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TITLE: Noncanonical Autophagy and Toll-Like Receptor Signaling in SLE

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13. SUPPLEMENTARY NOTES

14. ABSTRACT
There is increasing evidence that dysregulated toll-like receptor (TLR) signaling in response to endogenous nucleic acids contributes to SLE pathogenesis. We have recently identified a role for autophagy in regulation of TLR signaling in B cells and plasmacytoid DCs (pDCs) The overall aim of this project is to determine whether SLE risk variants in the autophagy component *ATG5* affect TLR-induced autophagy and whether this regulatory pathway is disrupted in lupus. We propose studies to analyze TLR signaling responses in pDCs from healthy individuals with ATG5 SNPs and in SLE patients, making use of genotyped samples from SLE patients and healthy volunteers collected as part of the BRI Immune Repositories. These experiments will be combined with genetic studies to determine the genes involved in TLR-induced autophagy in pDCs. Work in year one of this project has been focused on establishing robust assays to measure these processes and initial analysis of patient samples. Experiments are underway to determine whether cytokine responses are altered in pDCs from healthy controls and SLE patients.

15. SUBJECT TERMS
Toll-like receptors, autophagy, plasmacytoid DCs.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
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TABLE OF CONTENTS

1. **INTRODUCTION:** *Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.*

There is increasing evidence from human studies and animal models that dysregulated Toll like receptor (TLR) signaling in response to endogenous nucleic acids contributes to SLE pathogenesis. The purpose of this grant is to understand how gene polymorphisms in the gene SLE risk gene ATG5 influence TLR signaling in plasmacytoid dendritic cells (pDCs). We aim to test the hypothesis that SLE risk variants in ATG5 increase TLR signaling and determine whether ATG5 function is altered in SLE.

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

Toll-like receptor, ATG5, autophagy, SLE

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.

1. Measure TLR signaling and responses in healthy subjects
    Milestone: Analysis in risk and non-risk haplotypes: *Completed*
2. Genetic targeting of components of the TLR trafficking pathway
    Milestone: Identify candidate genes: 4 months: *complete*
    Milestone: Genetic targeting of genes in PBMCs: 18 months: *Adjusted to use cell lines instead of PBMCs. Completed for some genes*
3: Measure TLR signaling and responses in SLE subjects – *revised to transcriptional profiling of responses in ATG5 risk and non-risk cohorts. Completed*

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.

1: Major Activities:
Work over the course of the project involved 2 major research activities: (a) Analysis of TLR signaling in immune cells with polymorphisms in ATG5; (b) analysis of genes involved in the induction of non-canonical autophagy.

2: Specific Objectives:
The specific aims of the project are:
Aim 1: Test the hypothesis that SLE risk variants in *ATG5* alter TLR-induced autophagy and TLR signaling in primary human plasmacytoid DCs
Aim 2: Determine whether TLR-induced non-canonical autophagy and endosomal trafficking programs are altered in pDCs from human subjects with SLE.

3: Significant Results/ Key outcomes:

(a) assays of TLR signaling in immune cells with ATG5 polymorphisms

Measurement of cytokine production: Based on our preliminary experiments in mice, we hypothesized that risk variants in *ATG5* promote SLE through effects on this non-canonical autophagy pathway, affecting TLR7 and IRF7 signaling, leading to increased IFN α/β production by pDCs. In aim 1, we planned to test this hypothesis by determining how the ATG^R haplotype alters TLR7 signaling in pDCs from healthy subjects. We investigated the use of flow cytometry approaches to measure cytokine production and activation of non-canonical autophagy in pDCs and other cell types following stimulation. This required development of robust and reproducible assays for this process. We had success in year one detecting expression of interferon and TNF-α production in pDCs by flow cytometry, with the aim of developing a robust assay in which PBMCs from healthy controls are thawed and stimulated with the TLR7 and TLR9 agonists R848 and CpG DNA. Cells were then stained for surface markers of pDCs and for intracellular production of TNF-α and IFN-α. Although we had some initial success in detection of cytokines using this method, we found considerable variability in staining intensity, and were unable to extend this approach to measure activation of NF-κB and IRF7 transcription factors by pDCs as we had planned. We therefore decided to use a complementary approach using gene expression profiling (described below)

