AWARD NUMBER: W81XWH-20-1-0536

TITLE: Supersuppressive-Induced Regulatory T-Cell Populations

PRINCIPAL INVESTIGATOR: Gregory N. Tew

CONTRACTING ORGANIZATION: University of Massachusetts, Amherst, MA

REPORT DATE: July 2022

TYPE OF REPORT: annual

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

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2022	annual	01Jul2021-30Jun2022

4. TITLE AND SUBTITLE

Supersuppressive-Induced Regulatory T-Cell Populations

5a. CONTRACT NUMBER
W81XWH-20-1-0536

5b. GRANT NUMBER
PR190722 (GRANT12902477)

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Gregory Tew

E-Mail: tew@mail.pse.umass.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

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

University of Massachusetts,
Amherst
Office of Grants and
Contracts
Amherst, MA 01003

8. PERFORMING ORGANIZATION REPORT NUMBER
Unique Identifier: 153926712

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

This proposal is directly focused on studies that leverage immunological approaches to prevent and treat inflammatory bowel disease (IBD). IBD is a more encompassing term that includes two more widely recognized diagnoses: *ulcerative colitis* (UC) and *Crohn's disease* (CD). The number of both diagnoses are increasing. To date, there is no cure for IBD. Immune regulation of the gut is extremely complex. The gastrointestinal immune system must maintain the dexterity to both maintain tolerance towards food and the commensal microflora while mounting a rapid response against pathogens. It is the primary responsibility of regulatory T cells to maintaining this critical equilibrium. This capacity to maintain/restore balance establish regulatory T cells as the "master regulators" of intestinal balance. In fact, numerous animal studies have identified regulatory T cell deficiency as an important contributor to IBD. However, the *central critical question* to be addressed is: what is the exact regulatory T cell phenotype critical for immune regulation and therefore appropriate for IBD treatment remains unresolved? This proposal utilizes a proprietary combination of novel bio-inspired protein mimics to easily and effectively deliver antibodies intracellularly to CD4 T cells. The development of a stable, rapidly expandable, super-suppressive regulatory T cell phenotype will find widespread application in IBD.

15. SUBJECT TERMS
None listed.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRDC
Unclassified	Unclassified	Unclassified	Unclassified	30	19b. TELEPHONE NUMBER *(include area code)*

TABLE OF CONTENTS

1. Introduction

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the intestine; the two main pathogenicities are *ulcerative colitis* (UC) and *Crohn's disease* (CD). The incidence of IBD have risen by 50% from 1999 to 2015 with more than 3 million U. S. adult cases. To date, there is no cure for IBD. IBD pathogenesis has identified key features of dysfunction. The primary responsibility of Tregs is to maintaining gut homeostasis by discriminating between harmless (e.g., dietary compounds and intestinal microbes) and harmful stimuli (e.g., pathogens). Failure to orchestrate this equilibrium can lead to chronic IBD diseases. This proposal is directly focused on studies that leverage immunological approaches to prevent and treat IBD. It utilizes a proprietary combination of novel bio-inspired protein transduction domain mimics (PTDMs) to easily and effectively deliver antibodies intracellularly to T cells. Although naturally occurring (n)Tregs can be removed from patients and expanded *ex vivo*, nTregs make up a very small population (~2%) of circulating PBMCs. Therefore, the ability to convert CD4 T cells, which constitute approximately 15% of circulating T cells, into a super-suppressive iTreg population that expands rapidly *ex vivo* and remains stable *in vivo* will find widespread application in IBD. We hypothesize that PTDM-antibody delivery into CD4 T cells to inhibit PKC theta and/or SIRT1, prior to *ex vivo* iTreg differentiation, will generate super-suppressive iTregs that are resistant to Th17 conversion *in vitro* and *in vivo*. We predict these iTregs will provide symptomatic relief and durable remission when used as a cell-based therapy.

2. Keywords

Inflammatory bowel disease, protein transduction domain mimics, induced regulatory T cells, phosphorylated Protein Kinase C Theta, Sirtuin-1, ulcerative colitis, Crohn's disease, lamina propria, Forkhead box P3, peripheral blood mononuclear cell.

3. Accomplishments

a. What were the major goals of the project?

STATEMENT OF WORK – 01/01/2020-12/31/2022
PROPOSED START DATE - Jan. 1, 2020
UPDATED – July 2022

Site 1:
University of Massachusetts Amherst
120 Governors Dr.
Amherst, MA 01003
PI: Gregory N. Tew

Site 2:
University of Massachusetts Amherst
661 N. Pleasant St.
Amherst, MA 01003
PI: Lisa M. Minter

Site 3:
University of Massachusetts Amherst
102 Holdsworth Way
Amherst, MA 01003
PI: Guodong Zhang

Specific Aim 1 **Determine how inhibiting pPKCθ, SIRT1, or both, using PTDM:antibody delivery influences *ex vivo* iTreg differentiation**	**Timeline**	**Site 1**	**Site 2**	**Site 3**
Major Task 1 **Generate and characterize iTregs differentiated *ex vivo* under various conditions of PTDM:antibody delivery, with or without the addition of pro-inflammatory cytokines**				
Subtask 1 HRPO approval for use of commercially available healthy donor PBMCs	1-4 **COMPLETED**		Dr. Minter	
Subtask 2 HRPO approval for use of commercially available UC patient PBMCs	1-4 **COMPLETED**		Dr. Minter	
Subtask 3 Local IACUC approval	1-3 **COMPLETED**		Dr. Minter	Dr. Zhang
Subtask 4 ACURO protocol approval	4-6 **COMPLETED**		Dr. Minter	Dr. Zhang
Subtask 5 Produce synthetic protein transduction domain mimics (PTDMs)	1-36 **50% COMPLETE**	Dr. Tew		
Subtask 6 Complex PTDMs with: IgG, anti-pPKCθ, anti-SIRT	1-36 **COMPLETED**	Dr. Tew	Dr. Minter	Dr. Zhang
Subtask 7 Differentiate iTregs in the presence of: PTDM:IgG, PTDM:anti-pPKCθ, PTDM:anti-SIRT1, or both anti-pPKCθ and anti-SIRT1	4-12 **COMPLETED**	Dr. Tew	Dr. Minter Total # healthy PBMCs ~100-200x10^6 StemCell Tech. (Vancouver, BC)	
Subtask 8 Characterize iTregs differentiated in the presence of: PTDM:IgG, PTDM:anti-pPKCθ, PTDM:anti-SIRT1, or both anti-pPKCθ and anti-SIRT1 (phenotype, suppression assays, plasticity in the presence of pro-inflammatory cytokines)	4-12 **90% COMPLETE**	Dr. Tew	Dr. Minter Total # healthy PBMCs ~100-200x10^6) StemCell Tech.	

