix)	Down at 30 and 60 min.	Up at 30 min, down at 60 min.	F produce more IL-10 than M at all time courses, only significant at 30 min.
IL-12p40 (Luminix)	Down at 60 and 120 min.	No significant change.	F Sham produce less IL-12p40 than M Sham, no difference during I/R.
IL-12p70 (Luminix)	Down at 60 min.	No significant change.	No significant difference.
IL-8 (Luminix)	No significant change.	No significant change.	No significant difference.
TNF (Luminix)	No significant change.	No significant change.	No significant difference.
LTB4 (ECOS)	No significant change.	Up at 15 min, higher at 30 min, peak at 12 min.	F produce more LTB4 than M.
PGE2 (ECOS)	Up at 120 min.	No significant change.	M produce more PGE2 at 15 min and 120 min.
HIF-1a (RT-PCR)	Up at 15 min, kept high from 15-60 min, peak at 30 min.	No significant change from 15-60min, down at 120 min.	F Sham express more HIF-1a than M, but M express more than F from 15-30 min.
COX-2 (RT-PCR)	Up at 15 min, kept high from 15-60 min, peak at 15-30 min.	Up at 60 min, no significant change at other time points.	F Sham express more COX-2, but M express more COX-2 at 15-30 min after I/R.
A5-LOX (RT-PCR)	Up at 15 min, kept similar level from 15-120 min.	Up from 15-120 min, peak at 15 min.	F express more than M at 15 min.
C1q (RT-PCR)	Up at 15 min, kept similar level from 15-60 min, down at 120 min.	Down at 30 and 120 min.	M express more C1q than F at 15, 30, and 60 min.
MBL (RT-PCR)	Down at 60 and 120 min.	Up at 30 and 60 min, down at 120 min.	F Sham express more MBL than M. F express more MBL at 30, 60, and 120 min than M.
FB (RT-PCR)	Down at 30, 60, and 120 min.	Down at 120 min.	No significant difference.
IgM (IHC)	Up at 60 and 120 min.	Up at 30, 60, and 120 min.	F express more IgM than M.
C1q (IHC)	Up at 30 and 60 min.	Little change.	M express more C1q than F.

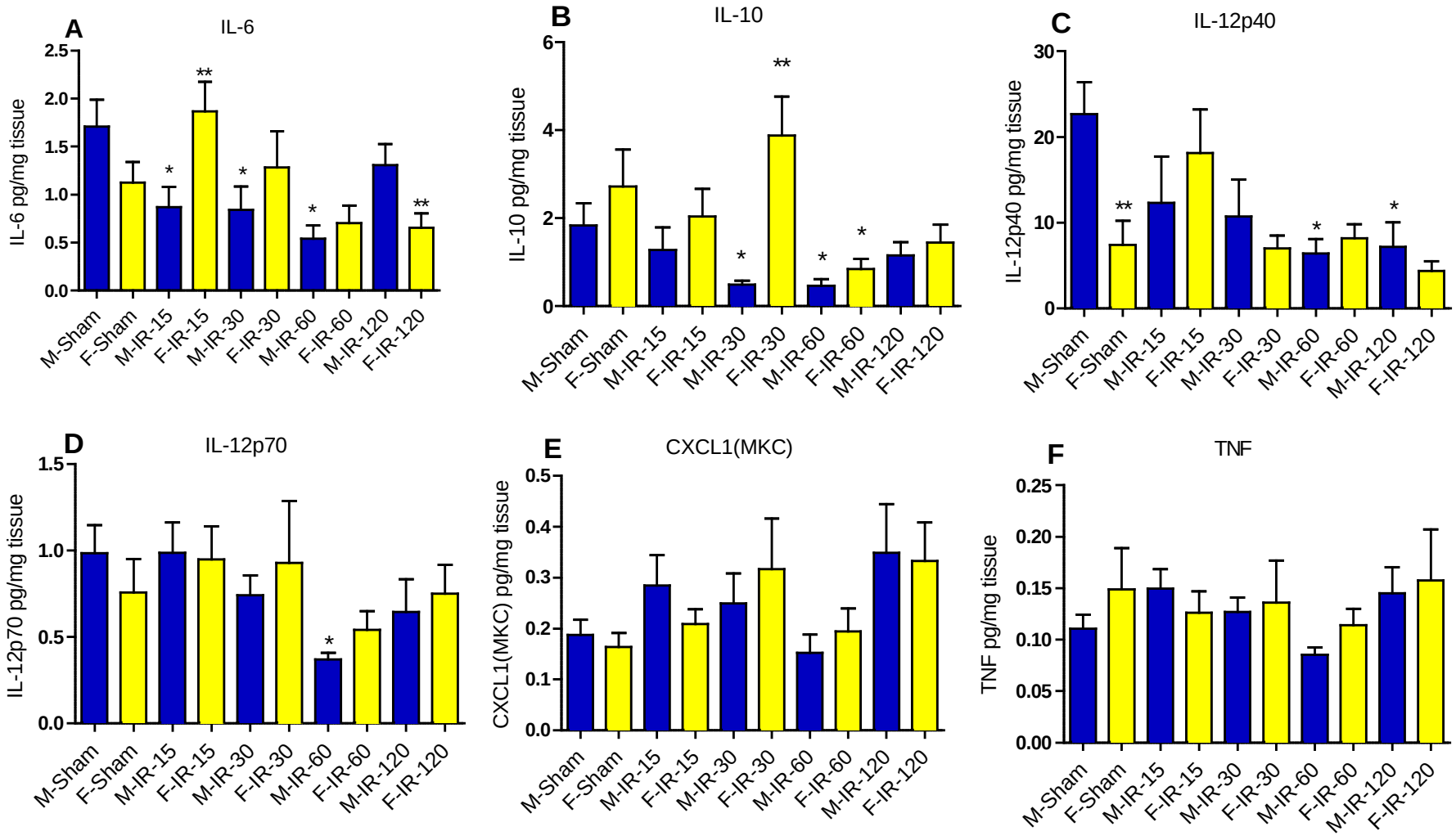
MBL (IHC)	Up at 60 and 120 min.	Up at 30, 60, and 120 min.	F express more MBL than M.
C4 (IHC)	Up at 30, 60, and 120 min.	Up at 30, 60, and 120 min.	No significant difference.
C3 (IHC)	Up at 60 and 120 min.	Up at 30, 60, and 120 min.	M express more C3 and later.
DAF(CD55) (RT-PCR)	Down at 15 min, but up at 30 and 60 min.	Down at 15 min, but up at 30 and 60 min.	No significant difference.
CRRY (RT-PCR)	No significant change.	Up from 15-120 min, significantly up at 120 min.	F express more CRRY at 15, 30 and 120 min than M.
FH (RT-PCR)	Up at 15 min, down at 60 min.	Up at 120 min.	M express more FH at 15 min, less FH at 60 and 120 min than F.
DAF (IHC)	Up at 15, 30, and 60 min.	Up at 15, 30, and 60 min.	M express more DAF than F.
CRRY (IHC)	Up at all time points.	Up at 60 and 120 min.	F express more CRRY than M.
FH (IHC)	Up at 15, 30, and 60 min.	Up at 60 and 120 min.	M express more FH than F.

