

AWARD NUMBER: **W81XWH-17-1-0363**

TITLE: **“Dextran Sulfate, Beta Cell Preservation, and Immune Regulation in Type 1 Diabetes”**

PRINCIPAL INVESTIGATOR: **Adolfo Garcia-Ocana, PhD**

CONTRACTING ORGANIZATION: **Icahn School of Medicine at Mount Sinai**
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REPORT DATE: **August 2019**

TYPE OF REPORT: **ANNUAL**

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

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REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. **PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.**

1. REPORT DATE (DD-MM-YYYY) August 2019		2. REPORT TYPE ANNUAL		3. DATES COVERED (From - To) 1 Aug 2018 - 31 Jul 2019	
4. TITLE AND SUBTITLE "Dextran Sulfate, Beta Cell Preservation, and Immune Regulation in Type 1 Diabetes"				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-17-1-0363	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Dirk Homann, MD; Adolfo Garcia-Ocana, PhD				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Icahn School of Medicine at Mount Sinai New York, NY 10029				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research Fort Detrick, Maryland				10. SPONSOR/MONITOR'S ACRONYM(S)	
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)	
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited					
13. SUPPLEMENTARY NOTES					
14. ABSTRACT Type 1 diabetes (T1D), with an increasing incidence worldwide over the past few years, poses a considerable challenge to afflicted individuals, to the development of effective prevention and treatment regimens, and to public health initiatives at large. In T1D, self-tolerance is lost leading to beta cell destruction. Autoreactive T cells acquire an effector inflammatory phenotype due to co-stimulatory signals leading to tissue invasion and beta cell destruction. Therapies focused on decreasing T cell activation to preserve functional beta cells are a priority for the treatment of the disease. We found that the semi-synthetic proteoglycan dextran sulfate (DS) decreases diabetes incidence in mice and preserves beta cells, but the therapeutic potential in humans is unknown. During the second year of the award, we have found that DS reduces human CD4+ T cell activation (decrease in interferon-gamma+ CD4+ T cells) when stimulated with anti-CD3/CD28. This correlates with changes in phenotypic markers of antigen presenting capacity in LPS-stimulated APC populations. Ongoing experiments are testing DS effects in human PBMCs from T1D donors.					
15. SUBJECT TERMS Type 1 Diabetes, Dextran sulfate, human PBMCs, CyTOF, immunomodulation					
16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT UU	18. NUMBER OF PAGES 17	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT U	b. ABSTRACT U	c. THIS PAGE U			19b. TELEPHONE NUMBER (include area code)

DoD progress report Year 2- Flow cytometry and CyTOF studies

Introduction:

Our overarching aim is "to dissect and stratify dextran sulfate (DS)-induced immunomodulatory effects on human T cell responses in health and T1D"; we hypothesize that DS exerts tolerogenic effects on human T cell responses by 1., skewing of APC maturation, phenotype and/or function, 2., direct modulation of T cell phenotypes/function, or 3., a combination thereof.

In pursuit of this hypothesis, we are using a combination of flow cytometric approaches and mass cytometry (MC/CyTOF technology) for a phenotypic and functional stratification of human PBMCs from healthy and T1D donors. hPBMCs from healthy donors are activated by several means (anti-CD3/CD28, PMA/ionomycin, LPA) and treated with different doses of dextran sulfate. Specifically, the studies developed during the second year of the award were designed as an approach to parse the general impact of DS on human T cell and APC populations, and we therefore performed flow cytometry analysis and designed and implemented a 35-parameter exploratory immunophenotyping MC staining panel to characterize the basic phenotypic properties of healthy donor PBMC cultured in the presence/absence of DS, polyclonal T cell stimulation with anti-CD3 and CD28 antibodies or LPS. Our initial analyses demonstrate a principal utility of the proposed experimental approach and feature data documenting that: 1) **DS decreases the activation of T cells by reducing the percentage of interferon-gamma-positive CD4+ T cells (Th1) and;** 2) **DS induces specific changes in the monocyte subset consistent with the possibility of reduced antigen-presentation capacity.** To further analyze this significant observation, we have therefore designed and built an APC-focused MC staining panel, the principal utility of which we have assessed and confirmed APC populations cultured in the presence absence of DS and LPS.