(b) Assays of TLR signaling by gene expression profiling in cells with ATG5 polymorphisms

Initial studies were performed in collaboration with Dr Mridu Acharya (Seattle Children's Research Institute) to measure gene expression profiles in an immune cell line (HBL-1) stimulated with TLR ligands. Analysis of gene expression profiles have showed that disruption of autophagy by gene deletion induces reproducible changes in gene expression responses to stimulation. Curiously, in this cell line, loss of autophagy results in consistent increases in basal gene expression of TLR-response genes, rather than increased transcriptional responses to TLR stimulation. These data suggest that a major role of autophagy may be to suppress constitutive low-level TLR signaling, as well as reducing the magnitude of responses after stimulation.
We have transferred these assays to cultures of pDCs and B cells from donors with ATG5 polymorphisms. We selected 2 cohorts of PBMCs from healthy donors for this study, designated risk and non-risk allele groups, based on haplotypes across multiple ATG SNP loci. Groups were

matched for age and sex. pDCs were sorted from PBMCs by flow cytometry, and stimulated with the TLR7 ligand R848. After 4 hours cells were harvested for RNA and analyzed by RNA-Seq. Genes induced by TLR stimulation were compared between risk and non-risk groups. Overall transcriptional responses in the 2 groups were similar. We did not observe significant differences in expression of *ATG5* or the nearby gene *PRDM1*, which has also been reported to be impacted by some of these polymorphisms. We did observe an increase in baseline expression of the type I IFN gene *IFNA1* in pDCs with the *ATG5* risk haplotype, and a small increase in expression of *IFNB1* and the autophagy component *ATG3* after stimulation.

To maximize our ability to identify transcriptional changes in immune cells due to ATG5 SNPs in these experiments, and build on our previous findings in B cells, we also assayed transcriptional responses in 2 populations of B cells (naïve and memory B cells) in parallel with our studies of pDCs. Sorted B cells were stimulated with the TLR9 ligand CpG DNA (B cells) and assayed by RNA-Seq after 4 hours. As with pDCs, we did not observe large differences in transcriptional responses between B cells from ATG risk and non-risk cohorts, or changes in ATG5 expression. We did detect small differences in expression of the neighboring gene PRDM1 in unstimulated naïve and memory B cells. Furthermore, naïve B cells carrying the ATG5 risk allele showed significant upregulation of several genes after stimulation with CpG DNA, compared with cells with the non-risk allele. These included Integrin β3, which we have previously shown is upregulated on B cells after stimulation, and promotes activation of non-canonical autophagy, and PD1, an inhibitory receptor implicated in autoimmunity.

We finally profiled all immune cell subsets in the ATG5 risk and non-risk haplotype cohorts by multiparameter mass cytometry (CyTOF). These analyses have identified increases in a subpopulation of transitional B cells previously associated with SLE, and in subpopulations of myeloid cells, suggesting that ATG5 risk haplotype may induce 'pre-autoimmune' phenotypes on healthy individuals.

(c) identification of candidate genes involved in non-canonical autophagy

To complement studies of natural gene variants in patient samples, we have used gene knockdown in cell lines to evaluate the role of specific genes in TLR-induced autophagy. In work in the first year, in collaboration with Dr Acharya (Seattle Children's Research Institute) we analyzed gene expression data from distinct primary human B cell populations (closely related to pDCs) to identify candidate genes involved in autophagy. We initially attempted to knockdown or edit expression of these genes in primary PBMCs. However, this was challenging, and we were unable to achieve efficient gene knockdown in primary cells. We had anticipated this eventuality in our original proposal, and as an alternative we have been using human cell lines. In collaboration with Dr Acharya, we targeted genes known to be involved in autophagy (including ATG5, Rubicon and Integrins alphav/ beta 3) in the HBL1 cell line, and extended these studies to new genes involved in non-canonical autophagy.

