The Effect of Immune Cell Activation on Glycogen Storage in the Context of a Nutrient Rich Microenvironment

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A Thesis

in

the Department

of

Biology

Presented in Partial Fulfillment of the Requirements

for the Degree of Master of Science (Biology) at

Concordia University

Montreal, Quebec, Canada

November 2015

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CONCORDIA UNIVERSITY
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Abstract

The Effect of Immune Cell Activation on Glycogen Storage in the Context of a Nutrient Rich Microenvironment

Mahdieh Tabatabaei Shafiei

Lymphocytes of the immune system become activated in order to fight pathogens. Activated lymphocytes absorb more glucose due to their high-energy demand. Glycogen is a branched polymer of glucose units that is formed in times of nutrient sufficiency and it is utilized in times of need. In the presence of high glucose, lymphocytes build up glycogen stores, but the fate of this content is not very well understood. The objective of this work is to demonstrate the presence of glycogen in activated human peripheral blood mononuclear cells (PBMCs) and to investigate the impact of low nutrient levels on the glycogen content of these cells. This was achieved by isolation of PBMCs from human blood, followed by *in-vitro* activation of the cells by a general activator and a T cell-specific activator. Glycogen concentrations were measured through periodic acid Schiff's staining (PAS) method and by using an enzymatic detection kit, in various time points. The role of glycogen in times of low nutrient availability was also examined. PBMC were found to contain glycogen by both methods. Upon stimulation of PBMCs with the general activator, there was an increase in glycogen formation in the activated lymphocytes as compared to the non-activated group using both techniques ($p<0.05$). The effect of T cell-specific activator was consistent with the effects of the general activator. This was confirmed through PAS staining and enzymatic detection kit techniques ($p<0.05$). Additionally, when the amount of nutrients was lowered, less glycogen was stored in PBMCs. This study demonstrated that activated PBMCs contain more glycogen stores as compared to non-activated cells. The excess glucose that is converted into glycogen may be used by the immune system when nutrients are low.

Acknowledgments

I would like to express the utmost appreciation to my supervisor Dr. Peter J. Darlington. He constantly and persuasively conveyed a spirit of adventure and enthusiasm in regard to research. Without his supervision and endless help this dissertation would not have been possible.

I would like to express my sincere gratitude to my committee members, Dr. Alisa Piekny and Dr. Malcolm Whiteway, who guided me throughout this project with their immense knowledge and insightful comments.

Lastly this study was not possible without the constant help and support of my siblings (Nasr, Zahra and Maryam), my parents, my dear friends Catalina Carvajal and Samiur Rahman. I would like to extend my heartfelt gratitude for all their assistance, encouragement and helpful discussions.

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List of Abbreviations:

APC: antigen-presenting cells

ATP: adenosine triphosphate

CD3 or CD28: cluster of differentiation 3 or 28

FBS: fetal bovine serum

Glut1: Glucose Transporter 1

HRP: Horseradish Peroxidase

Ils: Interleukins

MHC: major histocompatibility complex

PAS: periodic acid Schiff

PBMC: peripheral blood mononuclear cells

PhK: phosphorylase kinase

PKA: protein kinase A

PMA: phorbol myristate acetate

RBC: red blood cells

RPMI: Roswell Parks memorial institute

T-ALL: T cell acute lymphatic leukemia

TCR: T cell receptor

TMB: 3,3',5,5' tetramethylbenzidine

UDP-glucose: Uridine diphosphate glucose

Introduction

Human Immune Cells

Peripheral Blood Mononuclear Cells (PBMCs) are a subpopulation of immune cells composed of four main cell types; T Lymphocytes (T cells), B Lymphocytes, Natural Killer Cells and Monocytes. On average 70% of PBMCs are composed of T cells (Charles A Janeway, Travers, Walport, & Shlomchik, 2001). These cells originate from blood T cell precursors that are created in the bone marrow and migrate to thymus to mature. Once T cells enter the blood stream, they begin to circulate throughout the blood and lymphatic systems. In order to produce an adaptive immune response, T cells need to become activated, after which they proliferate (cell division), differentiate (change their properties), and coordinate other cells to eliminate the infection (Charles A Janeway et al., 2001; J. A. Maciolek, Pasternak, & Wilson, 2014; Pearce, 2010).

T Cell Activation

Activation of T cells is caused by two main signals sent through the T cell receptor (TCR) and the CD28 co-stimulatory receptor. These signals are initiated by specialized immune cells called antigen-presenting cells (APC), which are constantly inspecting the body for pathogens. When an APC encounters a foreign antigen (for example from a pathogen), it uptakes the antigen through endocytosis and performs antigen processing. Ultimately, the APC creates a fragment of that antigen, termed the epitope. This epitope is then presented to T cells through the antigen binding groove of major histocompatibility complex (MHC) of the APC (Charles A Janeway et al., 2001; Roche & Furuta, 2015).

The TCR scans the presented antigen and if it recognizes the epitope, it induces several cell signalling pathways in the T cell via cluster of differentiation 3 (CD3) which is attached to the TCR (Charles A Janeway et al., 2001). The engagement of TCR with the antigen-MHC complex is the first signal required for activation of T cells. However, this signal is not sufficient for proper activation of T cells. There is a second required signal that is mediated by a cell surface receptor called CD28. This co-stimulatory signal will ensure cell survival and differentiation ability of activated T cells. CD28 of T cells will engage with CD80 and CD86 on APCs to create the co-stimulatory signal and allow for full activation of T cells (Peach et al., 1995). One role of CD28 is to promote uptake of glucose, which is important because T Cells need sugar molecules, such as glucose, to survive (Candace M Cham et al. 2008; C. M. Cham and Gajewski 2005; Tripmacher et al. 2008; Chang et al. 2013).

Upon activation of CD3 component of TCR, many pathways such as MAPK, P38 and JNK become activated. These pathways will ultimately stimulate the transcription factors required for activation of T cells. CD28 co-stimulation will also initiate many signalling transductions, including AKT/PI3K pathway, and PKC, which activates further growth signals. One of the outcomes of this pathway is the increase in the expression of glucose channels, which will facilitate glucose absorption by the activated T cell (Fig. 1A) (Charles A Janeway et al., 2001).

Fig. 1) Activation Pathways and Glycogen Cycle

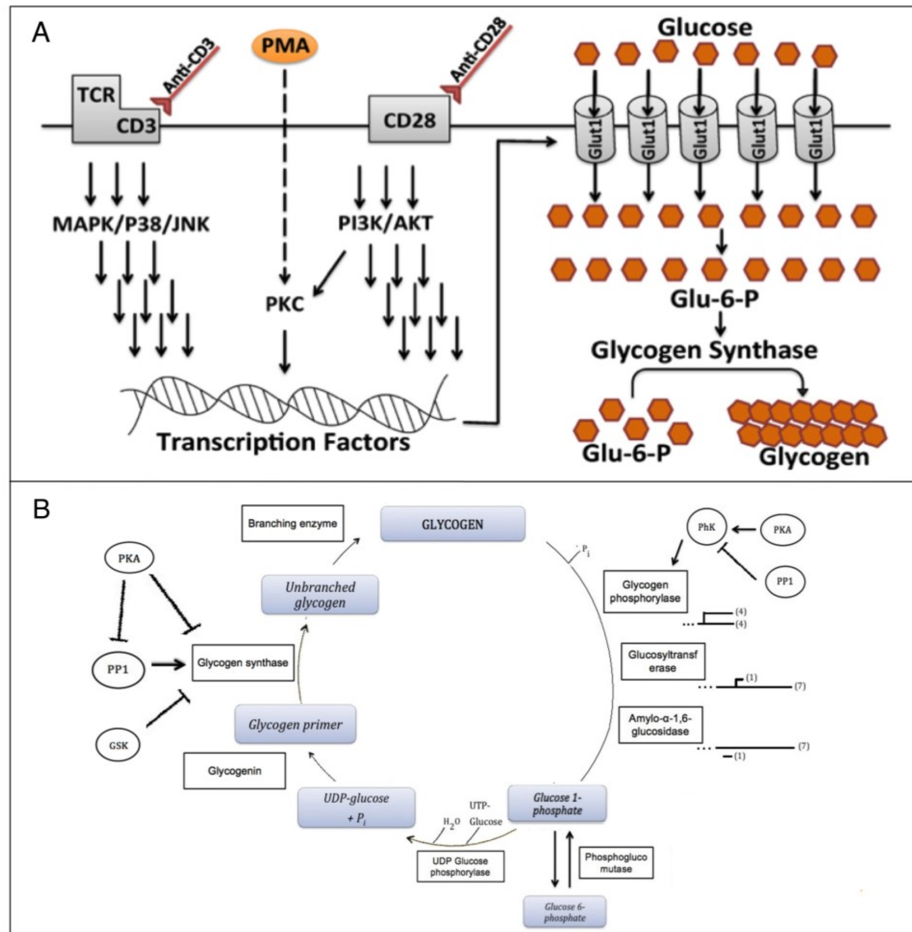


Figure. 1) A) Upon the addition of anti-CD3 and anti-CD28, many signalling pathways are initiated which ultimately activate transcription factors necessary for activation. PMA interacts with PKC, which is involved in the pathways that are triggered upon activation of T cells. Glu-6-P = Glucose-6-phosphorylase B) There are a number of enzymes involved in the breakdown and formation of glycogen. Upon activation of glycogen phosphorylase, the enzyme of the first and rate-limiting step of glycogen breakdown pathway, glucose units are released from glycogen. If blood glucose levels are high, the process reverses, and glycogen starts to build up again in the liver. Glycogen synthase, with the help of glycogenin, is responsible for adding glucose units to glycogen polymer.

Activation Markers

Activated immune cells secrete cytokines, which are cell-signalling proteins that stimulate cell growth, differentiation, survival, inflammation, and tissue repair (Fiore, Chaldakov, & Aloe, 2009; Galazios, Papazoglou, Tsikouras, & Kolios, 2009; Mailloux et al., 2013). Interleukins (ILs) are a subset of cytokines that were first discovered to be secreted by immune cells (Brocker, Thompson, Matsumoto, Nebert, & Vasiliou, 2010). The presence of cytokines and interleukins in the media of PBMC cultures provides information on the efficiency of activation treatment. Some types of ILs are specific to certain cell types. Helper T cells (Th cells) have several distinct subtypes, denoted as Th1, Th2, and Th17 cells. Each one of these subtypes secretes a different combination of ILs. For example, Th1 cells secrete IL-2 and IFN-γ , Th2 cells secrete IL-4, IL-5, IL-10 and IL-13, and Th17 cells secrete IL-17 (Saraiva et al., 2009; Toscano et al., 2007; Zhu & Paul, 2008). In PBMC cultures, IL-1β and IL-6 are likely to be secreted from monocytes, as compared to IL-17 which is specific to a subtype of T cells, and IL-2 which is secreted by all activated T cells (Brocker et al., 2010; Estruch et al., 2015). Another sign of activation is yellowing of the media, which is due to secretion of lactic acid as a byproduct of glycolysis, which makes the media more acidic and turns the phenol red from pink to yellow due to lactic acid excretion. Monitoring the pH of the culture and cytokine secretions are two methods of cell activation measurements. T cells can be activated in the laboratory with a general activator phorbol myristate acetate (PMA, which also activates other lymphocytes), or a specific activator that engages the signaling component of the T cell receptor (CD3) and the co-stimulatory receptor (CD28).

