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

TITLE: **Induction and Tolerization of Dystrophin Immunity**

PRINCIPAL INVESTIGATOR: **Mr. Gerald Coulis**

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

REPORT DATE: **APRIL 2022**

TYPE OF REPORT: **FINAL**

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

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
APRIL 2022	FINAL	01Jul2019 - 31DEC2021

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)

Mr. 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			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	14	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

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

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 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 (IFNγ+) 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 IFNγ 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

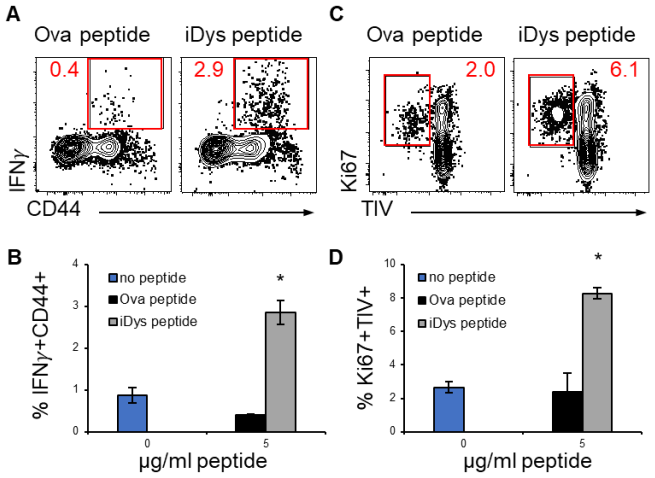


Figure 1. The immunization of WT mice with a dystrophin peptide induces a dystrophin-specific CD8+ T cell response. Immunization protocol: 8-wk-old C57BL/6 mice (WT) were immunized with an emulsion of dystrophin peptide in CFA and pertussis toxin. A pertussis toxin and dystrophin booster was provided 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 ug/ml) used for immunization or a control peptide (i.e. Ova= ovalbumin, 5 ug/ml). Cell proliferation and IFNg production in CD8+ T cells was determined by flow cytometry. (**A**) Representative contour plots showing the expression of IFNg and CD44 in CD8+ T cells following peptide stimulation for 3 days. (**B**) Quantification of the frequency of IFNg+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 IFNγ-producing CD44+ CD8 T cells in response to iDys peptide compared to Ova peptide (**Fig 2A**). Unexpectedly, this dystrophin-specific T cell 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).

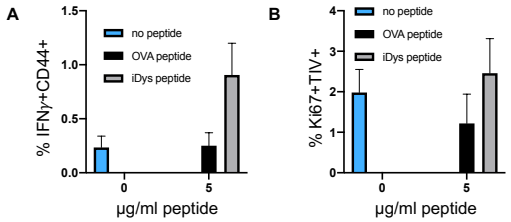


Figure 2. The immunization of mdx mice with a dystrophin peptide induces a dystrophin-specific CD8+ T cell response. Cell proliferation and IFNg production in CD8+ T cells was determined by flow cytometry. (**A**) Quantification of the frequency of IFNg+CD44+ CD8+ T cells in A. (**B**) Representative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

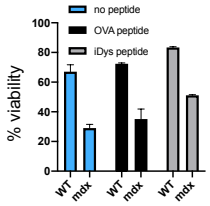


Figure 3. The immunization of mdx mice with a dystrophin peptide induces increased cell death. Cell viability was determined by flow cytometry in mdx or WT splenocytes.

Optimization of adjuvant for dystrophin immunization improves cell viability in mdx mice (Major Task 2)

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 Pertussis toxin (PTX) in a dystrophic setting where inflammatory signals are dysregulated might exacerbate apoptosis. We tested experimental conditions where various formulations of adjuvant were used. We used CD3/CD28 as an activation cocktail to recapitulate the recall antigen assay in this optimization approach. We anticipated improving cell viability and, therefore, dystrophin-specific immune response by modulating the adjuvant. This experiment revealed that CFA is the best adjuvant to preserve cell viability (Fig. 4C) while promoting a robust immune activation (Fig. 4A, B). Previously, we successfully demonstrated that we could induce dystrophin immunity in healthy mice. This approach was promising in dystrophic mice, but decreased cell viability posed a challenge. We suspected the cells to be in an activated state in dystrophic setting. The combination of CFA + mycobacterium and Pertussis toxin (PTX) will drive immune cells hyperactivation and subsequent increased cell death. We optimized a protocol in dystrophic setting that will require the use of CFA as an adjuvant. We found that this protocol caused an expansion of CD44+ effector T cells that produce IFNγ compared to Ova peptide, which was used as a negative control (Fig 5A and 5B). Following our adjustments, the dystrophin specific T cell response in the mdx mouse model is similar to the one observed in WT animals. This could be explained partly by a rescue of the viability (Fig. 6). With this issue resolved, we're now in the process of investigating Tregs' function to modulate dystrophin-specific immune response.