Results and Significance:

First, we have interrogated the effects of DS on polyclonal T cell activation (PBMC stimulated with α CD3/ α CD28 beads in the absence/presence of DS) using flow cytometry. We have made the significant observation that 100 ng/ml DS reduces the number of IFN-gamma-positive CD4+ T cells (Th1) while not altering other parameters such as T cell number or expression of other phenotypic markers such as PD-1. Results also show a trend towards increased number of Foxp3+CD25+ T cells what would suggest and increase in the number of regulatory T cells. On the other hand, CyTOF results indicate that DS promotes a selective increase of CXCR3 expression by activated CD8+T cells. Since expression of the chemokine receptor CXCR3 is upregulated on effector and memory as compared to naïve CD8+T cells, this set of observations was at first confounding. However, the kinetics of induced CXCR3 expression by activated CD8+T cells, analyzed in detail by us and others in murine model systems of infectious disease (Hu *et al.*, PMID: 21518913; Eberlein *et al.*, PMID: 27617858), are quite complex and do not permit a simple association between receptor expression levels and activation or developmental CD8+T cell status. Indeed, further stratification of the CXCR3+ CD8+T cell subset in DS cultures reveals that these cells significantly downregulated canonical T cell activation markers such as CD25, CD38 and HLA-DR. Importantly, the CXCR3 receptor and its ligands CXCL9, CXCL10 and CXCL11 have been implicated in both murine and human T1D pathogenesis, and the CXCR3: CXCL9/10/11 axis constitutes a promising target for T1D prevention and/or reversal (reviewed in an editorial we recently authored; van der Heide and Homann, PMID: 27401871). The unexpected confluence of DS-dependent CXCR3: CXCL9/10/11 modulation at the level of both T cells and islet cells (RNAseq analysis of human islets treated with cytokines + DS, studies independent of the current project) constitutes an exciting prospect, and we will leverage our established expertise in this field of T1D research to privilege the pursuit of this particular investigative avenue.

Second, an investigation into the specific impact of DS on APC maturation, we also adjusted our MC staining panel with a focus on monocytes and myeloid cells and have begun to interrogate the modulation of antigen presenting capacity in LPS-stimulated APC populations as deduced from the expression of HLA-ABC, HLA-DR and a variety of co-stimulatory/inhibitory molecules including PD-L1, PD-L2, CD80, CD86, CD40 and others. Collectively, a phenotype consistent with reduced antigen-presenting function adds to the potentially unique function of DS as an agent for the concurrent tolerogenic modulation of APCs, T cells and beta cells.

Third, we have adapted a recently published gene editing technique (Shifrut *et al.*, PMID: 30449619) that now permits us to effectively silence genes in primary human T cells; this technology will be uniquely useful to study specific pathways potentially relevant for immunomodulatory DS activities.

Conclusion, our studies developed during the second year highlight the therapeutic potential of DS for T1D since DS could clearly favor a "tolerogenic" phenotype in autoimmune T1D.

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

Our studies suggest that dextran sulfate (DS) can reduce the incidence of type 1 diabetes in non-obese diabetic (NOD) mice by decreasing inflammation, beta cell death and potentially inducing immune tolerance suggesting a therapeutic potential for this disease, but the therapeutic potential in humans is unknown. To address this, we plan to stratify DS-induced immunomodulatory effects on human immune cell responses. The hypothesis is that DS exerts tolerogenic effects on human T cell responses by 1) skewing APC maturation, phenotype and function; 2) direct modulation of T cell phenotypes/function; or 3) a combination thereof.

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

Type 1 diabetes, dextran sulfate, immune tolerance, T cell, antigen presenting cell, flow cytometry, CyTOF, human PBMCs.

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.

The goals this project are:

SA3.1: To parse the general impact of DS on human T cell and APC populations (6 months, Homann/Garcia-Ocana PIs). Unfractionated hPBMCs from healthy donors treated with dextran sulfate (DS) for 12-48h and phenotypic characterization of T cell and APC populations by CyTOF. Ongoing.