T cell Metabolism and Reprogramming upon Activation

Activated T cells have a high-energy demand due to processes such as proliferation, differentiation and the secretion of cytokines. Activated T cells require an increased source of carbon, such as glucose, for the synthesis of macromolecules essential for an efficient immune response (Wang & Green, 2012). In order for T cells to meet this energy and carbon demand, the cells will go through modifications for better efficiency. This reprogramming includes increased glucose uptake due to the increase in the expression of Glucose Transporter 1 (Glut1), which is a glucose channel, and increased level of insulin receptors on the activated cells surface, which promote insulin dependent glucose-uptake (Ader, Poulin, Yang, & Bergman, 1992; J. a. Maciolek, Alex Pasternak, & Wilson, 2014). One of the hallmarks of T cell activation is the switch from oxidative phosphorylation to anaerobic glycolysis in the presence of oxygen, known as the Warburg effect. This switch is required for cytokine production (Chang et al., 2013). Anaerobic glycolysis yields less ATP (Adenosine Triphosphate) and much more lactate as compared to oxidative phosphorylation. In order to compensate for this rapid use of glucose, activated T cells have an increased glucose uptake mediated through CD28 co-stimulatory signal (Frauwirth et al., 2002).

What is Glycogen?

Glycogen is a branched polymer of glucose, nucleated by the glycogenin protein (Berg, Tymoczko, & Stryer, 2002; Roach, Depaoli-Roach, Hurley, & Tagliabracci, 2012). The glucose-glycogen cycle has been extensively studied in the context of the liver. When blood glucose levels are low, glycogen breakdown is promoted in the liver, which releases glucose into blood. Protein Kinase A (PKA) phosphorylates and thus activates phosphorylase kinase (PhK). Upon

activation of PhK, glycogen phosphorylase enzyme is activated causing the release of glucose units from glycogen in the form of Glucose 1-phosphate. Glycogen phosphorylase can only break down glucose units up to 4 residues away from a branching point. At this point in the cycle, the debranching enzymes transfer 3 of the remaining residues to another chain and release the glucose unit at the branch. The released glucose units have to be in the form of glucose 6-phosphate so it can be used in metabolism (Fig. 1B) (Berg et al., 2002; Greenberg, Jurczak, Danos, & Brady, 2006; Johnson, 1992; Meléndez, Meléndez-Hevia, & Canela, 1999; Roach et al., 2012; Tavridou & Agius, 2003). Glycogen breakdown in the liver will lead to the increase in glucose concentrations in the blood. When intracellular glucose is too high, glucose-6-phosphate can be converted back to glucose 1-phosphate by phosphoglucomutase enzyme. This form of glucose can then convert into UDP-glucose (Uridine diphosphate glucose) and enter the cycle to produce glycogen. Glycogenin is an enzyme that self glycosylates and creates glycogen primer of 3-4 glucose residues long. Glycogen synthase starts adding residues to the primer and creates a long chain of glucose units. The branching enzyme is responsible to change some of the α-1,4-glycosidic linkages to α-1,6-glycosidic bonds to create branches in the glycogen molecule (Fig. 1B). (Berg et al., 2002, Chapter 21; Ceulemans & Bollen, 2004; Cid, Geremia, Guinovart, & Ferrer, 2002; Forde & Dale, 2007; Ragolia & Begum, 1998; Roach et al., 2012; Whitehouse, Tomkins, Lovegrove, Hopkinson, & McMillan, 1998).

Glycogen in Immune Cells

Glycogen metabolism is regulated by hormones such as glucagon and insulin in tissues such as muscle and liver (Roach et al., 2012; Villar-Palasi, 1968). There is evidence of the presence of glycogen in lymphocytes of the immune system as well. The existence of glycogen vacuoles in immune cells has been noted since 1950s, where researchers attempted to diagnose patients with various diseases based on the amount of glycogen that was accumulated in their lymphocytes (Drazancić & Janković, 1970; Hagemans et al., 2010; Jones, Goffi, & Hutt, 1962; Lilleyman et al., 1994; Sharvill, 1952). The use of glycogen content of immune cells as a diagnostic tool was inconclusive due to controversial findings (Jones, Goffi, and Hutt 1962; Leder and Donhuijsen 1978).

The biological relevance of glycogen in lymphocytes remains a mystery. The objective of this work was to fill this gap in the literature by demonstrating the presence of glycogen in human PBMCs, and investigating the impact of activation on the glycogen concentrations of these cells. The role of glycogen in times of low nutrient availability was also examined. It was hypothesized that upon activation of PBMCs, there will be an increase in glycogen expression and this storage can be used as a fuel source in times of nutrient insufficiency. The knowledge that was created in this project helps in better understanding of T cell fuel sources and the modifications these cells go through upon activation. This study is relevant in the field of T cell acute lymphatic leukemia (T-ALL) where glycogen content of lymphocytes is reduced in patients suffering from this disease (Andreewa, Huhn, Thiel, & Rodt, 1978; Huhn, 1984; Lilleyman et al., 1994). This suggests that the glycogen pathway is altered in this form of cancer (See Supplementary Table 1).

Hypothesis

PBMCs have glycogen stores that accumulate upon activation, and this content is proportional (responds) to changes in nutrient levels.

Aims

Aim 1: To demonstrate the presence of glycogen in PBMCs through two techniques of periodic acid Schiff (PAS) staining and enzymatic detection kit (glycogen assay).

Aim 2: To investigate the effect of two different activators (general and T cell-specific activation) on the glycogen content of PBMCs.

Aim 3: To address the influence of nutrient availability on the glycogen content of activated PBMCs.

Materials and Methods

Blood Draw and PBMC Purification

Concordia University Ethics Review Board, certificate number 10000618, approved research with human blood samples. Healthy human subjects participated in this study. Heath status was determined by self-reporting. A nurse drew venous blood from the subjects by venipuncture, into heparinized (anti-coagulant) blood collection tubes. All steps of PBMC purification were done in a biosafety cabinet using sterile technique and the equipment had been sterilized by the manufacturer, or sterilized by using the autoclave machine. The blood was diluted at a 1:1

ratio with phosphate buffer saline (PBS 1X) in a conical tube and then layered on top of Ficoll, a nonionic synthetic polymer of sucrose. The tube was centrifuged at room temperature for 30 minutes at 1800RPM (700 Xg) in a swinging bucket rotor with medium acceleration set to 5, and deceleration set to 0.

The buffy coat was carefully collected using a transfer pipette. The buffy coat was identified visually as a thin cloudy white layer, where PBMCs are known to be located. PBS was added to the buffy coat to achieve a final volume of 45ml. After mixing, the buffy coat was centrifuged at room temperature for 15 minutes at 1500RPM (480 X g) with both maximum acceleration and deceleration set to 9. This was done to create a pellet of PBMCs at the bottom of the conical tube. The supernatant was discarded and the pellet was loosened by gently racking the tube against an undulated surface. Fresh PBS was added to the PBMCs and the tube was centrifuged at room temperature for 12 minutes at 1500RPM (480 X g) with both maximum acceleration and deceleration set to 9. The supernatant was discarded and fresh PBS was added. At this step the number of viable PBMCs was estimated based on the trypan counting (see details below). The desired amount of cells was taken and centrifuged at room temperature for 12 minutes at 1200RPM (410 X g) with both maximum acceleration and deceleration set to 9. The supernatant was discarded and the PBMCs were re-suspended in the desired media.

Trypan Blue Counting

To check the number of viable cells, the trypan method was used. From the second to last step of the PBMC purification, 50µL of PBMCs in PBS was put in a microcentrifuge tube, to which an equal amount (50µL) of trypan blue, a vital stain, was added and mixed. This stain is

only taken up by dead cells, which will appear blue in the count, live cells will appear clear. 10µL was taken out and transferred to a haemocytometer. The number of live cells was calculated with the following formula taken from manufacturer instructions (VWR).

$$\left({}^{\textit{of live cells in hemacytometer grid}} \Big/ {}_{50\ mLs} \right) X\ 10^6 = \#\ of\ live\ cells/mL$$

Cell Culture Conditions

Purified PBMCs were suspended in complete media. The media contained Roswell Parks Memorial Institute (RPMI), 5% fetal bovine serum (FBS), 1% penicillin (10,000 units/mLs), 1% L-glutamine (200mM). After the treatments were added to the cells, the culture plate was kept in the incubator at 37°C and 5% CO_2.

PMA Activation of PBMCs

Phorbol myristate acetate (PMA) was chosen as a general activator. PBMCs received PMA treatment (SIGMA) at a final concentration of 2ng/mL diluted in media. Each time point had a negative control group that did not receive PMA treatment.

Anti-CD3, Anti-CD28 (T Cell-Specific) Activation of PBMCs

PBMCs were incubated with anti-CD3 and anti-CD28 antibodies (eBioscience) at a final concentration of 0.1µg/ml each. Each time point had a negative control group that did not receive the activation treatment.

Serum Titration Experiment

PBMCs were suspended in 2 mLs of complete media with T cell-specific activation mix at a final concentration of 0.1 µg/mL, in 6 wells of 24 well culture plate. Complete media was RPMI, 5% fetal bovine serum (FBS), 1% penicillin, 1% L-glutamine. After 24 hours of incubation, the cells from the first well were lysed for glycogen analysis. This was the control group. The rest of the 5 wells were labeled group 1 to 5. After 24 hours of incubation, PBMCs settle down to the bottom of the plate. Half of the complete media (1mL) was carefully removed from the top of each well and was replaced with 1mL of a new media with fresh activation mix. Each group had a different new media added; group 1 new media was serum free, group 2 new media had 1% FBS, group 3 the media had 5% FBS, group 4 the media had 10% FBS and for group 5 the new media was at 12% FBS. The plate was incubated for another 24 hours. After the incubation period was over, once again 1mL of the media was taken out from the top of each well and was replaced with 1mL of new media appropriate for each group with activation mix. At this point final concentration of FBS in the wells was 1%, 2% 5%, 8% and 11%. After incubating the plate for 24 hours one last time, the cells from each well are taken out and lysed for glycogen analysis.