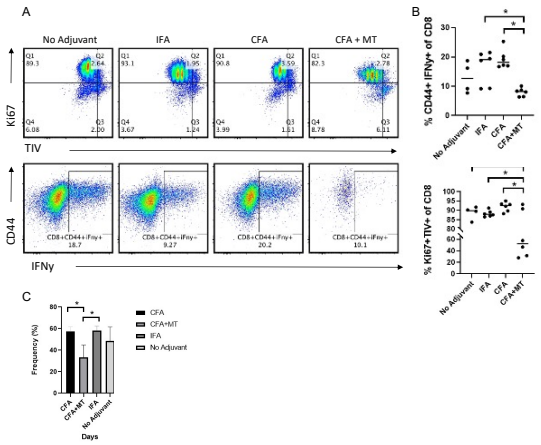


Figure 4. Optimization of adjuvant for dystrophin immunization. 8-wk-old mdx mice were immunized with an emulsion of dystrophin peptide in CFA and pertussis toxin. A pertussis toxin and dystrophin booster was provided on day 2 and 1 week after, respectively. **Recall antigen assay:** mice were euthanized on day 15, spleens were harvested and splenocytes were stimulated *in vitro* with a CD3/CD28 activation cocktail. Cell proliferation and IFNg production in CD8+ T cells was determined by flow cytometry. (**A**) Representative contour plots showing the dilution of Tag-it Violet (TIV) cell proliferation dye and the expression of Ki67 and representative contour plots showing the expression of IFNg and CD44 in CD8+ T cells following CD3/CD28 activation for 3 days. (**B**) Quantification of the frequency of IFNg+CD44+ CD8+ T cells and quantification of the frequency of Ki67+TIV+ CD8+ T cells in C. n= 3 mice per group. in A. (**C**). Quantification of the frequency of live cells n= 4-6 mice per group. *, p< 0.05; significantly different compared to no adjuvant group.

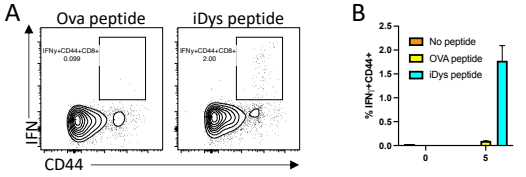
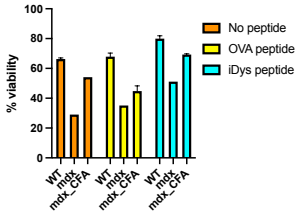
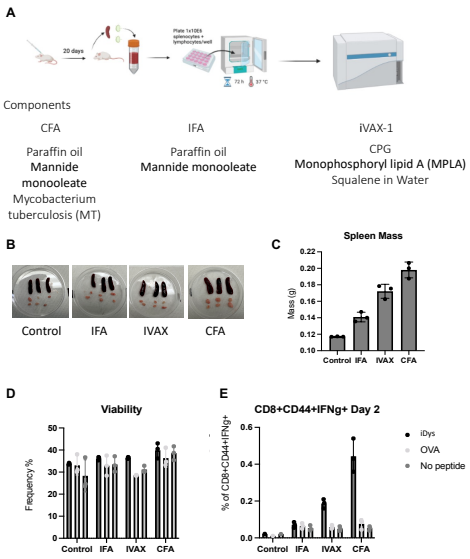


Figure 5. The immunization of mdx mice with a dystrophin peptide induces a dystrophin-specific CD8+ T cell response. Immunization protocol: 8-wk-old C57BL/6 mice (WT) were immunized with an emulsion of dystrophin peptide in CFA and pertussis toxin. A pertussis toxin and dystrophin booster was provided 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 ug/ml) used for immunization or a control peptide (i.e. Ova= ovalbumin, 5 ug/ml). Cell proliferation and IFNg production in CD8+ T cells was determined by flow cytometry. (**A**) Representative contour plots showing the expression of IFNg and CD44 in CD8+ T cells following peptide stimulation for 3 days. (**B**) Quantification of the frequency of IFNg+CD44+ CD8+ T cells in A.