			(Vancouver, BC)	
Major Task 2 **Test *in vivo* efficacy of iTregs using a humanized mouse model of IBD**				
Subtask 1 Differentiate iTregs from UC patient-derived PBMCs in the presence of: PTDM:IgG, PTDM:anti-pPKCθ, PTDM:anti-SIRT1, or both anti-pPKCθ and anti-SIRT1(phenotype, suppression assays, plasticity in the presence of proinflammatory cytokines)	12-24 **85%** **COMPLETE**	Dr. Tew	Dr. Minter total # patient PBMCs: ~310x10^6 (Prec. for Medicine, Frederick, MD)	
Subtask 2 Test *in vivo* efficacy of iTregs using a humanized mouse model of IBD generated from UC patient PBMCs	12-24 **COMPLETED**	Dr. Tew	Dr. Minter total # NSG mice:~25 (Jax Labs; Bar Harbor, ME)	Dr. Zhang
Specific Aim 2 **Expand PTDM structure-activity relationships to optimize antibody delivery into mouse T cells**	**Timeline**	**Site 1**	**Site 2**	**Site 3**
Major Task 3 **Expand SAR to optimize antibody delivery into mouse T cells**				
Subtask 1 Synthesize protein transduction domain mimics (PTDMs) and complex PTDMs with: IgG, anti-pPKCθ, anti-SIRT	1-36 **50%** **COMPLETE**	Dr. Tew	Dr. Minter	
Subtask 2 Differentiate iTregs in the presence of: PTDM:IgG, PTDM:anti-pPKCθ, PTDM:anti-SIRT1, or both anti-pPKCθ and anti-SIRT1	4-12 **90%** **COMPLETE**	Dr. Tew	Dr. Minter ~30 C57BL/6 mice (Jax Labs, Bar Harbor, ME)	
Subtask 3 Characterize iTregs differentiated in the presence of: PTDM:IgG, PTDM:anti-pPKCθ, PTDM:anti-SIRT1, or both anti-pPKCθ and anti-SIRT1 (phenotype, suppression assays, plasticity in the presence of pro-inflammatory cytokines)	4-12 **90%** **COMPLETE**	Dr. Tew	Dr. Minter ~30 C57BL/6 mice (Jax Labs, Bar Harbor, ME)	
Specific Aim3 **Test the therapeutic efficacy of PTDM:antibody-generated iTregs in mouse models of IBD**	**Timeline**	**Site 1**	**Site 2**	**Site 3**
Major Task 4 **Evaluate the effects of in vivo iTreg administration**				
Subtask 1 Generate iTregs and characterize their effects on the composition of normal microbiota	12-24 **IN-PROCESS**	Dr. Tew	Dr. Minter ~20 C57BL/6 mice (Jax Labs, Bar Harbor, ME)	Dr. Zhang

Subtask 2 Generate iTregs and test their *in vivo* efficacy using the DSS model of IBD	18-30 **IN-PROCESS**	Dr. Tew	Dr. Minter ~20 C57BL/6 mice (Jax Labs, Bar Harbor, ME)	Dr. Zhang
Subtask 3 Generate iTregs and test their *in vivo* efficacy using the IL-10$^{-/-}$ model of IBD	18-30 **IN-PROCESS**	Dr. Tew	Dr. Minter ~20 C57BL/6 mice (Jax Labs, Bar Harbor, ME)	Dr. Zhang
Subtask 4 Collate data, prepare, and submit scientific manuscript	30-36 **IN-PROCESS**	Dr. Tew	Dr. Minter	Dr. Zhang

b. **What was accomplished under these goals?**

1.) **Major activities**:

- Synthesizing new protein transduction domain mimics (PTDMs) with alcohol functionality.
- Studying PTDM:cargo complexes.
- Studying PTDM:antibody complex delivery to human and mouse CD4 cells.
- Quantifying impact of PTDM:anti-SIRT1 delivery on human iTreg plasticity specifically with respect to Th1, Th2, Th17 differentiation.
- Quantifying impact of PTDM:anti-SIRT1 delivery on murine iTreg plasticity specifically with respect to Th1, Th2, Th17 differentiation.
- Quantifying delivery in human IBD model.
- Training new personnel which included experiments, troubleshooting, central facilities, titrating reagents, handling sensitive cell types.
- Maintaining University of Massachusetts Amherst IACUC approval for protocols involving the use of human PBMCs and research animals.
- Maintaining HRPO approval for commercially available healthy donor PBMC and UC patient PBMCs.
- Maintaining ACURO for animal studies outlined in the award.

2.) **Specific objectives**:

- Maintaining all IACUC and DOD approvals for cell and animal experiments.
- Synthesized, complexed, and delivered PTDM:anti-SIRT1 complexes into human CD4 cells; differentiated into iTregs; characterized the impact of delivered cargo on iTreg population and phenotype.
- Synthesized, complexed, and delivered PTDM:anti-SIRT1 complexes into mouse CD4 cells; differentiated into iTregs; characterized the impact of delivered cargo on iTreg population and phenotype.
- Co-delivered PTDM:anti-SIRT1 and PTDM:anti-pPKCθ complexes into human CD4 cells.
- Developed humanized IBD model with iTregs.
- Developed new analytical technique to understand PTDM:cargo complex and molecular interactions.
- Developed biophysical models to understand PTDM:cargo release intracellularly to answer fundamental questions regarding cargo availability inside cells.
- New PTDMs with alcohol functionality, like the amino acid serine, provided new insight into PTDM design.

3.) Significant Results (See Appendix A for data):

- New analytical technique to understand PTDM:cargo complex (see figure 1)
- New PTDMs were designed, synthesized, purified, and characterized.
- Murine cells following PTDM:anti-SIRT1 delivery and differentiation toward iTregs. (see Figure 2)

- Human iTreg suppression assay following PTDM:anti-SIRT1. (see figure 3)
- Humanized IBD model. (see figure 4)

4.) Other Achievements:

- Graduated one PhD student; employed in Research Triangle Park, NC; brought new personal up to speed.
- Wrote, submitted, revised, and published three additional manuscripts in high impact journals. Funding from this grant was acknowledged.
- Other manuscripts in progress.

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

1.) **Describe opportunities for training and professional development**:

The team assembled for this grant includes physical and life sciences providing robust training and professional development opportunities for the students, postdocs, and faculty on the project. Biweekly meetings allow routine practice in communication to expertise beyond the students' discipline. Students are trained on instruments by professional staff; regular one-on-one and team mentor activities are conducted. Proficiency in virtual presentations and scientific exchange were developed.

d. **How were the results disseminated to communities of interest?**

1.) **Describe how the results were disseminated to communities of interest**.

Wrote, submitted, revised, and published three manuscripts. (https://doi.org/10.1021/acsbiomaterials.2c00125 ; https://doi.org/10.1039/D2PY00017B ; https://doi.org/10.1021/acs.biomac.1c00929).

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

- Produce PTDMs with alcohol functionality, complex these with antibodies, understand their delivery efficacy.
- Execute in vivo humanized mouse model studies; DSS model of IBD studies.
- Continue quantification of iTreg suppression assays to understand delivery efficacy.
- Evaluate the impact of FOXP3 protein delivery vs. antibody delivery.

4. Impact

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

This proposal utilizes a proprietary combination of novel bio-inspired protein transduction domain mimics (PTDMs) to easily and effectively deliver antibodies intracellularly to T cells. Given the role of Tregs in human immunity, a deeper understanding of their role in various diseases is critical. How widely can they be manipulated is a critical question. Because naturally occurring (n)Tregs can be removed from patients and expanded ex vivo, they represent an intriguing opportunity. However, nTregs make up a very small population (~2%) of circulating PBMCs. Therefore, the ability to convert CD4 T cells, which constitute approximately 15% of circulating T cells, into a super-suppressive iTreg population that expands rapidly ex vivo and remains stable in vivo will find widespread application in IBD. Given the widely recognized dysfunction of Tregs associated with IBD, considerable work is needed to understand Treg phenotype and stability associated with prevention of IBD. We expect to provide new insight in the development of stable, highly suppressive Treg populations that effectively expand ex vivo, enabling cell therapy solutions to IBD and autoimmune-related diseases. We expect the results of this proposal to set the stage for pre-clinical studies. Delivering multiple antibodies (or proteins) simultaneously to precisely regulate several biochemical pathways at once is extremely appealing. It has broad ramifications for basic science and human health.

b. **What was the impact on other disciplines?**

Given the widely recognized dysfunction of Tregs associated with IBD, considerable work is needed to understand Treg phenotype and stability associated with prevention of IBD. In the short-term, we expect to provide new insight in the development of stable, highly suppressive Treg populations that effectively expand *ex vivo*, are stable *in vivo*, and enabling cell therapy solutions to IBD and autoimmune-related diseases. We expect the results of this proposal to set the stage for pre-clinical studies. In the longer-term, delivering multiple antibodies (or proteins) simultaneously to precisely regulate several biochemical pathways inside cells is extremely appealing. It has broad ramifications for basic science and human health. Generating super-suppressive iTregs trained for tolerance against specific antigens also has broad implications for organ transplantation. Our research outcomes are expected to impact other areas including osteoarthritis, intestinal transplants, and immune rejection. This proposal will significantly impact the burgeoning field of cell-based therapies including new understanding of Treg phenotypes.

c. **What was the impact on technology transfer?**

Nothing to Report

d. **What was the impact on society beyond science and technology?**

We published three peer-reviewed journal articles in the last reporting period. The interdisciplinary nature of this team will train a generation of students with much broader team skills enabling them to more effectively solve major societal challenges. For example, Dr. Hazel Davis worked with immunologists regularly but also developed a collaboration with analytic chemists. Her ability to communicate and work with such interdisciplinary is the hallmark of how future scientists will be trained.

