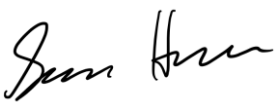
Regulation of Mitochondrial Mass by Host B Cell Receptors and Their Viral Mimics

A Senior Thesis Presented to
The Faculty of the Department of Molecular Biology,
Colorado College

By
Juliet Fink

Dr. Olivia Hatton
Primary Thesis Advisor



Dr. Sara Hanson
Secondary Thesis Advisor

Abstract

B cell lymphocytes are an immune cell able to generate antigen specific responses. B cell activation occurs through two different receptors, the B cell receptor and CD40, by interaction of antigen and CD40 Ligand, respectively. Following activation through the BCR and CD40, B cells undergo the energetically demanding processes of proliferation and differentiation into either memory cells, which provide a rapid and strong response to a secondary infection, or plasma cells, that produce large amounts of antigen specific antibodies. The immune cell most similar to B cells – T lymphocytes – require a metabolic change to aerobic glycolysis following activation to support effector function and memory differentiation. Due to the similarities in effector and memory classes of the two cell types, we hypothesized that a metabolic change must also occur in B cells to allow for their proliferation and differentiation to memory cells. Specifically, we examined alterations in mitochondrial mass to indicate a metabolic transition after activation. To do this, we stained cells with MitoTracker Green stain and quantified mitochondrial content by flow cytometry. We previously demonstrated that mitochondrial mass increased following stimulation through the BCR and CD40 in the Ramos cells – a germinal center-like Burkitt's Lymphoma B cell. To generalize these results, we repeated these experiments in BL41, another Burkitt's lymphoma B cell line. However, we found no increase in mitochondrial mass in BL41 following stimulation through BCR and CD40. We therefore investigated if strength of signaling could effect the cell types differently, and preliminary data suggests that increased BCR stimulation in BL41 may increase mitochondrial mass. We also asked whether a viral CD40 mimic – the Epstein-Barr Virus (EBV) protein LMP1 – can regulate mitochondrial mass in BL41 cells. LMP1 signaling did not increase mitochondrial mass. These results could motivate

future studies investigating the mechanism through which mitochondrial mass increases in Ramos cells in response to BCR and CD40 activation, using BL41 as a negative control.

Introduction

CD40 and BCR regulate B cell function

B lymphocytes are highly specialized immune cells that play a critical role in the adaptive immune system. Two critical regulators of B cell function are the B cell receptor (BCR) and CD40, which are both expressed on the B cell surface. Signaling through these two receptors during different stages of the B cell life cycle activate B cell response. The BCR is a membrane bound immunoglobulin, meaning it takes the same form as an antibody, which is unique to this cell type. B cells originate in the bone marrow where they undergo a genetic recombination to create a variable region of the BCR.[1] The cells are able to produce very specific BCRs to a wide range of antigens through different genetic recombination events.

During development in the bone marrow, signaling through the BCR indicates autoreactivity and results in apoptosis.[1] Surviving cells leave the bone marrow as mature naïve B cells and travel through the blood stream to secondary lymph organs; the lymph nodes and spleen. Here, T cell dependent activation in B cell follicles, called germinal centers, expose the BCR to antigen and produces the first activation signal, which induces changes in gene expression to begin the immune response. This leads to clonal expansion, and somatic hympermutation of the BCR.[2] Cells that produce a BCR with increased affinity for antigen undergo differentiation into plasma cells or memory cells, while those that do not undergo apoptosis. Plasma cells are released into the blood stream and secrete antigen-specific antibodies. The antibodies travel through the blood stream and carry out a number of roles including tagging foreign material for phagocytosis, neutralizing pathogens by interfering with their infection mechanism on

the surface, and activating the complement system.[3] Memory B cells remain quiescent until a secondary infection at which point they provide a faster and stronger antibody response. This characteristic of B cells is the targeted mechanism for vaccinations to work, especially as the ability to successfully fight some diseases is directly dependent on the amount of antibodies produced.[4] After the B cells undergo clonal expansion and differentiation, they migrate to a new zone of the germinal center where they receive differentiation and survival cues.[5] These signals are produced by CD40L on a T helper cell binding CD40 on the B cell surface. The BCR and CD40 signaling produce specific downstream effects in B cells, and may be connected to a larger network of dynamic cellular metabolic changes. We aimed to investigate the metabolic reprogramming events necessary to support metabolically demanding proliferation and differentiation, and therefore used germinal center-like B cells for these experiments.[6]

The importance of BCR and CD40 is highlighted by viral mimics of these receptor's signaling. Epstein Barr Virus (EBV), the most common human viral infection, has two membrane bound proteins, LMP2a and LMP1, which mimic BCR and CD40 signaling, respectively.[7] When expressed, these proteins are constitutively active, meaning they provide constant signaling within the cell.[8, 9] In fact, in B cell lymphomas that have lost the ability to express a high affinity BCR, most cells are EBV positive and express LMP2a.[10] In transgenic mice the LMP2a signal can replace the survival signals normally mediated by the BCR, allowing these cells to evade apoptosis.[11, 12] One model for EBV infection in B cells proposes that EBV infects naïve B cells inducing LMP1 and LMP2a expression, whose signaling is able to drive proliferation through a germinal center-like reaction to establish infection in memory B cells.[7] EBV infects approximately

95% of the population asymptomatically and is associated with a number of tumors, although its precise role in the oncogenic process is unclear.[13] Understanding a metabolic shift that allows EBV to undergo this germinal center-like reaction of clonal expansion and differentiation to establish infection could provide a target mechanism for EBV-related tumorigenesis.

