

AWARD NUMBER: W81XWH-19-1-0540

TITLE: Ex Vivo-Generated Autologous iTregs as a Cell-Based Therapy for Acquired Aplastic Anemia

PRINCIPAL INVESTIGATOR: Lisa M. Minter, PhD

CONTRACTING ORGANIZATION: University of Massachusetts Amherst, Amherst, MA 01003

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14. ABSTRACT

Patients with acquired AA show aberrant activation of T effector (Teff) cells and naturally occurring regulatory T cells (nTregs) that are frequently dysfunctional. Immunosuppressive therapies that use Tregs, rely on isolating and expanding rare populations of nTregs from the circulating blood. nTregs with *in vitro* suppressive functions have been successfully expanded from AA patients. However, the extremely low numbers of circulating nTregs, coupled with the long process (up to several months) of expanding these cells in culture, underscores the practical challenges of this approach for AA patients. We have developed a means of using synthetic cell-penetrating peptide mimics (CPPM) to deliver functional antibodies into human CD4 T cells. Delivering anti-pPKC θ into CD4 T cells generates iTregs (anti-pPKC θ -iTregs) with superior *in vitro* and *in vivo* suppressive functions and, in proof-of-concept experiments, provide a significant survival benefit in a humanized mouse model of AA, when given at the time of BMF induction. We determined that inhibiting pPKC θ or the protein repair methyltransferase, PCMT1, alters the cellular location and protein-protein association of key iTreg-destabilizing proteins, increases surface expression of the immune-inhibitory receptor, PD1, and increases demethylation the *FOXP3* promoter. We conclude that these alterations contribute to a more suppressive iTreg phenotype in vitro and in vivo.

15. SUBJECT TERMS

Aplastic anemia; immune-mediated bone marrow failure; intracellular antibody delivery; CPPM; ex vivo-generated iTregs; PKC θ ; PCMT1; cell-based therapy

16. SECURITY CLASSIFICATION OF:

a. REPORT	b. ABSTRACT	c. THIS PAGE
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17. LIMITATION OF ABSTRACT

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USAMRDC

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

Patients with acquired AA show aberrant activation of T effector (Teff) cells and naturally occurring regulatory T cells (nTregs) that are frequently dysfunctional. Immunosuppressive therapies that use Tregs, rely on isolating and expanding rare populations of nTregs from the circulating blood. nTregs with *in vitro* suppressive functions have been successfully expanded from AA patients. However, the extremely low numbers of circulating nTregs, coupled with the long process (up to several months) of expanding these cells in culture, underscores the practical challenges of this approach for AA patients. We have developed a means of using synthetic cell-penetrating peptide mimics (CPPM) to deliver functional antibodies into human CD4 T cells. Delivering anti-pPKC θ into CD4 T cells generates iTregs (anti-pPKC θ -iTregs) with superior *in vitro* and *in vivo* suppressive functions and, in proof-of-concept experiments, provide a significant survival benefit in a humanized mouse model of AA, when given at the time of BMF induction. We will investigate CPPM-antibody delivery as a therapeutic option to generate AA patient-derived iTregs, *ex vivo*. We will use a humanized mouse model of AA, optimized in our lab, *to test the hypothesis that combined delivery of anti-pPKC θ and anti-PCMT1 will generate iTregs with a “locked” phenotype*, refractory to the inhibitory actions of CsA *in vitro* and *in vivo*, and ask whether administering autologous anti-pPKC θ +anti-PCMT1-iTregs attenuates disease in a patient “avatar” model of AA.

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

Aplastic anemia; immune-mediated bone marrow failure; intracellular antibody delivery; CPPM; *ex vivo*-generated iTregs; PKC θ ; PCMT1; cell-based therapy

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 1 Determine how inhibiting pPKCθ, PCMT1, or both, using CPPM:antibody delivery influences <i>ex vivo</i> iTreg differentiation	Time line	Site 1	Site 2
Major Task 1 Generate and characterize iTregs differentiated <i>ex vivo</i> under various conditions of CPPM:antibody delivery, with or without the addition of CsA or Rapamycin			
Subtask 1 HRPO approval for use of commercially available healthy donor PBMCs	1-4	Dr. Minter 100% COMPLETED	
Subtask 2 Local IACUC approval	1-3	Dr. Minter 100% COMPLETED	
Subtask 3 ACURO protocol approval	4-6	Dr. Minter 100% COMPLETED	
Subtask 4 Produce synthetic cell penetrating peptide mimics (CPPMs)	1-18		Dr. Tew 100% COMPLETED
Subtask 5 Complex CPPMs with: IgG, anti-pPKC θ , anti-PCMT1	1-18		Dr. Tew 100% COMPLETED
Subtask 6 Differentiate iTregs in the presence of: CPPM:IgG, CPPM:anti-pPKC θ , CPPM:anti-PCMT1	3-9	Dr. Minter 100% COMPLETED	
Subtask 7 Characterize iTregs differentiated in the presence of: CPPM:IgG, CPPM:anti-pPKC θ , CPPM:anti-PCMT1 (phenotype; suppression assays; FOXP3 methylation)	3-9	Dr. Minter ONGOING 100% COMPLETED	
Subtask 8 Characterize iTregs differentiated in the presence of: CPPM:IgG, CPPM:anti-pPKC θ , CPPM:anti-PCMT1 with CsA or Rapamycin added (phenotype; suppression assays; FOXP3 methylation)	3-12	Dr. Minter ONGOING 90% COMPLETE	

Major Task 2 Test <i>in vivo</i> efficacy of iTregs using a humanized mouse model of AA with or without co-administration of CsA or Rapamycin			
Subtask 1 Test <i>in vivo</i> efficacy of iTregs using a humanized mouse model of aplastic anemia, with or without co-administration of CsA or Rapamycin	6-15	Dr. Minter ONGOING (50% COMPLETED)	
Specific Aim 2 Develop AA patient “avatar” mice and test patient-derived CPPM:antibody iTregs	Timeline	Site 1	Site 2
Major Task 3 Differentiate AA patient iTregs using the most effective combination(s) of CPPM:antibody and immunosuppressant (CsA or Rapamycin) as determined in Aim 1			
Subtask 1 Submit research proposal to NMDP for review and approval	1-4	Dr. Minter COMPLETED	
Subtask 2 Characterize AA patient iTregs differentiated under the most effective conditions, as determined in Aim1 (phenotype; suppression assays; FOXP3 methylation)	4-12	Dr. Minter ONGOING (20% COMPLETED)	
Major Task 4 Create “avatar” mice using AA patient samples and treat “avatar” mice with autologous iTregs			
Subtask 1 Create AA patient “avatar mice”	12-18	Dr. Minter ONGOING (10% COMPLETED)	
Subtask 2 Create AA patient “avatar mice” and treat with autologous patient iTregs, under conditions optimized in under Major Task 2, Subtask 1, above	12-18	Dr. Minter	
Milestone 1 Collate data, prepare and submit scientific manuscript	18-24	Dr. Minter	Dr. Tew

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.