Slide Preparation and Negative Control

The PBMCs of interest were centrifuged and re-suspended in PBS. PBMCs in PBS were placed onto a labeled microscope slide. A smear was made with the help of another slide. The slide was left in a biological safety cabinet to air dry.

Fixing the Slides

The fixative solution was prepared by mixing 0.5mLs of formaldehyde to 4.5mLs of ethanol. The final concentration of the fixative solution was 89.1% ethanol and 10.9% formaldehyde. When the slides were air dried, they were fixed with the freshly made fixative solution by immersing the slide in the solution for 1 minute and after, rinsing the slide for 1 minute.

Amylase Treatment

Amylase powder was dissolved in distilled water. The solution was then poured in a clean 100mL beaker. The slide was immersed in the beaker so that half of the slide received the treatment and the other half remained untreated. This is followed by incubation for 15 minutes at room temperature. After the incubation, the slide was taken out of the beaker and washed with ddH_2O to remove the amylase solution. The slide was left on the bench to air dry.

PAS staining, image acquisition and analysis

Staining the Slides

The slides were stained with PAS kit according to the manufacturer's instructions (SIGMA). The slides were first immersed in Periodic Acid Solution for 5 minutes followed by a wash with distilled water. The slides were then immersed in Schiff's reagent for 15 minutes. This step was followed by 5 minutes of washing the slides with distilled water. Once the slides were dried, a coverslip was placed on the slides with the help of mounting media. Slides were examined under Leica DM 2000 light microscope. For a summary of the steps above refer to figure 2.

Fig. 2) Step by Step Methodology of PAS Staining on PBMCs

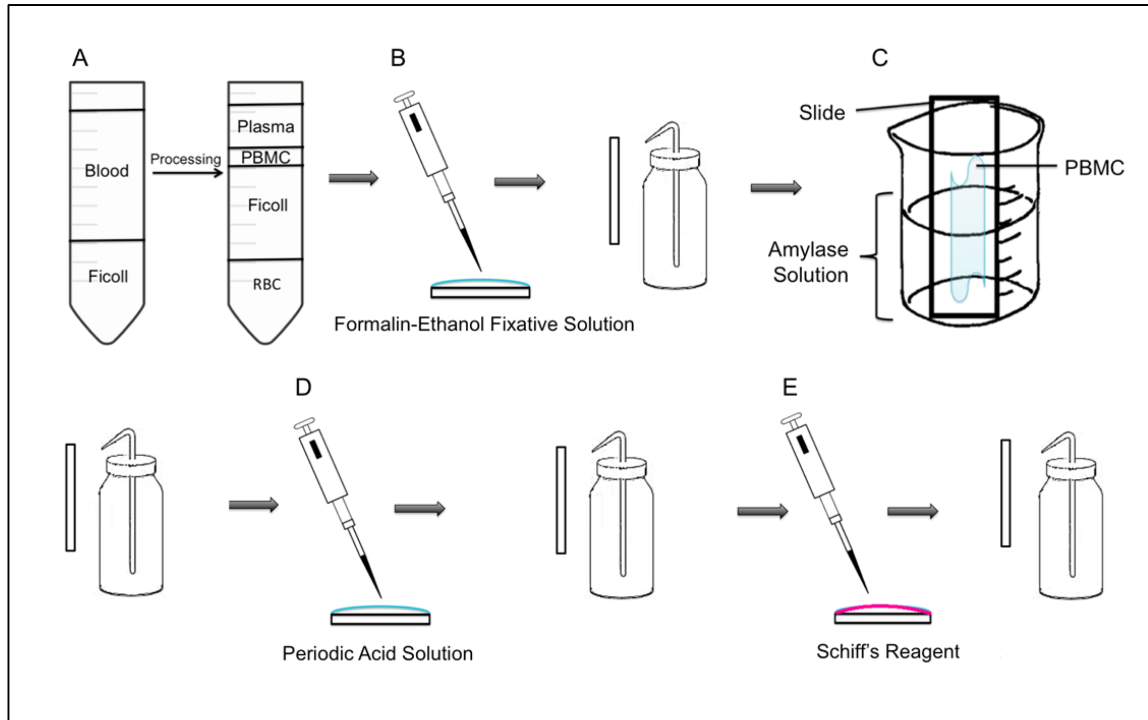


Figure. 2) Step by step methodology of PAS staining on PBMCs. A) Isolation of PBMC is achieved through ficoll gradient, the left panel shows the preparation before centrifugation, the right panel shows it after centrifugation where the buffy coat containing the PBMCs is observed in the center of the tube. B) Isolated PBMCs are fixed onto the slide using formalin-ethanol fixative solution. The slide is gently rinsed with distilled water from a plastic wash bottle. C) The slide is then placed in a 100mL beaker half way filled with amylase solution, which will dissolve glycogen. The slide is gently rinsed. D) The slide is treated with periodic acid solution, where oxidation of saccharides takes place. Slides are gently rinsed; this will remove the excess periodic acid and stop the oxidation step. E) When the Schiff reagent is added to the slides, it will react with aldehydes created during the oxidation step. This colorless reagent will then result in a deep red magenta product. Slides are gently rinsed to remove the excess Schiff reagent (Tabatabaei Shafiei et al., 2014).

ImageJ Software

To quantify the PAS positivity of the cells ImageJ, an open access public software published by NIH, was used (Schneider, Rasband, & Eliceiri, 2012). At least 50 cells from each slide was chosen at random and the intensity of the coloration of cells were measured. To remove the background signal, a value of the intensity of the background coloration was obtained. The value of each data point was subtracted from the background signal obtained from the area adjacent to each cell.

Glycogen Assay

This is an enzymatic detection kit (SIGMA) that measures the amount of glucose derived from glycogen in cells. The cells of interest first needed to be lysed to allow the internal glucose to release. A sample blank was also present to ensure the measured glucose was directly coming from glycogen.

Cell Lysis

The cells were transferred to a microcentrifuge tube and were centrifuged for 8 minutes at 0.5 xg at 4°C. Carefully without disturbing the pellet, the supernatant was removed and saved in -20°C freezer for future analysis. The cells were washed with PBS to ensure the media is washed off. After the wash, the supernatant was completely removed. The cells were incubated in milli-Q water for 5 minutes then transferred to a heating block that was at 100°C. The tube was kept on the heating block for 5 minutes then centrifuged at 13000 xg for 5 minutes at room temperature. After this step, the insoluble matter was discarded and the supernatant, which was the lysate, was poured into a cryo vial. The lysates were kept in liquid nitrogen tank. On the

day of the analysis, the cryo tubes were taken out of the liquid nitrogen and thawed in $37^{O}C$ waterbath.

Assay

The glycogen assay was done according to manufacturer's instructions (SIGMA). For each sample, a sample blank was made which did not receive the hydrolysis Enzyme Mix treatment. This was done in duplicates. The values of glycogen assay are reported as fold change in glycogen concentration. The control groups (Nil) of every time point were corrected to report a value of 1.00. The glycogen concentrations of the activated groups are in respect to the control groups.

Enzyme Linked Immunosorbent Assay (ELISA)

This technique was used to measure the cytokine released from PBMCs upon activation. The supernatants containing the cytokines of interest were analyzed by ELISA. This is a colorimetric assay that quantifies the exact amount of cytokine released in the media. The steps were done according to manufacturer's instructions (BD Bioscience, eBioscience). The wells of a 96 well flat bottom plate were coated with the capture antibody and left overnight. The next day the plate was first washed once with wash buffer then the remaining protein-binding sites in the coated well were blocked with assay dilutent. The plate was left at room temperature for 1 hour. Next the plate was washed three times before the diluted samples and the standards were added. The plate was incubated for 2 hours. Samples were removed and the plate was washed five times. Working detector, which is made of the combination of the detection antibody and secondary antibody conjugated with HRP (Horseradish Peroxidase), was added

and the plate was incubated for an hour at room temperature. The plate is then washed seven times. The detection substrate, TMB (3,3',5,5'-tetramethylbenzidine) solution was added to each well followed by an incubation of 15-30 minutes. An equal volume of stopping solution was added and the plate was read at wavelength of 450nm and at 570nm for correction.

Flow Cytometry

Cellular analysis was done using flow cytometry. Cells were stained with fluorescent antibodies against CD3, and a monocyte marker, CD14, then acquired on the flow cytometer (BD Accuri). Anti-CD3 and Anti-CD14 surface antibodies were mixed with the staining buffer and then added to the samples. The samples were put on ice for 30 minutes away from light. The samples were centrifuged at 13000 xg for 30 seconds to remove the supernatants. The cells were suspended in the staining buffer and analyzed by the flow cytometer. The flow cytometer excites cells individually with two lasers, and measures the fluorescence emission from the fluorochrome of any antibodies that attach to the marker of interest. Data analysis was done with FlowJo, a software for analyzing flow cytometry data, this package produces histograms and determines the percentage of positive cells expressing the marker.

Results

PAS Staining on Mouse Muscle Sections

Muscle sections from mouse were first used as a positive control, and to establish the protocol. PAS positive particles were visible inside of muscle cells (Fig. 3A). As a negative control, slides were treated with amylase, which dissolves glycogen. Reduced PAS positive cells were

observed when the muscle sections were pre-treated with amylase (Fig. 3B). The staining around the cell membrane remained after amylase treatment. From the stained muscle cells, the number of PAS positive cells was counted. A total of 15 images per treatment (each image had approximately 7 cells) were counted. The percentage of PAS positive cells was calculated for each treatment (Fig. 4). The results showed that the percentage of PAS positive cells in muscle sections was 37%. Amylase treatment reduced the PAS-signal to 4% ($p<0.001$, Student's t-test) (Fig. 4).