Mitochondrial functions beyond ATP

The metabolic processes that occur in the mitochondria, including the citric acid cycle, fatty acid oxidation and the electron transport chain, together serve the energetic demands of the cell. Regulation of these processes varies across immune cell subtypes. These subsets range from B cells to T cells to macrophages, but differences in metabolism also occur within these groups between even smaller classes, such as effector and memory cells. Generally, cells demonstrate low rates of oxygen and glucose consumption preceding activation through surface receptors, and then show a wide array of metabolic profiles after this event. These differences have led to the hypothesis that, in immune cells, mitochondrial metabolism may play an additional cell fate-determining role by acting as an input signal for differentiation rather than as a consequence to changing cell identity.[14]

In addition to generating ATP that supplies the bioenergetic needs of a cell to support proliferation and growth, mitochondrial intermediates are necessary to control signaling pathways and gene expression in the cell. For example, mitochondrial reactive oxygen species (mROS) are able to diffuse into the cytoplasm and change the activation of various transcription factors. In particular, in T cells, mROS can activate

NF-κB and NFAT, which change cell function by way of gene expression.[15] Similarly, the mitochondrial metabolites α-ketoglutarate and succinate levels are able to regulate gene expression by inhibition or promotion of histone demethylation.[16] This is because these molecules act as the substrate and product of histonedemethylase enzymes.[17] Finally acetyl-CoA is required for histone acetylation and is a central metabolite of mitochondrial metabolism.[14] The role of mitochondria as signaling organelles is still being explored, but the metabolic profiles and the role of mitochondria in different immune cell subtypes is better comprehended.

T cell phenotype is controlled by metabolism

In the closely related T cell, another cell in the adaptive immune system that has subsets of cells for attack and memory, metabolic reprogramming is well understood. Moreover, this reprogramming facilitates the immune cells unique functions. For example, oxidative phosphorylation is predominately used to make energy in a naïve cell, but following activation the cell switches to anabolic metabolism allowing for the generation of effector molecules, such as cytokines, and clonal expansion.[18] Essential for CD4+ helper T cell activation is the generation of mROS following T cell receptor (TCR) stimulation.[19] The importance of mROS in activation is further supported by the recruitment of mitochondria to the immune synapse.[20] In contrast, memory T cells develop through modulation of fatty acid metabolism and, in cytokine stimulated memory cells, rapid secondary response to infection is possible due to increased mitochondrial mass compared to naïve T cells.[21, 22] Mitochondrial remodeling also acts as a signaling mechanism in T cells. Effector T cells have independent mitochondria

while memory T cells have fused networks. Moreover, imposing mitochondrial fusion on effector T cells gives memory cell characteristics.[23] The distinction in metabolic profiles of effector and memory T cells, and the importance of mitochondrial mass in both, demonstrate how mitochondria may be signaling organelles in the germinal center processes of B cell proliferation and differentiation.

Metabolic Reprogramming in B cells is less characterized

It is reasonable to speculate that there may be similarities in the metabolic profile between short-lived plasma cells and memory B cells, and effector T cells and memory T cells, respectively. Studies in murine models have already demonstrated changes in cells that suggest these similarities to the T cell. For example, activation of mouse B cell through the BCR has been show to increase glucose metabolites, indicating increased glycolysis.[24] There is accumulating evidence that murine B cells can increase their mitochondrial mass in response to LPS, BCR, CD40 and L-4 stimulation. Mitochondrial ROS, a critical signaling metabolite for T cells, has also been shown to be important for germinal center and splenic B cell proliferation and activation, and increased production of mROS is supported by an accumulation of mitochondrial mass following CD40 and BCR stimulation.[25, 26] Additionally, naïve murine splenic B cells have been shown to increase mitochondrial mass following stimulation with lipopolysaccharide (LPS).[27] LPS binds toll-like receptor-4 (TLR-4), inducing a signaling cascade that culminates in the activation of transcriptional activator NF-κB. Another study observed two unique populations with regards to mitochondrial mass in murine splenic cells following stimulation with anti-CD40 and IL-4. One population had high mitochondrial content and

had undergone class switch recombination, while the other had intermediate levels of mitochondrial mass and expressed a surface marker of plasma cells, indicating differentiation.[28] Additionally, in murine germinal center cells that overexpress Myc, mitochondrial mass increases from both anti-CD40+IL-4 stimulation and anti-IgM stimulation, consistent with a role of Myc in mitochondrial biogenesis.[25] These results suggest a dynamic role for mitochondrial function and the importance of increased mitochondrial mass in directing cell activity following B cell activation through a variety of receptors.

It remains unknown if human B cells are able to modify their metabolism following stimulation through the BCR and CD40. Due to the similarity in murine and human cells, we anticipated that germinal center B cells would increase their mitochondrial mass to accommodate metabolic demands of clonal expansion and acquire a differentiated identity. We also expected that stimulation of the CD40 EBV mimic, LMP1, in BL41 cells will result in a mitochondrial mass increase to support clonal expansion. Correspondingly, initial results indicated a mitochondrial mass increase in the Ramos cell-line, and a potentially synergistic relationship between the BCR and CD40 in increasing mitochondrial mass. However, here, we attempt to generalize these results by duplicating the studies in a different cell line, BL41, and find that mitochondrial mass does not increase. In addition, we see no increase in mitochondrial mass increase as a result of LMP1 stimulation in BL41 cells.