- **Figure 6. The immunization of mdx mice with a dystrophin peptide induces increased cell death.** Cell viability was determined by flow cytometry in WT or mdx (previous protocol) and mdx_CFA (new adjuvant regimen) splenocytes.

Our preliminary results revealed that CFA was the best suitable adjuvant improving cell viability and promoting a robust immune activation. Then we wanted to test whether another adjuvant called IVAX could be more potent. IVAX (Fig.1A) is a squalene-emulsion based agent that induces a strong CD8 response. We compared 3 adjuvants i.e CFA, IFA and IVAX. IVAX and CFA injection led to a significant increase of the spleen mass (Fig 1B, 1C). Despite yielding similar results when investigation cell viability, CFA was the adjuvant generating the highest immune response as illustrated by the increase of CD8+CD44+ IFNg+ cells (Fig 7E). CFA appears as the most appropriate adjuvant to induce dystrophin-specific T cell response. Then, we investigated whether we could optimize our protocol to induce dystrophin-specific T cells in mice.



In vivo validation of the dystrophin-specific T cells induction (Major Task 2)

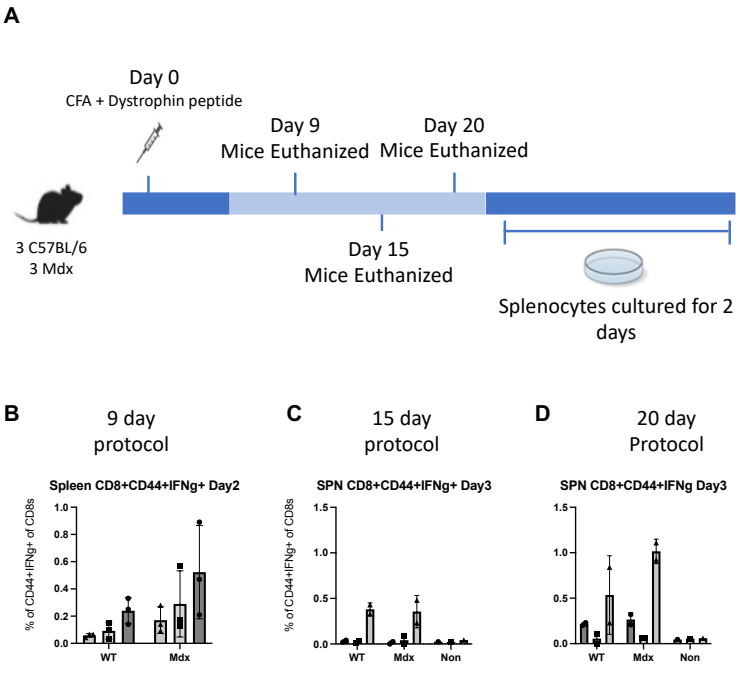


Figure 8: Protocol of immunization (A) with varying immunization time. Quantification of dystrophin-specific CD8+ T cells following 9 (B), 15 (C) and 20 days (D) post immunization.

We successfully identified CFA as the best adjuvant and we hypothesize that optimizing the immunization protocol could improve the immune response toward dystrophin. We established a protocol with 3 different immunization windows i.e. 9, 15 and 20 days. Following each immunization, our recall assay was performed after 2 days of culture (Fig. 8A). For each immunization timeframe, we were able to observe an immune response in mdx (Fig. 8B-D). Interestingly, our 20-day protocol provided a greater response in our mdx mice compared to WT mice (Fig. 8D). We found that this protocol caused consistently an expansion of CD44+ effector T cells that produce IFNγ compared to Ova peptide, which was used as a negative control (Fig 8D). Following our adjustments, the dystrophin specific T cell response in the mdx mouse model is reproducible and be applied to study how to mitigate this dystrophin-specific immune response.

