University of Nevada, Reno

Determining the Difference Between Circulating Follicular Regulatory T-cells and Follicular Helper T-cells in Subjects with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome-cells by Flow Cytometry

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science in the Biochemistry and Molecular Biology Major
and the
Honors Program

by

Ambreen Azim

Mentor: Dr. Vincent Lombardi

Supervisor: Shanti Rawat, MS

Department of Biochemistry and Molecular Biology/Pathology

May 2018

UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

AMBREEN A. AZIM

entitled

Determining the Difference Between Circulating Follicular Regulatory T-cells and Follicular Helper T-cells in Subjects with Myalgic Encephalomyelitis/Chronic Fatigue Syndrome-cells by Flow Cytometry

be accepted in partial fulfillment of the
requirements for the degree of

BACHELOR OF SCIENCE, BIOCHEMISTRY AND MOLECULAR BIOLOGY
and the
HONORS PROGRAM

_____
Vincent C. Lombardi, Ph.D., Thesis Advisor

_____
Tamara Valentine, Ph. D., Director, **Honors Program**

May 2018

Acknowledgements

I wish to express my sincere gratitude to Dr. Vincent Lombardi for mentoring me through conducting my experiments as well as helping me to write my thesis. The knowledge that I have gained throughout the semesters with Dr. Lombardi is immeasurable and the experiences we have had together are unforgettable. I would also like to thank Shanti Rawat for her help in the laboratory and the staff at the Nevada Center for Biomedical Research for allowing me to work in their laboratory.

Thank you to the Honors Program at the University of Nevada, Reno for enabling me to unlock my potential during my undergraduate career and in helping me create a thesis project that I am proud of.

Finally, this thesis is dedicated to my parents and brother, for without their love and support, my passion for science would not have blossomed.

Table of Contents

Abstract

The pathogenesis of myalgic encephalomyelitis/chronic fatigue syndrome (ME/CFS) is currently unknown; however, B cell dysregulation has been suggested to be involved. It is well known that balance between follicular helper T cells (TFH) and follicular regulatory T cells (TFR) is important in the regulation of B cell responses. The purpose of this study is to develop methodology to accurately and reproducibly evaluate circulating TFR and TFH in ME/CFS cases and compare this to healthy controls. Immunophenotyping kits from Beckman Coulter, Thermo Fisher and BD Biosciences were tested using the protocol adapted from Wang et al. [1]. The final results show that the kit from BD Biosciences was the best performing kit. The kit from Beckman Coulter showed less antibody binding to the nuclear protein Foxp3, which is used to identify T-regulatory cells (Tregs). Also, the kit from Thermo Fisher did not show significant differences between stimulated cells and unstimulated cells. With the finalized protocol validated, future testing will begin using peripheral blood (PBMCs) from 20 ME/CFS cases and 20 healthy controls. These donors will be examined for the frequencies of circulating TFH and TFR by flow cytometry. Alterations in circulating TFH and TFR cells shift the balance from immune tolerance to immune responsive state, potentially contributing to deregulation of B cell immunity and the pathogenesis of ME/CFS. We hypothesize that there will be a decrease in the frequency of Treg cells, particularly $CD45RA^{+}FoxP3^{+}$ Treg cells in ME/CFS patients when compared to healthy controls.

1.0 Introduction

1.1 Background on Myalgic Encephalomyelitis/Chronic Fatigue Syndrome

Myalgic encephalomyelitis, also known as chronic fatigue syndrome or ME/CFS, is a heterogeneous disorder of unknown etiology, commonly characterized by neurological symptoms, inflammatory sequelae and innate immune dysregulation [2]. Other notable symptoms include gastrointestinal and cardiovascular abnormalities [3]. According to the National Organization for Rare Diseases, the onset of ME/CFS is usually associated with a viral infection, such as a cold or respiratory illness, but can also be onset by non-viral factors, such as environmental toxins or trauma [4]. Current estimates propose that up to 2.5 million individuals are afflicted, with an annual productivity loss in excess of $9 billion in the United States alone, underscoring the significant impact of ME/CFS as a major public health concern both economically and socially [5]. There currently is no cure for ME/CFS, leaving thousands of those impacted, unable to perform basic day to day functions. ME/CFS is regularly misdiagnosed due to the lack of non-subjective biomarkers; therefore, identifying specific biological parameters that can be used to diagnose potential cases would dramatically improve patient care and reduce healthcare costs.