SA3.2: To delineate the specific impact of DS on APC maturation (8 months, Homann/Garcia-Ocana PIs). Unfractionated hPBMCs from healthy donors treated with DS for 24h in the absence or presence of LPS and phenotypic characterization of APC populations by CyTOF. Ongoing.

SA3.3: Analyze the impact on DS preconditioning on islet antigen-reactive CD4+T cell responses (6 months, Homann/Garcia-Ocana PIs). Immature DCs will be pre-conditioned with DS during maturation (LPS or inflammatory cytokine mix) for 48 hours; during the last 4h of maturation, \pm DS-conditioned DCs will be incubated with synthetic peptides, after which DCs will be cultured with CFSE-labeled antigen-reactive CD4 T cell clones. Proliferation and cytokine production (IL-2, IFN γ , IL-10) by the CD4 clones will be determined after 3d co-culture. Started.

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.

In SA3.1, we interrogated the effects of dextran sulfate (DS) on T cell activation (PBMC stimulated with α CD3/ α CD28 beads in the absence/presence of DS) using flow cytometry and our exploratory CyTOF staining panel. We found that DS decreases the number of IFN γ +CD4+ T cells (Th1 cells) as measured by flow cytometry. Surprisingly, CyTOF studies indicated that DS promotes a selective increase of CXCR3 expression by activated CD8+T cells. However, further stratification of the CXCR3+ CD8+ T cell subset in hPBMCs treated with DS revealed downregulated canonical T cell activation markers such as CD25, CD38 and HLA-DR. Next, we are designing a T cell-focused MC staining panel for an even more detailed characterization of DS-mediated effects on human CD8+T cell activation. In SA3.2, we are analyzing whether DS modulates antigen presenting capacity in LPS-stimulated APCs as deduced from expression of HLA-ABC, HLA-DR, PD-L1/L2, CD80, CD86, CD40 and others. Collectively, a phenotype consistent with reduced antigen-presenting function adds to the potentially unique function of DS as an agent for the concurrent tolerogenic modulation of APCs, T cells and beta cells. We just stated the palmification for performing the experiments in SA3.3 to analyze the impact on DS preconditioning on islet antigen-reactive CD4+T cell responses and the mechanisms induced by DS in SA3.4.

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.

Training on Flow Cytometry was provided by:

- Dr. Jordi Ochando, PhD; Assistant Professor, Oncological Sciences, Medicine, Nephrology and Pathology at Icahn School of Medicine at Mount Sinai.

Training on CyTOF data analysis was provided by:

- Dr. Adeeb Rahman, PhD, Assistant Professor, Genetics and Genomic Sciences, Director of Technology, Human Immune Monitoring Center at Icahn School of Medicine at Mount Sinai.

- Dr. Amir Horowitz, PhD, Assistant Professor, Oncological Sciences, Precision Immunology Institute at Icahn School of Medicine at Mount Sinai.

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.

Next year we will finish the experiments in SA 3.1, SA 3.2, and get deep on the studies in SA3.3 and SA3.4 as indicated in the SOW. We will delineate the specific impact of DS on T-cell activation, APC maturation and pro-inflammatory cytokine production. We will start interrogating metabolic alterations induced by DS in hPBMCs that could mechanistically explain the alterations in T cells and APCs. Thanks to our collaboration with Dr. Sally Kent at U. Mass, expert in human T cell clone generation, we have been trained on generating these clones for the studies in SA 3.3. Immature DCs will be pre-conditioned with DS and incubated with synthetic peptides, after which DCs will be cultured with CD4 T cell clones and proliferation and cytokine production by the CD4 clones will be determined.

4. **IMPACT:** Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:

What was the impact on the development of the principal discipline(s) of the project?

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

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

The unexpected confluence of dextran sulfate-dependent chemokine expression modulation at the level of both T cells and islet cells, the decrease in human T cell activation, together with a phenotype consistent with reduced antigen-presenting function highlights the unique function of dextran sulfate as an agent for the concurrent tolerogenic modulation of APCs, T cells and beta cells. In conclusion, our studies developed during the first two years highlight the specific immunomodulation by dextran sulfate treatment that could clearly favor a “tolerogenic” phenotype in autoimmune type 1 diabetes. This suggests the therapeutic potential of dextran sulfate for type 1 diabetes.