Figure 1. Female mice sustain less intestinal I/R-induced tissue injury and inflammation than male mice.



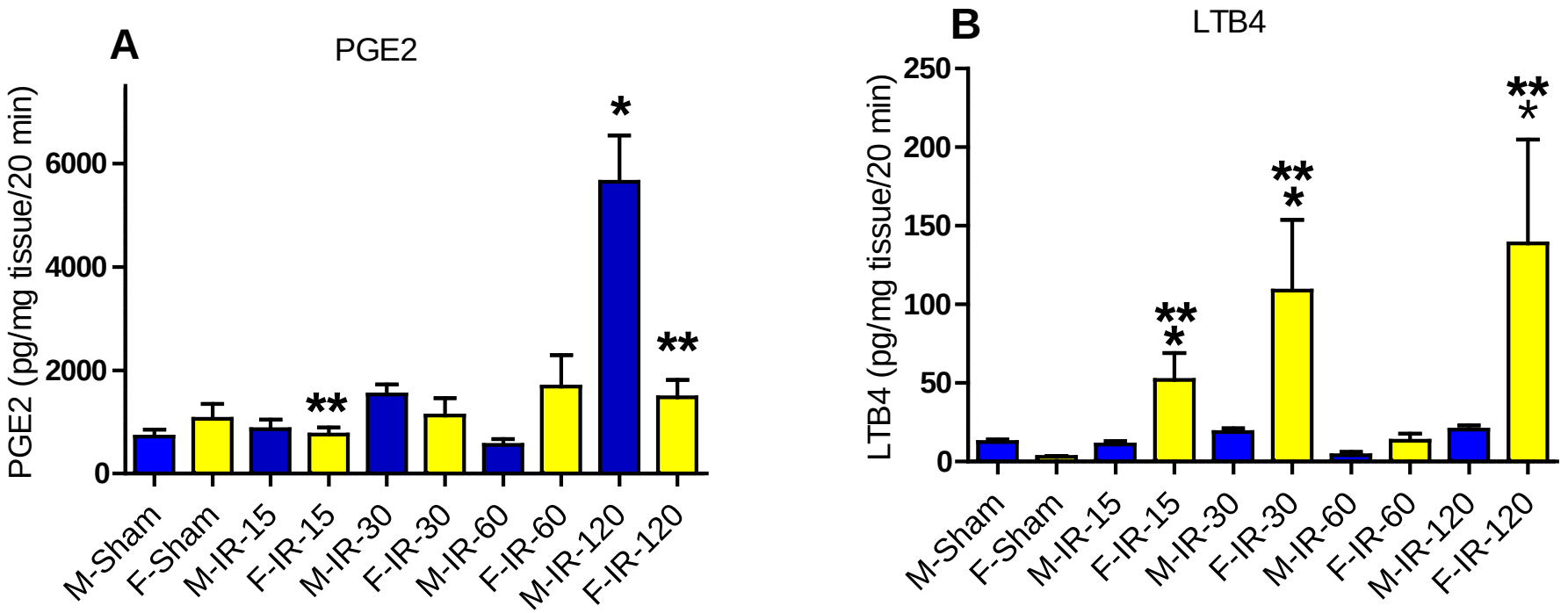
C57Bl/6 mice were subjected to Sham or different time course I/R treatment. (A) Mid-jejunal sections were H&E stained and scored for intestinal injury (75-150 villi per animal with 3-10 animals/treatment and each treatment was performed on at least 2 separate days). (B) Intestinal sections were analyzed ex-vivo for total peroxidase production. (C) Serum were analyzed by Elisa for myeloperoxidase (MPO). *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. Female mice produce more anti-inflammatory cytokines than male mice after intestinal I/R.



C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were analyzed ex-vivo for (A) IL-10, (B) IL-12p40, (C) IL-12p70, (D)CXCL1 (KC), and (E) TNF production. *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 3. Females produce LTB4 whereas males produce PGE2 in response to intestinal I/R, and they use different hypoxia pathways for different eicosanoid production.



C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were analyzed ex-vivo for (A) LTB4 and (B) PGE2. Mid-jejunal (C) HIF-1a, (D) A5-LOX, and (E) COX-2 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.