1.2 Research Goals

Gastrointestinal issues are common among those with ME/CFS to such an extent that many are initially misdiagnosed as having inflammatory bowel disease (IBD) [6]. Additionally, previous studies have suggested that IBD can be characterized as having alterations in the balance between TFH and TFR [1]. With this in mind, as well as the

previous observations that suggest subjects with ME/CFS have a B cell abnormality, the goal of my research project is to develop methodology that will be able to identify potential differences in specific TFH and TFR populations of immune cells in subjects with ME/CFS, when compared to healthy controls. The immune cells on which I will focus on are called T-regulatory cells (Tregs). They are a subset of CD4 positive T lymphocytes and are responsible for modulating the immune system with regard to tolerance of self-antigens and prevention of autoimmune disease. Recognizing differences in involved lymphocyte populations in ME/CFS pathophysiology will enable future research to focus on treating this disease and potentially finding a cure. The information derived as a result of this study may also benefit other diseases with overlapping pathology.

The protocol we are using was adapted from Wang et al. [1]. To accomplish this, we start with total lymphocytes and phenotype the cells of interest with fluorescently labeled monoclonal antibodies that bind to specific proteins expressed on and within those cells. The cells are then evaluated by flow cytometry and potential differences between cases and controls are determined.

2.0 Materials and Methods

2.1 Antibodies Specific to The Study

The antibodies used to surface-label specific cell populations are as follows: APC anti-human CD3 Clone: UCHT1, Alexa Fluor® 700 anti-human CD4 Clone: SK3, PE/Cy7 anti-human CD25 Clone: BC96, Pacific Blue™ anti-human CD45 Clone: HI30, FITC anti-human CD45RA Clone: HI100, and APC/Cy7 anti-human CD185 (CXCR5)

Clone: J252D4 (BioLegend, Inc, San Diego, CA). Additionally, the following antibody was used to intracellularly stain specific cell populations: PE anti-mouse/rat/human FoxP3 (forkhead-box P3, is a regulatory protein involved in the development and function of circulating follicular regulatory T cells involved in the immune response) Clone:150D. In order to differentiate non-specific binding of antibodies to Fc receptors, we also utilized the following isotype controls: FITC Mouse IgG1, x Isotype Ctrl (FC) Clone: MOPC-21, Mouse IgG1-PE (BioLegend, Inc, San Diego, CA).

2.1.1 Titration of Antibodies to Optimal Concentration

In order to determine the optimal concentration of antibodies to be used, titrations were run for the antibodies: FITC anti-human CD3, AF700 anti-human CD4, PB anti-human CD45, FITC anti-human CD45RA, APC/Cy7 anti-human CD185, and PE/Cy7 anti-human CD25. For each antibody, six sets of tubes were labeled as follows: 100, 50, 25, 12.5, 6.25, and 0, representing the respective concentrations of antibody. Then, 50 ul of fresh human whole blood was added to each tube. Next, four microcentrifuge tubes were then labeled 50, 25, 12.5, 6.25 and 2.5 ul of staining buffer was added to each tube. Then 2.5 ul of the respective stock antibody was added to each tube labeled ‘50’. The samples were mixed by vortexing, then centrifuged and 2.5 ul of this was pipetted into the ‘25’ microcentrifuge tube and the process repeated ending at the ‘6.25’ tube. Next, 2.5 ul of undiluted antibody was added to the blood tube 100 and 2.5 ul of diluted antibody was added to each respective blood tube, and mixed by vortexing at medium speed. No antibody was added to the ‘0’ tube but the tube was vortexed in order to maintain consistency between all the tubes. The samples were incubated in the dark for

15 minutes. After incubation, 1 mL of FACS lyse buffer was added to each test tube to lyse the red blood cells over the course of 10 minutes, or until the turbid solution turned transparent. After the solution was transparent, 3 mL of PBS was added to each tube and the samples were centrifuged at 1200 rpm for 8 minutes. The samples were then aspirated (being careful not to disturb the cell pellet at the bottom of the tube) and suspended in 300 ul PBS. The samples were transferred to a 96-well round-bottom plate and run on the BD LSR II Flow Cytometer. Data was analyzed using the cytometry analysis program FlowJo and the relative antibody concentrations were determined based on their level of fluorescence compared to each other and non-stained controls.