Tregs suppress antigen-specific T cells in dystrophic mice (Major Task 3)

We developed a diphtheria toxin (DT)-inducible Treg depletion mouse model of muscular dystrophy by crossing mdx mice with a transgenic mouse that carries a DT receptor (DTR) transgene inserted in the 3' UTR of the FoxP3 locus (mdx.FoxP3-DTR). The transgene is specifically expressed in FoxP3+Tregs and maintains endogenous FoxP3 expression. We isolated muscle draining lymph nodes following DT treatment and confirmed by flow cytometry that CD4+FoxP3+Tregs were depleted in mdx.FoxP3-DTR mice (DTR+), compared to control (DTR-) mice (Fig.9A and 9B). The depletion of Tregs increased CD44hi CD62Llo effector and memory T cells by 10-fold (Fig. 9C and 9D), indicating that Tregs actively suppress antigen-specific T cells in dystrophic muscle. We will apply this model, discussed above, to splenocytes to validate this hypothesis. We anticipate that depletion of Tregs will cause an increase in dystrophin specific T-cells, indicating that Tregs suppress dystrophin immunity.

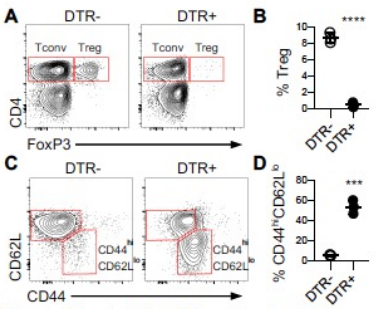


Figure 9. The depletion of Tregs in mdx mice causes in an increase in antigen-experienced T cells. Mdx.FoxP3-DTR mice were treated with diphtheria toxin (DT) every other day over a 9-day period and euthanized on day 10. The frequency of Tregs and antigen-specific T cells in the muscle draining lymph node were quantified by flow cytometry. (**A and B**) Representative contour plots (A) showing the expression of CD4 and FoxP3, and the quantification (B) of Tregs ($CD4^+FoxP3^+$). (**C and D**) Representative contour plots (C) showing the expression of CD44 and CD62L, and the quantification of $CD44^{hi}CD62L^{lo}$ antigen-experienced T cells (D). n= 3 mice per group. ****, p< 0.0001. ***, p< 0.001.

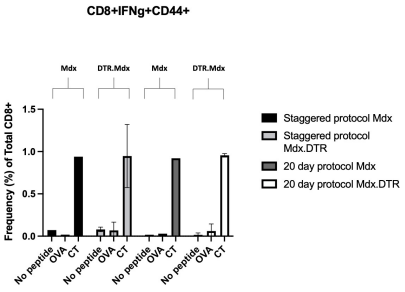


Figure 10 **Dystrophin-specific immune response in Tregs-depleted mdx mice**. Mdx and iTreg-depleted mdx mice were immunized with the iDys peptide and specific immune response was measured by using a recall assay with OVA or iDys (CT)

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.

During this reporting period, our trained student took over and made significant progress notably to validate a reproducible in vivo model to characterize dystrophin-specific immune response. He is admitted to the UCI graduate school program. He is conducting and designing experiments to test a potential immunosuppressive role of Tregs.

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.

Data were presented to local meetings and we are currently generating figures to share with the community the Dys peptide capable of breaking tolerance in dystrophic mice.

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.

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).

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.

Other immunosuppressive mechanisms may compensate for the loss of Tregs. For instance, PD-L1 (B7-H1), a negative costimulatory molecule that inhibits the activation of effector and memory T cells, is expressed in skeletal muscle. If Tregs depletion does not increase the number of dystrophin specific T cells, we will repeat it in the presence of isotype control antibody or a PD-L1-blocking antibody. If an increase in dystrophin immunity is seen with PD-L1 blockade, we will define what cells express PD-L1, to determine the mechanism of PD-L1-dependent inhibition of dystrophin immunity. The other potential caveat is the emergence of neutralizing antibodies against the DT injected to induce Tregs depletion

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).*

  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.

- **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: **Philip Farahat**
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12
Contribution to Project: Philip contributed to establish a consistent in vivo induction model. He has therefore designed an immunization protocol yielding the best results. He also initiated the Treg depletion studies and he is now addressing the issues mentioned above
Funding Support: Philip 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: The PI analyzed the data to generate the results and the figures. In addition, the PI provided some training regarding the Tregs depletion studies
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.