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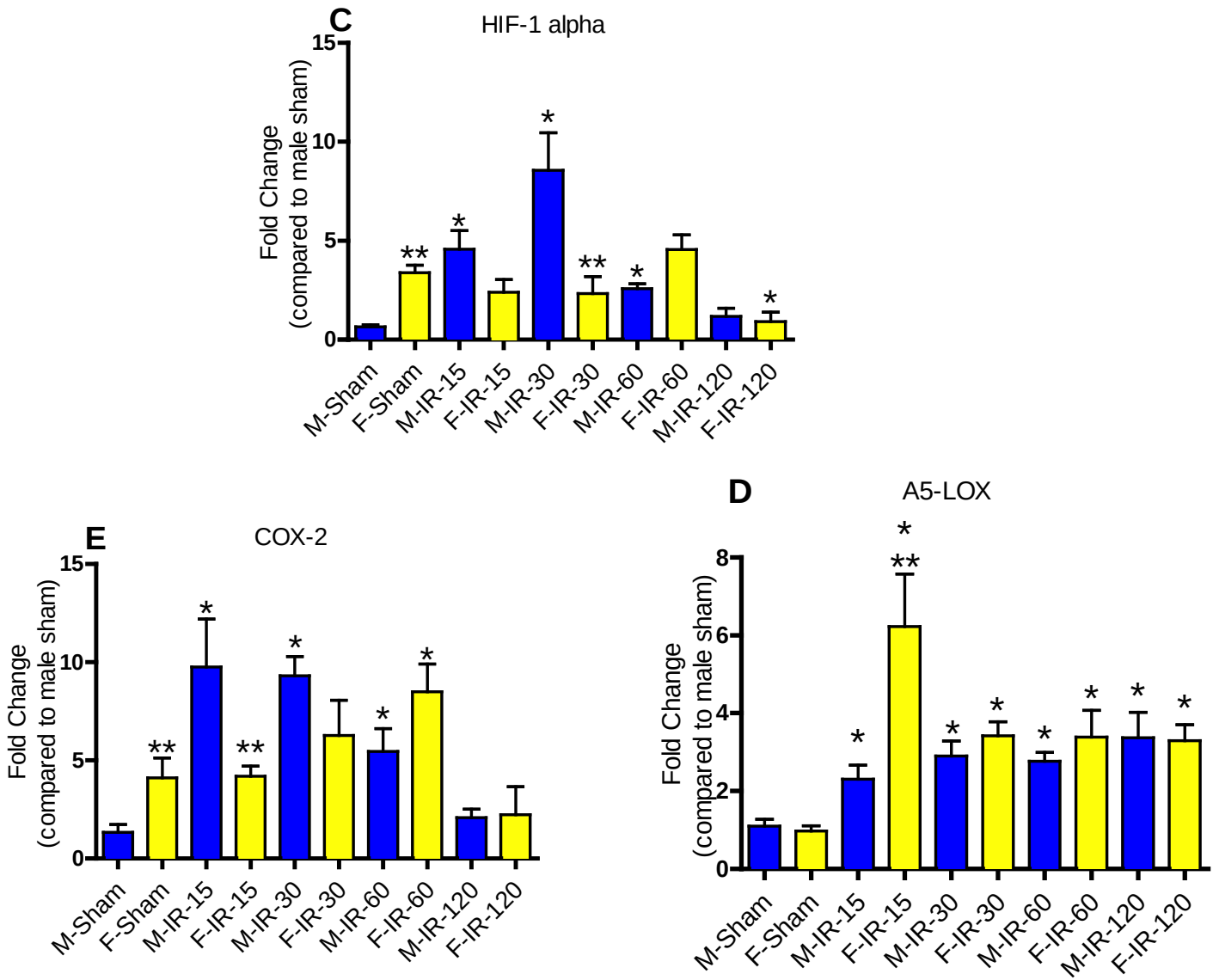
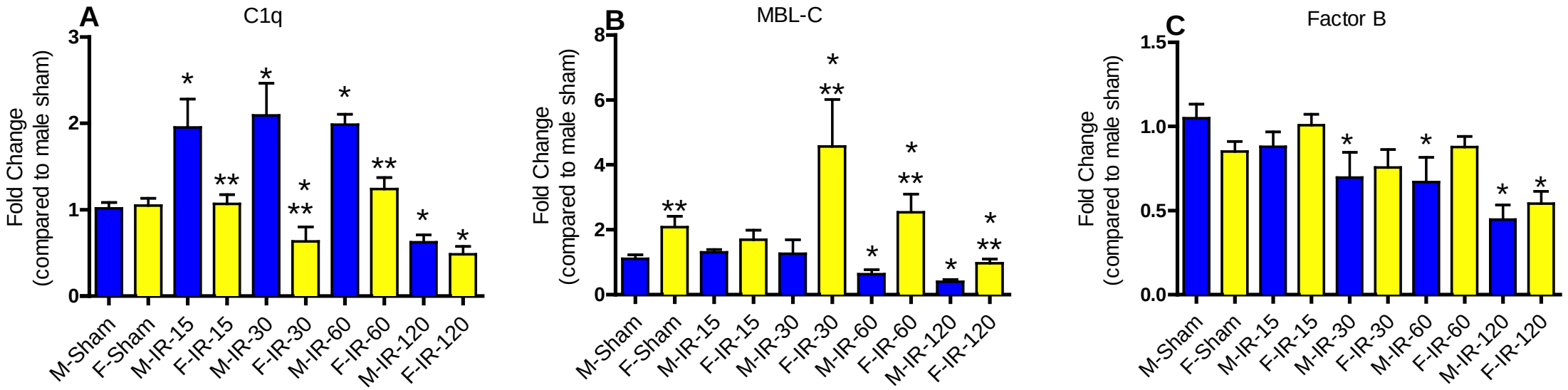
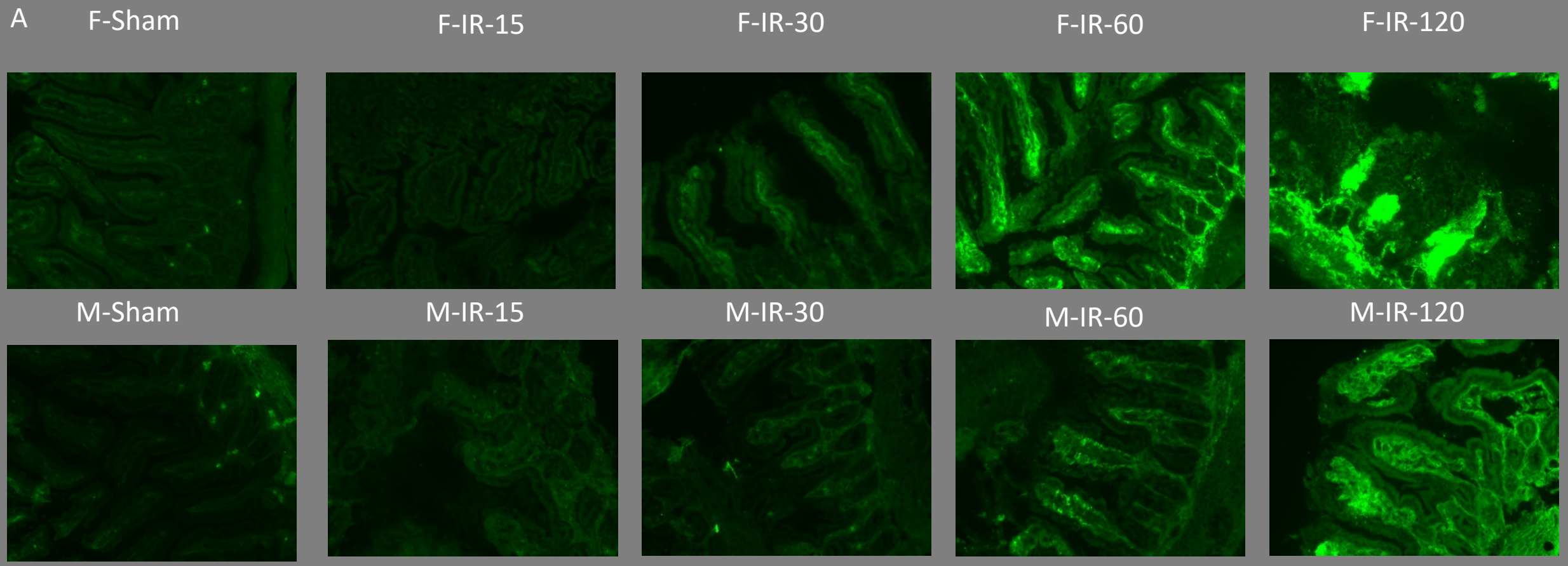


Figure 4. Complement initiation in response to intestinal I/R differs between sexes.