1. Major activities:

- a) Use CPPMs to deliver anti-pPKC θ , anti-PCMT1, and combined anti-pPKC θ -anti-PCMT1 to iTregs to determine if there are differences in iTreg suppressive function, based on antibody target or combination antibody delivery, compared to delivering irrelevant IgG.
- b) Use CPPMs to deliver anti-pPKC θ , anti-PCMT1, and combined anti-pPKC θ -anti-PCMT1 to iTregs to in the presence of cyclosporine or rapamycin, to determine how these immunosuppressive drugs affect iTreg differentiation *in vitro*, compared to delivering irrelevant IgG.
- c) Evaluate the effects of CPPM:antibody delivery on methylation of the *FOXP3* promoter
- d) Establish baseline data for optimized humanized mouse model of bone marrow failure
- e) Begin *in vitro* and *in vivo* experiments using patient samples.

2. Specific objectives:

- a) Complete evaluating the effects of differentiating iTregs in the presence of CPPM-IgG, CPPM-anti-pPKC θ , CPPM-anti-PCMT1, combined CPPM-anti-pPKC θ +CPPM-anti-PCMT1
- b) Complete evaluating the suppressive capacity of iTregs differentiated in the presence of CPPM-IgG, CPPM-anti-pPKC θ , CPPM-anti-PCMT1, combined CPPM-anti-pPKC θ +CPPM-anti-PCMT1
- c) Complete evaluating the effects of differentiating iTregs in the presence of CPPM-IgG, CPPM-anti-pPKC θ , CPPM-anti-PCMT1, combined CPPM-anti-pPKC θ +CPPM-anti-PCMT1, when exposed to low (40 ng/ml) and high (400 ng/ml) doses of cyclosporine A.
- d) Complete evaluating the effects of different antibody delivery conditions on methylation of the TSDR region of the *FOXP3* promoter.
- e) Begin *in vitro* experiments using PBMCs obtained from treatment-naïve patients diagnosed with Aplastic Anemia.
- f) Begin *in vivo* experiments using PBMCs obtained from treatment-naïve patients diagnosed with Aplastic Anemia.

3. Significant Results (See Appendix A for data, methodologies, and discussion):

- a) Differentiating CD4 T cells with anti-pPKC θ , anti-PCMT1, and combination anti-pPKC θ +anti-PCMT1 generates higher percentages of CD4+CD25+CD127-FOXP3+ iTregs and greater FOXP3+ expression than those differentiated with an irrelevant IgG (Figure 2). Delivering a combination of anti-pPKC θ + anti-PCMT1 resulted in the highest expression of FOXP3 among CD4+CD25+CD127- iTregs (data not shown).
- b) Differentiating CD4 T cells with anti-pPKC θ , anti-PCMT1, and combination anti-pPKC θ +anti-PCMT1 increases their suppressive capacity, compared to those differentiated with an irrelevant IgG (Figure 1). Consistent with the results above, delivering a combination of anti-pPKC θ + anti-PCMT1 resulted in the highest suppressive capacity for iTregs as determined in a standard suppression assay (Figure 1).

c) Complete data sets show low dose (40ng/ml) CsA exposure enhances iTreg differentiation. Using CPPM to deliver anti-pPKC θ , anti-PCMT1, or combination anti-pPKC θ +anti-PCMT1 protects FOXP3 expression in iTregs exposed to highdoses of (400 ng/ml) CsA (data not shown). However, CsA shows cytotoxicity at low (40ng/ml) and high (400ng/ml) concentrations, *in vitro*. Differentiating CD4 T cells using CPPM to deliver anti-pPKC θ , anti-PCMT1, or combination anti-pPKC θ +anti-PCMT1 does not increase FOXP3 expression above that of CPPM-IgG delivered iTregs. This is likely due to the presence of rapamycin in the iTreg differentiation kit. Cell viability for iTregs differentiated in the presence of additional rapamycin was high (data not shown).

d) Completed analysis of the methylation status of the TSDR region of the *FOXP3* promoter shows that in iTregs differentiated under different CPPM:antibody delivery conditions, delivering combination anti-pPKC θ +anti-PCMT1 increases the degree of demethylation of the TSDR region, both in unsorted populations of differentiated iTregs and especially in purified iTreg populations. Our data show that, following combination antibody delivery, the third CpG island of the TSDR is the most highly demethylated. Of note, this CpG island is found within an NF- κ B binding site, which has been shown to directly regulate *FOXP3* expression (Long et al., [2009] *Immunity*, 31, 921-931; Figure 2).

e) Initial experiments using CD4 T cells purified from archived samples of PBMCs from treatment-naïve patients diagnosed with Aplastic Anemia reveal extensive variation in iTreg differentiation potential. After culturing for 7 days in iTreg differentiation media showed that some patient samples showed high percentages of CD4+CD25+FOXP3 positive iTregs, while other samples showed an almost anergic phenotype, failing to upregulate CD4, CD25, or FOXP3, among the live cell population analyzed (Figure 3).

f) Together with the findings outlined in (e) above, we have learned that the cell concentration listed on the patient samples is unreliable, regarding the number of viable cells that can be recovered upon thawing. Although patient PBMCs were frozen back at concentrations of 5×10^6 cells/vial, cell recovery varied greatly, leaving us unable to use some samples to create “avatar” mice. This, coupled with the discovery that some patient samples completely failed to respond/expand in culture helps to explain why, of the 8 “avatar” mice that were reconstituted with patient PBMCs, none exhibited signs of disease as late as 35 days post-induction. (Figure 4). We have devised a plan to pre-screen samples used for generating “avatar” mice and for use in *in vitro* antibody delivery experiments.

Other Achievements:

Nothing to report.

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.

An animal technician, hired with separate funds, has been trained to induce disease in our humanized mouse model of bone marrow failure. He has established baseline data for the humanized BMF model and has begun to generate “avatar” mice.

How were the results disseminated to communities of interest?

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

Describe how the results were disseminated to communities of interest. Include any outreach activities that were undertaken to reach members of communities who are not usually aware of these project activities, for the purpose of enhancing public understanding and increasing interest in learning and careers in science, technology, and the humanities

Nothing to report.

Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives. If this is the final report, state “Nothing to Report.”

Plans are in place to:

1. Characterize patient iTregs differentiated in the presence of CPPM:anti-pPCK0, CPPM:anti-PCMT1, or CPPM:anti-pPKC0+anti-PCMT1.
2. Perform *in vivo* therapeutic experiments using a humanized model of bone marrow failure.
3. Create avatar mice and treat with patient iTregs.

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).

Nothing to report.

What was the impact on other disciplines?

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

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.

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.

4. 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.

There are no significant changes in the proposal 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.

Actual problems and actions taken:

DELAY IN OBTAINING ARCHIVED PATIENT SAMPLES FOR RESEARCH

Archived patient samples, to be supplied by the National Marrow Donor Program (NMDP), were requested in August 2020. Due to pandemic related reductions in staffing, backlog in workload, and staffing turn-over at the NMDP, coupled with corresponding delays at UMass Amherst, including mandatory staff furloughs and difficulties the UMass Amherst Procurement System has encountered classifying the NMDP as a recognized vendor, patient samples have not yet been made available for research purposes.