2.2 Running Compensation Beads

Compensation beads were run during each experiment and for each fluorescently labeled antibody. Compensation beads are polystyrene beads that are conjugated to goat-anti-mouse-Fc antibody. Therefore, these beads bind to fluorescently labeled mouse monoclonal antibodies and can simulate antibody-cell binding but in a consistent manner. Compensation beads are used so that the analysis software can calculate the amount of spectral overlap that occurs when the fluorescence of one fluorophore spills over into a detection channel of another fluorophore. For instance, the emission spectra of FITC overlaps into the detection range of the detector that measures PE making the PE signal appear greater that it actually is. This “spill-over” must be subtracted from each channel in order to obtain accurate results.

Compensation bead were prepared by added 50 ul of staining buffer to each test tube, 1 ul of beads, and 1 ul of a specific antibody. Only one type of antibody was added

to each tube. These beads were then incubated for 15 minutes at room temperature in the dark. After incubation, 4 mL of PBS was added to each tube and centrifuged at 1200 rpm for 8 minutes. The samples were then aspirated down to approximately 300 ul and loaded onto the 96-well round-bottom plate being used in the experiment. The beads were run on the flow cytometer and the data was used to automatically compensate the experimental results for the cases and controls.

2.3 Gating Protocol

Once the samples in each protocol were ran on the cytometer, the data were analyzed using a specific method, shown in Figure 1.

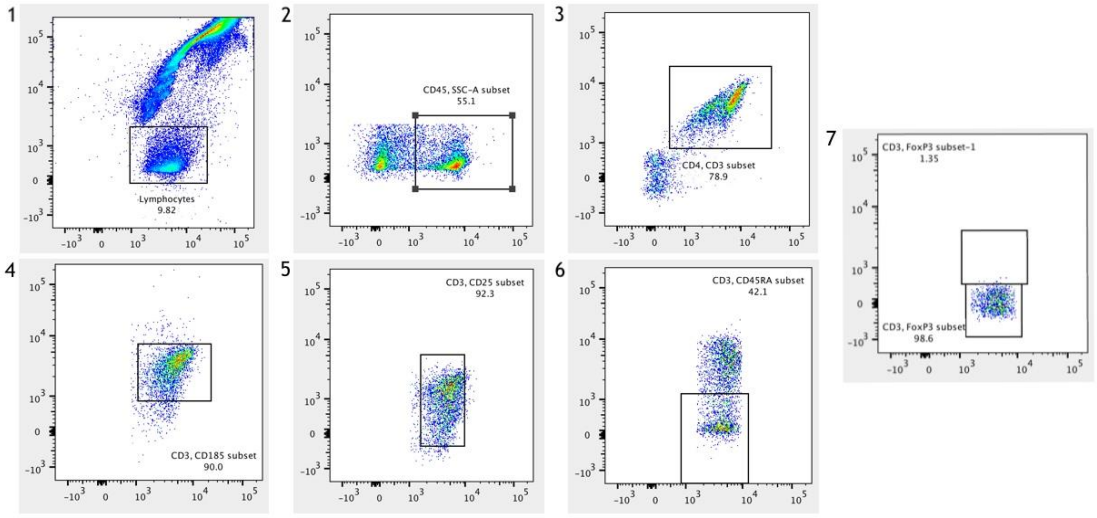

Figure 1. Gating Protocol to Determine TFR/TFH Expression in ME/CFS patients versus healthy controls. Cells were initially gated based on size and light scatter. Then the samples were gated on their expression of the leukocyte antigen CD45. Next, cells were selected based on their expression of the T cell marker CD3 and the T cell marker CD4. These CD3/CD4 positive cells were further gated to evaluate their expression of CD185, CD25, CD45RA and FoxP3.

2.4 Intraprep Permeabilization Reagent Kit from Beckman Coulter

2.4.1 Preparation and Count of Peripheral Blood Mononuclear Cells (PBMCs)

For this research, PBMCs (peripheral blood mononuclear cells) were used, which were taken from ME/CFS cases and healthy controls in Belgium and across the United States. PBMCs are any peripheral blood cells that have a round nucleus, such as lymphocytes or monocytes. Cryopreserved PBMCs were removed from liquid nitrogen storage and thawed on a heating block at 37 °C for 5 minutes. A sample of human AB serum was thawed in warm water for 15 minutes. The PBMCs were then transferred to a 15 mL centrifuge tube and 10 mL of PBS was added. The mixture was then centrifuged at 1200 rpm for 8 minutes. The supernatant was aspirated and the pellet was suspended in 2 mL of PBS.