C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Mid-jejunal (A) C1q, (B) MBL, and (C) FB 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. Complement components and IgM deposition in response to intestinal I/R differs between sexes.



C57Bl/6 mice were subjected to Sham or different time course I/R treatment. Intestinal sections were stained for (A) IgM, (B) C1q, (C) MBL, (D) C4, and (E) C3 by immunohistochemistry. Microphotographs (200x) are representative of 3-4 animals stained in at least 3 independent experiments.

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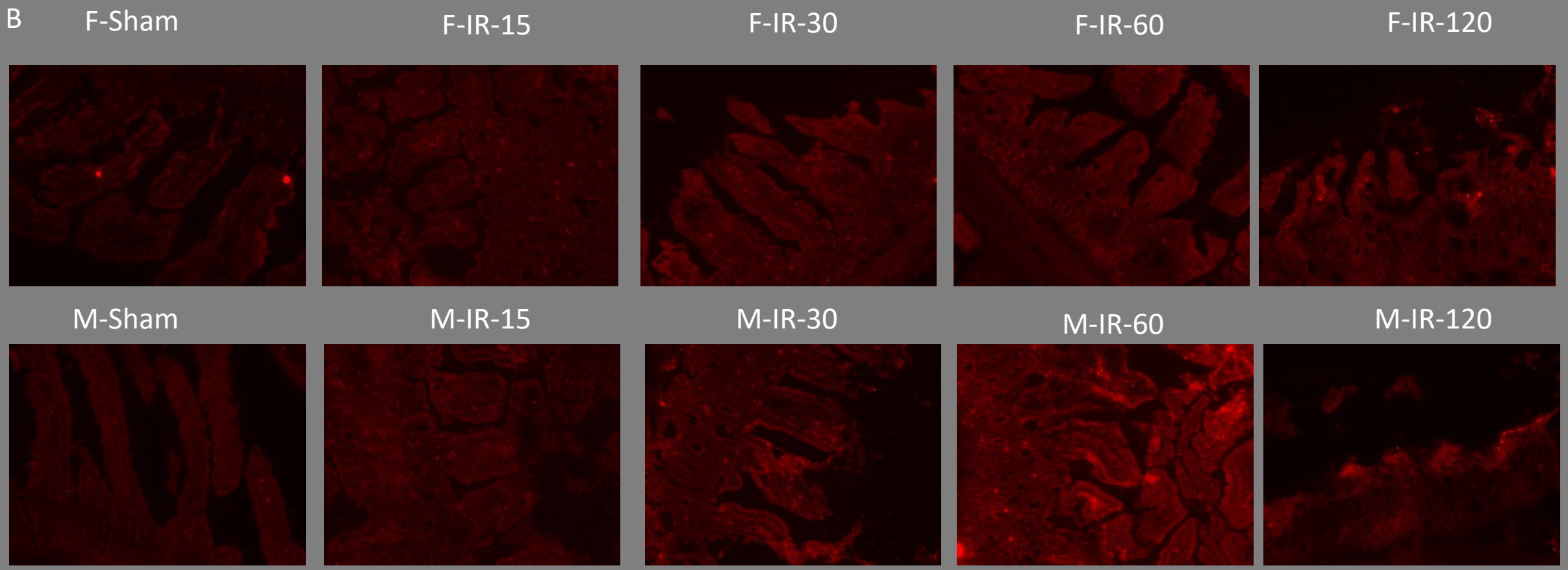

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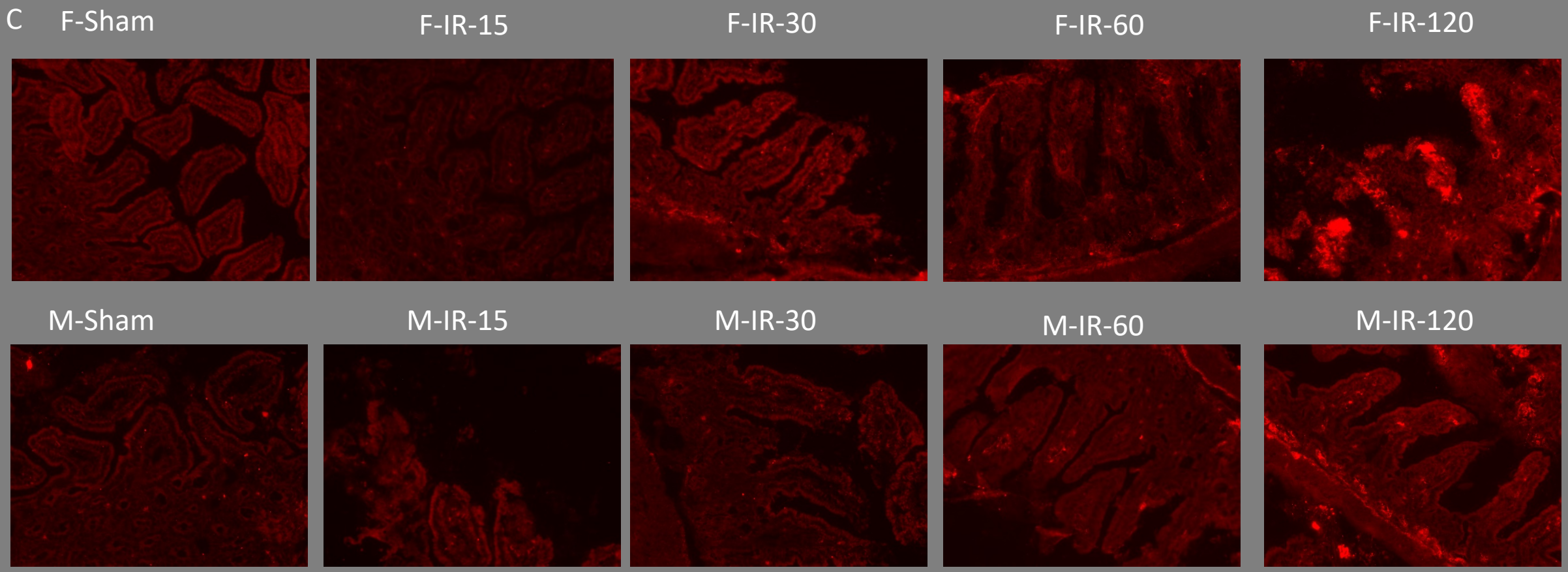