ACTIONS TAKEN:

Since August 2020, Minter Lab has been in constant correspondence with staff at the NMDP, as well as with departmental business office staff, to complete the steps necessary to obtain a purchase order to acquire the archived patient samples needed to complete experiments outlined in the SOW. The approval for this purchase order was finally processed for payment through the UMass Amherst Procurement System. Patient samples have been acquired and experiments using these materials are ongoing.

UNACCOMPANIED ACCESS TO OUR ON-SITE GAMMA-IRRADIATOR EXPIRED

The 10-year access period for Dr. Minter to use the on-site gamma-irradiator, which is necessary to complete ALL *in vivo* experiments, expired in August 2020. The process for approval involves an FBI background check – which itself requires updated fingerprinting. The UMass Police Department suspended all non-criminal fingerprinting services in April 2020, due to the pandemic. Dr. Minter was notified that this service would be reinstated once all UMass Police Department personnel were vaccinated.

ACTIONS TAKEN:

Dr. Minter learned in late 2020/early 2021 that the UMass PD suspended finger printing services. Since then, Dr. Minter has been in correspondence with the Radiation Safety Officer and with the Chief of UMass Police to monitor when fingerprinting services will be resumed. Dr. Minter has now been approved to resume use of the irradiator. Animal experiments have been initiated and are ongoing.

PARENT IACUC PROTOCOL, WHICH CONTAINS APPROVED DOD CDMRP ANIMAL EXPERIENTS, IS UP FOR 3-YEAR RENEWAL IN MAY 2021

Dr. Minter's IACUC-approved, 3-year parent protocol, Kuali 83, expired in May 2021. All animal experiments related to the DOD CDMRP BMF award were covered under this protocol.

ACTIONS TAKEN:

An updated 3-year parent protocol was submitted to the UMass IACUC by April 9, 2021, for review at their next meeting scheduled for April 14, 2021. Following approval, the following documents were uploaded to eBRAP: i) completed ACURO Appendix (July 2020 version); ii) updated IACUC protocol (with pertinent sections highlighted); iii) notification of protocol approval from UMass IACUC; iv) original IACUC protocol (with pertinent sections highlighted); and v) signed PI assurance form.

UNPREDICTABLE AND LOW CELL RECOVERY FROM AND RESPONSES OF ARCHIVED PATIENT SAMPLES

We have begun our experiments using archived treatment-naïve samples from patients diagnosed with Aplastic Anemia, obtained from the National Marrow Donor Program. The samples are archived with the number of cells/vial (approximately 5×10^6 cells/vial) on each vial. As shown in Figure 4A, there is great variability between the expected number of cells and the actual number of viable cells recovered. This poses a challenge in planning *in vitro* and *in vivo* experiments, both, as we cannot know, *a priori*, what will be the the number of viable cells recovered from a single vial.

Our early *in vivo* experiments utilizing archived treatment-naïve patient samples obtained from patients diagnosed with Aplastic Anemia failed to induce disease in our humanized model of bone marrow failure.

Following up with *in vitro* experiments using these patient samples, we have discovered that there is a wide range of responses to culturing these cells under conditions of stimulation (Figure 4B) or to iTreg differentiation (Figure 4).

ACTIONS TAKEN:

1. For *in vitro* experiments: We will review the inventory of patient samples and choose those for which there appear to be sufficient cell numbers to perform our intended *in vitro* experiments. We will thaw one vial and screen a small number of cells for their potential to differentiate into iTregs, before performing antibody delivery and characterizations on these samples.
2. For *in vivo* experiments: We will review the inventory of patient samples and choose those for which there appear to be sufficient cell numbers to induce disease in mice using our optimized protocol for bone marrow failure induction. We will thaw one vial and screen a small number of cells for their potential to respond to stimulation with anti-CD3+anti-CD28, before using these cells in our humanized mouse model of bone marrow failure.

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.

Changes that negatively impacted expenditures:

Low yield of CD 4 T cells from healthy human donor and some viability issues with batches of human healthy donor PBMCs has resulted in higher than budgeted spending on these resources. Addition of iTreg media during the 7-day differentiation period also resulted in increased cost of differentiating T cells.

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

Nothing to report. IACUC protocol (#1867) was approved on January 3, 2019.

Significant changes in use or care of vertebrate animals

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

4. 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).*

Nothing to report.

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.*

Nothing to report.

- **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.

Nothing to report.

- **Technologies or techniques**

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

Nothing to report.

- **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.

Nothing to report.

5. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate “no change”.

Example:

Name: Mary Smith

Project Role: Graduate Student

Researcher Identifier (e.g. ORCID ID): 1234567

Nearest person month worked: 5

Contribution to Project: Ms. Smith has performed work in the area of combined error-control and constrained coding.

Funding Support: The Ford Foundation (Complete only if the funding support is provided from other than this award.)

Name:	Lisa M. Minter
Project Role:	Principal Investigator
Researcher Identifier (ORCID ID)	0000-0002-1728-6389
Nearest person month worked:	2 (summer)
Contribution to Project:	Dr. Minter analyzed final data sets, wrote two manuscripts that were peer reviewed and published, oversees current experimental plan, execution, data acquisition, analysis, and troubleshooting.
Name:	Gregory N. Tew
Project Role:	Co-Principal Investigator
Researcher Identifier (ORCID ID)	0000-0003-3277-7925
Nearest person month worked:	1 (summer)
Contribution to Project:	Dr. Tew provided critical input on the two published manuscripts and supervised synthesis of cell-penetrating peptide mimic polymers used for antibody delivery.
Name:	Sudarvili Shanthalingam
Project Role:	(Senior Research Fellow)
Researcher Identifier (ORCID ID)	N/A
Nearest person month worked:	12 months
Contribution to Project:	Dr. Shanthalingam assisted in the collection of final data sets needed for manuscripts, provided critical input on manuscripts, performed in vitro differentiation experiments, aided in trouble shooting proliferation and suppression assays.
Name:	Christopher Hango
Project Role:	Graduate Student Research Assistant
Researcher Identifier (ORCID ID)	0000-0002-4066-9548
Nearest person month worked:	5 months
Contribution to Project:	Mr. Hango assisted in the synthesis of cell-penetrating peptide mimic polymers used for antibody delivery.

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.

The PI is listed as a co-PI on a second, recently funded DOD grant (CDMRP Log Number: PR190722, PI TEW) to explore the use of *ex vivo*-generated iTregs as a cell-based treatment for Irritable Bowel Disease. The PI will decrease compensation from the current DOD BMF grant from 1.8 mo summer salary to 1.5 mo summer salary, to comply with University of Massachusetts Amherst summer salary policy, but the PI will not decrease actual effort on the current funded project.

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.