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.

Dextran sulfate can have potential applicability for other autoimmune diseases such as lupus, rheumatoid arthritis, thyroiditis, etc. since our results indicate an immunomodulatory effect of dextran sulfate in antigen presenting cells and T cells highlighting the therapeutic potential of this agent not only for autoimmune type 1 diabetes but for other autoimmune diseases. Therefore, our current studies can have an important impact in other medical disciplines

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.

No changes in scope, objectives or approach.

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.

No anticipated problems or delays during the project.

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.

No significant impact on expenditures.

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

No changes in use of human subject samples.

Significant changes in use or care of vertebrate animals

No animals are involved in this project.

Significant changes in use of biohazards and/or select agents

No changes in biohazards and/or select agents.

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).*

- G Lu, T Zhang, D. Homann and A. Garcia-Ocaña. Dextran Sulfate and HGF Ameliorate Type 1 Diabetes. *Diabetes* 2019 Jun; 68(Supplement 1): 2131-P; DOI: 10.2337/db19-2131-P

- G. Lu, F. Rausell-Palamos, Z. Zheng, T. Zhang, S. Valle, C. Rosselot, C. Berrouet, P. Conde, M.P. Spindler, J.G. Graham, D. Homann, A. Garcia-Ocaña. Dextran Sulfate Ameliorates Type 1 Diabetes, Pancreatic Beta Cell Death and Autoimmunity. Submitted.

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).*

No books or other non-periodical, one time-publications for this first year of the award.

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.*

- Oral presentation: G. Lu, D. Homann and A. Garcia-Ocana. Dextran Sulfate Ameliorates Autoimmune Diabetes via Reprogramming Mitochondria's Metabolic Activities of T Cells. 27th Annual Boston Ithaca Islet Club Meeting. New York, NY.

- Poster presentation: G. Lu, D. Homann and A. Garcia-Ocana. Dextran Sulfate Ameliorates Type 1 Diabetes by Enhancing Beta Cell Survival and Mitochondrial Function and Increasing Regulatory T Cells. 11th nPOD Meeting, Hollywood, FL.

- **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.

No websites or internet sites.

- **Technologies or techniques**

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

No new technologies or techniques.

- **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.

No inventions, patent applications, and/or licenses.

- **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.*

No other products.

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: Dirk Homann; Project Role: PI; Researcher Identifier: orcid.org/0000-0002-7622-5754
Nearest person month worked: 4.0
Contribution to Project: Directing, reviewing and interpreting the results of the project.
Funding Support: No additional funding for this project.

Name: Adolfo Garcia-Ocana; Project Role: PI; Researcher Identifier: orcid.org/0000-0002-6883-6176
Nearest person month worked: 3.0
Contribution to Project: Directing, reviewing and interpreting the results of the project.
Funding Support: No additional funding for this project.

Name: Zihan Zhang; Project Role: Technician; Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6.0
Contribution to Project: hPMBCs treatments, Flow cytometry and analysis of CyTOF data.
Funding Support: No additional funding for this project.

Name: Jessica Wilson; Project Role: Technician; Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 4.0
Contribution to Project: Cell culture and hPMBCs treatments.
Funding Support: No additional funding for this project.

Name: Geming Lu; Project Role: Senior Scientist; Researcher Identifier (e.g. ORCID ID): N/A
Nearest person month worked: 6.0
Contribution to Project: hPMBCs treatments, Flow cytometry and analysis of CyTOF data.
Funding Support: No additional funding for this project.

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.

Previous Grant Support that Ended:

NIH/NIDDK DK-012893 (Garcia-Ocana: Co-I): “Osteoprotegerin and the Pancreatic Beta Cell” ended on 01/31/2019

Pending Grants that are now Active:

JDRF 201305402 (Homann: MPI): “Center for mass cytometry analysis of TrialNet samples”
Period: 11/01/2018 - 10/31/2020

Pfizer, Inc. (Homann: Co-I) “Rat Diabetes Microgliosis Investigation”
Period: 9/1/18 – 8/31/19

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.