For each sample, 10 ul of the PBMC sample was mixed with 10 uL of Trypan Blue stain 0.4% from which 10 uL was loaded onto a hemocytometer. The number of viable (non-blue) PBMCs in the top row of the middle square of the hemocytometer were averaged as shown in Figure 2 (red). This number was then multiplied by twenty-five, to give the total number PBMCs in the middle square of the hemocytometer (shown in green in Figure 2). This number was then multiplied by 10,000 to give the number of PBMCs in 1 mL of the stained solution and multiplied by two to yield the total number of PBMCs per milliliter of prepped sample. The total was then used to calculate the amount of human AB serum needed to adjust the cell count to 1 million PBMCs per 1 mL of solution.

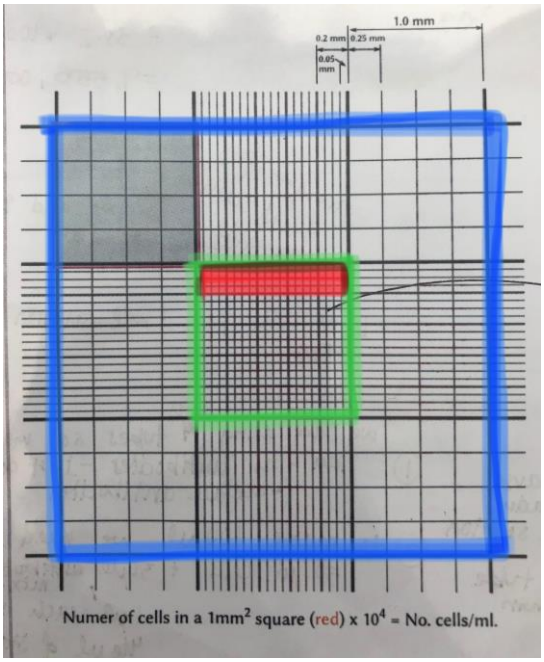

Figure 2. Hemocytometer grid for counting cells. Total number of PBMCs in one row shown in red. Total number of PBMCs in one square shown in green. Total number of PBMCs in original sample shown in blue.

2.4.2 Surface Antibody Labelling

Three test tubes for the isotype control, antibody control, and experimental were prepared and labeled “ISO”, “Blank” and “EXP”, respectively, shown in Figure 3. Three test tubes for the isotype control, antibody control, and experimental were prepared and labeled “ISO”, “Blank” and “EXP”, respectively. In the ISO, EXP and Blank tubes, the following were added: 50 ul of the prepared PBMCs; 2.5 ul of CD3, CD4, CD25, and CD45; and 40 ul of staining buffer. In the EXP tubes, CD45RA and CD185 were added as well. The samples were then vortexed and incubated in the dark for 15 minutes.

#/ Bio-Ab (Lymphs 50 uL blood)	Pac Blue (460)	Pac Orange (585)	(605)	FITC (515)	PerCP (675)	PE (575)	PE-TxR (625)	PE-Cy5 (670)	PE-Cy 5.5 (730)	PE-Cy7 (780)	APC AF647 (670)	AF700 (730)	APC-Cy7/AF750 (780)
ISO	CD45			*Iso		*Iso				CD25	CD3 APC	CD4	*Iso
						IgG1,k							
Blank	CD45			—		—				CD25	CD3 APC	CD4	—
						IgG1,k							
EXP	CD45			CD45RA		*FoxP3				CD25	CD3 APC	CD4	CD185 APC-Cy7
						IgG1,k							IgG1,k

Figure 3. Antibody Panel showing the contents of ISO, EXP, and Blank tubes.

The tubes were then treated with Reagent #1 from the Intraprep kit to fix the antibodies to the surface of the cells. 100 ul of Intraprep Reagent #1 was added to each test tube. The samples were vortexed and incubated in the dark for 15 minutes. After incubation, 4 mL of PBS was added to each sampled and centrifuged art 1200 rpm for 8 minutes. The supernatant was aspirated and the pellet was suspended in 300 ul of PBS.