5. Changes/Problems

a. **Changes in approach and reasons for change**

- The delivery of antibodies, anti-PKCθ and anti-SIRTI, has proved temperamental. When a new student starts delivery experiments, there is a significant learning curve. Our understanding remains that this is a product of the complexity involved in primary cells and the delicate differentiation into iTregs. As a result, we will also consider delivering the protein FOXP3. Previous results suggest protein delivery is less temperamental than antibody delivery although protein delivery has not focused on iTreg differentiation.

b. **Actual or anticipated problems or delays and actions or plans to resolve them**

1.) **Actual problems:**

-Covid-19 continue to cause delays stretching through the Fall of 2021. By summer 2022, it appears many of these delays have been resolved. It finally appears the University, suppliers, etc are returning to normal..

-Technical challenges/new personnel- As new personnel were brought onto the project, unforeseen technical challenges related to (a) cell isolation during protocol steps and (b) iTreg differentiation conditions (cell density, temporal) slowed progress. Careful analysis at each step and step-by-step re-training has provided progress in both areas (which are related to each other). Some challenges remain, specifically in the heparin-washing step that follows PTDM:cargo delivery. Also, outcome of iTreg differention and the IgG control experiments. We have made good progress with the low cell count but antibody delivery remains temperamental. This is frustrating when experiments are scaled to generate larger iTreg cell populations for animal studies. We continue to explore team-wide solutions including more careful protocols, reduced heparin-washing, or even eliminating this step completely.

c. **Changes that had a significant impact on expenditures**

i. Covid-19 slowed personnel hiring/training.

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

i. Nothing to report.

6. Products

a. **Publications, conference papers, and presentations**

i. **Journal publications.**

7. Davis H, Posey N, Tew G Protein Binding and Release by Polymeric Cell-Penetrating Peptide Mimics. *Biomacromolecules*, 23(1):57–66. https://doi.org/10.1021/acs.biomac.1c00929

8. Hango CR, Davis HC, Uddin EA, Minter LM, Tew GN (2022) Increased block copolymer length improves intracellular availability of protein cargo. *Polymer Chemistry*, 13(13):1901–1910. https://doi.org/10.1039/d2py00017b

9. Davis HC, Pan X, Kirsch ZJ, Vachet RW, Tew GN (2022) Covalent Labeling-Mass Spectrometry Provides a Molecular Understanding of Noncovalent Polymer-Protein Complexation. *ACS Biomaterials Science and Engineering*, 8(6):2489–2499. https://doi.org/10.1021/acsbiomaterials.2c00125

ii. **Other publications, conference papers, and presentations.**

10. nothing to report.

b. **Technologies or techniques**

11. nothing to report.

c. Other Products

12. nothing to report.

7. Participants & Other Collaborating Organizations

a. **What individuals have worked on the project?**

Name:	Hazel Davis
Project Role:	Graduate Student

Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Hazel synthesized PTDMs, studied their complexes with IgG and other proteins, delivered anti-PKCθ into human CD4 cells.
Funding Support:	
Name:	Kayla Kock
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Kayla designed and synthesized new PTDMs, delivered anti-PKCθ into human CD4 cells.
Funding Support:	
Project Role:	Senior Research Fellow
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Sudar delivered anti-PKCθ into human CD4 cells, studied their differentiation into iTregs. Improved iTreg protocol in several areas including cells recovery and iTreg time-evolution.
Funding Support:	
Name:	Deeksha Mohan
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Deeksha delivered anti-PKCθ into mouse CD4 cells, studied their differentiation into iTregs.
Funding Support:	
Name:	Lisa M. Minter

Project Role:	Co-Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0002-1728-6389
Nearest person month worked:	1.5 summer
Contribution to Project:	Lisa directs experimental plan, execution, data acquisition, analysis, and troubleshooting. Lisa trains and mentors all researchers. Lisa develops, reviews, edits, all figures and text for publications.
Funding Support:	
Name:	Eric Decker
Project Role:	Co-Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1.0 summer
Contribution to Project:	Eric directs experimental plan, execution, data acquisition, analysis, and troubleshooting related to two IBD animal models.
Funding Support:	
Name:	Gregory N Tew
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	0000-0003-3277-7925
Nearest person month worked:	2.0 summer
Contribution to Project:	Greg directs all aspects of the project. Greg directs experimental plan, execution, data acquisition, analysis, and troubleshooting. Greg trains and mentors all researchers. Greg develops, reviews, edits, all figures and text for publications.
Funding Support:	

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

-Guodong Zhang left UMass. We replaced him with Prof. Eric Decker from the same department who will oversee the proposed animal experiments along with Prof. Hang Xiao and their research groups.

c. **What other organizations were involved as partners?**

-nothing to report.

8. Special Reporting Requirements

- **QUAD CHARTS:**

Supersuppressive-Induced Regulatory T-Cell Populations for IBD Enabled by Intracellular Antibody Delivery

(W81XWH-20-1-0536)

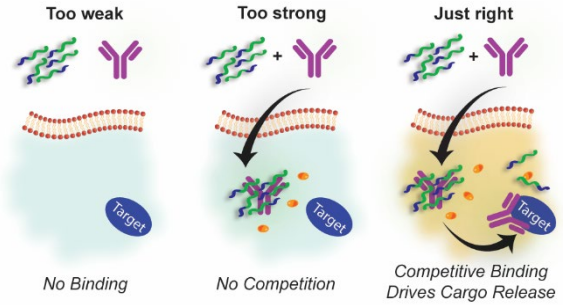
PI: Tew, Gregory N. **Org:** University of Massachusetts, Amherst **Award Amount:** $1,876,523.40

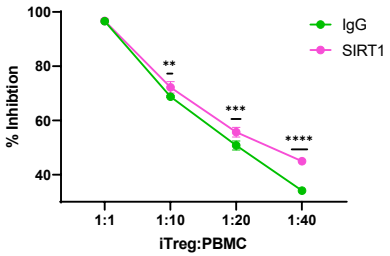
Study/Product Aim(s)

- Synthesize PTDMs for intracellular antibody delivery
- Characterize PTDM:antibody complexes for delivery into CD4 cells.
- Evaluate PTDM:antibody delivery with one, or more, antibody including anti-PKCθ and anti-SIRT1.
- Quantify iTreg phenotype for human CD4 with respect to CD127, Foxp3, IFN-γ, suppression activity, and plasticity toward Th17.
- Quantify iTreg phenotype for mouse CD4 with respect to CD127, Foxp3, IFN-γ, suppression activity, and plasticity toward Th17.
- Determine in vivo activity in animal models of IBD.

Approach

PTDMs are synthesized using living ring-opening metathesis polymerization (2005 Nobel Prize). These bio-inspired synthetic vectors enable intracellular antibody delivery. CD4 cells are isolated and treated with PTDM:antibody, differentiated into iTregs over 5-7days. This iTreg phenotype, which appears to be super-suppressive, is characterized by key markers and suppression activity.