Fig. 3) PAS Staining of Mouse Muscle Sections

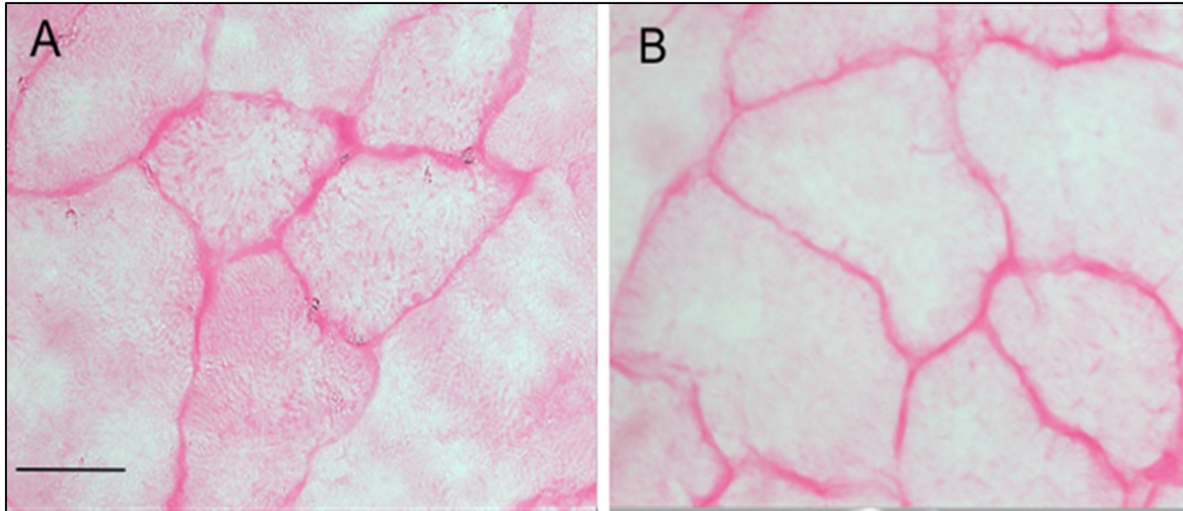


Figure. 3) PAS Stained Mouse Muscle Sections. Mouse soleus muscle sections were stained with PAS and analyzed with light microscopy. Some samples were pre-treated with amylase. A) PAS-positive particles were visible inside of muscle cells. B) Less PAS positive cells were observed when the muscle sections were pre-treated with amylase. The staining around the cell membrane remained after amylase treatment. (Scale bar= 100µm) (Tabatabaei Shafiei et al., 2014)

Fig. 4) Quantification of PAS positive Muscle Cells

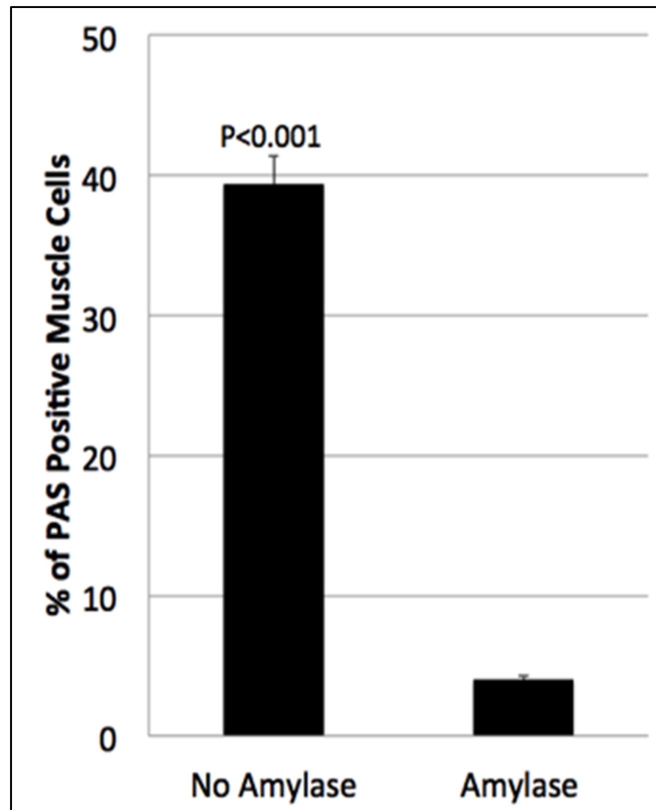


Figure. 4) Quantification of PAS positive Muscle Cells. The proportion of muscle cells that were PAS positive was counted from a representative slide without or with the amylase pre-treatment. As expected, 37% of muscle cells were positive for glycogen. Amylase treatment significantly reduced the PAS-signal to 4%. (*p<0.001, Student's t-test) (Tabatabaei Shafiei et al., 2014)

Glycogen Analysis of Human Immune Cells

PAS staining was attempted on whole human blood, which contains lymphocytes, red blood cells (RBCs), platelets and neutrophils. Blood films were made and stained with PAS. Differential interference contrast images confirmed the presence of mostly RBCs by revealing the characteristic disk-like shape of these cells. RBCs represented almost 99% of the entire field of view of the microscope, making it challenging to find and observe immune cells (Fig. 5C).

Fig. 5) PAS Staining on Human PBMCs

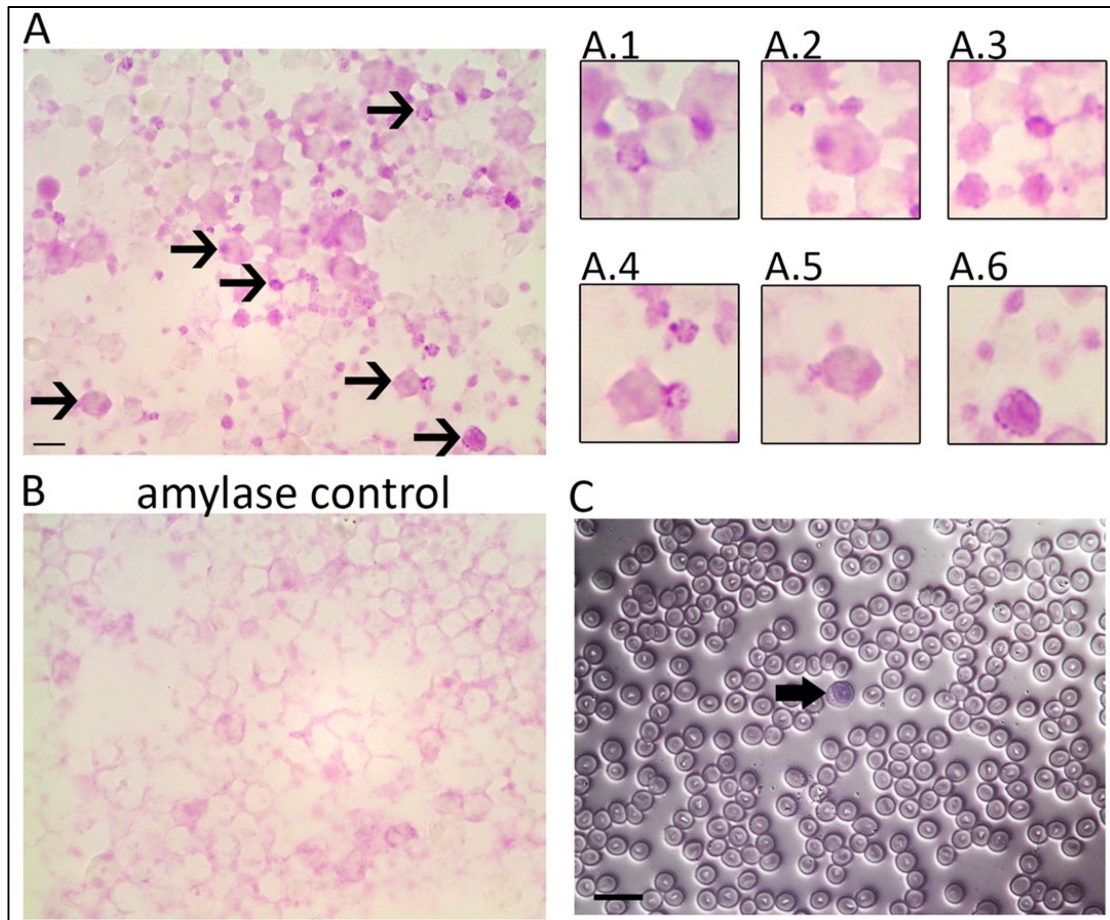


Figure. 5) PAS Staining on Human PBMCs. A) PAS-staining on PBMCs was performed. Two types of cells were observed. A.1-6) smaller cells (at 5µm) in non-amylase treated slides showed magenta particles consistent with glycogen. These cells could be resting cells. Larger cells (more than 5µm) in non-amylase treated cells had diffuse PAS-positive staining. These cells could be activated lymphocytes. B) PBMCs were treated with amylase for 15 minutes prior to staining, which diminished the PAS signal. Representative of seven different healthy human subjects. C) The PAS and hematoxylin staining done on whole blood slide. The arrow shows a PBMC surrounded by many red blood cell. (Scale bar= 10µm)

Next, PBMC were prepared from whole blood, which eliminates RBCs, platelets and neutrophils. PBMC fractions are majority T cells, with monocytes, natural killer cells, and B cells making up the remainder. The PAS staining was performed on both amylase treated cells and non-amylase treated cells. Different sizes and shapes of PBMCs were visualized on the slides, and varying patterns of PAS positivity was seen among cells of different sizes (Fig. 5A.1-A.6). Cells that did not receive the amylase treatment appeared to have more PAS positive particles compared to amylase-treated slides (Fig. 5A and 5B).

PAS positivity was calculated in both amylase treated and non-amylase treated slides. The cells observed on the slides were divided into 2 groups; larger cells (>5µm) and smaller cells (<5 µm). This size was chosen because 5µM is the approximate size of a lymphocyte. The proportion of PBMCs that were PAS positive was counted from a representative slide with or without the amylase pre-treatment. 98% of the small-sized cells were positive for PAS. 40% of the larger cells were positive for PAS. Amylase treatment eliminated the PAS signal in the small cells ($p<0.001$, Student's t-test) and significantly diminished the PAS signal in the larger cells to 7% ($p<0.001$, Student's t-test) (Fig 6).

Fig. 6) Quantification of PAS positive PBMCs

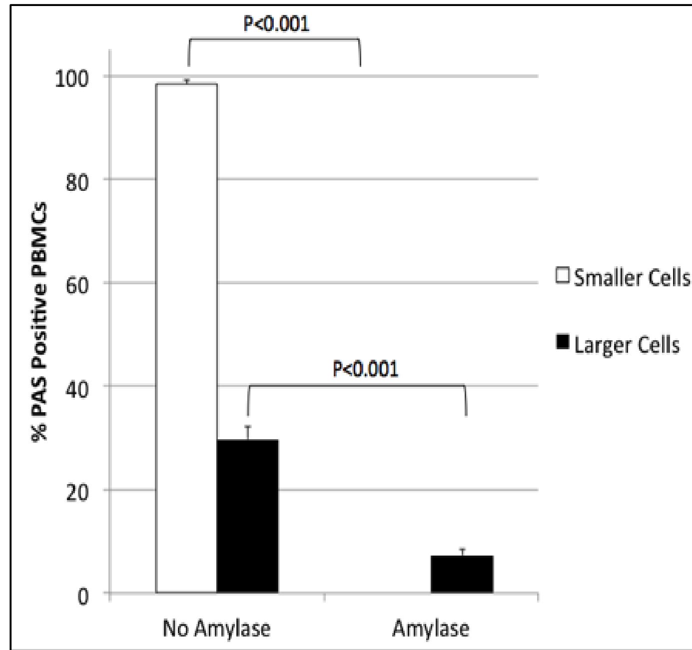


Figure. 6) Quantification of PAS positive PBMCs. The proportion of PBMCs that were PAS positive was counted from a representative slide without or with the amylase pre-treatment. 98% of the small-sized cells were positive for PAS. 40% of the larger cells were positive for PAS. Amylase treatment eliminated the PAS signal in the small cells ($p<0.001$, Student's t-test) and significantly diminished the PAS-signal in the larger cells to 7%. ($p<0.001$, Student's t-test) (Tabatabaei Shafiei et al., 2014).