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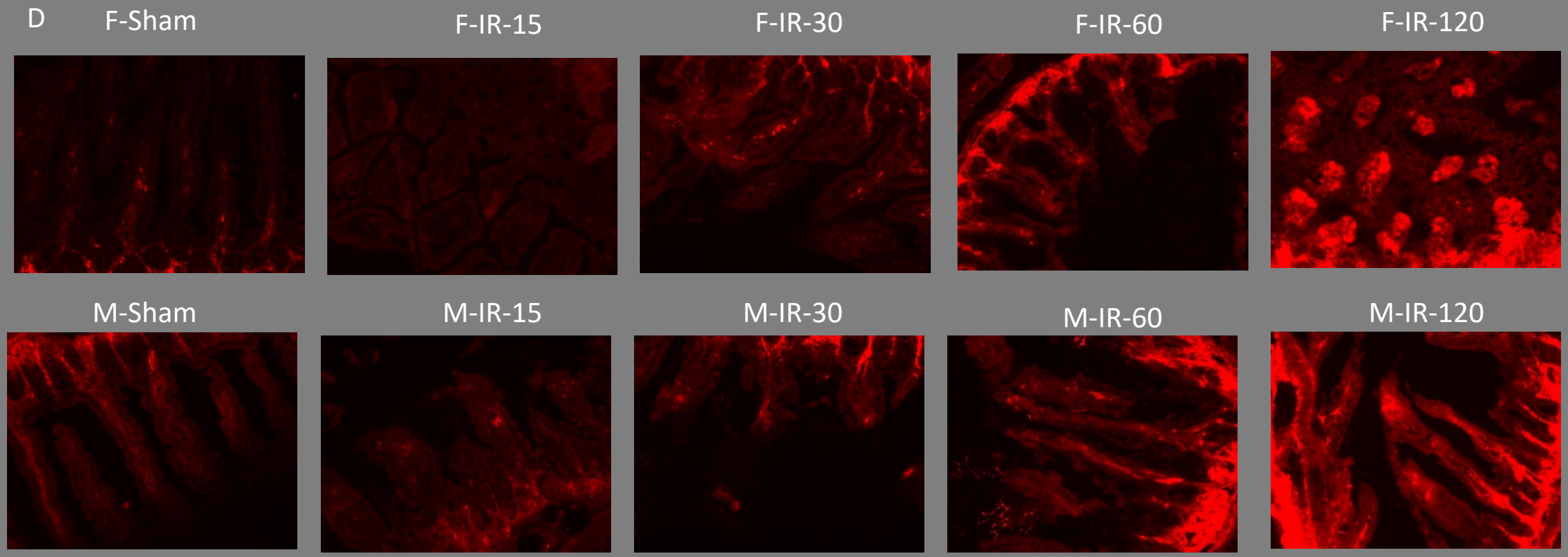

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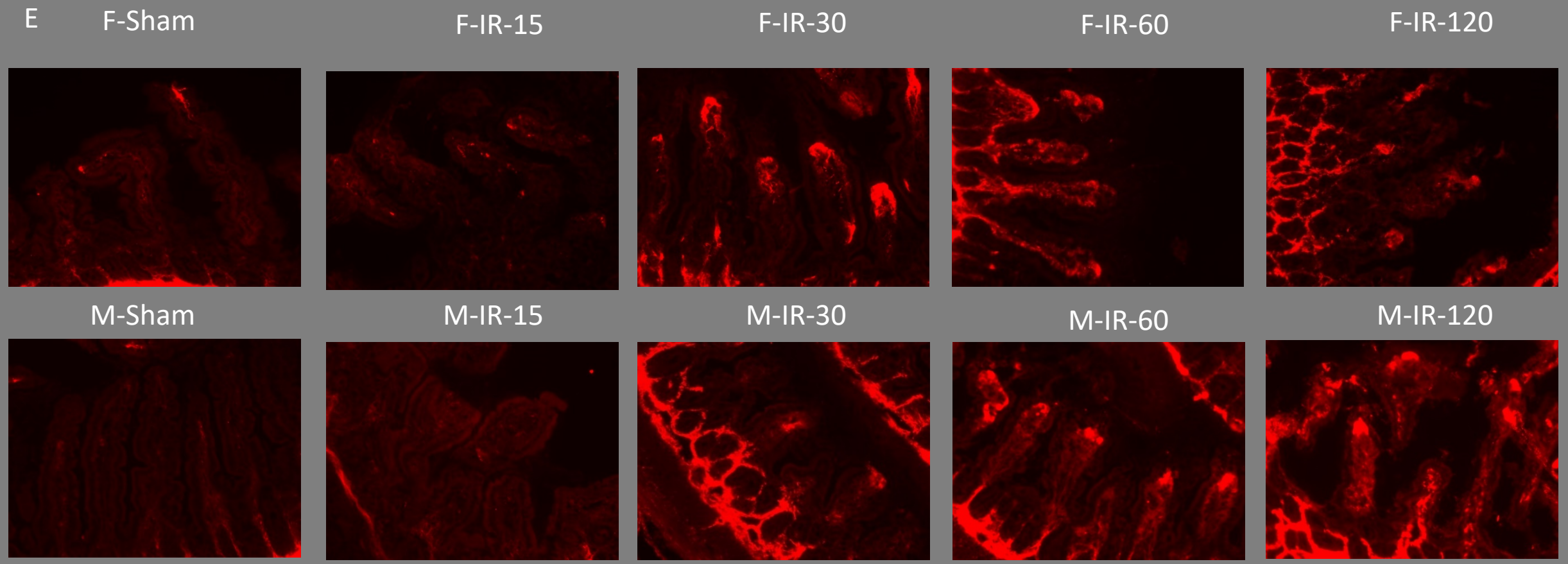

