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

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

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REPORT DATE: **July 2021**

TYPE OF REPORT: **Annual**

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
July 2021	annual report	01Jul2020-30Jun2021

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)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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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)*

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1. INTRODUCTION:

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:

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

3. ACCOMPLISHMENTS:

What were the major goals of the project?

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?

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. 1C) while promoting a robust immune activation (Fig. 1A, 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 2A and 2B). 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. 3). With this issue resolved, we're now in the process of investigating Tregs' function to modulate dystrophin-specific immune response.

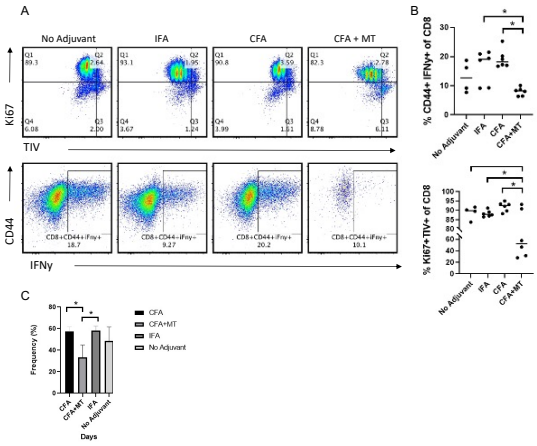


Figure 4. Optimization of adjuvant for dystrophin immunization. 8-wk-old mdx mice were immunized with an emulsion of dystrophin peptide in CFA and pertussis toxin. A pertussis toxin and dystrophin booster was provided on day 2 and 1 week after, respectively. **Recall antigen assay:** mice were euthanized 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.

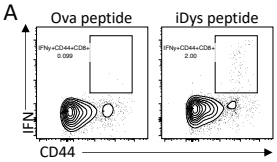
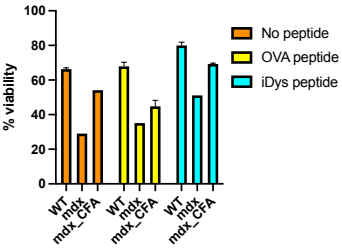


Figure 1. The immunization of mdx mice with a dystrophin peptide induces a dystrophin-specific CD8+ T cell response. Immunization protocol: 8-wk-old C57BL/6 mice (WT) were immunized with an emulsion of dystrophin peptide in CFA and pertussis toxin. A pertussis toxin and dystrophin booster was provided 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 2. 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.

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.4A and 4B). The depletion of Tregs increased CD44hi CD62Llo effector and memory T cells by 10-fold (Fig. 4C and 4D), 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. We are currently performing Tregs depletion combined to dystrophin immunization. Our first attempt failed due to an unfortunate bacterial contamination. In addition, we plan on using a Tregs expansion system to show that increased number of Tregs will contribute to reduce dystrophin-specific T cells.

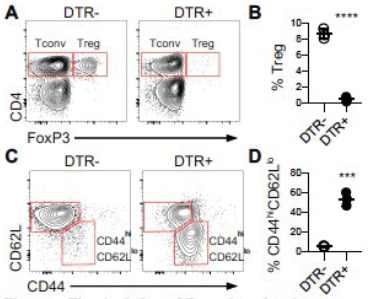


Figure 4 The depletion of Tregs in mdx mice causes in an increase in antigen-experienced T cells. Mdx.FoxP3-DTR mice were treated with diphtheria toxin (DT) every other day over a 9-day period and euthanized on day 10. The frequency of Tregs and antigen-specific T cells in the muscle draining lymph node 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.

What opportunities for training and professional development has the project provided?

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 has trained a new lab member to the immunization techniques and the necessary analysis to evaluate the dystrophin T cell immune response. He has made substantial progress and will soon enter the UCI graduate school program.

How were the results disseminated to communities of interest?

Results were only discussed during internal meetings. We are making good progress and we foresee a publication of this work for the beginning of next year.

What do you plan to do during the next reporting period to accomplish the goals?

We successfully demonstrated than we could improve cell viability by selecting the CFA adjuvant (Fig 1). This optimization enhances dystrophin immunity in dystrophic mice (Fig. 2) and reduces cell death (Fig. 3). With this promising approach, we are in a good position to elucidate the immunosuppressive function of Tregs toward dystrophin immunity. We are mastering the Tregs depletion technique (Fig. 4) that will allow us to test the hypothesis. We've already started some trials and in the first months of the next reporting period, we will assess the dystrophin specific T cell response when you deplete Tregs. Conversely, in the final months of the award, we hope to evaluate this response when you expand Tregs. Last, we are constantly trying to improve our dystrophin immunity mouse model. One area of improvement is the number of dystrophin specific T cells. To do that, we will test novel adjuvants that do not use emulsifying agents but use STING ligands to selectively prime Th1 cells. We do have all the reagents and the mice readily available in the lab to complete the task.

4. IMPACT:

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

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?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report..

5. CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to report..

Actual or anticipated problems or delays 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. Last, we are constantly trying to improve our dystrophin immunity mouse model. One area of improvement is the number of dystrophin specific T cells. To do that, we will test novel adjuvants that do not use emulsifying agents but use STING ligands to selectively prime Th1 cells.

Changes that had a significant impact on expenditures

Nothing to report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

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:

- **Publications, conference papers, and presentations**

  Journal publications.

  Books or other non-periodical, one-time publications.

Other publications, conference papers and presentations.

- **Website(s) or other Internet site(s)**

- **Technologies or techniques**

- **Inventions, patent applications, and/or licenses**

- **Other Products**

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name: **Philip Farahat**
Project Role: Research technician
Researcher Identifier (e.g. ORCID ID):
Nearest person month worked: 12
Contribution to Project: Philip contributed to define the best adjuvant to optimize a specific dystrophin immune response. He has performed the immunization work and the flow cytometer work on WT and mdx mice. He is currently conducting the Tregs depletion experiment to assess the role of Tregs in suppressing dystrophin immunity.
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 frominstitutional 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?

Nothing to report.

What other organizations were involved as partners?

Nothing to report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

QUAD CHARTS:

9. APPENDICES: