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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.

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a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
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1. INTRODUCTION:

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:

Toll-like receptor, ATG5, autophagy, SLE

3. ACCOMPLISHMENTS:

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: Initial Cohort Testing: 18 months: *30% complete*
2. Genetic targeting of components of the TLR trafficking pathway
    Milestone: Identify candidate genes: 4 months: *75% complete*
    Milestone: Genetic targeting of genes in PBMCs: 18 months: *50% complete*
3: Measure TLR signaling and responses in SLE subjects
    Milestone: Initial Cohort Testing: 18 months: *50% complete*

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 in year two involved 2 major research activities: (a) Analysis of TLR signaling in plasmacytoid DCs (pDCs); (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.

Specific objectives in year two were:
(a) beginning assays of TLR signaling in cells from healthy controls
(b) developing assays for measuring TLR signaling by gene expression profiling
(c) identification and characterization of candidate genes involved in non-canonical autophagy

3: Significant Results/ Key outcomes:

(a) beginning assays of TLR signaling in cells from healthy controls

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 plan to test this hypothesis by determining how the ATG^R haplotype alters TLR7 signaling in pDCs from healthy subjects. We are making use of flow cytometry approaches to measure cytokine production and activation of non-canonical autophagy in pDCs and other cell types following stimulation. This requires robust and reproducible assays for this process. We had success in year one developing assays for measuring interferon and TNF-α production in pDCs by flow cytometry. We now have 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. In the past year we have begun assessing TNF-α and IFN-α production in PBMCs from healthy subjects.

(b) developing assays for measuring TLR signaling by gene expression profiling

Measurement of transcription factor activation: We had planned to measure activation of NF-κB and IRF7 transcription factors by pDCs after stimulation with TLR ligands. Although we have previously had some success with this approach in B cells, it has proved more challenging in pDCs. In particular, it has been difficult to obtain the reproducible staining that we anticipate needing to allow accurate comparison of samples from different patient groups. In year 2 of the project, we have been developing approaches to use RNA analysis to measure gene activation in PBMCs and pDCs stimulated with TLR ligands.

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 whole PBMC cultures and are in the process of evaluating dose effects of TLR stimulation on transcriptional profiles. We will then analyse profiles of gene expression responses in whole PBMCs and sorted pDCs from ATG5-SNP healthy controls and SLE patients.

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

To complement studies of natural gene variants in patient samples, we are using gene knockdown to evaluate the role of specific genes in TLR-induced autophagy. In work in year one we analyzed gene expression data from distinct primary human B cell populations (closely related to pDCs). We compared gene expression in memory/ GC cells that undergo TLR induced autophagy with gene expression in naïve B cells that do not, to identify candidate genes involved in autophagy. In year one, we had 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 Mridu Acharya,
we have targeted genes known to be involved in autophagy (including ATG5, Rubicon and Integrins alphav/ beta 3) in the HBL1 cell line. In year 2 these studies have been extended to new autophagy related genes, identifying several new genes involved in non-canonical autophagy. We are establishing similar CRISPR targeting in the pDC line, Cal1 to test the role of these and other genes in autophagy in these cells.

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.

We plan to broadly follow the experimental timeline as set out in the original statement of work. Our main objectives in Year 3 are: 1. Complete tests of healthy and SLE cohorts for effects of ATG5 SNPs on cytokine production and autophagy induction 2. Determine whether changes in TLR signaling and autophagy are associated with increased ISG signature 3. Evaluate transcriptional profiling for analysis of TLR signaling changes in healthy and SLE patient pDCs

4. IMPACT:

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).

Nothing to Report

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 have impacted our progress in year two. We moved some lab-based work to online analysis of available gene expression data sets during the height of restrictions. We had anticipated completing our initial analysis of healthy control and SLE patient cytokine production by the end of year two, but these experiments are still ongoing. We have now implemented new safe working protocols to allow work to proceed. We anticipate being able to complete many of the planned studies by the end of the original project period.

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

- **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

  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.

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

Title: Identification of Host Drug Development Targets in Influenza Using Transposon Mutagenesis (R33AI119341)
Effort: 2.4 calendar
Supporting Agency: NIH/ NIAID
Grants Officer: Amy Krafft
Performance Period: 07/1/2015 – 06/30/2020
Funding Amount: (Annual Directs)
Project Goals: The overall aim of this grant is to utilize the power of cell-based genetic screening to identify host factors that contribute to resistance to influenza infection, with the ultimate aim of developing strategies to target these pathways for potential therapeutic benefit.

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.*

Organization Name: *Seattle Children's Research Institute*
Location of Organization: *Seattle, WA*
Partner's contribution to the project: *Collaboration*. Dr Acharya provides intellectual input inot the project (eg identification of genes involved in autophagy processes).

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