Methods

Reagents

The recombinant human CD40 ligand MEGACD40L (CD40L; Enzo Life Sciences) was reconstituted with sterile water to 0.1 mg/mL and stored at -20˚C. $F(ab')_2$ goat anti-human IgM+IgG (anti-IgM; Bioscience, 1 mg/mL), stimulated BCR signaling, was stored at 4˚C. Mouse anti-human NGFR (BioLegend, 0.5 mg/mL) and goat anti-mouse IgG (Jackson ImmunoResearch, 1.8 mg/mL) were used to crosslink the chimeric NGFR.LMP1 molecule. Phycoerythrin (PE) mouse anti-human NGFR (BioLegend, 100 µg/mL) stained for the expression of NGFR. PE mouse anti-human CD54/ICAM (BioLegend, 100 µg/mL), PE mouse anti-human CD69 (BioLegend), and their isotype, PE mouse IgG1κ (BioLegend 200 µg/mL) were used to stain to confirm cell stimulation. MitoTracker Green (ThermoFisher) was reconstituted with DMSO to 1 mM and stored at -20˚C. Propidium Iodide (Sigma-Aldrich, 1 mg/mL) was diluted in sterile water to 10 µg/mL. Propidium Iodide and 7-AAD (BioLegend, 50 µg/mL) were used as viability stains.

Cell Lines

The EBV^- Ramos Burkitt's Lymphoma cell line was acquired from ATCC. The EBV^- BL41 Burkitt's Lymphoma cell line as well as the BL41 line stably expressing NGFR.LMP1 (BL41 NGFR.LMP1) were kindly provided by Dr. Olivia Martinez at Stanford University. Ramos and BL41 cells were cultured in RMPI-1640 with 10% heat-inactivated fetal calf serum and 50 units/mL penicillin-streptomycin added, and kept in a

5% CO_2 humidified 37˚C incubator. Growth media for BL41.NGFR-LMP1 was additionally supplemented with 0.7 mg/mL Geneticin/G418 (Sigma).

Cell Stimulation

To stimulate CD40 or the BCR, a final concentration of 50 ng/mL MEGACD40L or 5 µg/mL anti-IgM, respectively, was added unless otherwise indicated. Cells expressing NGFR.LMP1 were stimulated with 1 µg/mL mouse anti- human NGFR and incubated at room temperature for 30 minutes before crosslinking with 2 µg/mL goat anti-mouse IgG. For all stimulations, cells were plated at a final concentration of 1 million cells/mL in a final volume of 1.8 mL for all BCR and CD40 experiments, and 1 mL for NGFR crosslink experiments. Cells were incubated in a 5% CO_2 humidified 37˚C incubator.

Flow Cytometry for Surface Markers

PE mouse anti-human CD54/ICAM, PE mouse anti-human CD69 or PE mouse IgG1κ were used to measure activation 24 hours post stimulation. Samples were first washed with FACS buffer (0.1% NaN_3, 1% Bovine Serum Albumin in phosphate-buffered saline (PBS)) and then stained for 30 minutes on ice. Following staining samples were washed again with FACS buffer. Data (10,000-20,000 events per sample) was collected on a Millipore Guava easyCyte 5 Flow Cytometer. Data was analyzed using the Millipore Guava inCyte software. All samples were first gated on forward and side scatter to exclude cellular debris.

Flow Cytometry for Mitochondrial Mass

After simulation, cells were washed with FACS buffer then stained with MitoTracker Green (100 nM) for 30 minutes in at 37˚C in a 5% CO_2 humidified incubator. Following that incubation, samples were washed again with FACS buffer then stained with 7-AAD (50 µg/mL) for 5 minutes at room temperature in the dark prior to data (10,000-20,000 events per sample) being collected on a Millipore Guava easyCyte 5 Flow Cytometer. Data was analyzed using the Millipore Guava InCyte software. Samples were gated on forward and side scatter to exclude cellular debris and then gated to exclude 7-AAD$^+$, non-viable cells.

Microscopy

At 48 hours post-stimulation, cells were stained with MitoTracker Green (100 nM) for 30 minutes in at 37˚C in a 5% CO_2 humidified incubator. Following that incubation, samples were stained with propidium iodide (10 µg/mL) for 5 minutes at room temperature. Cells were viewed at 1000X magnification with the Zeiss Axio Scope.A1 light microscope. Exposure was set to 60 for light photos and 460 for fluorescence photos. Pictures were overlaid using ImageJ Fiji image processing application.

Results

ICAM and CD69 upregulation indicates CD40 and BCR activation, respectively

First, it was necessary to ensure that MEGACD40L (CD40L) and F(ab')$_2$ goat anti-human IgM+IgG (anti-IgM) activate our B cells through CD40 and the BCR as expected. ICAM upregulation has previously been used to confirm successful activation of CD40 and the BCR[29, 30]; therefore, we analyzed ICAM expression by flow cytometry 24 hours post-CD40L, anti-IgM and CD40L+anti-IgM treatment (Figure 1A). In the germinal center-like BL41 Burkitt's lymphoma B cell line, ICAM expression increases approximately 2.4-fold from CD40L treatment relative to unstimulated cells. Treatment with both anti-IgM and CD40L increased ICAM expression 2.6-fold compared to unstimulated cells. However, ICAM expression does not increase after anti-IgM treatment alone in BL41 cells (Figure 1B). Similar results were found in the germinal center-like Ramos Burkitt's lymphoma B cell line (data not shown). Together, these results indicate that CD40L activates CD40 in BL41 and Ramos cells.