Accomplishment: New PDTMs were synthesized. PTDM:antibody complexes were studied. New analytic methods were developed to understand their molecular interactions. Biophysical assays studied release which is essential for intracellular antibody activity. Treated CD4 cells (human and murine) differentiate into iTregs following anti-SIRT1 delivery. Suppression assays supported anti-SIRT1 is a more effective target than anti-pPKCtheta. Humanized IBD models established

Timeline and Cost

Activities	CY 21	22	23
Major Task 1 (subtasks 1-8)			
Major Task 2 (subtasks 1-2)			
Major Task 3 (subtasks 1-3)			
Major Task 4 (subtasks 1-4)			
Estimated Budget ($K)	**$475**	**$700**	**$700**

Updated: (07/31/2022)

Goals/Milestones

CY22 Goal – PTDM synthesis, complex with antibody, deliver to CD4 cells

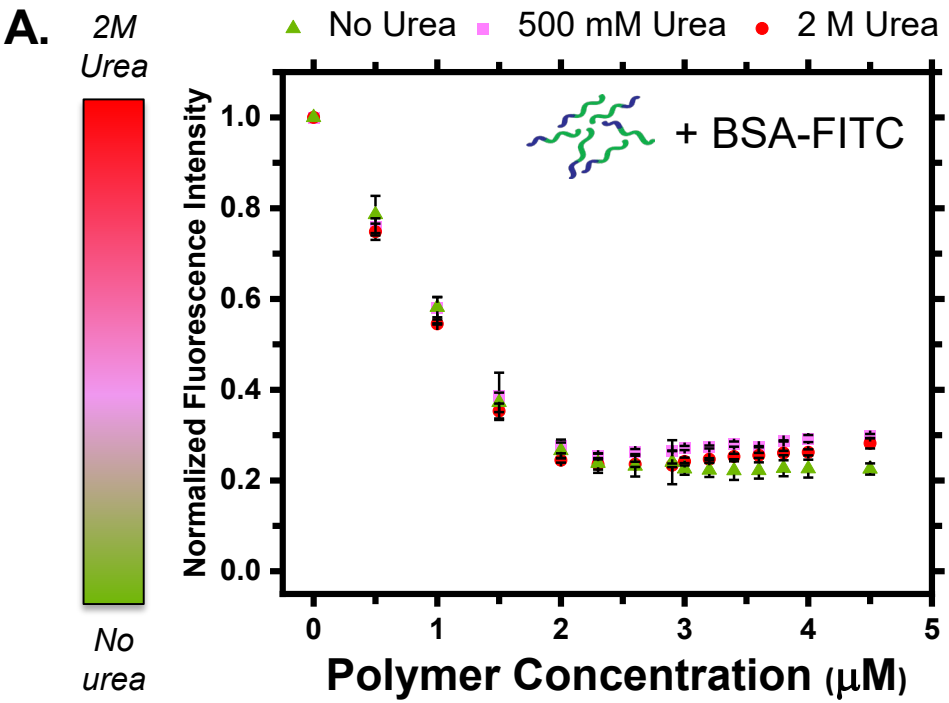
- ☑ Produced several new PTDMs and antibody complexes
- ☑ Delivered to human and mouse CD4 cells, differentiated into iTregs
- ☑ Characterized with respect to CD127 and Foxp3
- ☑ iTreg suppression activity
- ☑ Humanized IBD model
- ☐ Optimized iTreg activity versus antibody concentration, types of antibody delivered, and differentiation time.
- ☐ iTreg in vivo activity in IBD models

Comments/Challenges/Issues/Concerns

- Covid-19 pandemic slowed all aspects and promoted Prof. Guodong Zhang's departure from UMass.
- Total recovered CD4 cells following continues to require optimization. Several improvements were implemented; a few more to explore.
- Spending remains behind schedule (~$750,000 ending July).

