

AWARD NUMBER: **W81XWH-19-1-0012**

TITLE: **Induction and tolerization of dystrophin immunity**

PRINCIPAL INVESTIGATOR: **Gerald COULIS**

CONTRACTING ORGANIZATION: **University of California, Irvine**

REPORT DATE: **JULY 2020**

TYPE OF REPORT: **ANNUAL**

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

DISTRIBUTION STATEMENT: **Approved for Public Release;
Distribution Unlimited**

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

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 JULY 2020		2. REPORT TYPE ANNUAL		3. DATES COVERED 07/01/2019-06/30/2020	
4. TITLE AND SUBTITLE Induction and tolerization of dystrophin immunity				5a. CONTRACT NUMBER	
				5b. GRANT NUMBER W81XWH-19-1-0012	
				5c. PROGRAM ELEMENT NUMBER	
6. AUTHOR(S) Gerald COULIS E-Mail:				5d. PROJECT NUMBER	
				5e. TASK NUMBER	
				5f. WORK UNIT NUMBER	
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University California Irvine Irvine, CA 92697				8. PERFORMING ORGANIZATION REPORT NUMBER	
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Development Command Fort Detrick, Maryland 21702-5012				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 Duchenne muscular dystrophy (DMD), the most common form of muscular dystrophy, is a lethal X-linked recessive disorder caused by the dysfunction of a protein called dystrophin. Our growing understanding of DMD has led to an accurate characterization of this genetic disease yet an effective cure remains to be found. Therefore, there have been tremendous efforts from the scientific community to slow down or halt disease progression in DMD. Dystrophin gene therapy is an attractive avenue that has yet to overcome: the challenge posed by dystrophin immunity: an elicited immune response when restoring dystrophin. Herein, we obtained promising results that demonstrate that we can recapitulate this unwanted immune response in a dystrophic mouse model. A better characterization of this immune response is paramount to identify selective regulatory targets to dampen dystrophin immunity.					
15. SUBJECT TERMS NONE LISTED					
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	14	19b. TELEPHONE NUMBER (include area code)

TABLE OF CONTENTS

Page

- 1. Introduction**
- 2. Keywords**
- 3. Accomplishments**
- 4. Impact**
- 5. Changes/Problems**
- 6. Products**
- 7. Participants & Other Collaborating Organizations**
- 8. Special Reporting Requirements**
- 9. Appendices**

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

Duchenne muscular dystrophy (DMD) is a genetic disease caused by mutations in the dystrophin gene that leads to the absence of dystrophin expression in muscles. To date, there is no effective therapy to cure this devastating disease although the administration of glucocorticoids can temporarily slow down the progression of the pathology. Promising therapies, now entering clinical trials for the treatment of DMD, include micro dystrophin gene transfer, exon skipping or gene editing to correct or splice out the nonsense mutations. However, recent clinical trials identified significant T-cell immune responses directed at dystrophin epitopes in patients treated to restore dystrophin expression. These Dystrophin-specific T cells possess the ability to eliminate all genetically modified muscle resulting in an ineffective therapy, as well as presenting a general concern for the community. Currently, there is no preclinical model to study this specific immune response in order to develop an effective immunosuppressive strategy to avoid dystrophin T cell responses. This proposal aims at (1) providing the community with a novel preclinical model to study this very specific immune barrier; (2) Identifying putative targets with a focus on regulatory T cells that could block this process leading to dystrophin immunity; (3) Proposing a safe and effective therapy alongside the dystrophin gene delivery to ensure successful tolerance to dystrophin and its restoration.

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

Duchenne muscular dystrophy, dystrophin, gene therapy, dystrophin immunity, preclinical model, regulatory T cells, tolerance

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: Establish a pre-clinical system to induce dystrophin immunity in a mouse model of muscular dystrophy that enables the testing of tolerogenic therapies to mitigate dystrophin-specific T cells

Major Task 1 *In silico* design and synthesis of immunogenic peptides that induce a specific T cell response. 0-6 months

Major Task 2 Screen predicted immunogenic peptides and validate in vivo induction of dystrophin-specific T cells. 6-12 months

Major Task 3 Determine the capacity of Tregs to induce dystrophin tolerance. 12-18 months

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.

Predicted immunogenic dystrophin peptides. We used *in silico* approaches, guided by known mutational hotspots, to predict immunogenic dystrophin peptides. This method provides a significant advancement in our ability to address the role of dystrophin immunity by using innovative computational methods to predict, with high confidence, a set of immunogenic dystrophin peptides that induce dystrophin-specific T cells in mice. The *in silico* design of immunogenic dystrophin peptides was performed with the T Cell Epitope Prediction Tool at the Immune Epitope Database, which is supported by the National Institute of Allergy and Infectious Diseases. The database is equipped to predict peptides for multiple MHC types and over 75 HLA variants in human. The selection of MHC-I and MHC-II-restricted peptides is based on the peptides binding affinity to major histocompatibility complex (MHC), which is responsible for the presentation of peptides to T cells. Peptides are further ranked based on their predicted immunogenicity and processing by the proteasome and transporter associated with antigen processing (TAP) system. We only included MHC-I and MHC-II-restricted peptides that shared 100% homology with human dystrophin to establish an immunization protocol that can be used in mdx and ultimately in humanized mdx mice.