Since ICAM staining was unable to indicate activation of the BCR in our cell lines, it was necessary to use another stain to confirm successful BCR activation. CD69 is a cell surface activation marker of B cells[31]; consequently, we analyzed CD69 expression by flow cytometry 24 hours post-treatment with different concentrations of anti-IgM (Figure 1C). In BL41 cells, CD69 expression increases in a dose-dependent manner after anti-IgM treatment, relative to unstimulated cells (Figure 1D). However, under the same stimulation conditions, CD69 expression remained unchanged in Ramos cells (data not shown). Taken together, these data show that CD69 upregulation indicates successful activation of BCR in BL41. Therefore, these stains were performed

during subsequent experiments to confirm that activation was achieved before investigating mitochondrial mass.

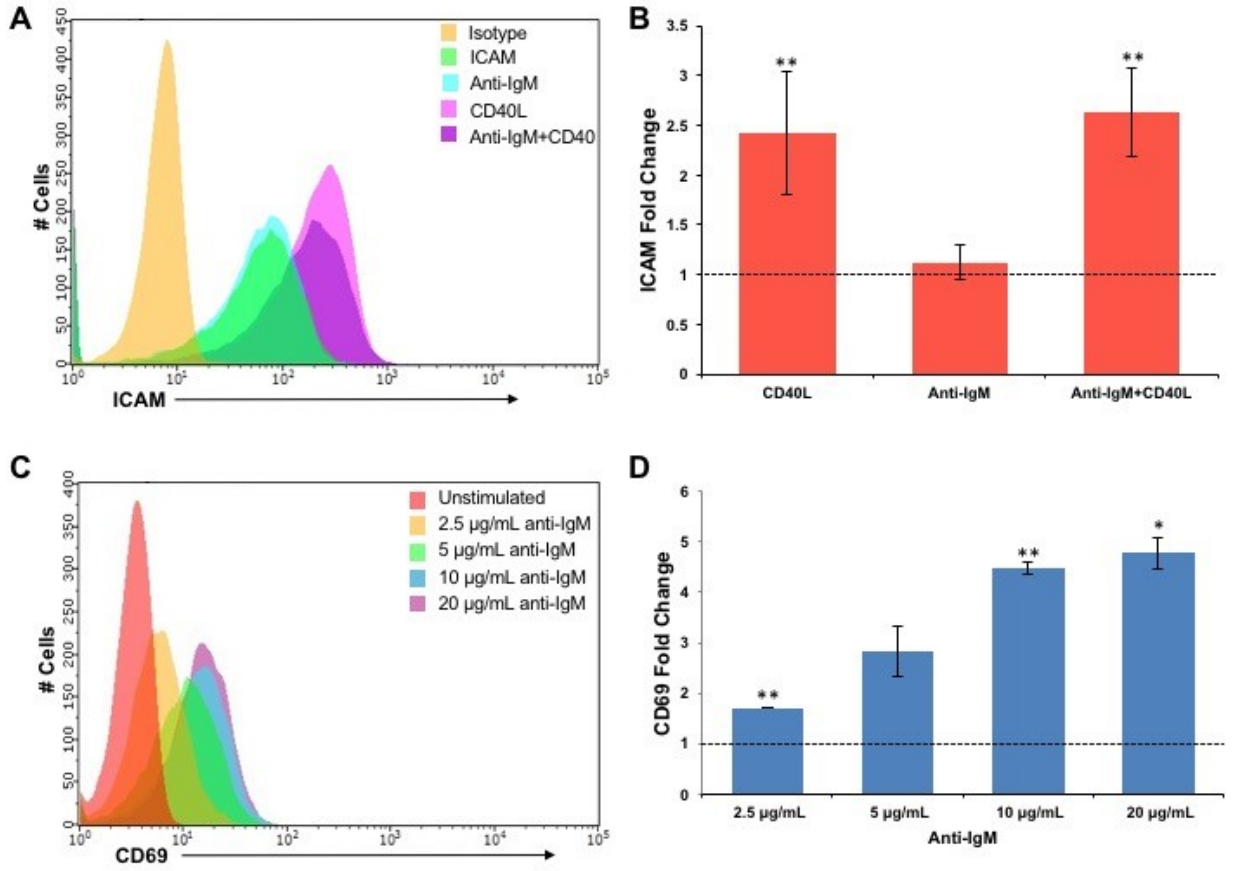


Figure 1: CD40 and BCR stimulation upregulates ICAM and CD69, respectively. (A-B) BL41 cells were stimulated using 50 ng/mL MEGACD40L (CD40L), 5 µg/mL anti-IgM or both (anti-IgM+CD40L). After 24 hours, ICAM expression was analyzed by flow cytometry. (A) Representative histogram plot. (B) ICAM fold change was calculated as median fluorescence intensity (MFI) of stimulated cells divided by that of unstimulated cells. The dotted line represents normalized ICAM expression of unstimulated cells. Mean fold change is shown from n=4 experiments and error bars represent standard deviation. (C-D) BL41 cells were stimulated as indicated using 2.5, 5, 10, or 20 µg/mL anti-IgM. After 24 hours, CD69 expression was analyzed by flow cytometry. (C) Representative histogram plot. (D) CD69 fold change was calculated as MFI of stimulated cells divided by that of unstimulated cells. The dotted line represents CD69 expression of unstimulated cells. Mean fold change is shown from n=2 experiments and error bars represent standard deviation. Mean and SDs are shown, and statistical significance was determined by Student's t test (* $p < 0.05$, ** $p < 0.01$).