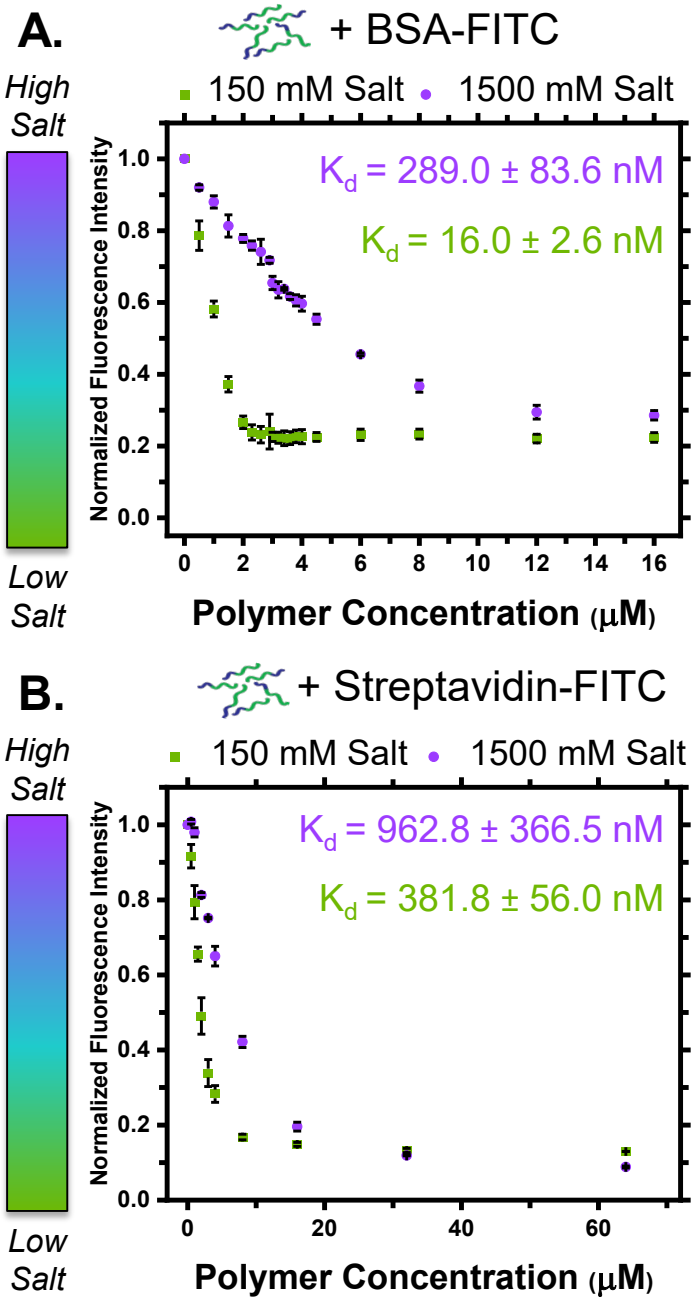
Appendix A: Significant Results

Figure 1



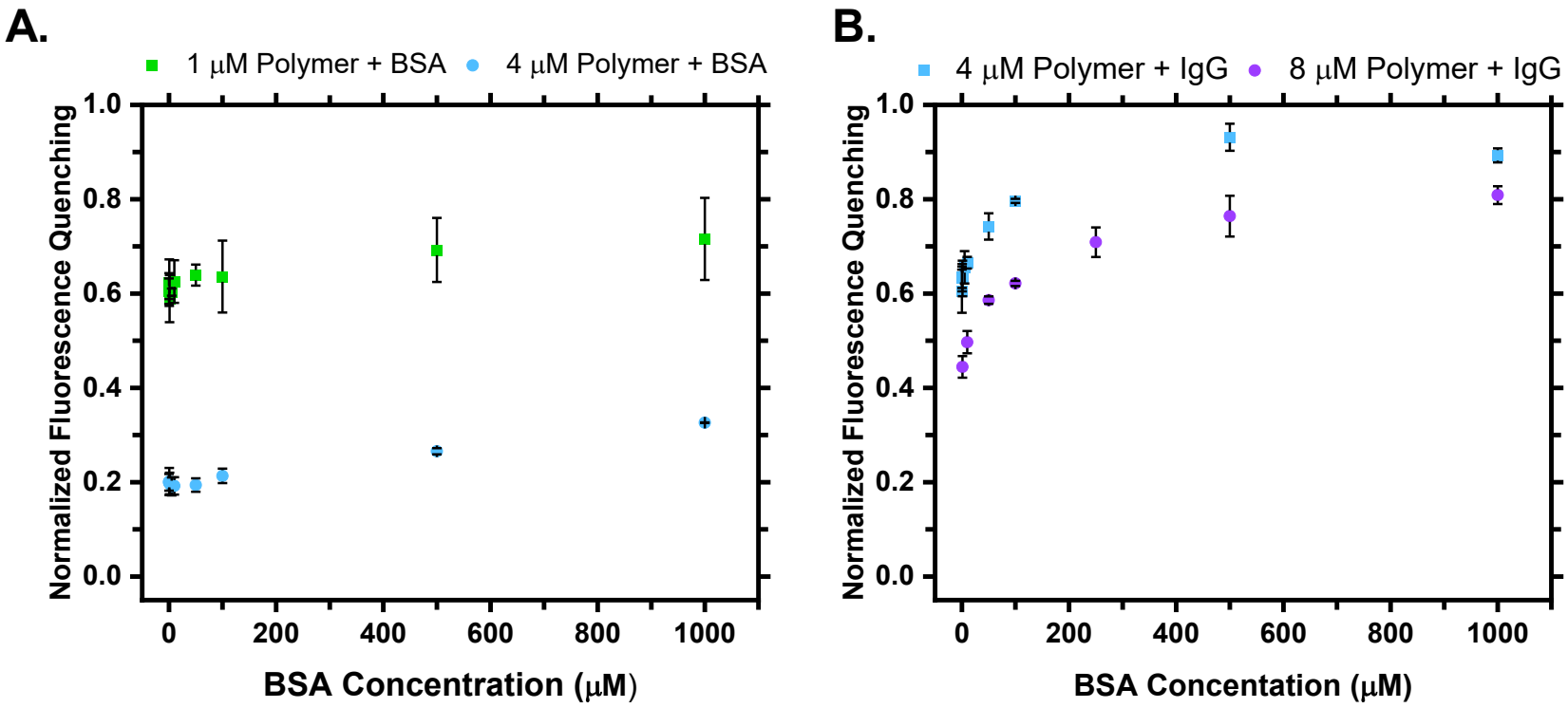
Comparison of A) BSA-FITC and B) IgG-FITC and polymer *MePh10-b-dG5* binding curves measured using equilibrium fluorescence quenching experiments. For A) BSA-FITC, three urea conditions were measured: no urea (green), 500 mM urea (pink), and 2M urea (red). For B) IgG-FITC, two urea conditions are measured: no urea (green) and 500 mM urea (pink). Scale bars to left of each plot represent gradient in urea added. Quenching is normalized to a positive control of BSA-FITC only. Experiments were conducted in duplicate (with the exception of BSA-FITC with no urea, which was conducted in triplicate) and error bars represent +/- one standard deviation.

Figure 1-continued



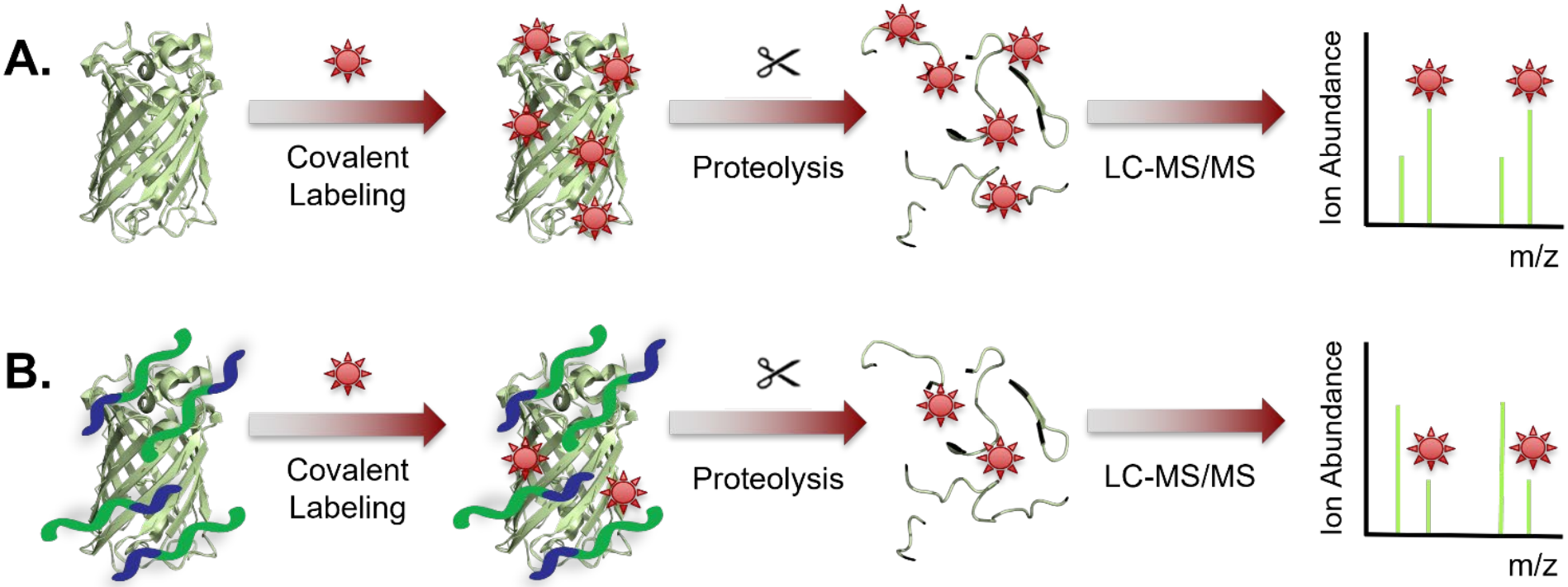
Comparison of A) BSA-FITC and B) Streptavidin-FITC and polymer *MePh10-b-dG5* binding curves measured using equilibrium fluorescence quenching experiments. For each protein, two salt conditions are shown here: 1x PBS containing approximately 150 mM total salts (green), and 10x PBS containing approximately 1500 mM total salts (purple). Scale bars to left of each plot represent gradient in salt content of PBS. Fluorescence quenching experiments were conducted in duplicate (with the exception of BSA-FITC in 1x and 10x PBS, which were conducted in triplicate) and error bars represent +/- one standard deviation. Quenching is normalized to a positive control of BSA-FITC only. Fluorescence intensity plots are translated to fractional saturation plots for fitting to calculate Kd values as described in a previous report. Calculated Kd values are shown with +/- standard error associated with the fitting.

Figure 1-continued



Utilizing BSA as a competitor for polymer:BSA-FITC or polymer:IgG-FITC binding. Binding of A) BSA-FITC or B) IgG-FITC with *MePh10-b-dG5* with increasing concentrations of competitor BSA as measured using equilibrium fluorescence quenching experiments. Various concentrations of *MePh10-b-dG5,* representing different positions of the initial binding curves measured in Figure 3A and **SI.** For BSA-FITC, 1 μM (green) and 4 μM (blue) polymer were measured, and for IgG-FITC, 4 μM (blue) and 8 μM (purple) were measured. The concentration of fluorescently labelled protein in all experiments was 200 nM. Fluorescence quenching experiments were conducted in duplicate and error bars represent +/- one standard deviation. Values are normalized to the fluorescence intensity of labelled protein with each respective BSA concentration in the absence of polymer.