Lysate samples were collected and the glycogen was measured using glycogen assay, which provides a quantitative measure of glycogen concentration. The sample contained a concentration of 0.6μg of glycogen in samples that were diluted 1:1, which means that 1 million cells contained 1.2μg of glycogen (Fig. 7). To confirm that the amount of glycogen measured was directly proportional to the amount of lysate present in the sample, the lysate was further diluted, which produced a corresponding reduction in glycogen (Fig. 7). These results confirm that PBMC have glycogen stores.

Fig. 7) Measurement of Glycogen Using Glycogen Enzymatic Detection

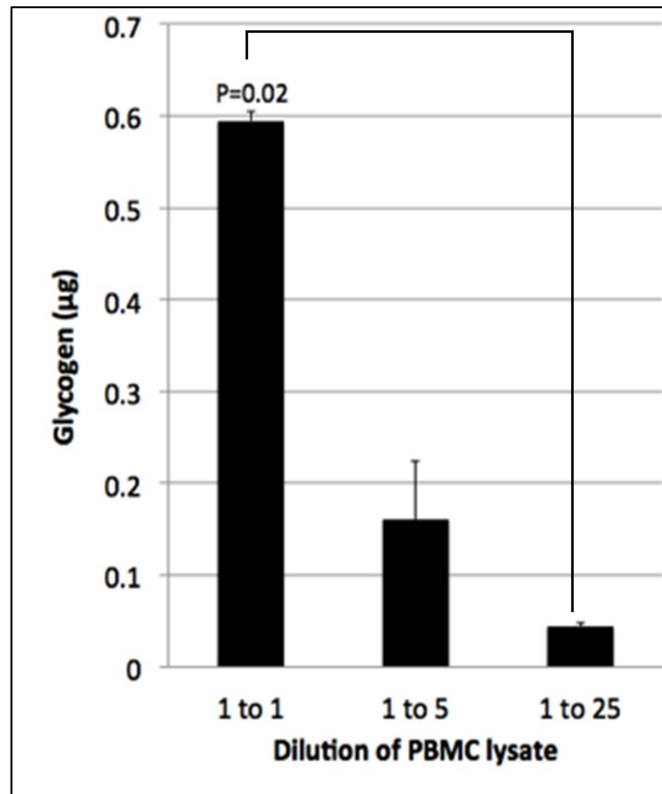
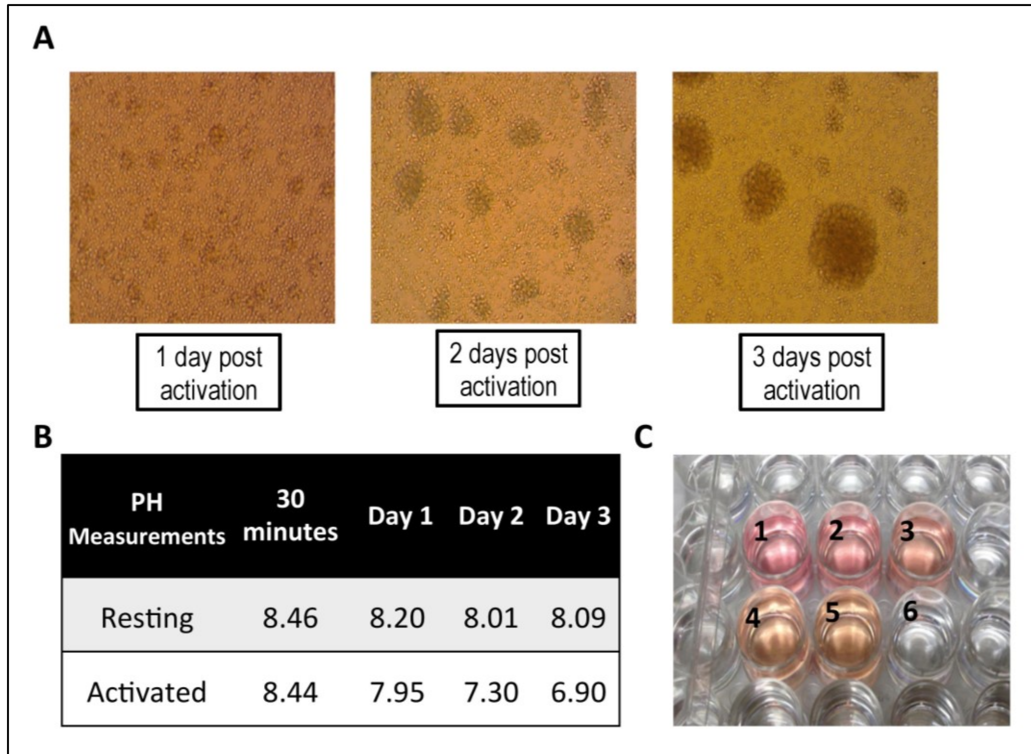


Figure. 7) Measurement of Glycogen Using Glycogen Enzymatic Detection. Glycogen was measured according to manufacturer's instructions. In brief, the protocol entails hypotonic lysis of $1x10^6$ PBMCs followed by pelleting of insoluble material that contains glycogen. The pellet was washed and digested with hydrolysis enzymes yielding glucose, which was measured by spectrophotometry and compared to a standard curve. The cell lysate was diluted at the indicated ratios with hydrolysis buffer. Data is representative of three experiments. Significant levels of glycogen were detected in the 1:1 dilution (p=0.02, Student's t-test), and this signal titrated out as the lysate was further diluted (Tabatabaei Shafiei et al., 2014).

Glycogen Measurements upon PMA Activation

PBMCs were activated with PMA, a chemical that activates most immune cells. To ensure the activation treatment was working, the culture plate was examined under the microscope for signs of activation at each time point. The appearance of clumped cells indicated that PMA activated the samples (Fig. 8A). The pH of the media was lower when cells were activated, confirming that activation had occurred (Fig. 8B). The colour of the media was also more yellow, due to the presence of pH indicator in the cell culture media, which is consistent with the pH values (Fig. 8C). Both pH measurements and microscope observations of the cultures established the effective activation of these cells with PMA.

Fig. 8) Evidence of Activation of PBMCs with PMA



PH Measurements	30 minutes	Day 1	Day 2	Day 3
Resting	8.46	8.20	8.01	8.09
Activated	8.44	7.95	7.30	6.90

Figure. 8) Evidence of Activation of Immune Cells with PMA. A) The cells in the culture plate were analyzed under a light microscope (20X objective) at day one, day two and day three. Activated Immune cells aggregate into clumps at the bottom of the culture plate. B) pH was measured in each sample. The cells that had the activation treatment for longer times had a more acidic media than the nil non-activated (resting) condition. C) The pH indicator, phenol red, of the media provides an indication of the acidity of each group, where yellow means more acidic. Top Row from left to right: 1) no activation, 2) 30 minutes post activation, 3) 1 day post activation. Bottom Row from left to right: 4) 2 days post activation, 5) 3 days post activation and 6) PBS, no cells.

Glycogen measurements, PAS staining and glycogen assay, were done at time points of 30 minutes, 1 day, 2 days and 3 days post PMA activation treatment. Microscopy analysis of PAS stained cells revealed the gradual increase in glycogen in activated cells (Fig. 9A). The intensity of the PAS positivity of the cells was measured and recorded for each time point. There was an increase in glycogen concentrations of the PMA activated cells of 30 minutes, day 1 and day 3 groups, as compared to the resting cells ($p<0.05$)(Fig. 9B). On Day 2 there was a trend towards higher glycogen that did not reach significance. Using the glycogen assay, the glycogen concentrations were significantly higher in each time point as compared to resting cells ($p<0.05$). These findings from glycogen assay are consistent with the results of PAS staining and demonstrate that there is an increase in glycogen concentrations of the activated cells as compared to the resting cells.