Mitochondrial mass increases in Ramos cells following CD40 and BCR stimulation

Next, we sought to determine if activation of CD40 and BCR changes mitochondrial mass in our human germinal-center B cell lines. We have previously

demonstrated that the Ramos cells increased mitochondrial mass following stimulation through the BCR and CD40. Maximum activation was achieved at 48 hours post-CD40 stimulation (Figure 2A). At this timepoint, mitochondrial mass increased approximately 1.5 and 1.3-fold following BCR and CD40 stimulation, respectively, compared to unstimulated cells (Figure 2B). Mitochondrial mass also increased approximately 2-fold following a combination of CD40 and BCR stimulation compared to unstimulated cells, which was significantly greater than CD40 alone.

To confirm these results, the experiments were replicated. Confirming our previous results, CD40L treatment resulted in a 1.4-fold increase in mitochondrial mass compared to unstimulated cells (Figure 2C). Similarly, mitochondrial mass increased approximately 2-fold after treatment with a combination of CD40L and anti-IgM, compared to unstimulated cells; this increase was again significantly greater than stimulation with CD40L alone. However, given the large variation in standard deviation anti-IgM treatment did not significantly increase mitochondrial mass as we had previously shown. No quantitative data is provided when describing T cell mitochondrial mass increase, and therefore cannot be used as a comparison. Together, these results confirm that mitochondrial mass increases following CD40 activation.

Cellular proliferation and apoptosis are associated with changes in mitochondrial mass and mitochondrial processes. For example, mitochondrial proliferation appears to be an early event in the apoptotic pathway and mitochondrial mass also increases before proliferation to increase energy supply.[32, 33] Additionally, increased mitochondrial production of the reactive oxygen species superoxides signals cellular proliferation, but an overwhelming amount triggers cell death.[34] This close relationship between

