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TITLE: Peptidylarginine Deiminase 2 and Citrullination of IgG in Immunity and Rheumatoid Arthritis

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14. ABSTRACT

The purpose of this application is to identify mechanisms by which peptidylarginine deiminases (PADs) and citrullination regulate antibodies in immunity and rheumatoid arthritis. To this end, this project will (1) determine how PADs and IgG citrullination regulate a normal antibody response to immunization and normal antibody-based immunity to influenza, (2) identify how PADs and citrullinated IgG pathologically contribute to rheumatoid arthritis, and (3) determine if smoking increases IgG citrullination leading to autoimmune antibodies in genetically susceptible people. In the first year of this award, all administrative protocols were approved and experiments were initiated and are progressing well. No sets of experiments have been completed and there are no findings to report and discuss at this time.

15. SUBJECT TERMS
Rheumatoid arthritis, Antibodies, Anti-citrullinated protein antibodies, Rheumatoid factor, Citrullination, Peptidylarginine Deiminase 2

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1. INTRODUCTION:

Rheumatoid arthritis, an autoimmune arthritis with a lifetime risk of about 3 percent, can lead to pain, disability, and early mortality despite lifelong treatment. Moreover, many of the treatments are unpleasant to inject and extremely costly. With about 1 percent of Veterans affected by rheumatoid arthritis often costing more than $15,000 per year, this is a major problem. Many people with rheumatoid arthritis produce antibodies against immunoglobulin (Ig) G, called rheumatoid factor (RF), and anti-citrullinated protein antibodies (ACPAs). These autoantibodies underpin the main diagnostic tests for rheumatoid arthritis. Unfortunately, about 25% of rheumatoid arthritis patients are seronegative for these tests, which delays diagnosis and treatment. In addition to these clinical dilemmas in rheumatoid arthritis, important pathophysiologic mysteries remain. Despite decades of research on ACPAs and RF, why these two different types of autoantibodies develop or why immune tolerance is broken against IgG is unknown. Further, the peptidylarginine deiminases (PADs) catalyze citrullination, the post-translational conversion of arginines to citrullines, and PAD2 and PAD4 are found in immune cells. However, our understanding of how citrullination and PADs regulate immunity and arthritis beyond simply generating the targets for ACPAs is rudimentary at best. Identifying the mechanisms by which the PADs and citrullination impact the immune system is critical to define fundamental pathways in immunity and aberrant pathways in rheumatoid arthritis. Moreover, gaps in our understanding of pathophysiology hinder the development of optimal diagnostics and treatments. The objective of this application is to identify mechanisms by which PADs and citrullination regulate antibodies in immunity and rheumatoid arthritis. The central hypothesis is that PAD2 regulates antibody-secreting plasma cells and citrullinates IgG, enhancing immunity and exacerbating rheumatoid arthritis. To test this hypothesis, Aim 1 will determine how PADs and IgG citrullination regulate a normal antibody response to immunization and normal antibody-based immunity to influenza. Aim 2 will identify how PADs and citrullinated IgG pathologically contribute to rheumatoid arthritis as well as determine how smoking, a major problem among Veterans, may increase IgG citrullination leading to autoimmune antibodies in genetically susceptible people. The successful completion of these Aims, in the short term, will establish a new mechanistic basis for how PAD2 and IgG citrullination regulate immunity and drive inflammation through immune cell function and citrullinated antigen generation. In the long term, these advances will usher in new translational opportunities to innovate diagnostics incorporating novel autoantibodies and therapeutics targeting the PADs ultimately to allow for faster diagnosis and more effective treatment of rheumatoid arthritis.

2. KEYWORDS:

Rheumatoid arthritis
Antibodies
Anti-citrullinated protein antibodies
Rheumatoid factor
Citrullination
Peptidylarginine Deiminase 2

3. ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Obtain appropriate approvals
- Target completion Date: January 31, 2019
- Completed: April 19, 2019

Major Task 2: Determine which arginines in IgG are citrullinated by PAD2 to extend IgG half-life.
- Target completion Date: September 29, 2021
- Percent Completed: 15%

Major Task 3: Determine how PAD2 regulates plasma cell numbers.