Fig. 9) Glycogen Content of PBMCs upon PMA Activation

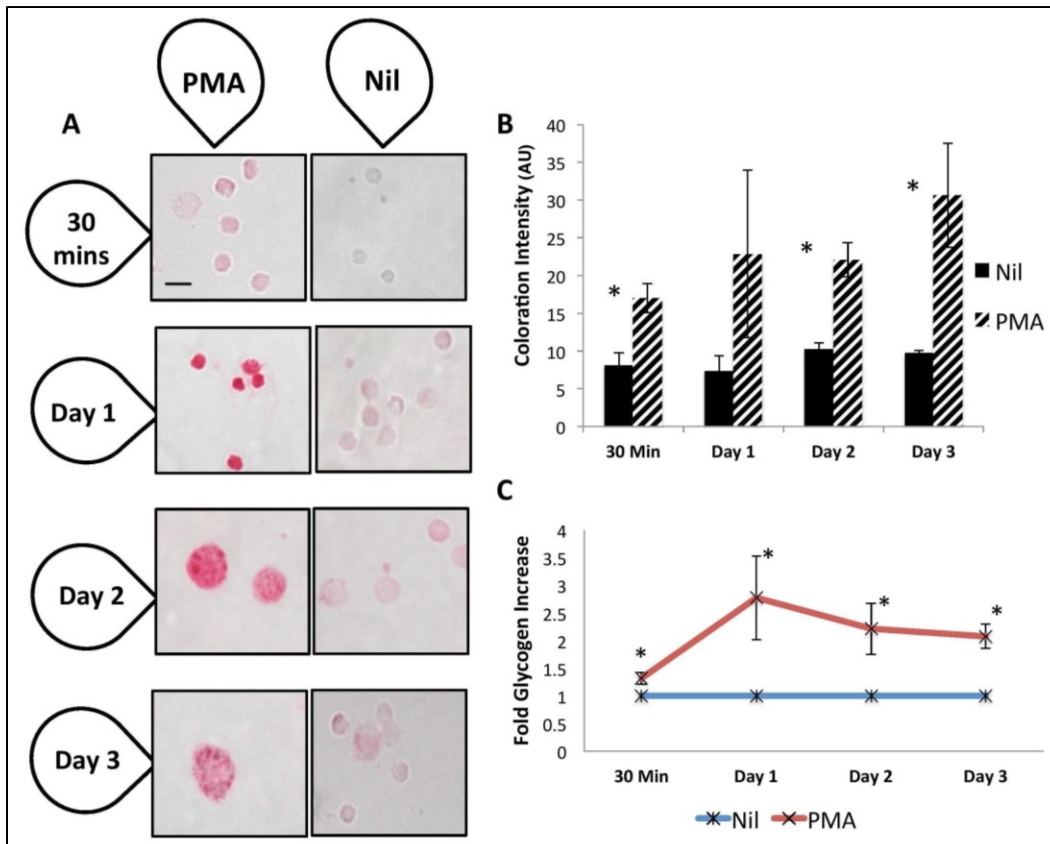


Figure. 9) Glycogen Content of PBMCs upon PMA Activation. A) PAS staining was done on PBMCs. The experiment was done in time points of 30 minutes, Day 1, Day 2 and Day 3. The images on the left column are the cells that received the PMA activation treatment and the images on the right column are the cells that are in resting state (Nil). (Scale bar= 10µm). B) The intensity of the coloration of the cells was analyzed with Image J software. Significant increase of coloration intensity was observed in 30 min, day, 2, and day 3 activated groups compared to non-activated cells (AU= Arbitrary Units). The data is pooled from 3 individual experiments. C) The glycogen content of PMA activated and non-activated (nil) PBMCs was measured with glycogen assay. There was a significant increase in the fold change of glycogen accumulation upon PMA activation. Pooled from 3 individual experiments. (*p<0.05, One-way ANOVA followed by Fisher LSD).

Glycogen Measurements upon T Cell-Specific Activation

To determine the contribution of T cells, PBMCs were activated with T cell-specific activator that provides the two necessary signals for proper T cell activation (CD3 and CD28, which are only found on T cells). Glycogen measurements was done through PAS staining and glycogen assay at time points of 30 minutes, 1 day, 2 days and 3 days post activation treatment. To ensure the activation was effective, supernatants of the cultures were collected at each time point and they were measured for various interleukins (ILs). ELISA was used to measure the amounts of IL-1B and IL-6, to determine if the monocytes were activated, alongside IL-17 and IL-2 to determine if the T cells were activated (Figure 10.A-D). The level of T cell-specific cytokines, IL-2 and IL-17 increased. The monocyte cytokines increased as well, which indicates that they were activated as a by-product of the T cell-specific activation. Glycogen measurements by PAS staining demonstrated glycogen accumulation 30 minutes post-activation.

Fig. 10) Evidence of Activation of PBMCs with T Cell-Specific Activation (IL-1β, IL-6, IL-17, IL-2)

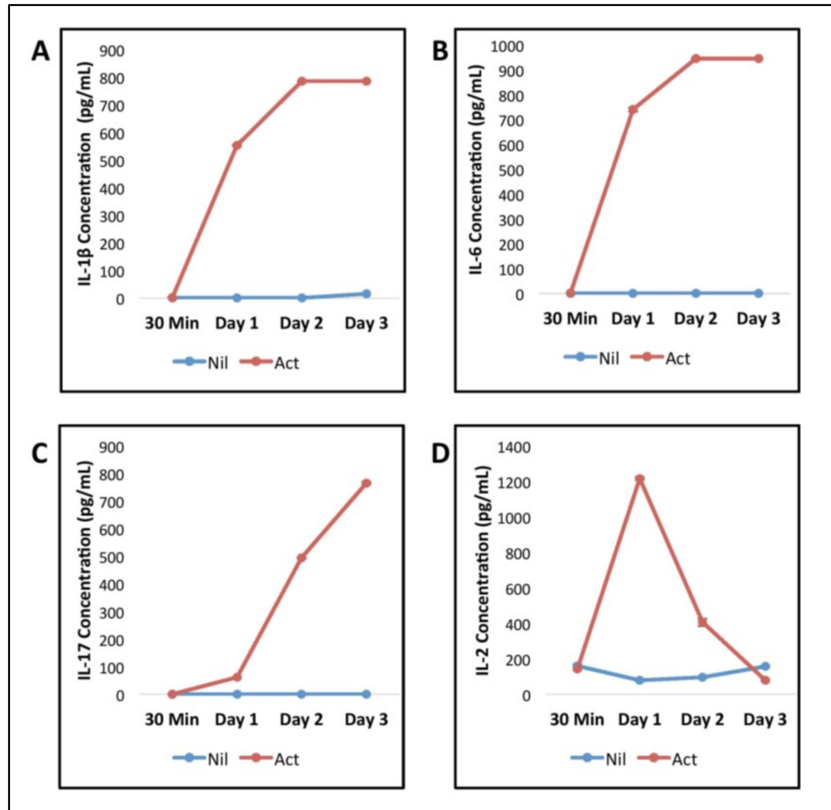


Figure. 10) Evidence of Activation of PBMCs with T Cell-Specific Activation (IL-1β, IL-6, IL-17- IL-2). A): ELISA results done on the supernatants of activated (Act) and non-activated (Nil) groups. The ILs secretion was used as a validation of activation. A) IL-1β was tested to determine monocyte activation. IL-1β β started to increase from 30 minutes post activation treatment and remained high through day 3. The concentration of IL-1β reached a plateau by day 3. B) IL-6 concentration was measured as a second activation marker for monocytes. There is an increase in secretion of IL-6 starting from 30 minutes post activation treatment and reaching a plateau by day 3. C) IL-17, a T cell activation marker, was used to validate the activation of T cells in the cultures. D) IL-2 was measured as a second activation marker for T cells. As expected, IL-2 levels peak at day 1. Representative of 3 individual experiments.

The cells exhibited the gradual increase of PAS positivity of the cells upon treatment with T cell-specific activation (Fig. 11.A). The intensity of PAS positivity of the cells was measured at each time point (Fig. 11.B). Results show activated groups of 30 minutes, day 2 and day 3 had a greater PAS positivity compared to their respective non-activated groups ($p<0.05$, One-way ANOVA). When glycogen levels were measured with glycogen assay, a higher concentration of glycogen was found in activated cells as compared to the non-activated groups ($p<0.05$, One-way ANOVA) (Fig 11.C). The results from glycogen assay are consistent with the results of PAS staining. Thus, activating T cells specifically promotes glycogen formation.

Fig. 11) Glycogen Content of PBMCs Upon T Cell-Specific Activation

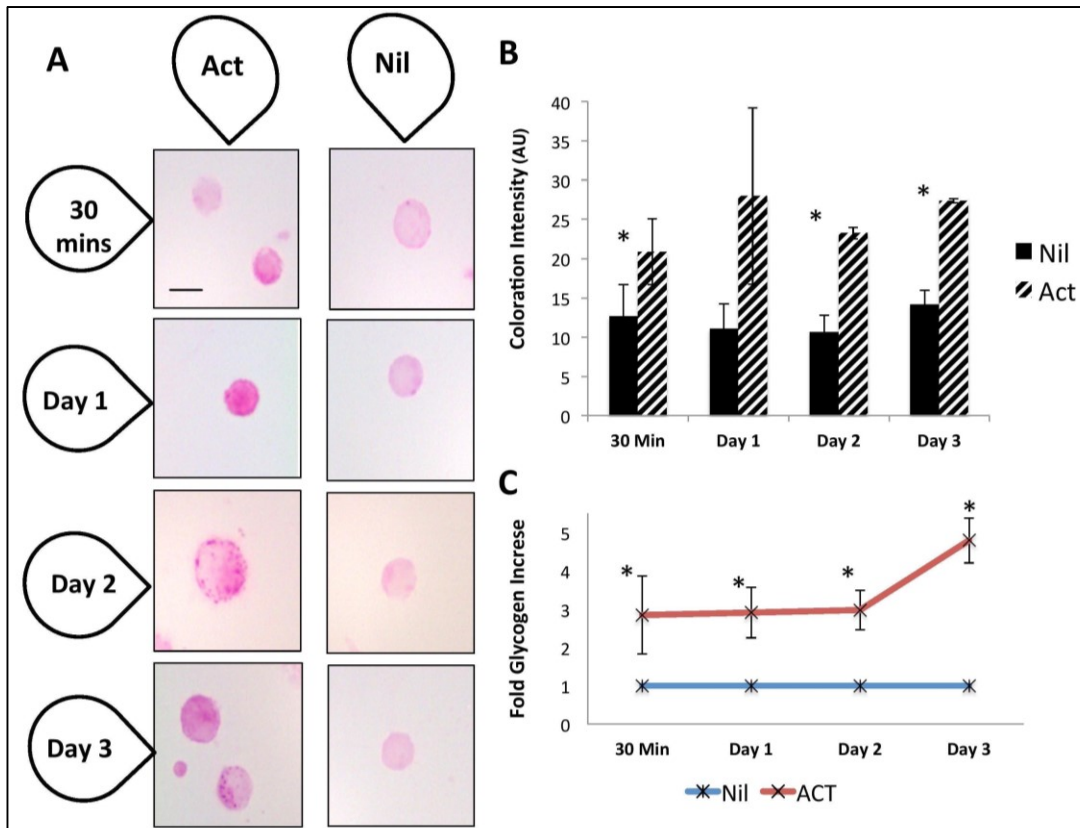


Figure. 11) Glycogen Content of PBMCs Upon T Cell-Specific Activation. A) PAS staining was done on activated (Act) and resting PBMCs (Nil). The experiment was done in time points of 30 minutes, Day 1, Day 2 and Day 3. The images on the left column are the cell that received the activation mix and the images on the right column are the cells that are in resting state. (Scale bar= 10µm). B) The intensity of the coloration of the cells was analyzed with Image J software. Significant increase of coloration intensity was observed in 30min, day 2 and day 3 activated groups compared to non-activated groups. Data pooled from 3 individual experiments (AU= Arbitrary Units). C) The glycogen content of T cell-specific activated and non-activated PBMCs was measured with glycogen assay. There was a significant increase in the fold change of glycogen accumulation upon PMA activation. Pooled from 3 individual experiments. (*$p<0.05$, One-way ANOVA followed by Fisher LSD).