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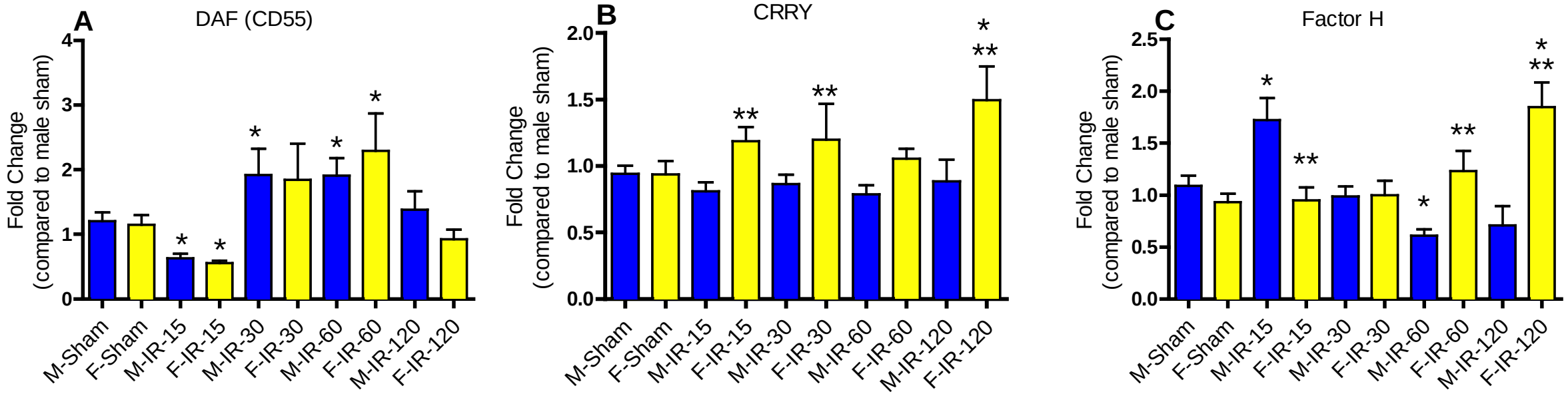
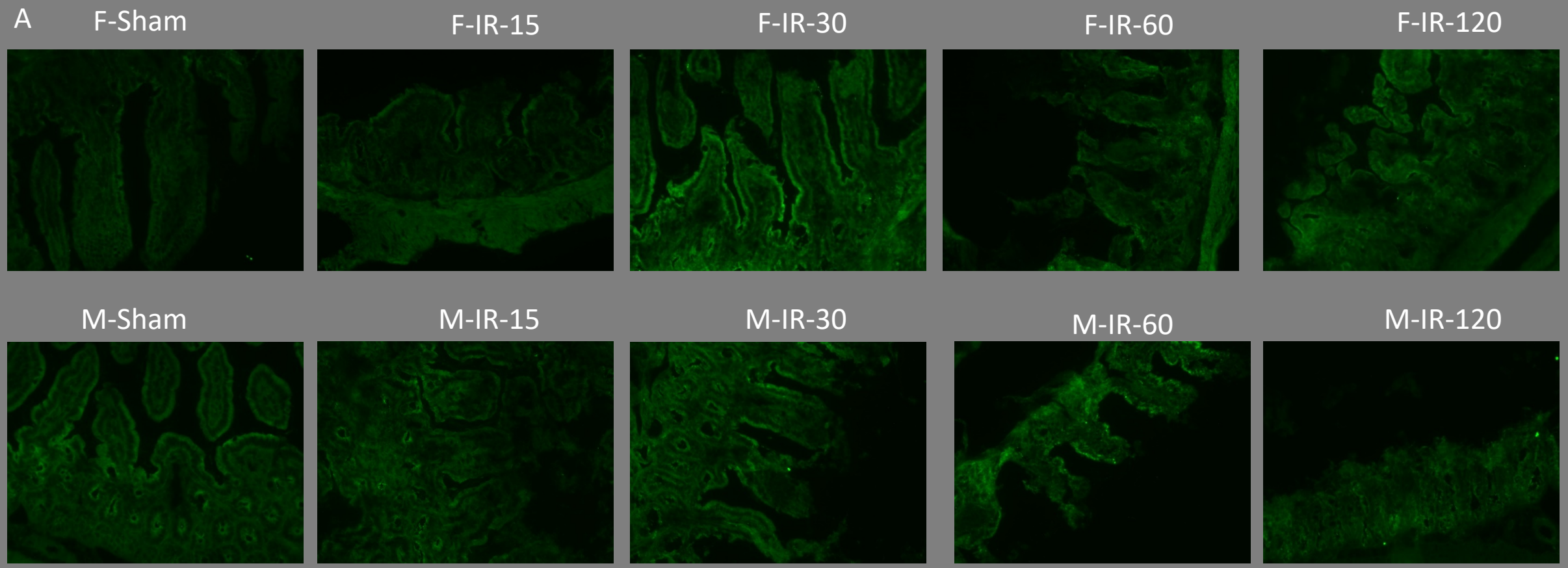
Figure 6. Complement inhibitors in response to intestinal I/R differs between sexes.



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 7. Complement inhibitor deposition in response to intestinal I/R differs between sexes.



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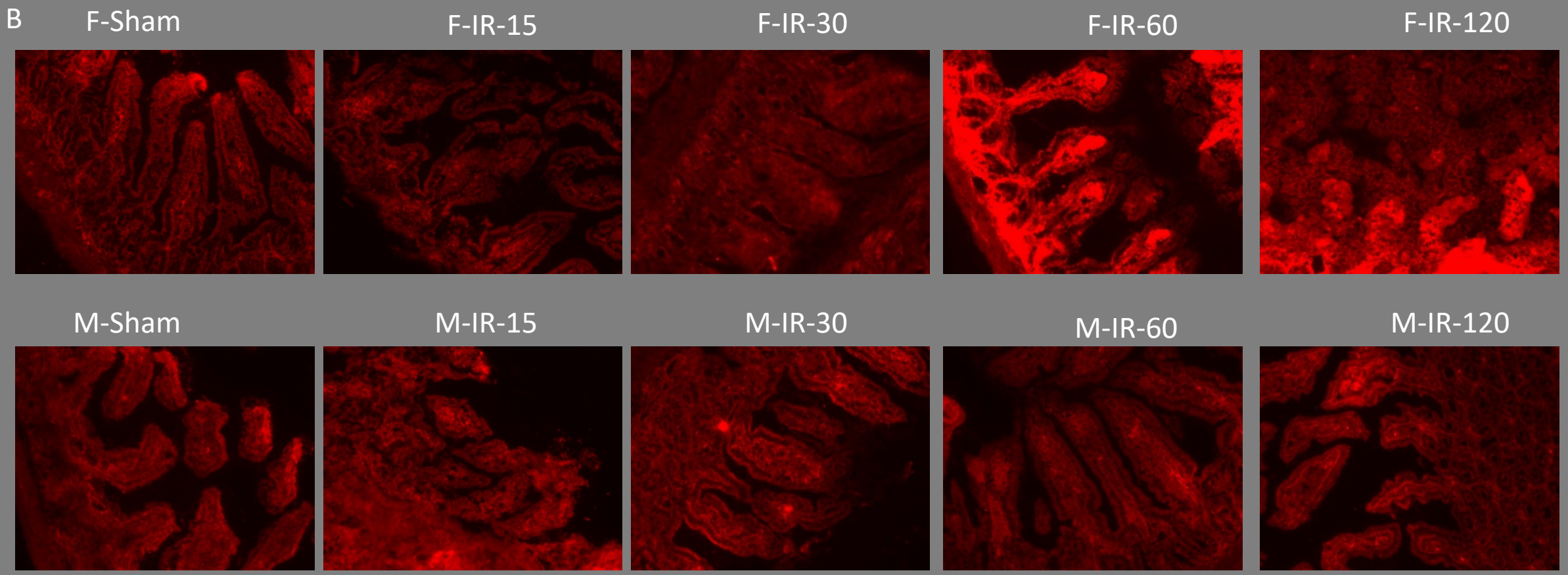

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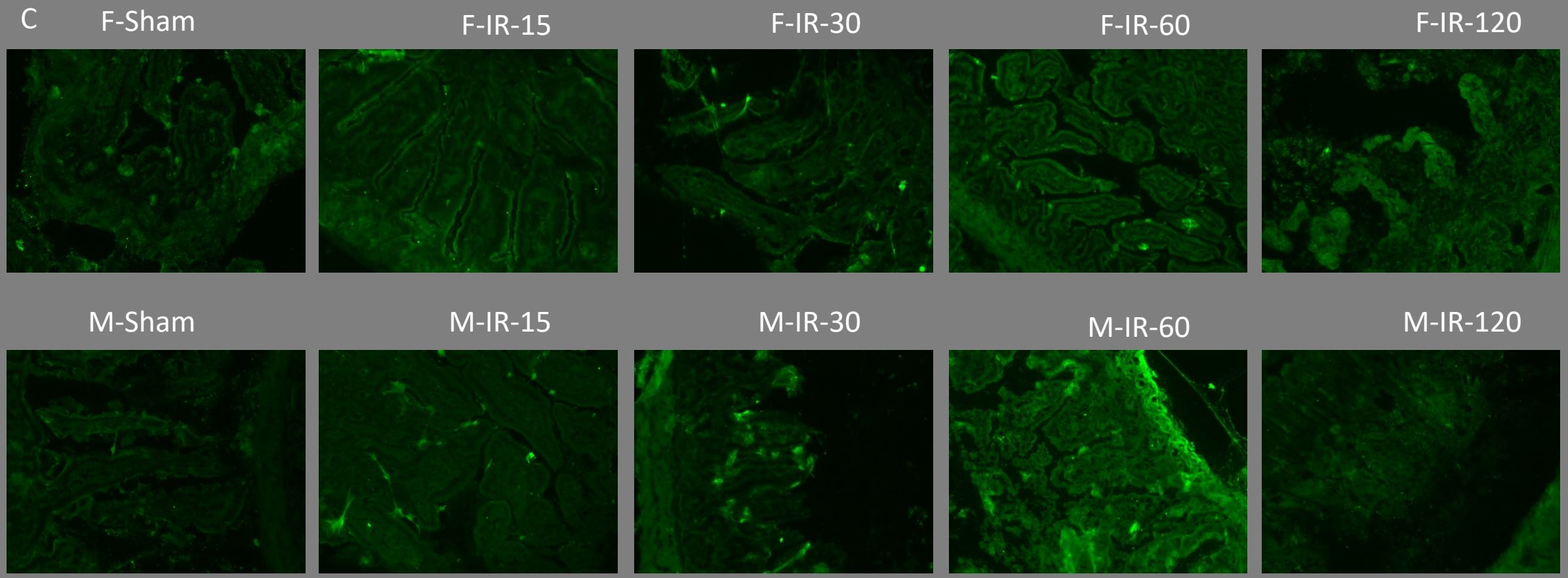