Adjustment of goals and goals not met: As discussed above, we were not able to establish reliably robust and sensitive assays to assess TLR signaling by analysis of transcription factor activation by flow cytometry in heathy individuals and SLE patients, as we had originally intended. Instead, we refocused our efforts on measuring comprehensive transcriptional responses to TLR stimulation by

RNA-Sequencing. Due to the increased time needed for experiments and resources for sequencing and bioinformatics analysis, we focused these experiments on cohorts of healthy donor samples with ATG5 risk and non-risk alleles.

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.

Nothing to Report

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

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

Final report – nothing to report

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).

The results from this study increase our understanding of the potential role of a cellular process called autophagy in systemic lupus erythematosus and autoimmunity. We have made new connections between genetic variants in this pathway that are linked with SLE and immune signals known to be involved in activation of the immune system in other diseases. These results extend our findings in animal models into human autoimmune disease, and identify new areas for future research.

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:*

Nothing to report

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.

The COVID pandemic and resulting restrictions on workplace access, resources and reagents impacted our progress in years two and three. We moved some lab-based work to online analysis of available gene expression data sets during the restrictions. Despite these delays and challenges, we were still able to complete the transcriptional profiling of TLR responses in pDCs in ATG5 risk and non-risk cohorts.

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.

None

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

None

Not applicable

Significant changes in use of biohazards and/or select agents

None

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).*

A manuscript including some work from this project is under revision for publication in the journal *Autophagy.* A manuscript describing the transcriptional profiling of ATG5 risk haplotype cells is in preparation.

  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.*

Nothing to Report

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

Nothing to Report

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

Nothing to Report

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

Nothing to Report

- **Other Products**
  Identify any other reportable outcomes that were developed under this project. Reportable outcomes are defined as a research result that is or relates to a product, scientific advance, or research tool that makes a meaningful contribution toward the understanding, prevention, diagnosis, prognosis, treatment and /or rehabilitation of a disease, injury or condition, or to improve the quality of life. Examples include:
  - *data or databases;*
  - *physical collections;*
  - *audio or video products;*
  - *software;*
  - *models;*
  - *educational aids or curricula;*
  - *instruments or equipment;*
  - *research material (e.g., Germplasm; cell lines, DNA probes, animal models);*
  - *clinical interventions;*
  - *new business creation; and*
  - *other.*

Nothing to Report

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

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

Example:

Name:	*Mary Smith*
Project Role:	*Graduate Student*
Researcher Identifier (e.g. ORCID ID):	*1234567*
Nearest person month worked:	*5*
Contribution to Project:	*Ms. Smith has performed work in the area of combined error-control and constrained coding.*
Funding Support:	*The Ford Foundation (Complete only if the funding support is provided from other than this award.)*

Name: *Adam Lacy-Hulbert*
Role: Principal Investigator
Identifier (e.g. ORCID ID): *0000-0003-2162-0156*
Nearest person month worked: *2*
Contribution to Project: Dr Lacy-Hulbert supervised and directed the research, analyzed data and wrote reports.
Funding Support: *Not Applicable*

Name: *Emily Gilbertson*
Role: *Research Technician*
Identifier (e.g. ORCID ID): *none*
Nearest person month worked: *12*
Contribution to Project: *Ms Gilbertson performed experiments in all areas of the research project.*
Funding Support: *Not Applicable*

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.

The PI is co-investigator on a new award, providing 0.24 calendar months of effort:
Title: Type 1 Diabetes in Acute Pancreatitis Consortium – Pacific Northwest Clinical Center: Immune Pathogenesis of Post-Pancreatitis T1D, U01 DK127404 NIDDK, **PI:** Greenbaum, Carla
Performance Period: 09/16/2020 – 07/31/2025
Total Award Amount (Including Indirect Costs):

A previously active grant for the PI has closed. This has not affected effort or resources available for this project.

Title: Epithelial control of responses to allergen challenge and viral exacerbation, U19 AI125378 NIAID. PI: Dr Steven Ziegler
Effort: 2.4 calendar months
Performance Period: 07/15/2016 – 06/30/2021
Total Award Amount (Including Indirect Costs):

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*

Organization Name: *Seattle Children's Research Institute*
Location of Organization: *Seattle, WA*
Partner's contribution to the project: *Collaboration*. Dr Acharya provided intellectual input int the project (including identification of genes involved in autophagy processes and analysis of CyTOF data).

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.*

No Appendices Included