Manipulation of Nutrient Availably through Serum Titration

The major source of glucose in these cultures is derived from the fetal bovine serum (FBS) supplement. To manipulate the nutrient availability, PBMCs were activated and cultured in media containing 5% FBS for 1 day, followed by a gradual change in the level of FBS in the media over the course of 3 days (Fig 12.A). The initial amount of glycogen at 5% serum was 8 ng/mL. After 3 days with 1% serum, the amount of glycogen was reduced to 3ng/mL ($p<0.05$, One-way ANOVA). There were higher levels of glycogen in the other serum values (2%, 5%, 8%, and 11%) as compared to 1% serum. This demonstrates that glycogen concentration was influenced by the decrease in nutrient availability.

Fig. 12) Manipulation of Nutrient Availably through Serum Titration

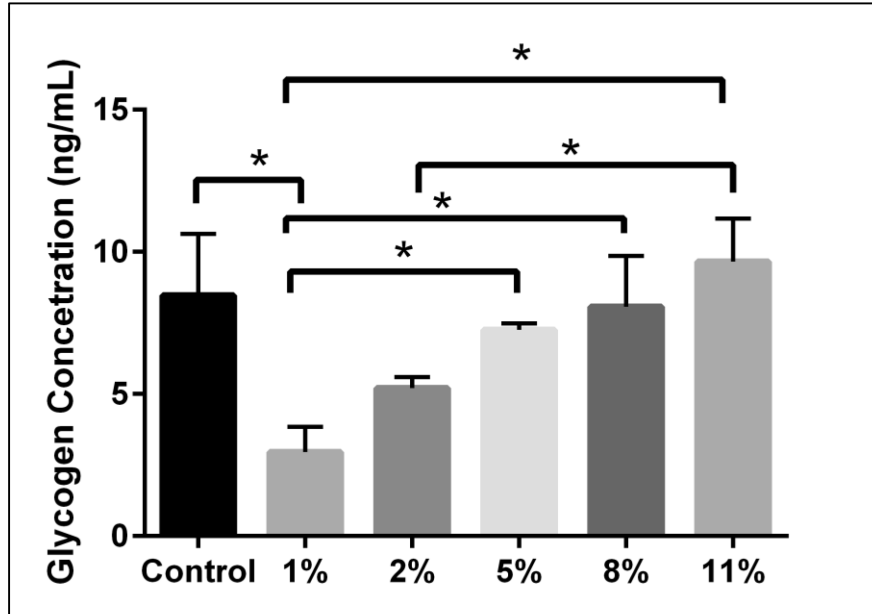
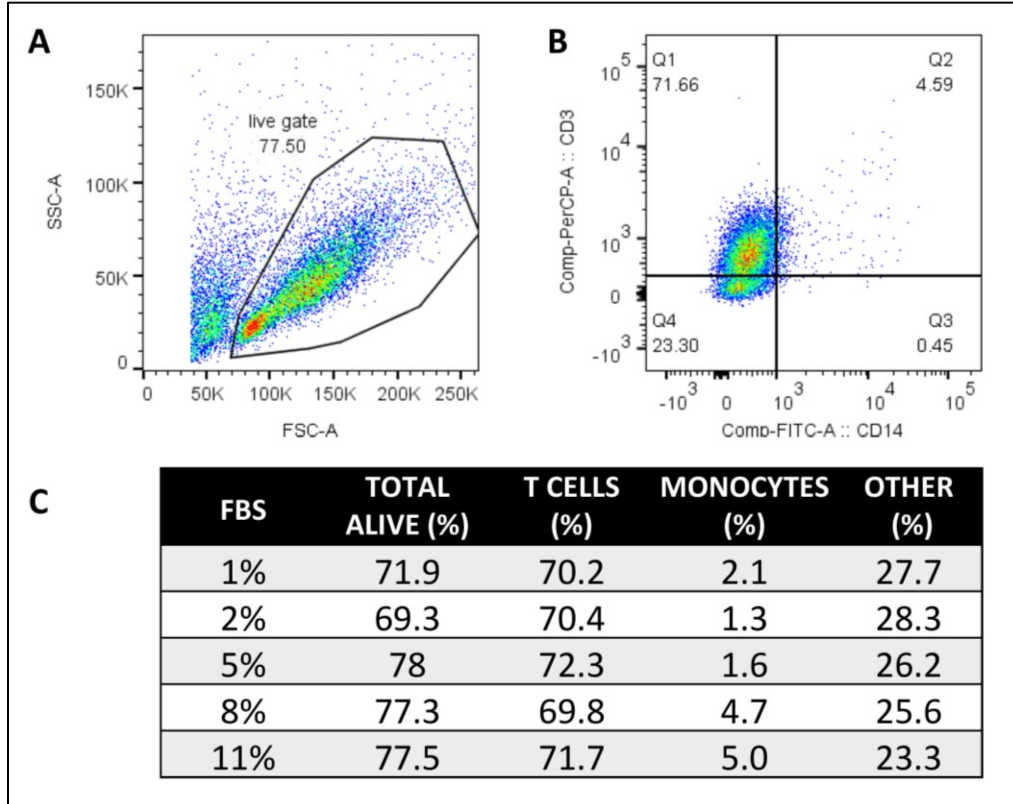


Figure. 12) Manipulation of Nutrient Availably through Serum Titration. Glycogen Content of PBMCs that were cultured in different nutrient levels and measured with glycogen assay. Prior to changing the media, all groups were cultured and treated with T cell-specific activation in 5% media for 24 hours (Control). Following that, the cells had their media changed gradually over 3 days to reach the final FBS concentrations of 1%, 2%, 5%, 8% and 11%. The media that was changed included fresh activation to hold the activation stimulus constant. On day 3, the cells were lysed and glycogen analysis was done with glycogen assay. (*p<0.05, One-way ANOVA). Pooled from 2 individual experiments.

One caveat of this experiment is that manipulating nutrient levels could result in more dead cells, or a change in the proportion of lymphocyte subtypes. To address this issue, flow cytometry was used to obtain an estimate for the amount of live cells, and the proportion of T cells and monocytes in the samples. In all serum levels tested (1%, 2%, 5%, 8%, and 11%), the proportion of T cells was ~70%, with less than 5% being monocytes, and the proportion of live cells on average was 74% (± 5%) (Fig 13).

Fig. 13) Cellular Composition Of PBMC Used for Glycogen Analysis in Serum Titration Experiment



FBS	TOTAL ALIVE (%)	T CELLS (%)	MONOCYTES (%)	OTHER (%)
1%	71.9	70.2	2.1	27.7
2%	69.3	70.4	1.3	28.3
5%	78	72.3	1.6	26.2
8%	77.3	69.8	4.7	25.6
11%	77.5	71.7	5.0	23.3

Figure. 13) Cellular Composition of PBMC Used for Glycogen Analysis in Serum Titration Experiment. Before glycogen analysis, the cellular composition of the samples was determined by flow cytometry using fluorescent antibodies to detect a T cell-specific marker (CD3) and a monocyte-specific marker (CD14). A) The laser scattering properties of the cells is plotted in a representative sample (11% serum). Each dot is an event, the heat map indicates multiple cells in the same spot. Based on this pattern, the cells that are alive were gated (live gate). B) The fluorescent emission was plotted from the cells of the live gate. In quadrant 1 (Q1) the T cells are found, in Q2 (and Q3) the monocytes are found, and any other type of lymphocyte falls into Q4. C) The different percentage FBS in serum conditions are presented in table format.

Discussion

The purpose of my Master's thesis was to demonstrate the presence of glycogen in lymphocytes and investigate how glycogen content is affected upon activation. The role of nutrient availability on glycogen concentration was also addressed.

Techniques Used

To measure glycogen, two main techniques were used; periodic acid Schiff's (PAS) staining and enzymatic detection kit (glycogen assay). This work is the first to use both techniques on T cells. These techniques are complimentary to each other because PAS staining provides information on the intensity of glycogen positivity of intact cells, while the glycogen assay provides information about glycogen concentrations of the whole sample.

To analyze PAS stained images, ImageJ software was used to measure the overall intensity of the purple coloration in each cell. This approach differs from the literature which used a scoring system based on the number of granules in the cells, where a certain number of granules was used as a cut-off point for positivity (Mitus et al. 1958; Gärtner and Nordén 2009). Scoring granules in this fashion introduces a bias because glycogen patterns can be subjective, which is avoided by measure the overall intensity of the purple coloration. The scaling systems present in literature are inconsistent and there is no universal scaling system that would address all different PAS positivity patterns and also report the intensity of the positivity.

PAS Staining on Mouse Muscle Sections

PAS staining was first done on mouse soleus muscle sections as a positive control. The human soleus muscle consists of 60% slow twitch and 40% fast twitch muscle fibres. This means 40% of the muscle fibres have more glycogen storage compared to the other 60%. A mouse (Mus spretus) soleus muscle has similar percentage compositions; 64% slow twitch fibres and 36% fast twitch (Drummond, 1967; Totsuka et al., 2003). The analysis revealed 37% PAS positive muscle cells, which is similar to the percentage composition of fast twitch fibres in the muscle used (Fig 4). There was a clear staining visible on the cell membrane; this was because PAS also stains elements such as glycoproteins, glycolipids and mucins that are found on the cell membrane. However, Amylase used as a negative control in the slides can only dissolves glycogen. This supports the fact that the dark particles in the non-amylase treated cells are in fact glycogen particles.

Glycogen Analysis of Human Immune Cells

Healthy human subjects were recruited based on the following selection criteria: anyone above the age of 18 who was comfortable with blood draws and needles, not taking any prescriptions medications that affect blood pressure or immune function, not suffering from chronic medical conditions (autoimmune disease, cancer, lung or heart disease) and did not receive any vaccinations within the past 2 months prior to participation.

The initial experiment was done using human whole (un-fractioned) blood. PAS staining on whole blood films was not efficient, because of the presence of mostly RBCs that weren't of interest for this study. Therefore, it was decided to isolate the cells of interest (PBMCs) from

the whole blood samples and stain them separately, since this technique eliminates RBC (Fig.5C).

Amylase was chosen as a negative control treatment. Even when amylase treatment was used, there was a dark rim stained at the outline of cells, similar to what was seen with the muscle cells. This was likely due the fact that PAS also stains other molecules of the cell membrane. Unlike glycogen polymers, these molecules are not digested by amylase treatment, resulting in them picking up the stain particles of PAS staining (Fig. 5A and 5B). These results indicate that PBMCs contain glycogen positive particles and diffuse glycogen throughout the inside of the cell that are dissolved once the cells are treated with amylase. PBMCs show different patterns of glycogen positivity, suggesting the varying amounts of glycogen in these cells. Some of the patterns shown in Fig. 5A where the cells show diffused or granular patterns covering the entire cell, diffused pattern on one side and granular pattern on the other side of the cell, a mixture of both patterns or no pattern of glycogen positivity. Conventional antibody staining yields a brown or purple colour, which is difficult to discriminate from the PAS staining.