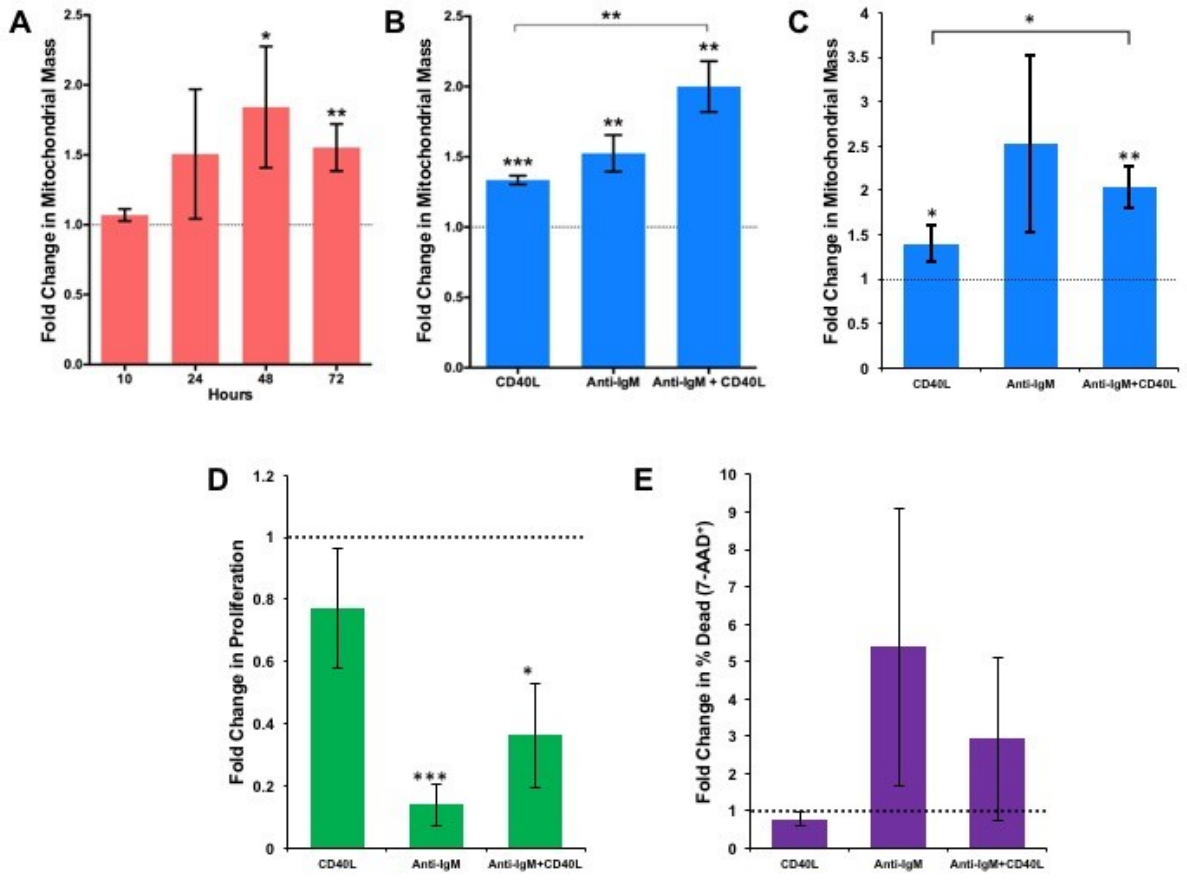


Figure 2: Ramos cells increase mitochondrial mass following CD40 and BCR stimulation. Ramos cells were stimulated using 50 ng/mL CD40L, 5 µg/mL anti-IgM or both. At 24 hours, stimulation was confirmed by upregulation of ICAM as in Figure 1. Fold change in mitochondrial mass, proliferation and viability were determined by dividing the MFI of stimulated cells by that of unstimulated. The dotted line represents normalized mitochondrial mass, proliferation, or percent dead of unstimulated cells. Error bars represent standard deviation. (A-C) Samples were stained with 50-100 nM MitoTracker Green at indicated time points. (A) Mean fold change in mitochondrial mass is shown from n=2 experiments for 10 hours, n=5 experiments for 24 and 48 hours and n=4 experiments for 72 hours. (B) Mean fold change in mitochondrial mass is shown from n=4 experiments; experiments performed by Jay Hartman. (C) Mean fold change in mitochondrial mass is shown from n=3 experiments, aiming to replicate results of part (B). (D) Ramos cell concentration was determined by Trypan blue exclusion. Data is shown from n=3 experiments. (E) At 48 hours post stimulation samples were stained with 50µg/mL 7-AAD for 5 minutes. Mean fold change is shown from n=3 experiments. Mean and SDs are shown, and statistical significance was determined by Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

mitochondrial function and these processes lead us to ensure mitochondrial mass changes could be attributed to the CD40 and BCR stimulation protocol and not cell death or proliferation. Stimulation of the BCR and BCR+CD40 showed significant decrease in proliferation (Figure 2D), so the increase in mitochondrial mass is not

attributed to a proliferating cell population. Interestingly, BCR+CD40 stimulation rescued cell proliferation over BCR stimulation alone. There was no significant change in dead cells following any stimulation (Figure 2E). However, BCR stimulation showed approximately 5.4-fold average increase in percent dead cells over unstimulated while CD40 stimulation lead to a 0.8-fold change relative to unstimulated. Stimulation of both receptors together resulted in a 2.5-fold average increase in dead cells over unstimulated, which may imply a rescuing affect of CD40 stimulation. Since these changes were not statistically significant, the increases in mitochondrial mass were not attributed to changes in proliferation or cell death.

BL41 cells do not increase mitochondrial mass following CD40 and BCR stimulation

To confirm that CD40 and BCR stimulation increase mitochondrial mass in a different Burkitt's lymphoma cell line – BL41 – we used the same stimulation and staining protocol described above. Here, mitochondrial mass did not increase following treatment with CD40L, anti-IgM, or the combination of CD40L and anti-IgM over unstimulated cells (Figure 3A). Visualization with microscopy also demonstrated no change in mitochondrial mass from stimulation (Figure 3B). Unlike Ramos cells, stimulation did not result in a change in proliferation compared to unstimulated cells (Figure 3C). Additionally, there was no significant change in BL41 viability following stimulation (Figure 3D). Together these results indicate that mitochondrial mass does not increase in BL41 cells, creating a distinction between Ramos and BL41 in response to the same stimulation of CD40 and BCR with 50 ng/mL CD40L and 5 μg/mL anti-IgM.