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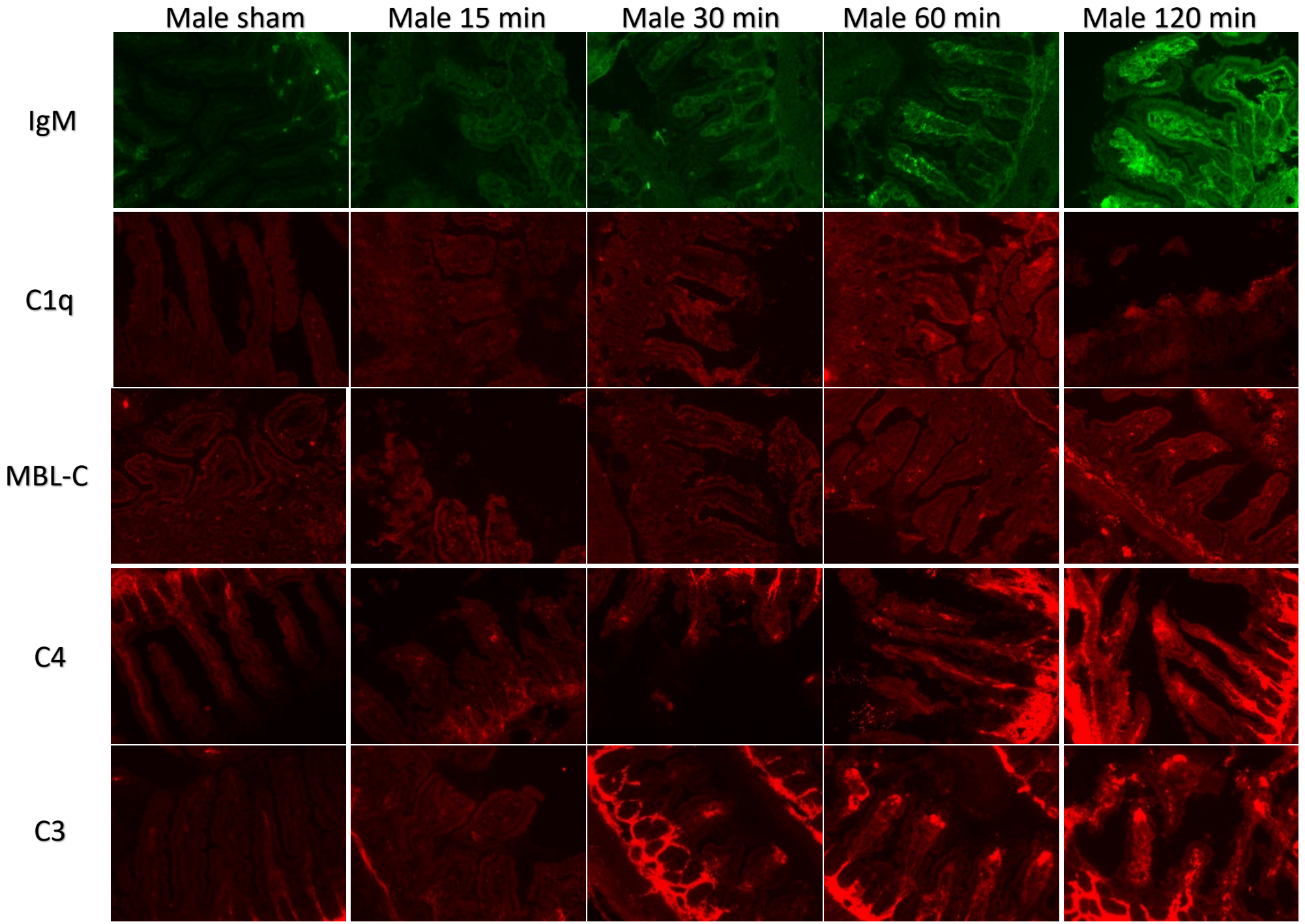
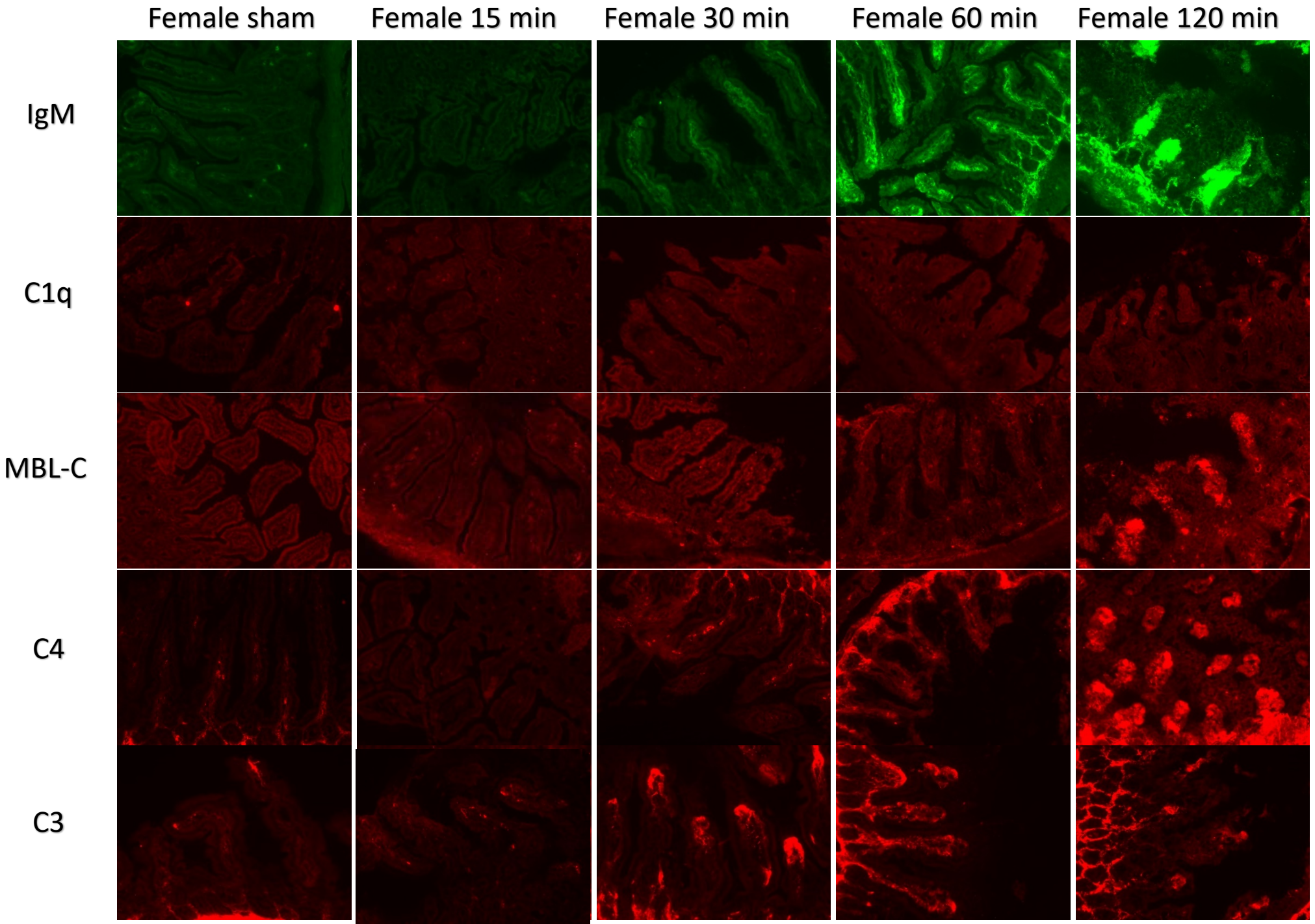

Male sham
Male 15 min
Male 30 min
Male 60 min
Male 120 min
IgM
C1q
MBL-C
C4
C3


Female sham
Female 15 min
Female 30 min
Female 60 min
Female 120 min
IgM
C1q
MBL-C
C4
C3