2.4.3 Intracellular Antibody Labelling

The tubes were then treated with 100 ul of Intraprep Reagent #2 from the Intraprep kit to perforate the cells and allow internal stains to enter the cells. The samples were mixed gently, no vortex, and incubated in the dark for 5 minutes. The samples were mixed gently after incubation. 2.5 ul of FITC Iso-control (Mouse IgG1) and 2.5 ul of Mouse IgG1-PE was added to the ISO tube. 2.5 ul of PE-anti-mouse/rat/human FoxP3 was added to the ‘EXP’ tube. The samples were gently mixed and incubated in the dark for 15 minutes. 4 mL of PBS was added to each test tube and centrifuged at 1200 rpm for 8 minutes. The supernatant was aspirated and the pellet suspended in 300 ul of staining buffer.

2.4.4 Running Samples in BD LSR II Flow Cytometer and Analyzing Data in FlowJo Program

The samples were loaded into a 96-well round-bottom plate and run in the flow cytometer for fluorescence detection. Data was collected for each sample for 130 seconds or until 1 million events were collected, whichever occurred first. The list mode files (raw cytometry data files) were annotated and analyzed using the computer program FlowJo program.

2.5 Invitrogen™ eBioscience™ Foxp3/Transcription Factor Staining Buffer Set from ThermoFisher Scientific

2.5.1 Thawing, Count and Stimulation of Cells

Samples were thawed in 10 mL of complete culture media and centrifuged at 1200 rpms for 8 minutes. The samples were then aspirated and pellets were suspended in 10 mL of fresh culture media. A cell count was taken of each sample by mixing 10 ul of the PBMC sample with 10 uL of Trypan Blue stain 0.4% and quantitating the mix in the Countess automated cell counter (Invitrogen). Cells were diluted with fresh culture media to 1 million cells/mL of solution and half of each donor was loaded on a 24-well plate, 1 mL per well. 1 ul/mL of Leukocyte Activation Cocktail with GolgiPlug™ by BD Biosciences was added to the remaining half of each donor and loaded alongside unstimulated controls. The samples were incubated at 37 °C in 5% CO_2 for 4 hours. The cells were then harvested and distributed across flow tubes, 50 ul per tube.

2.5.2 Preparing Buffers/Solutions for Cell Staining

The FoxP3 Fixation/Permeabilization working solution was made by combining one part FoxP3 Fixation/Permeabilization Concentrate (4X) to three parts FoxP3 Fixation/Permeabilization diluent. Enough FoxP3 Fixation/Permeabilization working solution was created for 1 mL per tube. The 1X Permeabilization Buffer was made by combining one part Permeabilization Buffer (10X) with nine parts distilled water. Enough 1X Permeabilization Buffer was created for 4.25 mL per tube.

2.5.3 Surface and Intracellular Labelling

Six test tubes were prepared; three for the stimulated and three for the unstimulated cells. For each condition, an isotype control, an antibody control, and an experimental tube were prepared for surface staining of the antibodies, shown in Figure 4. The tubes were labeled “ISO”, “Blank” and “EXP”, respectively, with either a “S” for stimulated or an “U” for unstimulated designation on each tube. In the ISO, EXP and Blank tubes, the following were added: 50 ul of the prepared PBMCs, stimulated and unstimulated; 2.5 ul of CD3, CD4, CD25, and CD45; and 40 ul of staining buffer. In the EXP tubes, CD45RA and CD185 were added as well. The samples were then vortexed and incubated in the dark for 30 minutes.

#/ Bio-Ab (Lymphs 50 uL blood)	Pac Blue (460)	Pac Orange (585)	(605)	FITC (515)	PerCP (675)	PE (575)	PE-TxR (625)	PE-Cy5 (670)	PE-Cy 5.5 (730)	PE-Cy7 (780)	APC AF647 (670)	AF700 (730)	APC-Cy7/AF750 (780)
ISO, S	CD45			*Iso		*Iso				CD25	CD3 APC	CD4	*Iso
						IgG1,k							
Blank, S	CD45			—		—				CD25	CD3 APC	CD4	—
						IgG1,k							
Exp, S	CD45			CD45RA		*FoxP3				CD25	CD3 APC	CD4	CD185 APC-Cy7
						IgG1,k							IgG1,k
ISO, U	CD45			*Iso		*Iso				CD25	CD3 APC	CD4	*Iso
						IgG1,k							
Blank, U	CD45			—		—				CD25	CD3 APC	CD4	—
						IgG1,k							
Exp, U	CD45			CD45RA		*FoxP3				CD25	CD3 APC	CD4	CD185 APC-Cy7
						IgG1,k							IgG1,k

Figure 4. Antibody Panel showing the contents of ISO, EXP, and Blank tubes for stimulated and unstimulated cells.