Figure 1-continued



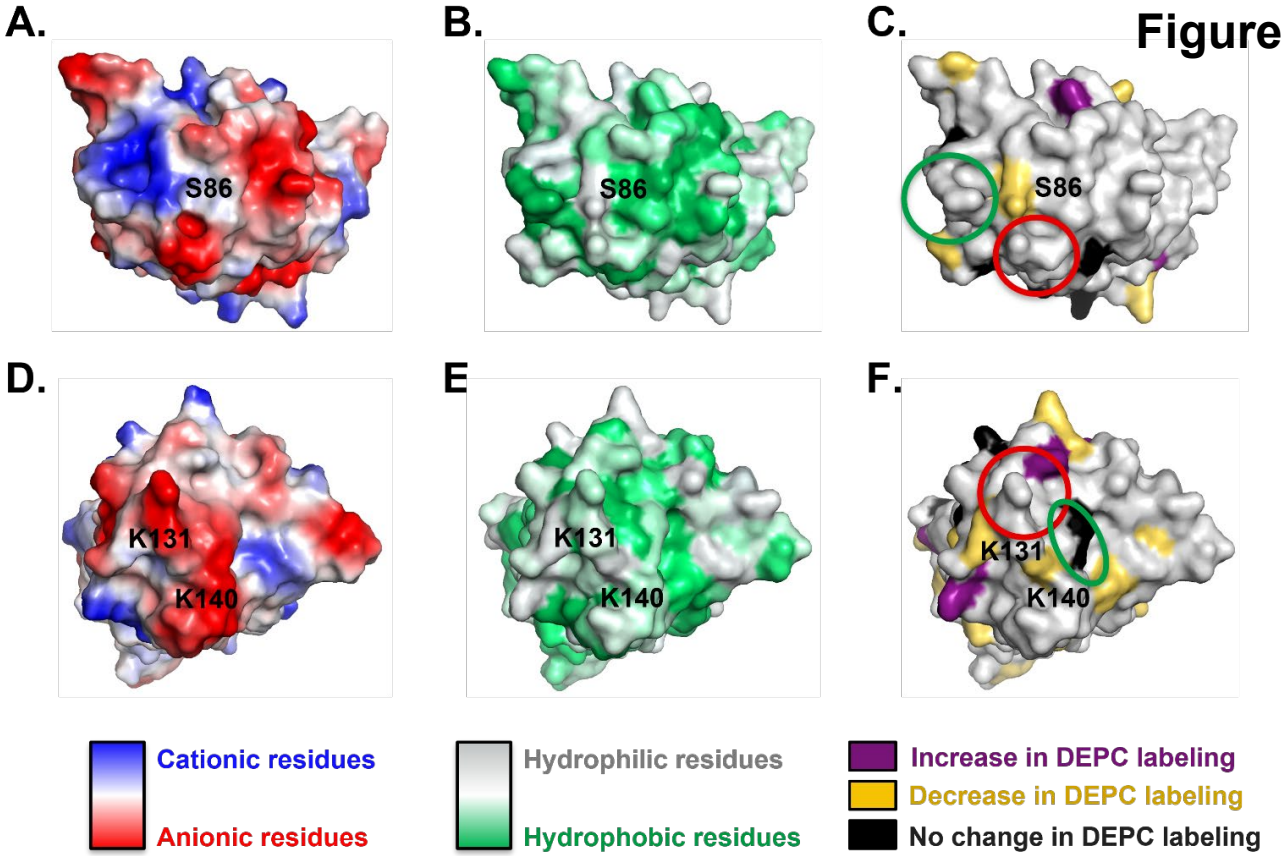
Cartoon of experimental workflow of CL-MS experiment. A) Native sfGFP protein (PDB: 2B3P) is labeled with reagent of choice (red star) wherein the reagent will react with surface-exposed residues. Following proteolysis, the labeled protein is analyzed by LC-MS/MS in order to identify the labeled residues in the protein. B) In a parallel experiment, native sfGFP protein complexed with polymer is labeled with the same reagent and sites occupied by polymers are protected from labeling. Interactions between polymers and protein are depicted as cartoons, and actual complex sizes may be much larger. Following proteolysis, the labeled residues are identified by LC-MS/MS analysis. A comparison of the data from (A) and (B) reveal polymer-protein surface binding sites.

Figure 1-continued

DEPC-labeled Residue	3:1 Polymer:sfGFP	20:1 Polymer:sfGFP	40:1 Polymer:sfGFP
K3	No Change	Decrease	Decrease
T9	Decrease		
H25		Increase	Increase
K26		Decrease	Decrease
S28		Increase	Decrease
T38	No Change	Increase	Decrease
K41	No Change	Decrease	Decrease
H81		No Change	Decrease
K85	No Change	No Change	Decrease
S86		Decrease	Decrease
Y92	No Change	Decrease	Decrease
S99		Decrease	Decrease
K101	No Change	Increase	Decrease
T105		No Change	Decrease
K107	No Change	No Change	No Change
T108		No Change	No Change
K113	No Change	No Change	Decrease
T118	No Change		
K126	No Change	No Change	Decrease
K131		Decrease	Decrease
H139		No Change	Decrease
K140	Increase	Decrease	Decrease
Y143	Decrease	Decrease	Decrease
S147		Decrease	Decrease
H148	No Change		
Y151	No Change	Decrease	Decrease
T153		Decrease	Decrease
K156	No Change	Decrease	Decrease
K158		No Change	No Change
K162	No Change	Increase	Decrease
K166	No Change	No Change	Decrease
H217	Decrease	Decrease	Decrease
H231		Decrease	Decrease

Summary of DEPC labeling changes in sfGFP as a function of increasing polymer:sfGFP ratio, originally plotted as each amino acid residue with its corresponding percentage of labeling change in Figure 6. Modified residues experiencing a decrease in DEPC-labeling in the presence of polymer are highlighted in gold, modified residues with an increase in DEPC-labeling are shown in purple, and modified residues experiencing no statistically significant change to the DEPC-labeled sfGFP control are highlighted in gray. Blank rows indicate residues that did not experience DEPC-labeling per respective condition. Determination of significant changes in labeling in the presence of polymer was based upon Student's *t*-test with 95% confidence interval.

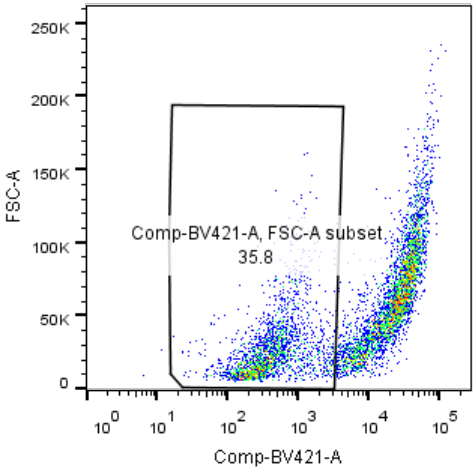
Figure 1-continued



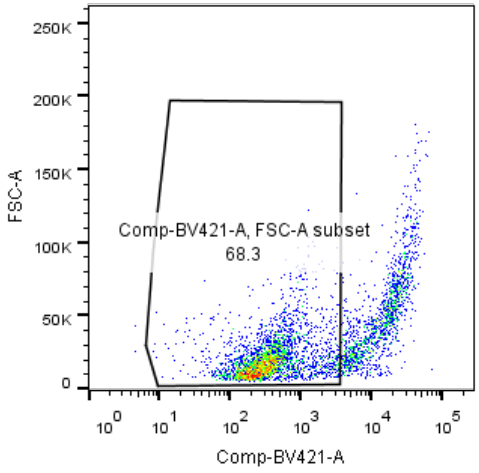
sfGFP surface maps showing the locations of residues that experience changes in DEPC-labeling in comparison to anionic and hydrophobic patches on sfGFP surface. A, B, and C) represent one orientation of sfGFP with D, E, and F) representing a second view. A) and D) portray electrostatic surface maps with positively charged residues highlighted in blue and negatively charged residues highlighted in red. B) and E) denote hydrophobic patches on the sfGFP surface, adapted from Eisenberg hydrophobic moments,[42] where more hydrophobic residues are highlighted in green. Finally, C) and F) represent amino acid residues that experience either an increase (purple), a decrease (gold), or no change (black) in DEPC-labeling in the presence of 20:1 polymer:sfGFP as compared to DEPC-labeling of sfGFP alone. Red and green circles are used to draw attention to specific anionic and hydrophobic regions of sfGFP surface, respectively, which are located particularly close to residues with observed decreases in DEPC labeling.