- Target completion Date: January 31, 2021
- Percent Completed: 15%

Major Task 4: Define the role of PAD2 in antibody-based immunity to influenza.
- Target completion Date: September 29, 2021
- Percent Completed: 15%

Major Task 5: Define the pathologic role of PAD2 in autoantibody-dependent inflammatory arthritis.
- Target completion Date: September 29, 2021
- Percent Completed: 15%

Major Task 6: Determine how anti-citrullinated IgG antibodies develop including the role of smoking and HLA variants.
- Target completion Date: September 29, 2021
- Percent Completed: 5%

Major Task 7: Prepare/publish manuscripts
- Target completion Date: September 29, 2021
- Percent Completed: 10%

What was accomplished under these goals?

Major Task 1:
1. Specific Objectives: Obtain appropriate approvals
2. Major activities: ACURO protocol submitted and approved. HRPO protocol submitted and approved.
3. Significant results: All protocols approved.
4. Other achievements: None
5. Goals not met: None

Major Task 2:
1. Specific Objectives: Determine which arginines in IgG are citrullinated by PAD2 to extend IgG half-life.
2. Major activities: Mass spectrometry optimization and identification of some arginines and citrullines of the 4 murine isotypes of IgG. Also, IgG from PAD2 WT and KO mice was purified with protein G, citrullinated *in vitro* and transferred to several pairs of mice with serum collection for half-life experiments.
3. Significant results: Several citrullines were identified, but data sets and analyses are not complete.
4. Other achievements: None
5. Goals not met: Mass spectrometry experiments to identify arginines and citrullines of murine IgG were not completed in the predicted timeframe. Dr Coon and Dr Hebert identified some citrullines, but had technical difficulties so that they were unable to identify all arginines and citrullines. They recommended that we work with Dr Lingjun Li (Professor of Pharmacy, University of Wisconsin) and her trainees who have recently created new methodology to enhance identification of citrullination. Dr Lingjun Li and her trainees will perform some pilot experiments in their lab related to this project and, if successful, they will join the study team.

Major Task 3:
1. Specific Objectives: Determine how PAD2 regulates plasma cell numbers.
2. Major activities: Backcrossing to the C57BL/6 background was completed. Flow cytometry was optimized and experiments to determine why plasma cells are reduced in PAD2-/- mice were initiated. To complement the flow cytometry experiments, ELISpot and limiting dilution assays were optimized and initiated.
3. Significant results: None
4. Other achievements: None
5. Goals not met: None

Major Task 4:
1. Specific Objectives: Define the role of PAD2 in antibody-based immunity to influenza.
2. Major activities: Dosing of influenza was optimized for DBA1/J background mice, which we discovered were much more sensitive to influenza than would be expected from the published studies on C57BL/6

mice. Also, the first set of PAD2 WT/KO mice were infected with influenza and challenged. Serum was collected. HI assay and HA ELISA protocols were optimized.
3. Significant results: None.
4. Other achievements: None.
5. Goals not met: None.

Major Task 5:

1. Specific Objectives: Define the pathologic role of PAD2 in autoantibody-dependent inflammatory arthritis.
2. Major activities: Collagen induced arthritis was induced in several pairs of mice and arthritis is being scored and serum/tissue collected. Anti-collagen ELISA, ELISpot, flow cytometry assays, and a complementary limiting dilution assay were optimized.
3. Significant results: None.
4. Other achievements: None.
5. Goals not met: None.

Major Task 6:

1. Specific Objectives: Determine how anti-citrullinated IgG antibodies develop including the role for smoking and HLA variants.
2. Major activities: Mass spectrometry was initiated and is being optimized as discussed above. Experiments to identify anti-IgG antibodies in clinical subsets have been initiated.
3. Significant results: None.
4. Other achievements: None.
5. Goals not met: HLA typing has not been completed.

Major Task 7:

1. Specific Objectives: Publish manuscripts
2. Major activities:
    a. An invited editorial was written and accepted for publication that includes many of Dr. Shelef's theories described in the grant application that led to the experiments in this project.
    b. A manuscript that contains a small amount of data funded by this award related to anti-citrullinated IgG antibodies was accepted for publication.
3. Significant results: Publication
4. Other achievements: None

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

Aisha Mergaert and Michael Denny received one-on-one mentoring and have attended seminars at the UW. Aisha Mergaert attended the American College of Rheumatology annual meeting.

How were the results disseminated to communities of interest?

Nothing to report.