The cells observed on the slides were analyzed based on their size; larger cells (>5µm) and smaller cells (<5 µm). The reason for this classification is that PBMC population is mostly made up of lymphocytes including, T cells, natural killer cells, B cells, and myeloid cells including monocytes (myeloid) and a very small proportion of dendritic cells. The cells that are larger than 5µm are anticipated as monocytes and some dendritic cells, and cells that are the same size or smaller than 5µm are expected to be resting lymphocytes (Downey et al., 1990; Goya et al., 2008; Marguerat & Bähler, 2012). Nearly all of the small cells were positive for glycogen,

while only a proportion of the larger cells were positive, suggesting that lymphocytes express more glycogen than myeloid cells. Glycogen levels in cell lysates were analyzed by glycogen assay. The amount detected was 1.2µg per 10^6 lymphocytes. This value was comparable to the report from Hedeskov et al, who found that 10^{10} lymphocytes from healthy humans contained 31 mg of glycogen, which is equivalent to 3.1µg per 10^6 lymphocytes (Hedeskov & Esmann, 1967). The glycogen in lymphocytes represents between 2-4% of the glucose that is used by human lymphocytes, the rest of the glucose is used for glycolysis, aerobic respiration (Krebs cycle), and pentose phosphate pathway (Hedeskov 1968; Quaglino & Hayhoe 1965; Hedeskov and Esmann 1967). To put that value in perspective, glycogen content in neutrophils is 13.6 µg per 10^6 cells (Gahrton & Yataganas, 1976).

Glycogen Measurements upon PMA Activation

All activation experiments were analyzed at 30 minutes, day 1, day 2 and day 3 to allow enough time for PBMCs to become fully activated (Ling and Holt 1967). It also provided the advantage of observing the gradual change in glycogen concentrations of the cells. PMA was initially used to activate because it stimulates most cells and was thus a positive control. PMA-activated Immune cells were observed to cluster at the bottom of the culture plate, while resting cells were evenly distributed on the plate (Pulvertaft & Pulvertaft, 1967). This pattern is expected for PMA activated cells (Fig. 8A). The activation was also measured by the change in the pH of the media; the pH was lower in activated samples. This occurs due to lactic acid as a by-product of glycolysis.

Glycogen is higher at 30 minutes post PMA treatment as compared to resting cells, a pattern that remains through 3 days post PMA activation. This data demonstrates the direct association of glycogen build-up and PMA activation, suggesting that PMA induces the glycogen synthesis pathway

Glycogen Measurements upon T Cell-Specific Activation

Using a non-specific activator as positive control revealed that lymphocytes accumulate glycogen, however, it is not clear which cells are responding. Upon activation of PBMCs with T cell- specific activators, there was a similar increase in glycogen concentrations. This suggests that due to activation, immune cells increase the glycogen production, possibly because of the increase in glucose uptake in activated PBMCs.

The findings from glycogen assay and PAS staining are consistent. At each time point there is an increase in glycogen concentrations of the activated cells as compared to the resting cells. The results of both activation treatments (PMA and T cell-specific activation) are consistent thus validating the effect of activation on the increase of glycogen concentration. The production of glycogen is enzymatic. The rapid rise in glycogen confirms that the enzymes are already present and that there is likely no gene expression required. With PMA activation, this rapid rise was delayed and that could be due to the fact that PMA is a general activator and activation is accelerated when it is obtained through T cell- activators.

To confirm that T cells were being activated, cytokines were measured. As expected T cell-specific cytokines were found including IL-1, an early cytokine, and IL-17, a cytokine that peaks later than IL-2. Other cytokines that are derived from monocytes, IL-1, IL-6, showed that

monocytes were also activated. Despite the presence of activated monocytes, there were very few monocytes detected in the samples used for glycogen assay. This is likely because activated monocytes stick very strongly to plastic surfaces, but to analyze glycogen, only the floating cells are taken.

Previous studies tested the activation effects of lymphocytes on glucose metabolism, using phytohaemagglutinin (PHA) as an activator of T cells. PHA is a lectin-derived chemical extracted from legumes especially kidney beans. PHA acts as a mitogen on lymphocyte cells, which causes them to clump and proliferate much like they do in their activated state. Glycogen was found to accumulate in the PHA-activated activated lymphocytes (Hamelryck et al., 1996; Hedeskov, 1968; Helderman, 1981). This is consistent with the findings of the current study where more direct activation treatments (PMA and T cell-specific activators) were used. One limitation of the previous studies was that they only used one technique to measure glycogen, while in this study two techniques were used to measure glycogen.

Arrizabalaga et al. investigated the glycogen content by measuring glycogen concentration in a human T cell line (Arrizabalaga, Lacerda, Zubiaga, & Zugaza, 2012). The authors found that glycogen decreased when the cell line was activated. In contrast, in the current study glycogen accumulated in activated lymphocytes. One key difference between Arrizabalag and the current study is that primary lymphocytes from healthy donors are more physiologically relevant than a T cell line, which has chromosomal abnormalities.

Manipulation of Nutrient Availably through Serum Titration

The change in the serum concentration in the media of the culture had to be gradual so as to allow the cells to adapt to the new environment and prevent shocking the cells. In low serum levels there was less glycogen. The possible explanation for the higher glycogen concentration in the cells that were cultured in high nutrient levels (8% and 11% FBS) was that they had more glucose available. However, all groups were first cultured in 5% FBS and thus they all had the same amount of initial glycogen concentrations prior to suspension in medias with different nutrient availabilities. With lower nutrient levels (1% and 2% FBS), the cells expressed lower amounts of glycogen concentrations suggesting glycogen may have been used a fuel source by the cells. The proportion of live cells on average was 74% with a range of only 5%, ruling out the possibility that cells died more in the low serum levels. The proportion of T cells was approximately the same in all serum conditions (~70%) and very few monocytes were present (less than 5%). Once PBMCs are incubated in culture plates, most of the monocytes present in this mixed population will stick to the bottom of the well and will not detach unless trypsinization techniques are used. This was to our advantage since the cells that were in suspension would end up being enriched in T cells and low levels of monocytes are present.

Formation of glycogen upon activation could be beneficial for activated T cells in many ways. With the increased glucose uptake upon activation, the concentration of glucose inside the cytoplasm of T cells is higher than the outside environment. This is harmful due to the fact that the difference in the osmotic pressure could cause the cells to burst. On the other hand, if all of the glucose inside the cytoplasm is processed at once, it will cause the activated T cells to go towards a state called hyperactivity (Maciver et al., 2008). At this state healthy T cells will start

to promote programmed cells death, which is called apoptosis. Cancer cells are resistance to apoptosis and thus due to hyperactivity they become pathogenic (Maciver et al., 2008; Nicot, 2015). Formation of glycogen reduces the amount of soluble glucose thus it may prevent hyperactivity and osmotic imbalances.

Future Directions

I speculate that the blood glucose levels would correlate to the amount of glycogen in the lymphocytes. A future direction of this project is to measure blood glucose levels from participants prior to the blood draw. An interventional study could also look at the effects of controlled diets and how eating different amounts of glucose would alter the lymphocyte glycogen levels. Another direction is to inhibit key enzymes in glycogen metabolism such as glycogen phosphorylase, or glycogen synthase, and investigate how that will affect activation and cell survival and cytokine production. In this way, one could test if glycogen is required for immune cell activation; if a glycogen phosphorylase inhibitor prevented immune cell activation that would be a way to demonstrate the function of glycogen.

Conclusion

This project demonstrates the presence of glycogen in resting immune cells from healthy people through two independent techniques (PAS staining and glycogen assay). The effect of activation on glycogen content of immune cells was examined through the use of two different immune cell activators (PMA and anti-CD3/anti-CD28 mixture). Upon activation of immune cells, there is a significant increase in glycogen accumulation. Finally, the role of nutrient availability

was studied for this project. The findings suggest that activated immune cells may utilize glycogen in times of low nutrient availability.

This thesis is relevant in the context of T-cell acute lymphoblastic leukemia (T-ALL). T-ALL patients have less glycogen positivity than other types of cancer (Lilleyman et al, Andreewa et al., and Huhn et al.; supplemental table 1). The cancer cell could interrupt the normal regulation of the glycogen pathway in order to obtain energy to grow aberrantly, while in healthy people T cells store glycogen until it is needed. In T-ALL the PI3K/Akt pathway, which is initiated by CD28 co-stimulatory signal in healthy cells, is always on. One of the functions of this pathway is to regulate the increase in glucose uptake of the cells. This implies that in this disease there is abundance of glucose available to the cancer cell. Since reduced levels of glycogen are expressed in these cells, it is indicated that the glucose inside these leukemic cells is being processed to create energy, which may have caused their hyperactivity (Barata et al., 2004; Durinck et al., 2015; Nicot, 2015). Glycogen stores could prevent harmful outcomes that follow high levels of glucose and ATP inside the cytoplasm. The more we understand the purpose and the role of glycogen, the better we can control these unfavourable outcomes.

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Appendix

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Supplementary Table 1: Comparison of three papers from the literature that measured PAS Positivity of Lymphocytes in Patients Suffering from T-ALL

	Lilleyman et al.		Andreewa et al.		Huhn et al.	
T-ALL		Patients		Patients		Patients
	<1% PAS+ cells	58 (70%)	Granular PAS+ cells	2 (11%)	% of PAS+ Patients	6 (18.5%)
	10% PAS+ cells	20 (24%)	Total	14 (100%)	Total	34 (100%)
	>10% PAS+ cells	5 (6%)	The mean score: 19			
	Total	83 (100%)				
non-T-ALL		Patients		Patients		Patients
	<1% PAS+ cells	74 (38.7%)	Granular PAS+ cells	15 (46%)	% of PAS+ Patients	54 (53.5%)
	10% PAS+ cells	74 (38.7%)	Total	32 (100%)	Total	101 (100%)
	>10% PAS+ cells	43 (22.6%)	The mean score: 99			
	Total	191 (100%)				
Conclusion	Negative PAS reaction is significantly more common in T-ALL: p<0.0005		Significant difference between the mean% and scores: p<0.001		Significant difference was found between the groups: p<0.01	

Supplementary Table 1: PAS positivity of lymphocytes was measured in patients suffering from T-ALL and non-T-ALL. Each study had a different scaling system however the conclusion of the analysis was consistent between all studies. A reduction in glycogen positivity of lymphocytes was observed in patients suffering from T-ALL as compared to non-T-ALL patients. **Data adapted from Lilleyman *et al.*, Andreewa *et al.*, and Huhn *et al.***