Immunization with a dystrophin peptide (iDys) induces dystrophin-specific T cells in mice. We

designed an immunization protocol based on methods widely used in the autoimmunity field to break tolerance to self-antigens. Briefly, WT mice were immunized with a dystrophin peptide (iDys) emulsified in Complete Freund's Adjuvant (CFA). The rationale for using CFA was based on the success of numerous studies using CFA with myelin oligodendrocyte glycoprotein or collagen peptides to induce experimental autoimmune encephalomyelitis (EAE) or collagen-induced arthritis (CIA), to model multiple sclerosis and rheumatoid arthritis, respectively. Fifteen days after the initial immunization, mice were euthanized, splenocytes were isolated and stimulated *in vitro* with iDys or ovalbumin (Ova) peptide for 3 days. The induction of dystrophin-specific CD8⁺ T cells was assessed by measuring the frequency of activated (CD44⁺), cytokine-producing (IFN γ ⁺) and proliferating T cells (Ki67⁺ and low signal for TIV proliferation dye) by flow cytometry (Fig. 1). We found that the dystrophin immunization protocol caused an expansion of CD44⁺ effector T cells that produce IFN γ in response to iDys peptide compared to Ova peptide, which was used as a negative control (Fig. 1A and 1B). Activation of T cells in response to antigen encompasses a proliferative response. Accordingly, we found that the frequency of Ki67⁺CD8⁺ T cells that

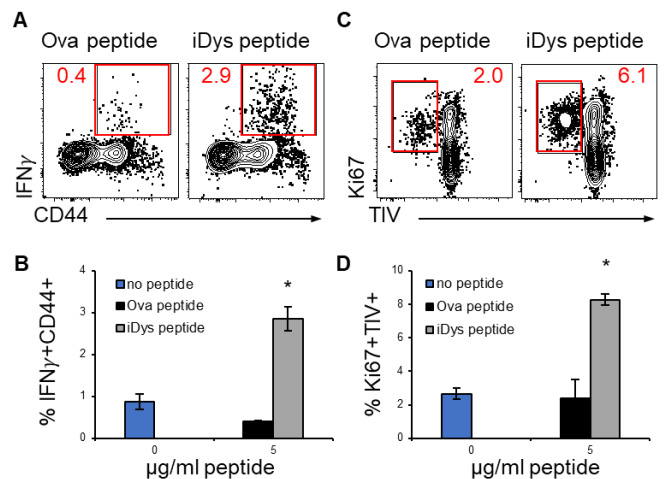


Figure 1. The immunization of WT mice with a dystrophin peptide induces a dystrophin-specific CD8⁺ T cell response. Immunization protocol: 8-wk-old C57BL/6 mice (WT) were immunized with an emulsion of dystrophin peptide in CFA and pertussis toxin. A pertussis toxin and dystrophin booster was provided on day 2 and week, respectively. **Recall antigen assay:** mice were euthanized on day 15, spleens were harvested and splenocytes were stimulated *in vitro* with the same dystrophin peptide (5 μ g/ml) used for immunization or a control peptide (i.e. Ova= ovalbumin, 5 μ g/ml). Cell proliferation and IFN γ production in CD8⁺ T cells was determined by flow cytometry. (A) Representative contour plots showing the expression of IFN γ and CD44 in CD8⁺ T cells following peptide stimulation for 3 days. (B) Quantification of the frequency of IFN γ +CD44⁺ CD8⁺ T cells in A. (C) Representative contour plots showing the dilution of Tag-it Violet (TIV) cell proliferation dye and the expression of Ki67. (D) Quantification of the frequency of Ki67+TIV⁺ CD8⁺ T cells in C. n= 3 mice per group. *, p< 0.05; significantly different compared to ova peptide group.

diluted TIV proliferation dye was increased in mice immunized with iDys peptide (Fig. 1C and 1D). With each round of division, a cell labeled with TIV dye will approximately dilute the TIV signal by half. Thus, a reduction in TIV signal, along with an increased proportion of Ki67+ cells, is indicative of enhanced proliferation. Collectively, these data show that our immunization protocol breaks dystrophin tolerance in WT mice, leading to the induction of dystrophin-specific T cells. Importantly, Tregs were not expanded by immunization with iDys. This protocol was applied in mdx mice, where we expected an even larger induction of dystrophin-specific T cells. Briefly, we observed an expansion of IFN γ -producing CD44+ CD8 T cells in response to iDys peptide compared to Ova peptide (Fig 2A). Unexpectedly, this dystrophin-specific T cell

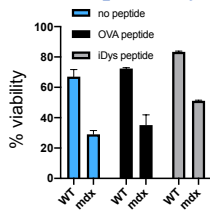


Figure 3. The immunization of mdx mice with a dystrophin peptide induces increased cell death. Cell viability was determined by flow cytometry in mdx or WT splenocytes.

response is somewhat lesser than the response obtained in WT mice. In line with this, T cell proliferation measured by the TIV signal and the Ki67 marker was found in mdx mice at more modest levels than healthy mice (Fig 2B). Altogether, our data demonstrate that we established an immunization protocol using an immunogenic peptide iDys that enables a specific immune response in both healthy and dystrophic mice. However, in mdx mice the immune response is reduced likely due to the muscle pathology. In fact, we could observe a decreased cell viability in dystrophic setting compared to healthy mice (Fig 3). Therefore, some optimization will be required to potentiate this response in mdx mice prior to modulate it using Tregs (major task 3).

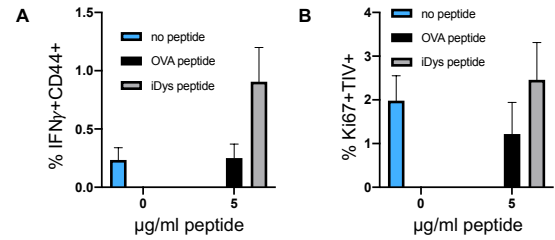


Figure 2. The immunization of mdx mice with a dystrophin peptide induces a dystrophin-specific CD8+ T cell response. Cell proliferation and IFN γ production in CD8+ T cells was determined by flow cytometry. (A) Quantification of the frequency of IFN γ +CD44+ CD8+ T cells in A. (B) Representative contour plots showing the dilution of Tag-it Violet (TIV) cell proliferation dye and the expression of Ki67. n= 3 mice per group.

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.

This project generated several opportunities for training and professional development. The P.I for instance benefited some one-on-one work with his mentor to increase his knowledge in immunity and tolerance. Another opportunity was given to a trainee to develop his skills in mouse immunization and in vitro antigen recall assay. All the data acquired during this time period originates from his work. He’s now on a path to become a MD/PhD. Finally, the P.I is now training a new lab member to the immunization techniques and the necessary analysis to evaluate the dystrophin T cell immune response.

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.

Results were only discussed during internal meetings. However, this project will be presented to local and national meetings during the next reporting period.

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.

We successfully demonstrate that we can induce dystrophin immunity in healthy mice (fig 1). This approach is promising in dystrophic mice (Fig 2) but we need to increase cell viability (Fig 3). We suspect the cells to be in an activated state in dystrophic setting. By using CFA and/or Pertussis toxin (PTX), the immune cells become hyperactivated, which will in turn increase cell death. In the first months of the next reporting period, we will optimize an immunization protocol that requires a different adjuvant. Once the model established, we'll be able to modulate this immune response essentially by using regulatory T cells (Tregs). We do have all the reagents and the mice readily available in the lab to complete the task.

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).

Conventional gene-based strategies aiming to deliver functional copies of DMD in patients have turned out problematic, mostly due to poor delivery and the activation of an immune response specific to the dystrophin gene. To face this challenge, there is a lack of an animal model to study dystrophin immunity. Herein, we generated promising results **and soon we will be able to provide the musculoskeletal disorders field with a novel mouse model mimicking dystrophin immunity.** This tool will be useful to our lab and other groups to test potential targets to enable dystrophin tolerance.

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.

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.

Our preliminary results showed a reduced dystrophin-specific immune response in mdx mice, a dystrophic mouse model. Dystrophin immunity is likely to be hampered by aberrant cell death in mdx mice as shown in figure 3. The use of CFA or PTX in a dystrophic setting where inflammatory signals are dysregulated might lead to an exacerbated apoptosis. To address it, we planned on testing experimental conditions where CFA

and/or PTX concentrations are reduced. We anticipate that we will improve cell viability and therefore dystrophin-specific immune response.

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.

Nothing to report.

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

N/A

Significant changes in use or care of vertebrate animals

No significant changes

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

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).

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.*

- **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.

- **Technologies or techniques**

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

- **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.*

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: **Ali Maanna**
Project Role: Undergraduate Student
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 6

Contribution to Project: *Ali contributed to the identification of peptides of interest. Ali identified Ali has performed the immunization work and the flow cytometer work on WT and mdx mice.*

Funding Support: *Ali was supported by the start funds of my mentor: Dr Villalta*

Name: **Dr Armando VILLALTA**
Project Role: Mentor
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 2

Contribution to Project: *Dr Villalta provided guidance and mentoring to the PI and performed data analysis*

Funding Support: *Dr Villalta was covered by cost sharing from institutional funds*

Name: **Dr Gerald COULIS**
Project Role: PI
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12

Contribution to Project: *Dr Coulis elaborated the in silico design approach and identified dystrophin peptides of interest. Dr Coulis performed immunization and flow cytometry on WT and mdx mice. Dr Coulis also analyzed the data to generate the results and the figures.*

Funding Support:

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.

Nothing to report.

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.*