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

Major Task 1: Completed

- Nothing to do.

Major Task 2: Determine which arginines in IgG are citrullinated by PAD2 to extend IgG half-life.

- Optimize mass spectrometry and identify citrullines in IgG (likely with Dr Lingjun Li and her trainees).
- IgG transfer experiments will continue to determine if PAD2 is required for IgG half-life.
- Once all citrullines are identified and IgG half-life studies are completed to ensure a requirement for PAD2 in IgG half-life, mutation studies will begin.

Major Task 3: Determine how PAD2 regulates plasma cell numbers.

- Murine plasma cell studies will continue.
- Breeding will continue for B cell studies.

Major Task 4: Define the role of PAD2 in antibody-based immunity to influenza.

- More mice will be infected with influenza and re-challenged. Serum will be collected and plaque assays initiated.
- HI assays and HA ELISA will be performed to quantify antibody response to flu in PAD2-/- and PAD2+/+ mice.
- Serum transfer experiments will be initiated.

Major Task 5: Define the pathologic role of PAD2 in autoantibody-dependent inflammatory arthritis.

- More PAD2-/- and PAD2+/+ mice will undergo arthritis induction, scoring, and serum/tissue collection
- ELISA will be performed to quantify anti-collagen antibody levels in PAD2-/- and PAD2+/+ mice with arthritis.
- Flow cytometry, limiting dilution assay, and ELISpot will be used to evaluate plasma cells in PAD2-/- and PAD2+/+ mice with arthritis.
- IgG will be evaluated by mass spectrometry.

Major Task 6: Determine how anti-citrullinated IgG antibodies develop including the role for smoking and HLA variants.

- Identify citrullines in purified IgG from clinical subsets by mass spectrometry
- Identify anti-IgG antibodies in clinical subsets
- Determine which HLA types correlate with anti-citrullinated IgG antibodies
- Determine which IgG peptides bind which HLA types

Major Task 7: Prepare manuscripts

- No tasks anticipated in the next year.

4. IMPACT:

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

Nothing to Report. All data is still preliminary.

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

There were no significant changes in objectives or scope.

Actual or anticipated problems or delays and actions or plans to resolve them

Major Task 1: Obtain appropriate approvals
- **Problem/Delay:** HRPO protocol was not approved by the projected time.
- **Action/Plan:** Protocol was ultimately approved and work has been initiated, but some time was lost.

Major Task 2: Determine which arginines in IgG are citrullinated by PAD2 to extend IgG half-life.
- **Problem/delay:** Mass spectrometry experiments to identify arginines and citrullines of murine IgG were not completed. Dr Coon and Dr Hebert identified some citrullines, but had technical difficulties so that they were unable to identify all arginines and citrullines.
- **Action/Plan:** Dr Coon and Dr Hebert recommended that they no longer participate in the project and that Dr Lingjun Li and her trainee (University of Wisconsin) replace them. Dr Li's team has recently created new methodology to enhance identification of citrullination that is being prepared for publication. This methodology involves using a probe to bind to citrulline to increase the molecular mass enough to more easily identify citrullines by mass spectrometry. Dr Li's group will perform some pilot experiments in their lab related to this project and, if successful, they will join the study team. If these experiments are unsuccessful, we will contact Dr Eranthie Weerapana (Boston College), with whom we have published in the past, about collaborating on this project given her expertise in detecting citrullines by mass spectrometry.

Major Task 3: Determine how PAD2 regulates plasma cell numbers.
- **Problem/delay:** None
- **Action/Plan:** N/A

Major Task 4: Define the role of PAD2 in antibody-based immunity to influenza.
- **Problem/delay:** None
- **Action/Plan:** N/A

Major Task 5: Define the pathologic role of PAD2 in autoantibody-dependent inflammatory arthritis.
- **Problem/delay:** None
- **Action/Plan:** N/A