1 mL of the FoxP3 Fixation/Permeabilization working solution was added to each tube and vortexed. The samples were then incubated for 30 minutes at room temperature in the dark. After incubation, the samples were then washed with 2 mL of the 1X Permeabilization Buffer and centrifuged at 500 rpm for 5 minutes at room temperature. The supernatant was discarded and the cells were resuspended in residual 1X Permeabilization Buffer (approximately 100 ul). For internal staining, 2.5 ul of FITC Iso-control (Mouse IgG1) and 2.5 ul of Mouse IgG1-PE were added to the ISO tube and 2.5 ul of PE-anti-mouse/rat/human FoxP3 was added to the 'EXP' tube. The samples were incubated at room temperature for 30 minutes in the dark.

After incubation, the samples were washed again with 2 mL of the 1X Permeabilization Buffer and centrifuged at 500 rpm for 5 minutes at room temperature. The supernatant was aspirated until there was approximately 300 ul left in each tube. The samples were then loaded onto a 96-well round-bottom plate and run in the cytometer and analyzed in FlowJo as described above.

2.6 BD Pharmingen™ Transcription Factor Buffer Set from BD Biosciences

2.6.1 Thaw and Count PBMCs

PBMCs and human AB serum were thawed on a heating block for 1-3 minutes until completely defrosted. 100 ul of PBMCs were transferred to a 15 mL centrifuge tube with 900 ul of the human AB serum and gently vortexed. For each sample, 10 ul of the PBMC sample was added to 10 uL of Trypan Blue stain 0.4% and counted in the Countess machine by ThermoFisher Scientific.

2.6.2 Preparing Buffers/Solutions for Cell Staining

Solutions were created according to Figure 5. Enough Fixation/Permeabilization Buffer was created to provide 1 mL of buffer for each tube and enough Permeabilization/Wash Buffer was created to provide 7.2 mL of buffer for each tube.

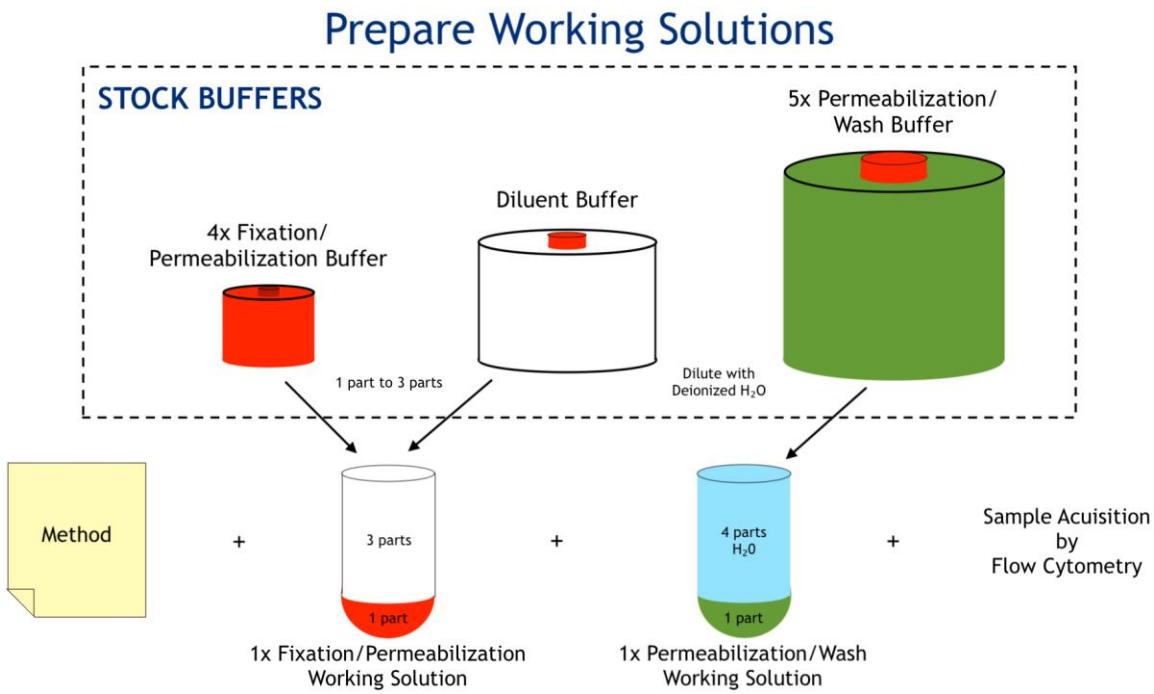

Figure 5. Guide to Preparing Working Solutions with Stock Buffers. Buffers were provided in the Transcription Factor Buffer Set from BD Biosciences (Catalog No. 562574).