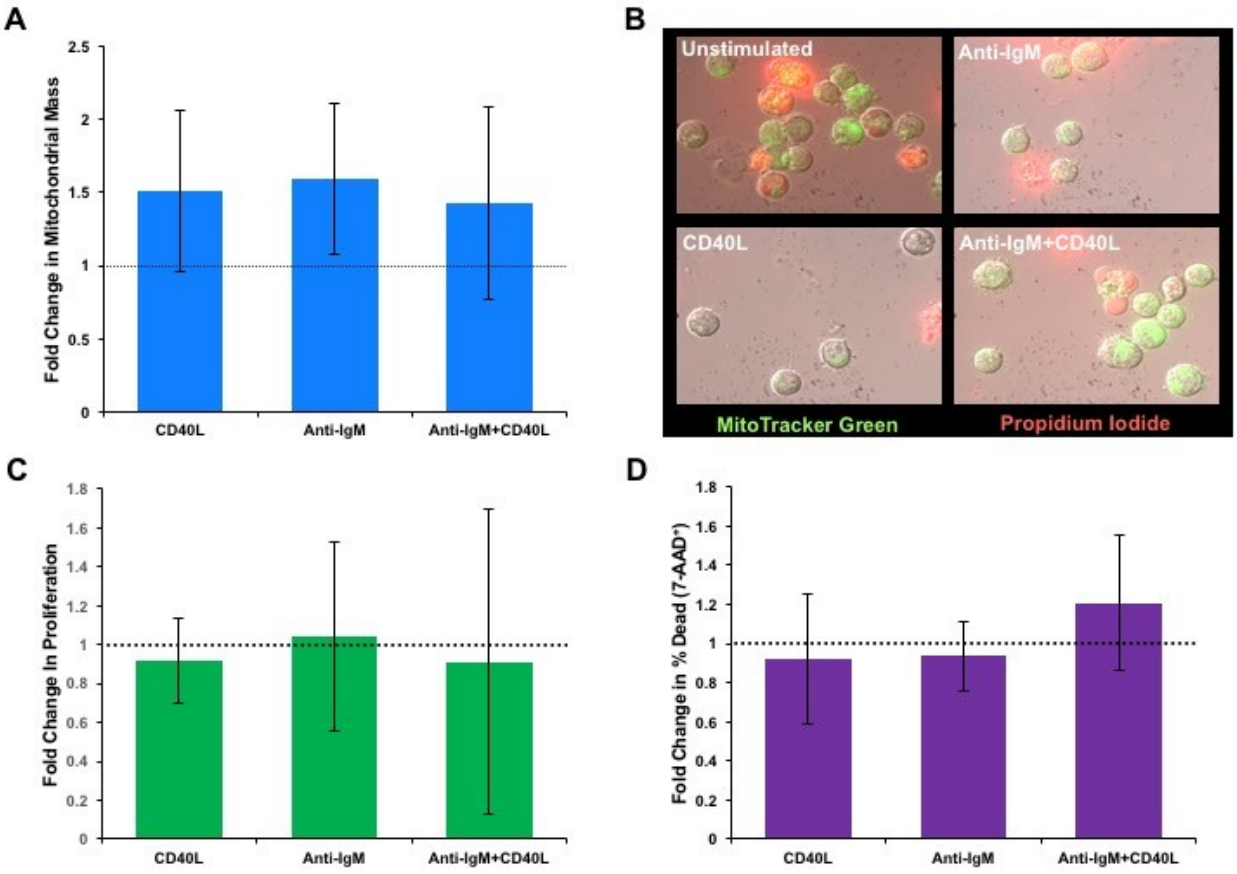


Figure 3: Mitochondrial mass does not increase in BL41 cells following CD40 and BCR stimulation. BL41 cells were stimulated using 50 ng CD40L, 5 µg/mL anti-IgM or both. At 24 hours, stimulation was confirmed by upregulation of ICAM and CD69 as in Figure 1. Fold change in mitochondrial mass, proliferation, and viability were determined by dividing the MFI of stimulated cells by that of unstimulated. The dotted line represents normalized mitochondrial mass, proliferation, or percent dead of unstimulated cells. Error bars represent standard deviation. (A, C, D) At 48 hours post-stimulation, all samples were stained with 100 nM MitoTracker Green for 30 minutes and 50 µg/mL 7-AAD for 5 minutes. (A) Mean fold change of mitochondrial mass is shown from n=4 experiments. (B) At 48 hours post-stimulation, samples were stained with 100 nM MitoTracker Green (green) for mitochondria and 10 µg/mL Propidium Iodide (red) for viability. Cells were viewed at 1000X magnification on the Zeiss Axio Scope.A1 light microscope. Data is representative of n=2 experiments. (C) BL41 cell concentration was determined by Trypan blue exclusion. Data is shown from n=3 experiments (D) Mean fold change is shown from n=3.

Increased BCR stimulation induced mitochondrial mass increase in BL41 cells

We next sought to determine if mitochondrial mass increase in response to anit-IgM treatment might be dependent on the concentration of anti-IgM used in BL41 cells. Mitochondrial mass may depend on the strength of BCR signaling, much like CD69

expression (Figure 1D). Anti-IgM treatment increased mitochondrial mass in a dose-dependent manner (Figure 4A). In experiments described above, 5 µg/mL anti-IgM was used, but stimulation with 10 µg/mL anti-IgM increased mitochondrial mass most at 3-fold larger compared to unstimulated cells (Figure 4B). However, treatment with 20 µg/mL leads to only a 2.1-fold increase in mitochondrial mass over unstimulated cells, indicating that the dose dependency exists between 0 and 10 µg/mL before plateauing. Therefore, strength of BCR signaling affects mitochondrial mass, and in future experiments, 10 µg/mL anti-IgM may be a better concentration to use to stimulate the BCR in BL41 cells.

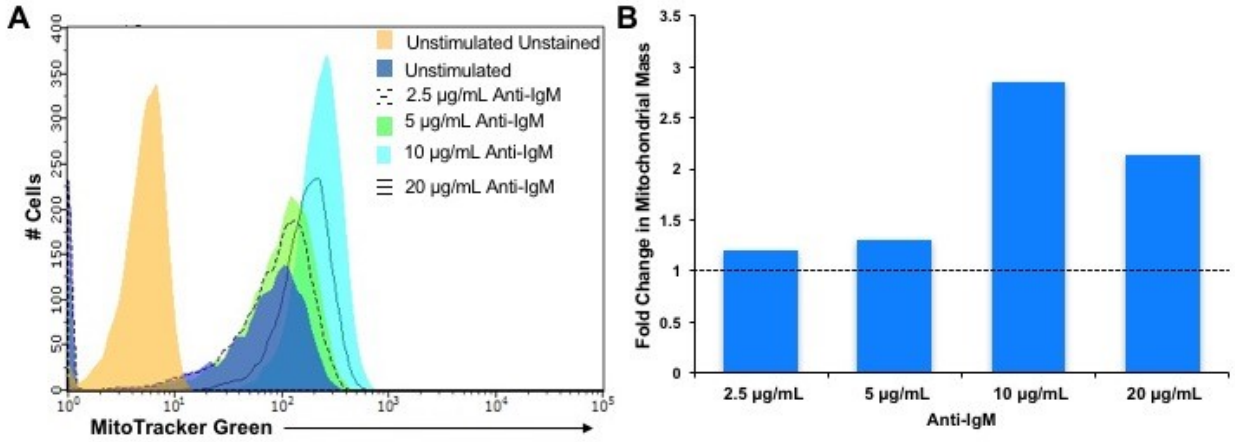


Figure 4: Upregulation of mitochondrial mass depends on strength of BCR signaling. BL41 cells were stimulated with 2.5, 5, 10, or 20 µg/mL anti-IgM as indicated. Stimulation was confirmed by upregulation of CD69 as in Figure 1. At 48 hours after stimulation, samples were stained with 100 nM MitoTracker Green and analyzed by flow cytometry. (A) Representative histogram plot. (B) Fold change was determined by dividing MFI of stimulated cells by that of unstimulated. The dotted line represents normalized mitochondrial mass of unstimulated cells. Mean fold change is shown from n=1 experiments.