Live/Dead

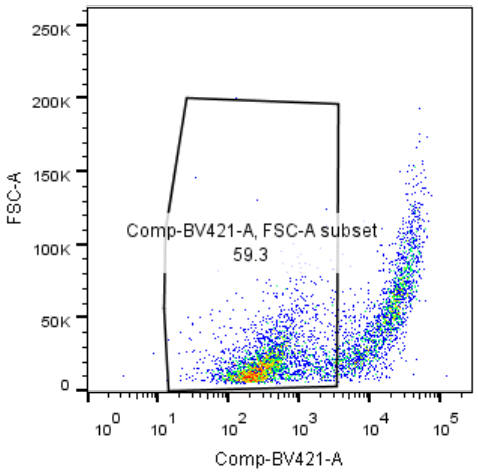
CPPM



CPPM+IgG

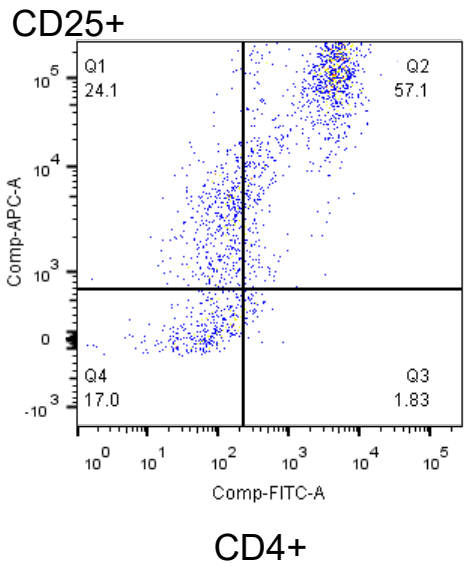


CPPM+anti-Sirt1

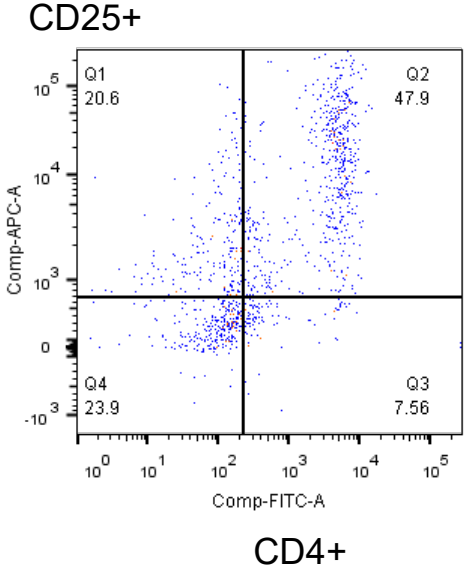


CD4+CD25+ population

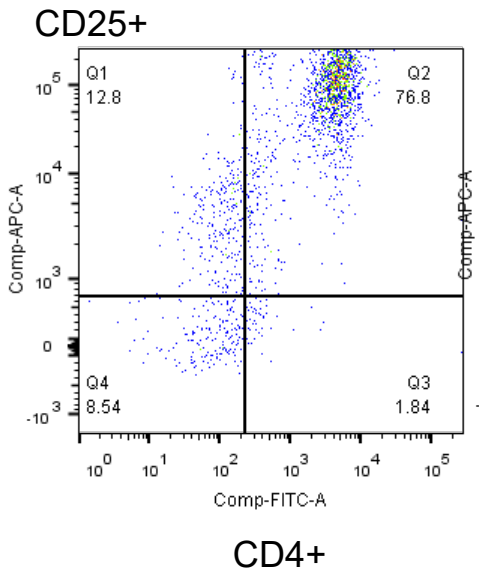
Non-polarized-1



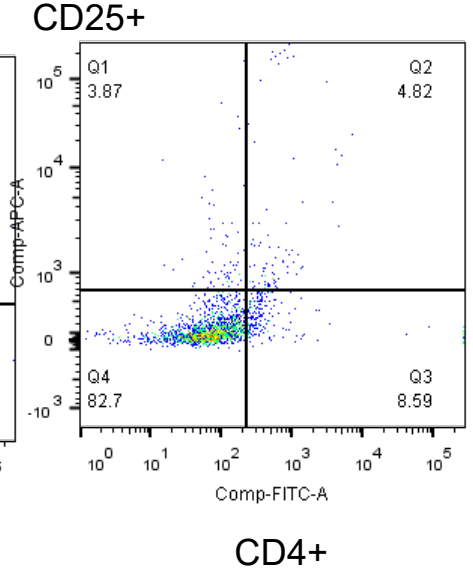
Treg



Non-polarized-2

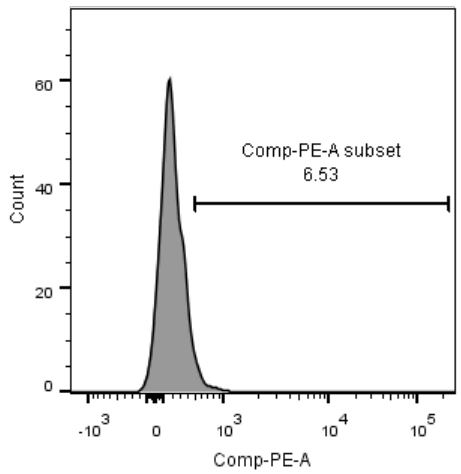


Th17



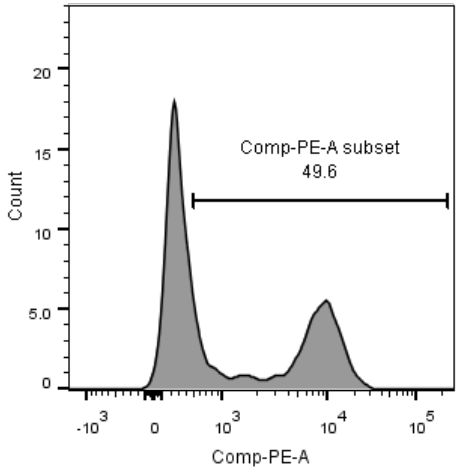
FOXP3+ within CD4+CD25+CD127-

Non-polarized-1



Foxp3+

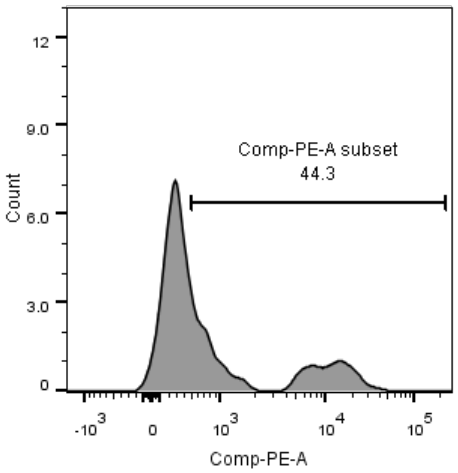
Treg



Foxp3+

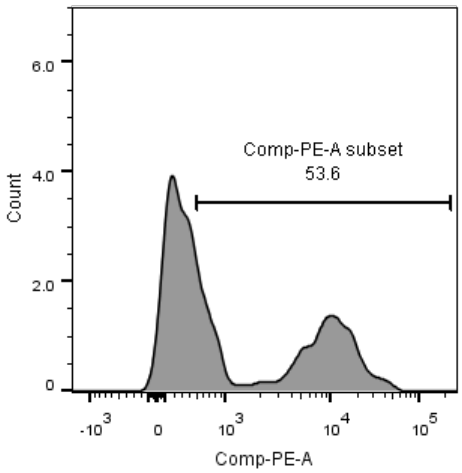
FOXP3+ within CD4+CD25+CD127- population