Major Task 6: Determine how anti-citrullinated IgG antibodies develop including the role for smoking and HLA variants.
- **Problem/Delay:** HRPO protocol was not approved by the projected time.
- **Action/Plan:** Work has been initiated, but some time was lost.
- **Problem/Delay:** Challenges with mass spectrometry (as above)
- **Action/Plan:** As above.
- **Problem/Delay:** Roche dissolved Nimblegen, the portion of the company that offers the array service that would allow us to identify anti-IgG antibodies in clinical subsets by peptide array.
- **Action/Plan:**
    - Prior to Nimblegen dissolving, we fortuitously and previously unexpectedly were able to include IgG-derived peptides into a different array for projects unrelated to this CDMRP award that broadly evaluate antibody binding in lupus and Sjogren's Syndrome. By doing so, we have some data (less than proposed in this award) that allows us to design a library of peptides to dissect the binding patterns of antibodies against IgG in rheumatoid arthritis vs lupus by ELISA, a second method originally proposed to characterize anti-IgG antibodies in this award. Thus, we have expanded the ELISA portion of this Task and have focused on ELISA instead of array at least for the immediate future. Due to the large number of ELISA experiments, additional personnel may be required to accomplish this task.

    - A new company called Nimble Therapeutics has been created, which may be able to offer the original array services. We will continue to follow developments at Nimble Therapeutics to evaluate this option.
- **Problem/Delay:** HLA typing has not been completed due to the late approval of the HRPO protocol.
- **Action/Plan:** HLA typing will be completed.

Major Task 7: Prepare/publish manuscripts
- No delays

Changes that had a significant impact on expenditures

The HRPO protocol was not approved by the projected time. Thus, all human subjects work was delayed creating fewer expenditures in year 1. These expenditures are expected to be made in subsequent years and thus total expenditures are expected to be unchanged over the whole period of the grant.

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

No significant changes were made.

Significant changes in use or care of vertebrate animals

No significant changes were made.

Significant changes in use of biohazards and/or select agents

No significant changes.

6. PRODUCTS:

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

Journal publications.

1. **Shelef MA.** New Relationships for Old Autoantibodies in Rheumatoid Arthritis. Arthritis & Rheumatology. 2019 Sep;71(9):1396-1399. Federal support acknowledged.

2. Zheng Z, Mergaert AM, Fahmy L, Bawadekar M, Holmes CL, Ong I, Bridges AJ, Newton MA, **Shelef MA.** Disordered antigens and epitope overlap between anti-citrullinated protein antibodies and rheumatoid factor in rheumatoid arthritis. Arthritis and Rheumatology. Accepted. Federal support acknowledged.

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

None

Other publications, conference papers and presentations.

None.

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

  None.

- **Technologies or techniques**

  None. All are still to preliminary to share.

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

  None.

- **Other Products**

  None.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Name:	*Miriam Shelef*
Project Role:	*PI*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*4*
Contribution to Project:	*Dr Shelef has been leading the projects, working on all protocols, coordinating all scientists, reviewing all ongoing experiments, participating in data analysis, and writing manuscripts.*
Funding Support:	*NIH, Doris Duke Charitable Foundation, UW-Madison*

Name:	*Marulasiddappa Suresh*
Project Role:	*Co-Investigator*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*<1*
Contribution to Project:	*Once ACURO approval was obtained, Dr Suresh oversaw, guided, and optimized influenza infection experiments.*
Funding Support:	*NIH, UW-Madison*

Name:	*Aisha Mergaert*
Project Role:	*Graduate Student (replaced proposed postdoctoral fellow)*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*8*
Contribution to Project:	*Ms. Mergaert has been inducing CIA and performing related experiments, infecting mice with flu and performing related experiments, working on serum transfer experiments, performing*

	plasma cell experiments, and working on anti-IgG ELISA experiments.
Funding Support:	*UW-Madison*

Name:	*Michael Denny*
Project Role:	*Scientist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*12*
Contribution to Project:	*Dr. Denny has been working on purifying IgG for mass spec as well as optimizing protocols for ELISA and limiting dilutions assays as well as working on anti-IgG ELISA experiments.*
Funding Support:	*None*

Name:	*Alex Hebert*
Project Role:	*Assistant Scientist*
Researcher Identifier (e.g. ORCID ID):	*N/A*
Nearest person month worked:	*3*
Contribution to Project:	*Dr. Hebert has been performing and analyzing mass spec experiments.*
Funding Support:	*UW-Madison*

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Miriam Shelef: NIH NIAMS K08 AR065500 and the Clinical Scientist Development Award from the Doris Duke Charitable Foundation ended.

What other organizations were involved as partners?

Nothing to Report.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

N/A

QUAD CHARTS:

Quad chart and generic award chart are included.

APPENDICES:

Publications are included.