6. 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.*

7. **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.*

Appendix A: SignificantResults_MINTER_8-2021_Annual and Technical Report

Appendix B: QuadChart_MINTER_8-2021_Annual and Technical Report

Annual and Technical Report
(8/1/2020 – 7/31/2021)
MINTER – W81XWH1910540

APPENDIX A
SIGNIFICANT RESULTS

Figure 1

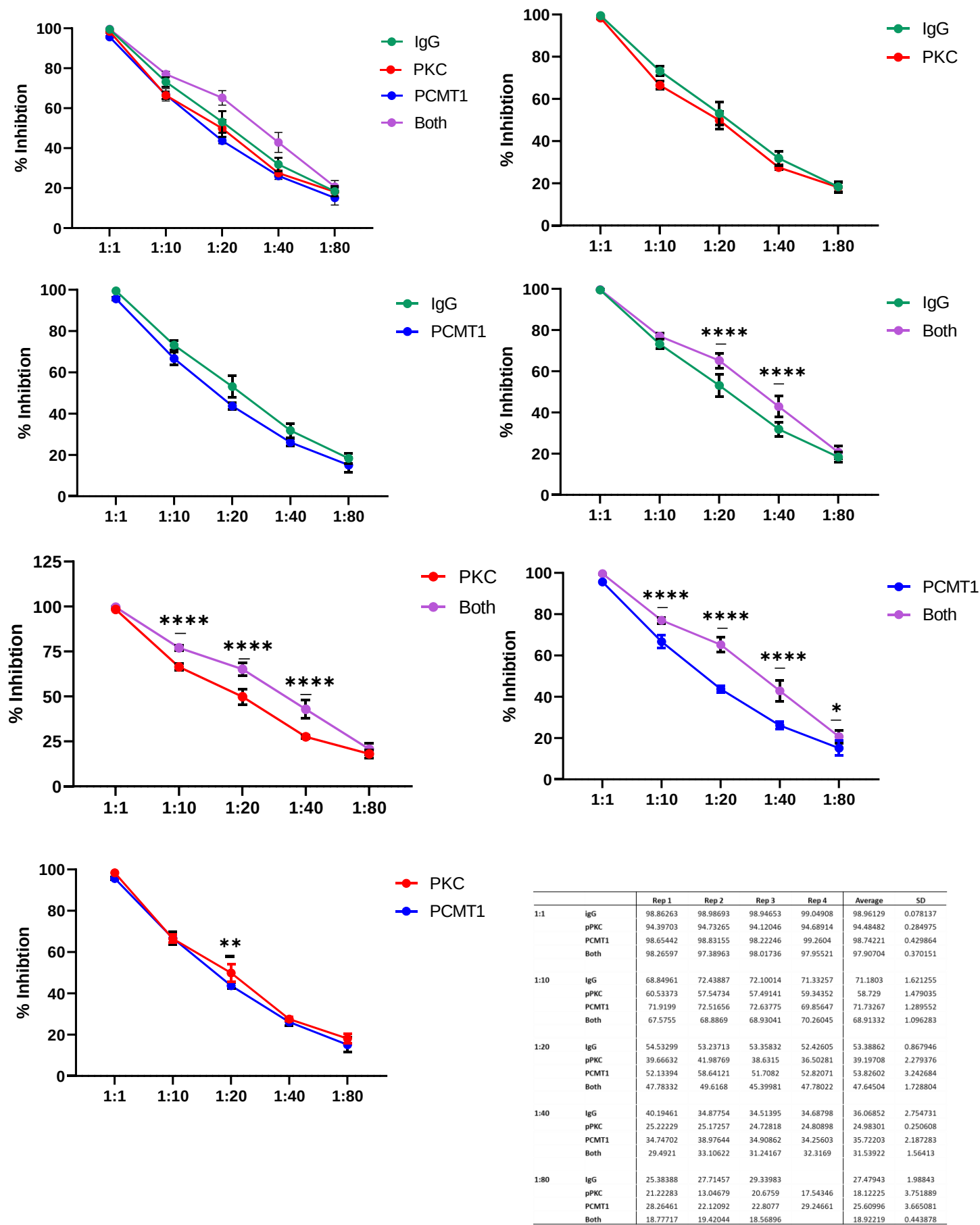


Figure 1

Figure 1. CD4 T cells differentiated in the presence of CPPM:anti-pPKC θ +anti-PCMT1 generate iTregs that have superior suppressive capacity at low iTreg:Responder Cell ratios.

Human CD4 T cells were isolated from Peripheral Blood Mononuclear Cells (PBMCs) obtained from healthy donors. Cells were pre-incubated with CPPM:IgG, CPPM:anti-pPKC θ , CPPM:anti-PCMT1, or both CPPM:anti-pPKC θ and anti-PCMT1. iTregs were cultured for seven days, labeled with Red650 cell tracker dye, then mixed with (responder) PBMCs that were stimulated with soluble anti-human CD3 plus anti-human CD28, cross-linked with anti-mouse IgG, and labeled with UltraGreen cell tracker dye. Cells were mixed at ratios of 1:1 and 1:25 iTreg:Responder cells, respectively, and co-cultured for (A) 6 or (B) 7 days. At the indicated timepoints cells were harvested and Ultragreen fluorescence was measured by flow cytometry. Percent of highly proliferated cells are indicated by inserts in the flow cytometry histograms. (C) Percent suppression was calculated and is presented graphically for co-cultures of cells mixed at ratios of 1:1, 1:10, 1:20, 1:40, and 1:80 iTreg:Responder cells, respectively. Data are the mean + SD of technical replicates of at least two independent experiments that showed similar results.

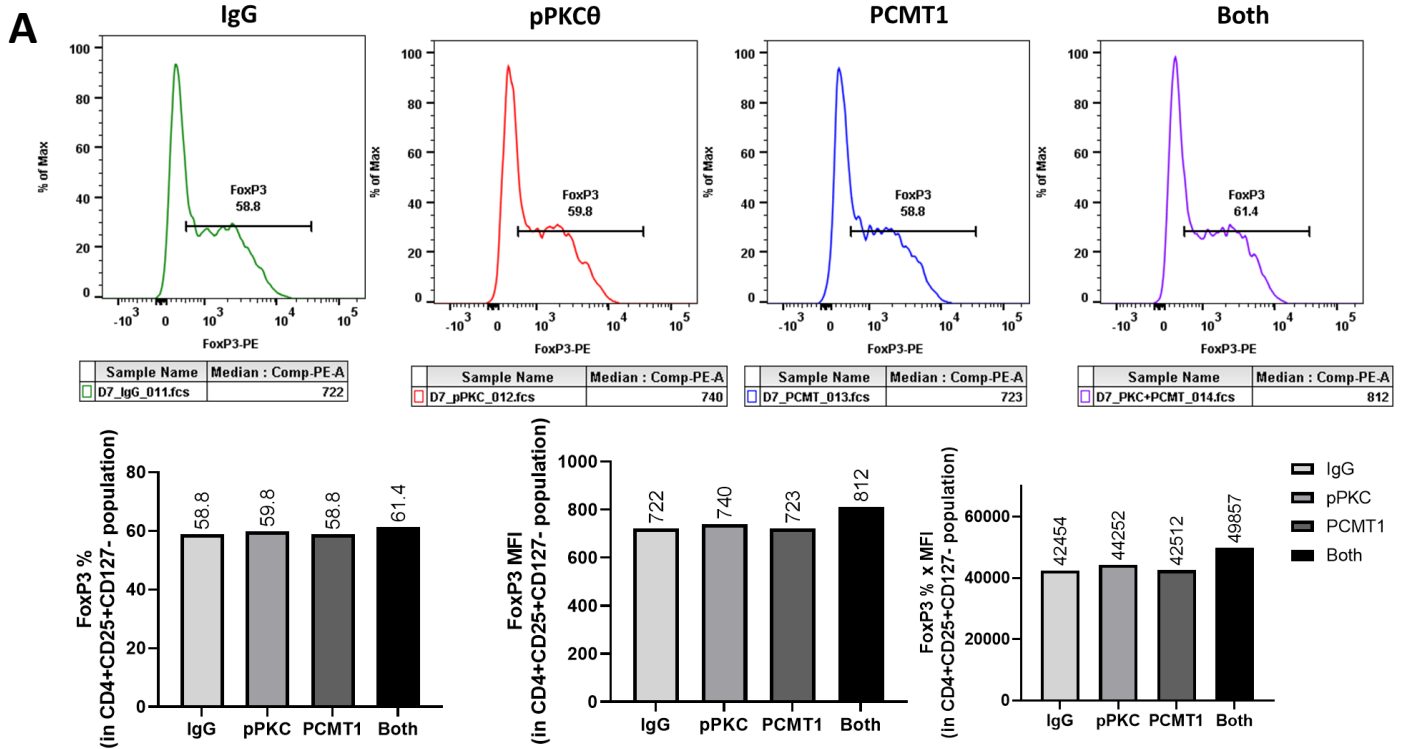
Methodology:

iTreg differentiation: Human PBMCs were thawed, plated in media, and rested overnight at 37C. On the next day CD4 T cells were positively isolated using anti-CD4 magnetic beads. CPPM was complexed for 30 minutes at RT with antibodies (anti-pPKC θ , anti-PCMT1, or both), or with isotype control Rabbit IgG, at a molar ratio of 40:1 (1 μ M P₁₃D₅ CPPM + 25nM antibody). CPPM:antibody complexes (100 μ l) were added drop-wise to CD4 T cells (1x10⁶/900 μ l media) and incubated for 4 hours at 37C, after which cells were washed twice with ice cold heparin (20U/ml) to remove surface-bound CPPM:antibody complexes. CD4 T cells (1x10⁶/ml media) were plated in iTreg differentiation media in single wells of a 12-well plate that had been pre-coated with anti-human CD3 (5 μ g/ml) plus anti-human CD28 (2.5 μ g/ml). Cells were split 1:1 on day 3 and fresh iTreg media was added to plates. iTregs were harvested on day 7 of culture. A small sample was analyzed by flow cytometry to confirm iTreg differentiation. Zombie-BV421 was used for live/dead staining; iTreg cells were stained for CD4 (FITC), CD25 (PECy7), CD127 (AF700) and FoxP3 (PE). Compensation was set using fluorescent beads and negative gates were set using a Fluorescence Minus One (FMO) approach.

Suppression Assay: Human PBMCs (responder cells) were thawed and rested for 1 hour at 37C. Cells were stimulated with soluble anti-human CD3 (5 μ g/ml) plus anti-human CD28 (2.5 μ g/ml) at 4C for 30 minutes. Antibodies were cross-linked by incubating cells with anti-mouse IgG (5 μ g/ml) at RT for 30 minutes. Cells were washed once in cold PBS and labeled for 37C for 20 minutes with CytoTell UltraGreen cell tracker dye (0.5x10⁵ cells were stained 1:1500 in 1500 μ l of PBS). Cells were washed twice with cold PBS and counted. iTregs, differentiated as described above, were labeled for 20 minutes at 37C with CytoTell Red 650 cell tracker dye (0.5x10⁵ cells were stained 1:2000 in 2000 μ l of PBS). Cells were washed twice with cold PBS and counted. Responder cells were added to wells of a 48-well plate; then iTregs were added at the following ratios: 1:1, 1:10, 1:20, 1:40, 1:80.

A proliferation control well was set up containing 0.5x10⁵ cells, but no iTregs; 30U/ml IL-2 was added to all wells at time of plating. After 5 days of co-culture, cells were harvested, and cell proliferation was measured by flow cytometry as determined by loss of UltraGreen fluorescence. Percent suppression was calculated using the area under the curve to account for the variances in degree of differentiation among cells in an entire population.

Figure 2



FOXP3 TSDR CpG Islands

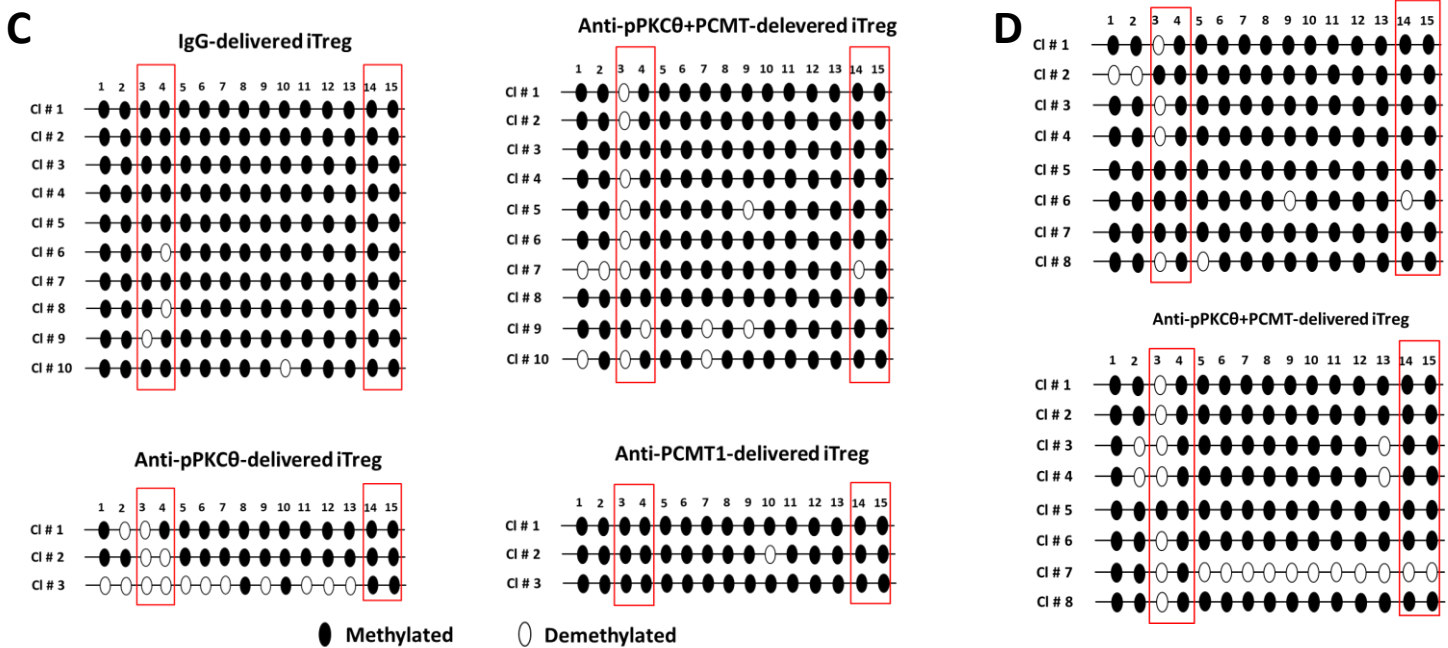


Figure 2

Figure 2. CD4 T cells differentiated in the presence of CPPM:anti-pPKC θ +anti-PCMT1 generate iTregs that have a higher percentage of demethylated sites in the TSDR region of the *FOXP3* promoter.