CPPM



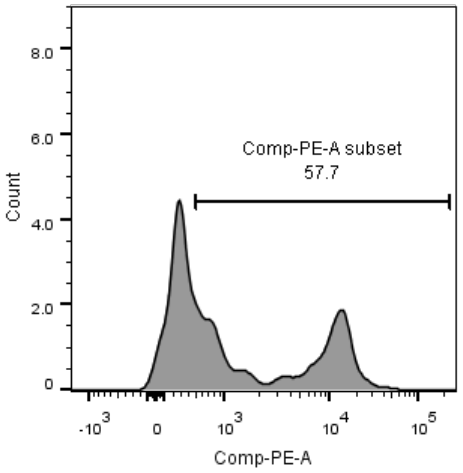
Foxp3+

CPPM+IgG



Foxp3+

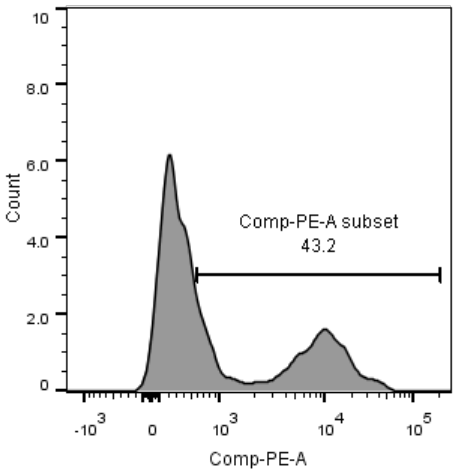
CPPM+anti-Sirt1



Foxp3+

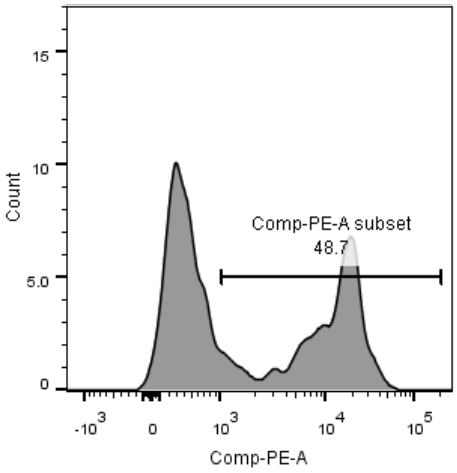
FOXP3+ within CD4+CD25+ population

CPPM



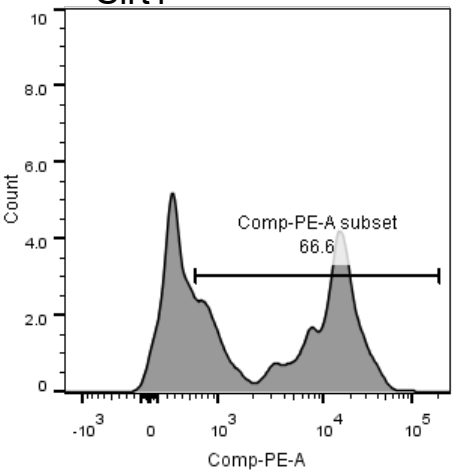
FOXP3+

CPPM+IgG



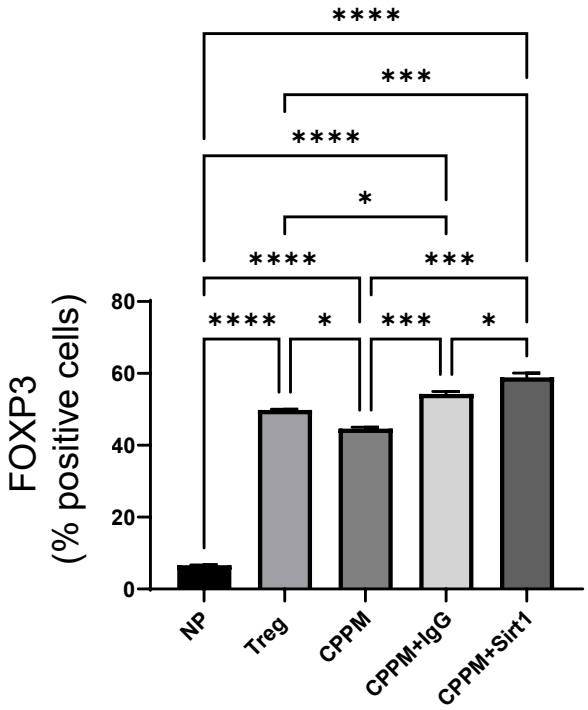
FOXP3+

CPPM+anti-Sirt1



FOXP3+

Percent FOXP3+ and MFI within CD4+CD25+CD127- population



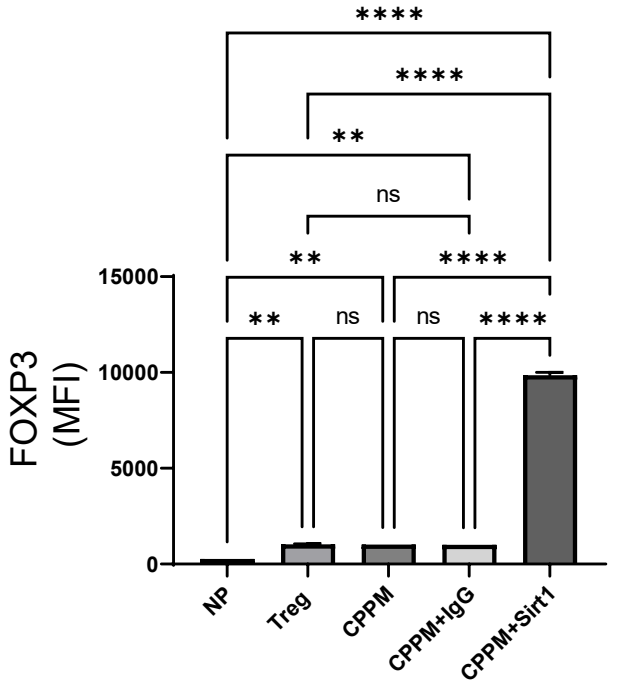
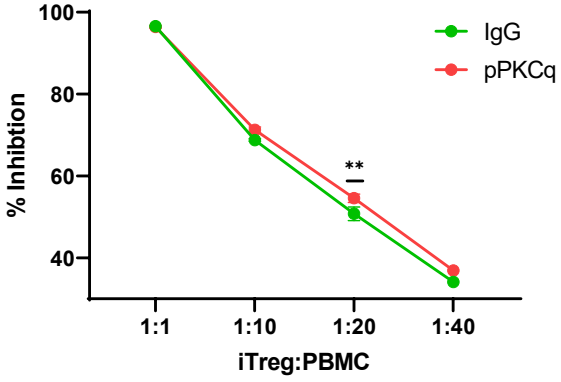
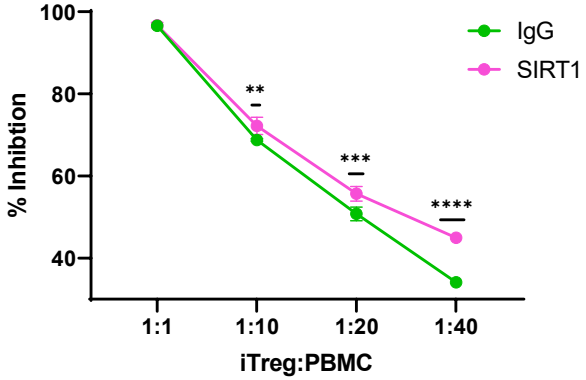


Figure 3

Suppression Assays

Comparison with IgG





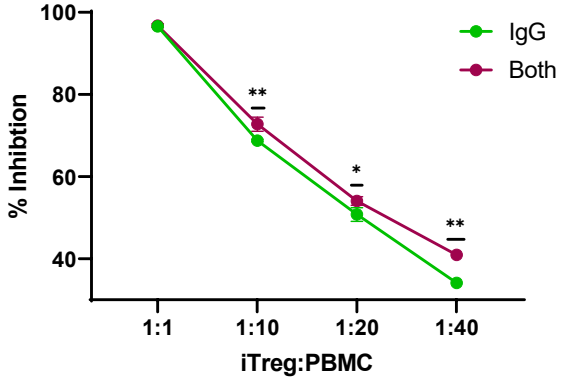


Figure 4

Colon Length

Mouse	Length (cm)	Clinical score (D18)	Splenocyte count
Blue	6.5	5	1.2*10^6
Green	7.5	x	>1.5*10^5
Red	7.0	8	1.6*10^5
Black	7.5	4	3.2*10^6
White	7.7	2	21.2*10^6

Splenocytes