2.6.3 Surface and Intracellular Antibody Labelling

Three test tubes for the isotype control, antibody control, and experimental were prepared and labeled “ISO”, “Blank” and “EXP”, respectively, shown in Figure 6. In the ISO, EXP and Blank tubes, the following were added: 50 ul of the prepared PBMCs; 2.5 ul of CD3, CD4, CD25, and CD45; and 40 ul of staining buffer. In the EXP tubes, CD45RA and CD185 were added as well. The samples were then vortexed and incubated in the dark for 30 minutes.

#/ Bio-Ab (Lymphs 50 uL blood)	Pac Blue (460)	Pac Orange (585)	(605)	FITC (515)	PerCP (675)	PE (575)	PE-TxR (625)	PE-Cy5 (670)	PE-Cy 5.5 (730)	PE-Cy7 (780)	APC AF647 (670)	AF700 (730)	APC-Cy7/AF750 (780)
ISO	CD45			*Iso		*Iso				CD25	CD3 APC	CD4	*Iso
						IgG1,k							
Blank	CD45			—		—				CD25	CD3 APC	CD4	—
						IgG1,k							
EXP	CD45			CD45RA		*FoxP3				CD25	CD3 APC	CD4	CD185 APC-Cy7
						IgG1,k							IgG1,k

Figure 6. Antibody Panel showing the contents of ISO, EXP, and Blank tubes.

After incubation, 2 mL of stain buffer was added to each tube and then centrifuged and aspirated. Then 1 mL of the Fixation/Permeabilization (1X) buffer was added to each tube and vortexed for 3 seconds. The samples were then incubated at room temperature for 40 minutes in the dark.

After the second incubation, 1 mL of Permeabilization/Wash (1X) buffer was added to each tube and centrifuged and aspirated. The pellet was then washed with an additional 2 mL of Permeabilization/Wash (1X) buffer and centrifuged and aspirated.

After the wash, 100 ul of Permeabilization/Wash (1X) buffer was added to all tubes, 2.5 ul of FITC Iso-control (Mouse IgG1) and 2.5 ul of Mouse IgG1-PE were added to the ISO tubes and 2.5 ul of PE-anti-mouse/rat/human FoxP3 was added to the ‘EXP’ tubes. The samples were vortexed for 10 seconds and incubated at room temperature for 40 minutes in the dark.

After the third incubation, the samples were vortexed for 3 seconds to loosen the pellet and 2 mL of Permeabilization/Wash (1X) buffer was added to each tube, centrifuged, and aspirated. After the wash, 2 mL of Permeabilization/Wash (1X) buffer was added again to wash. The cells were centrifuged and aspirated to approximately 300

ul and loaded onto the 96-well round-bottom plate. The samples were run on the flow cytometer and the data was analyzed the FlowJo program, again as describe previously.

3.0 Results

When the Intraprep Reagent Kit from Beckman Coulter was tested and the data analyzed, our results showed that there was little internal staining. Initially, when the cells were counted the viability was also determined by the Countess machine, and only cells with a viability percentage of 80% or higher were used. This rules out that the cells had lysed and were not viable. Analyzing the samples for surface antibody staining showed significant staining. When the samples were gated for PE-FoxP3, the results show little expression. This suggests that the internal antibody was unable to enter the nuclease and bind to its antigen.

Since the first kit yielded little results, we used the Foxp3/Transcription Factor Staining Buffer Set from Thermo Fisher. This kit provided a protocol that called for the stimulation of the cells prior to staining. After analyzing the data in FlowJo, we were able to see that while there was a small number of cells that were stained for PE-FoxP3, there was no significant difference between the stimulated cells and the unstimulated cells. Using the data published by Wang et al. as a reference, we were not satisfied with the magnitude of Foxp3 expression observed.