Human CD4 T cells were isolated from Peripheral Blood Mononuclear Cells (PBMCs) obtained from healthy donors. Cells were pre-incubated with CPPM:IgG, CPPM:anti-pPKC θ , CPPM:anti-PCMT1, or both CPPM:anti-pPKC θ + anti-PCMT1, before differentiating into iTregs. After seven days, iTregs were harvested and **(A)** the percent and median fluorescence intensity (MFI) of FOXP3+ cells within the CD4+CD25+CD127- population was measured by flow cytometry. CD4 T cells differentiated under different antibody delivery conditions were harvested and the **(B)** CpG islands of the TSDR region of the *FOXP3* promoter were analyzed for their degree of methylation in **(C)** unsorted and **(D)** sorted populations of iTregs from within the culture of differentiated cells. Red boxed region: NF- κ B binding site. Blue underlined regions: STAT5 binding sites.

Methodology:

We will assess TSDR Sodium bisulfite modification of genomic DNA using the EZ DNA Methylation Direct Kit (Zymo Research) according to the manufacturer's protocol. Bisulfite-treated DNA will be PCR-amplified using the following methylation-specific primers and ZymoTaqTM DNA polymerase (Zymo Research): forward primer: 5'-TGTTTGGGGGTAGAGGATTT-3' and reverse primer: 5'-TATCACCCACCTAAACCAA-3'. PCR conditions are as follows: initial denaturation at 95C for 10 min, 40 cycles of denaturation at 95C for 30 sec + annealing at 55C for 40 sec + extension at 72C for 1 min, and final extension at 72C for 7 min. Amplified DNA products will be gel purified using GeneJET gel extraction kit (Thermo Scientific) and cloned into pMiniTTM 2.0 cloning vector using NEB PCR Cloning Kit (New England Biolabs). Competent cells will be transformed with the vector. 10 individual positive bacterial colonies will be selected, from which recombinant plasmid DNA will be purified and sequenced with Sanger sequencing (Genewiz, South Plainfield, NJ).

Figure 3

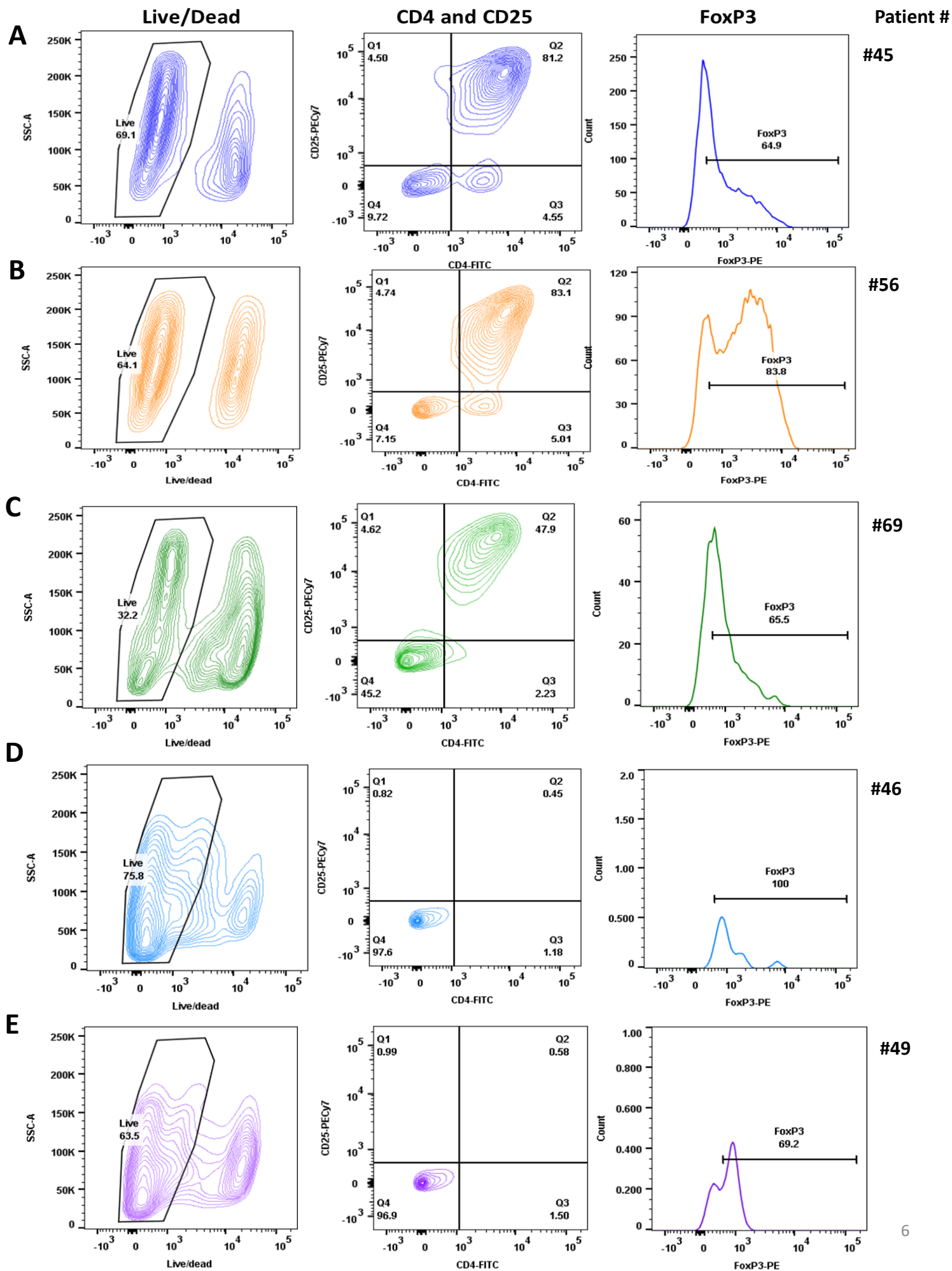


Figure 3

Figure 3. Patient CD4 T cells show diverse capacity to differentiate into iTregs.

Archived Peripheral Blood Mononuclear Cells (PBMCs) from treatment-naïve patients diagnosed with Aplastic Anemia were obtained from the National Marrow Donor Program. PBMCs were thawed and CD4 T cells were isolated and differentiated into iTregs. After seven days, (A-E) iTregs were harvested and the percent of live cells, CD4+CD25+, and FOXP3+ cells within the CD4+CD25+CD127- population was measured by flow cytometry.

Methodology:

iTreg differentiation: Human PBMCs obtained from archived patient samples were thawed in a 37C water bath. CD4 T cells were positively isolated using anti-CD4 magnetic beads. CD4 T cells (1×10^6 /ml media) were plated in iTreg differentiation media in single wells of a 12-well plate that had been pre-coated with anti-human CD3 (5µg/ml) plus anti-human CD28 (2.5µg/ml). Cells were split 1:1 on day 3 and fresh iTreg media was added to plates. iTregs were harvested on day 7 of culture. Samples were analyzed by flow cytometry to confirm iTreg differentiation. Zombie-BV421 was used for live/dead staining; iTreg cells were stained for CD4 (FITC), CD25 (PECy7), CD127 (AF700) and FoxP3 (PE). Compensation was set using fluorescent beads and negative gates were set using a Fluorescence Minus One (FMO) approach.

Figure 4

A

Cane #	/vial (10^6)	Total number of PBMC recovered	Injected?	Cells for <i>in vitro</i> assay?
26	4.7	1.54×10^6 / 3 vials	No	No
45	4.93	8.19×10^6 / 2 vials	Cohort 1	Yes
46	4.7	10.31×10^6 / 3 vials	Cohort 1	Yes
32	4.75	15.53×10^6 / 2 vials	Cohort 1	Yes
41	4.93	9.32×10^6 / 3 vials	Cohort 1	No
56	4.58	16.66×10^6 / 3 vials	Cohort 2	Yes
69	4.8	9.24×10^6 / 2 vials	Cohort 2	Yes
49	4.4	10.72×10^6 / 3 vials	Cohort 2	Yes
50	4.33	16.55×10^6 / 3 vials	Cohort 2	Yes
77	4.78	6.63×10^6 / 3 vials	No	Yes
48	4.96	7.26×10^6 / 3 vials	No	Yes

B

	Cane #	# of PBMCs	# of CD4s isolated	# of cells plated /well	Tcon plated	CD4+ CD25+
Cohort 1	45	1.5×10^6	0.675×10^5	0.675×10^5	-	Yes
Cohort 1	46	0.56×10^6	0.3×10^5	0.3×10^5	-	No
Cohort 1	32	3.67×10^6	0.375×10^5	0.375×10^5	-	No
Cohort 1	41	Not enough cells				
Cohort 2	56	1.5×10^6	1.65×10^5	1×10^5	-	Yes
Cohort 2	69	4.67×10^6	4.3×10^5	1×10^5	1×10^5	Yes
Cohort 2	49	1.75×10^6	0.6×10^5	0.6×10^5	-	No
Cohort 2	50	1.31×10^6	2.9×10^5	1×10^5	1×10^5	No
Not injected	77	2.4×10^6	1.56×10^5	1×10^5	0.56×10^5	No

Figure 4

Figure 4. Archived patient samples provide lower than expected numbers of cells upon thawing

Archived Peripheral Blood Mononuclear Cells (PBMCs) from treatment-naïve patients diagnosed with Aplastic Anemia were obtained from the National Marrow Donor Program. PBMCs were thawed and the number of live cells/vial was determined using Trypan Blue exclusion. **(A)** Summary of cell numbers used to induce disease in two cohorts of NSG mice. Mice were lightly irradiated (2Gy); 4 hours later, 7.5×10^6 patient PBMCs were administered via retro-orbital injection using patient samples from which sufficient live cells could be obtained. **(B)** Summary of patient samples analyzed post-disease induction to determine their capacity to respond to anti-CD3+anti-CD28 stimulation (Tconv), and to differentiate into iTregs. Cells were stained using antibodies specific for CD4, CD25, and FOXP3 and analyzed by flow cytometry.

Methodology:

Cell recovery: Human PBMCs obtained from archived patient samples were thawed in a 37C water bath. When sample was a slurry, cells were washed twice in 10ml each of pre-warmed media. A sample of cells was stained in 0.4% Trypan Blue and live cells were counted in a hemacytometer using a Trypan Blue exclusion assay.

Tconv assay: Human PBMCs obtained from archived patient samples were thawed in a 37C water bath. CD4 T cells were positively isolated using anti-CD4 magnetic beads. CD4 T cells (1×10^6 /ml media) were plated in media in single wells of a 12-well plate that had been pre-coated with anti-human CD3 (5µg/ml) plus anti-human CD28 (2.5µg/ml). Cells were split 1:1 on day 3 and fresh media was added to plates. Tconv cells were harvested on day 7 of culture. Samples were analyzed by flow cytometry to confirm stimulation. Zombie-BV421 was used for live/dead staining; Tconv cells were stained for CD4 (FITC), CD25 (PECy7), CD127 (AF700) and FoxP3 (PE). Compensation was set using fluorescent beads and negative gates were set using a Fluorescence Minus One (FMO) approach.

iTreg differentiation: Human PBMCs obtained from archived patient samples were thawed in a 37C water bath. CD4 T cells were positively isolated using anti-CD4 magnetic beads. CD4 T cells (1×10^6 /ml media) were plated in iTreg differentiation media in single wells of a 12-well plate that had been pre-coated with anti-human CD3 (5µg/ml) plus anti-human CD28 (2.5µg/ml). Cells were split 1:1 on day 3 and fresh iTreg media was added to plates. iTregs were harvested on day 7 of culture. Samples were analyzed by flow cytometry to confirm iTreg differentiation. Zombie-BV421 was used for live/dead staining; iTreg cells were stained for CD4 (FITC), CD25 (PECy7), CD127 (AF700) and FoxP3 (PE). Compensation was set using fluorescent beads and negative gates were set using a Fluorescence Minus One (FMO) approach.

Discussion of stated goals not met

Several unexpected issues were encountered during the second year of funding for this DOD grant that delayed reaching some experimental goals laid out in the SOW. As a result, a one-year no-cost extension to this grant was requested and approved.

DELAY IN OBTAINING ARCHIVED PATIENT SAMPLES FOR RESEARCH

Archived patient samples, to be supplied by the National Marrow Donor Program (NMDP), were requested in August 2020. Due to pandemic related reductions in staffing, backlog in workload, and staffing turn-over at the NMDP, coupled with corresponding delays at UMass Amherst, including mandatory staff furloughs and difficulties the UMass Amherst Procurement System has encountered classifying the NMDP as a recognized vendor, patient samples have not yet been made available for research purposes.

ACTIONS TAKEN:

Since August 2020, Minter Lab has been in constant correspondence with staff at the NMDP, as well as with departmental business office staff, to complete the steps necessary to obtain a purchase order to acquire the archived patient samples needed to complete experiments outlined in the SOW. The approval for this purchase order was finally processed for payment through the UMass Amherst Procurement System. Patient samples have been acquired and experiments using these materials are ongoing.

UNACCOMPANIED ACCESS TO OUR ON-SITE GAMMA-IRRADIATOR EXPIRED

The 10-year access period for Dr. Minter to use the on-site gamma-irradiator, which is necessary to complete ALL *in vivo* experiments, expired in August 2020. The process for approval involves an FBI background check – which itself requires updated fingerprinting. The UMass Police Department suspended all non-criminal fingerprinting services in April 2020, due to the pandemic. Dr. Minter was notified that this service would be reinstated once all UMass Police Department personnel were vaccinated.

ACTIONS TAKEN:

Dr. Minter learned in late 2020/early 2021 that the UMass PD suspended finger printing services. Since then, Dr. Minter has been in correspondence with the Radiation Safety Officer and with the Chief of UMass Police to monitor when fingerprinting services will be resumed. Dr. Minter has now been approved to resume use of the irradiator. Animal experiments have been initiated and are ongoing.