After receiving the results from the last kit, we decided to use the exact kit that was used in the paper by Wang et al. paper. The Transcription Factor Buffer Set from BD Biosciences had a protocol similar to that of the kit from Thermo Fisher minus the stimulation of the cells prior to staining. The data analyzed showed expression of PE-

FoxP3 in both patients and healthy controls. Further analysis of the patient samples showed expression of TFR/TFH cells at approximately 10x that of healthy controls.

4.0 Discussion

There are multiple factors that contribute to Myalgic Encephalomyelitis/Chronic Fatigue Syndrome. These factors include environmental and genetic factors but the exact causes have not been determined. ME/CFS occurs more commonly in women, but the cause is unknown [1]. The main symptoms are neurocognitive dysfunction and exercise intolerance. Those with ME/CFS also have significant fatigue that persists for over six months and does not improve with rest [1]. There is no cure for this condition but the symptoms can be managed to provide some relief.

Considering the data collected from using the three different kits, the kit from BD Bioscience is the kit we will use in further studies of determining the TFR/TFH expression in ME/CFS patients versus healthy controls. Now that we have evaluated the different kits and have determined the best one for our purposes, we will be able to move forward with this project and start analyzing 20 ME/CFS patient samples and 20 healthy control samples. We will be looking at whether the frequency of circulating TFR cells is down-regulated or up-regulated in ME/CFS cases when compared to healthy controls. We will also be looking at whether the frequency of circulating Treg subsets were differentially regulated in ME/CFS patients versus healthy controls. From there, we will be able to determine whether circulating TFR and TFH cells are differentially correlated with clinical parameters in ME/CFS patients.

Several studies have suggested the involvement of CD45RA-FoxP3+ Treg cells in the development of autoimmune disease and cancer [7][8]. Future studies will seek to elucidate the multiple mechanisms within TFR and TFH cells in the peripheral circulation that are functionally important in ME/CFS development. Measuring the levels of these T cell subpopulations will provide valuable information on the severity of the disease. In addition, targeting these alterations may provide novel approaches and present therapeutic efficacy in ME/CFS patients [9].

References

1. Wang, X., Jiang, Y., Zhu, Y., Zhang, M., Li, M., Wang, H., & Gao, P. 2016. Circulating memory B cells and plasmablasts are associated with the levels of serum immunoglobulin in patients with ulcerative colitis. *J Cell Mol Med*, 20(5), 804–814.
2. Devanur, L.D., and Kerr, J.R. 2006. Chronic fatigue syndrome. *J Clin Virol*, 37(3), 139-50.
3. Chronic fatigue syndrome. Mayo Clinic. https://www.mayoclinic.org/diseases-conditions/chronic-fatigue-syndrome/symptoms-causes/syc-20360490. Published January 5, 2018. Accessed January 8, 2018.
4. Myalgic Encephalomyelitis. NORD (National Organization for Rare Disorders). https://rarediseases.org/rare-diseases/myalgic-encephalomyelitis/. Accessed May 8, 2018.
5. Jason, L. A., Benton, M. C., Valentine, L., Johnson, A., & Torres-Harding, S. 2008. The economic impact of ME/CFS: individual and societal costs. *Dyn Med*, 7, 6.
6. Blomberg J, Gottfries C-G, Elfaitouri A, Rizwan M, Rosén A. 2018. Infection Elicited Autoimmunity and Myalgic Encephalomyelitis/Chronic Fatigue Syndrome: An Explanatory Model. Frontiers in Immunology. 9.
7. Sun, W., Li, W. J., Wu, C. Y., Zhong, H. & Wen, W. P. 2014. CD45RA-Fox3 high but not CD45RA+Fox3low suppressive T regulatory cells increased in the peripheral circulation of patients with head and neck squamous cell carcinoma and correlated with tumor progression. *J Exp Clin Cancer Res*, 33, 35.
8. Montoya J. G., Holmes T. H., Anderson J. N., Maecker H. T., Rosenberg-Hasson Y., Valencia I. J., Chu L., Younger J. W., Tato C. M., Davis M. M. 2017. Cytokine signature associated with disease severity in chronic fatigue syndrome patients. *Proc Natl Acad Sci*, 114.
9. Matsuki, F., Saegusa, J., Miyamoto, Y., Misaki, K., Kumagai, S., Morinobu, A. 2013. CD45RA-FoxP3 (high) activated/effector regulatory T cells in the CCR7 + CD45RA-CD27 + CD28+central memory subset are decreased in peripheral blood from patients with rheumatoid arthritis. *Biochem Biophys Res Commun.* 438, 778-783.