PARENT IACUC PROTOCOL, WHICH CONTAINS APPROVED DOD CDMRP ANIMAL EXPERIENTS, IS UP FOR 3-YEAR RENEWAL IN MAY 2021

Dr. Minter's IACUC-approved, 3-year parent protocol, Quali 83, expired in May 2021. All animal experiments related to the DOD CDMRP BMF award were covered under this protocol.

ACTIONS TAKEN:

An updated 3-year parent protocol was submitted to the UMass IACUC by April 9, 2021, for review at their next meeting scheduled for April 14, 2021. Following approval, the following documents were uploaded to eBRAP: i) completed ACURO Appendix (July 2020 version); ii) updated IACUC protocol (with pertinent sections highlighted); iii) notification of protocol approval from UMass IACUC; iv) original IACUC protocol (with pertinent sections highlighted); and v) signed PI assurance form.

UNPREDICTABLE AND LOW CELL RECOVERY FROM AND RESPONSES OF ARCHIVED PATIENT SAMPLES

We have begun our experiments using archived treatment-naïve samples from patients diagnosed with Aplastic Anemia, obtained from the National Marrow Donor Program. The samples are archived with the number of cells/vial (approximately 5×10^6 cells/vial) on each vial. As shown in Figure 4A, there is great variability between the expected number of cells and the actual number of viable cells recovered. This poses a challenge in planning *in vitro* and *in vivo* experiments, both, as we cannot know, *a priori*, what will be the the number of viable cells recovered from a single vial.

Our early *in vivo* experiments utilizing archived treatment-naïve patient samples obtained from patients diagnosed with Aplastic Anemia failed to induce disease in our humanized model of bone marrow failure.

Following up with *in vitro* experiments using these patient samples, we have discovered that there is a wide range of responses to culturing these cells under conditions of stimulation (Figure 4B) or to iTreg differentiation (Figure 4).

ACTIONS TAKEN:

For *in vitro* experiments: We will review the inventory of patient samples and choose those for which there appear to be sufficient cell numbers to perform our intended *in vitro* experiments. We will thaw one vial and screen a small number of cells for their potential to differentiate into iTregs, before performing antibody delivery and characterizations on these samples.

For *in vivo* experiments: We will review the inventory of patient samples and choose those for which there appear to be sufficient cell numbers to induce disease in mice using our optimized protocol for bone marrow failure induction. We will We will thaw one vial and screen a small number of cells for their potential to respond to stimulation with anti-CD3+anti-CD28, before using these cells in our humanized mouse model of bone marrow failure.

Ex Vivo-Generated Autologous iTregs as a Cell-Based Therapy for Acquired Aplastic Anemia (W81XWH1910540)



PI: MINTER, Lisa M.

Org: University of Massachusetts Amherst

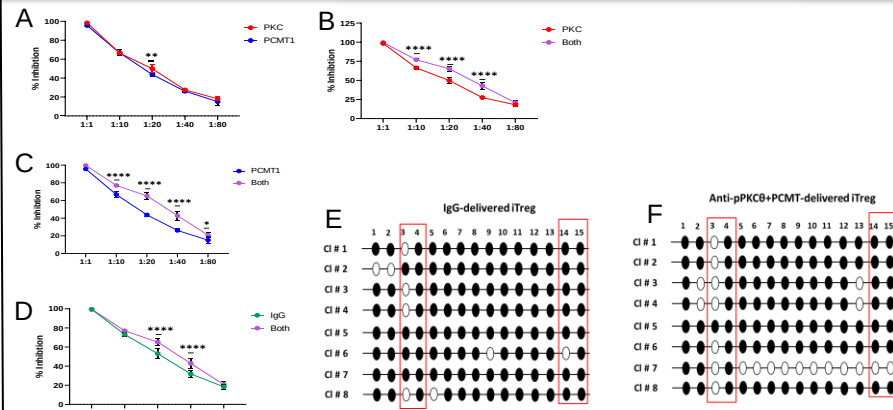
Award Amount: \$509,356.00

Study/Product Aim(s)

- Complex CPPMs with: IgG, anti-pPKC θ , anti-PCMT1, combination anti-pPKC θ +anti-PCMT1
- Differentiate iTregs in the presence of: CPPM:IgG, CPPM:anti-pPKC θ , CPPM:anti-PCMT1, and combination of CPPM:anti-pPKC θ +CPPM:anti-PCMT1
- Characterize iTregs differentiated in the presence of: CPPM:IgG, CPPM:anti-pPKC θ , CPPM:anti-PCMT1, and combination of CPPM:anti-pPKC θ +anti-PCMT1 (phenotype; suppression assays; *FOXP3* methylation)
- Evaluate patient samples for iTreg differentiation capacity
- Begin *in vivo* animal studies

Approach

We isolated human CD4 T cells isolated from PBMCs and differentiated them into iTregs for 5-7 days following addition of different CPPM:antibody combinations, or IgG as control, then characterized them by flow cytometry. We evaluated the suppressive capabilities of iTregs differentiated with different CPPM:antibody combinations. We sorted differentiated iTregs and analyzed the methylation status of the TSDR region of the *FOXP3* promoter using bisulfite sequencing. We cultured patient samples under iTreg polarizing conditions to assess their differentiation capacity. We established baseline data for our humanized bone marrow failure model. We performed an initial *in vivo* experiment to generate patient sample-derived "avatar" mice.



Accomplishments: (A-D) We differentiated human CD4 T cells into iTregs under different CPPM-antibody delivery conditions, then evaluated their ability to suppress responder cells using a standard suppression assay. (E, F) We purified iTregs differentiated under different CPPM-antibody delivery conditions, then analyzed the methylation status of the TSDR region of the *FOXP3* promoter using bisulfite sequencing. Numbers refer to the CpG island; closed circles indicate methylated sites; open circles indicate demethylated sites.

Timeline and Cost

Activities	CY	2019	2020	2021	2022
Major Task 1 (sub tasks 1-3)					
Major Task 1 (sub tasks 4-5)					
Major Task 1 (sub tasks 6-7)					
Major Task 1 (sub tasks 8)					
Major Task 2 (sub task 1)					
Major Task 3 (sub task 1)					
Major Task 3 (sub task 2)					
Major Task 4 (sub task 1)					
Estimated Budget (\$K)		\$80K	\$160K	\$85K	

Updated: (8/31/2021)

work completed

work in progress

Goals/Milestones

CY 2020/CY 2021 Goals – Complete *in vitro* testing and characterization studies Completed:

- ☒ Synthesized Cell Penetrating Peptide Mimics
- ☒ Suppression assays for iTregs differentiated with CPPM:antibodies
- ☒ Characterize iTregs differentiated with CsA or Rapamycin
- ☒ Perform promoter methylation studies for iTregs differentiated with CPPM:IgG or CPPM:anti-pPKC θ +antiPCMT1
- ☒ Collected baseline data for our humanized bone marrow failure model

Comments/Challenges/Issues/Concerns

- Irradiator access expired during Covid pandemic, delaying initiation of *in vivo* studies
- Parental IACUC protocol expired; new parent protocol approved by ACURO
- Patient samples show low viability/low cell recovery upon thawing
- NCE requested and approved
- Additional personnel hired to complete *in vivo* animal studies

Budget Expenditure to Date

Projected Expenditure: Y2 = \$165,047.00

Actual Expenditure: Y2 = \$158,752