Mitochondrial mass does not increase following LMP1.NGFR stimulation

The Epstein-Barr Virus viral oncogene LMP1 is a constitutively active mimic of CD40.[35] We demonstrated that CD40 stimulation in Ramos cells increased mitochondrial mass (Figure 2A-C). Therefore, we hypothesized that LMP1 signaling

could also increase mitochondrial mass in human B cells. To test this, we used genetically modified BL41 cells, which stably express a chimeric NGFR.LMP1 molecule that contains the extracellular domain of nerve-growth factor receptor (NGFR) and the

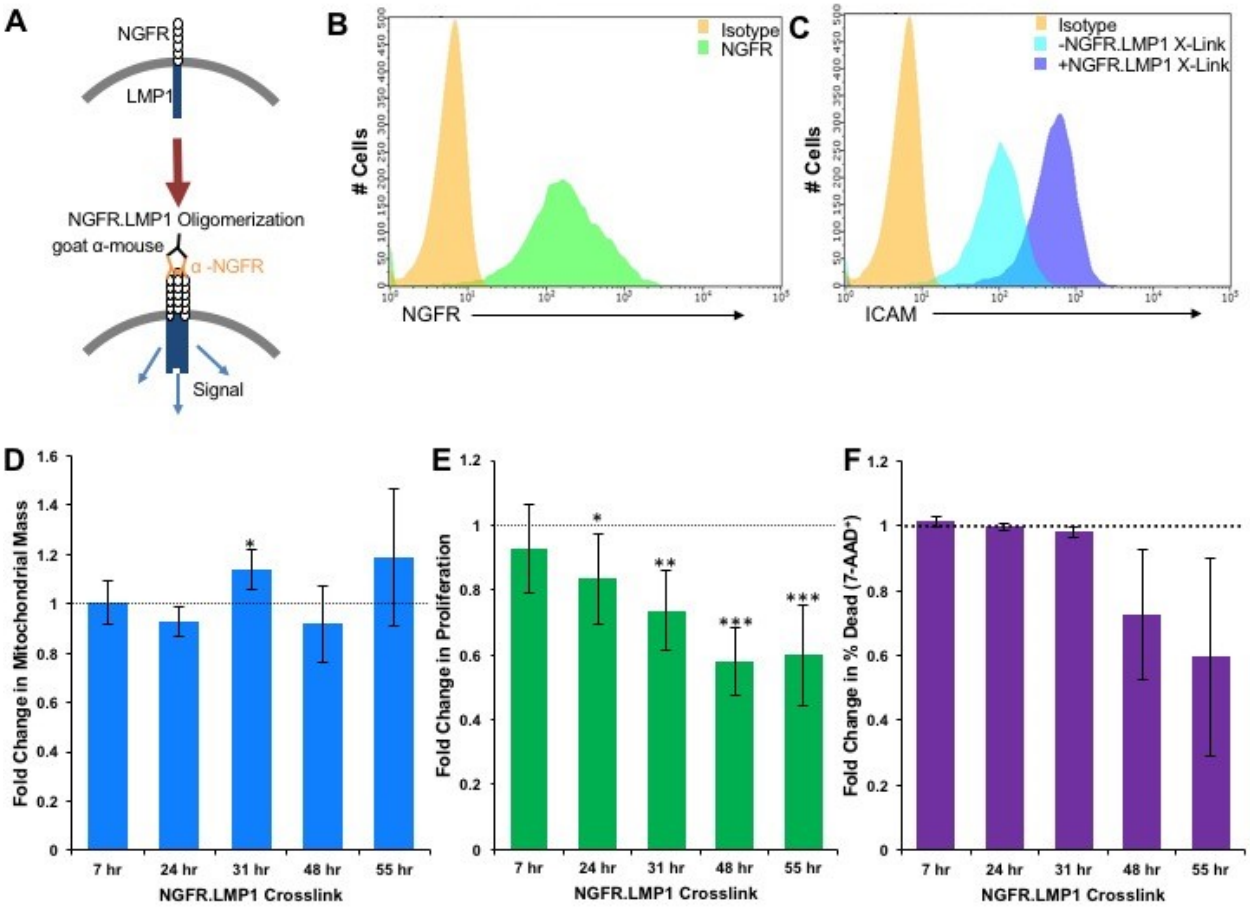


Figure 5: Mitochondrial mass does not change after LMP1 signaling. BL41 cells expressing a chimeric NGFR.LMP1 molecule were stimulated by adding 0.5 µg/mL mouse anti-NGFR antibody followed by the addition of 1.8 mg/mL goat anti-mouse antibodies. At 24 hours, stimulation was confirmed by upregulation of ICAM. (A) Cartoon of NGFR.LMP1 stimulation. (B) At 24 hours, stable expression of NGFR was confirmed. Histogram plot is representative of n=3 experiments. (C) Histogram plot is representative of n=3 experiments. (D-F) Fold change in mitochondrial mass, proliferation, and viability were determined by dividing the MFI of stimulated cells by that of unstimulated. The dotted line represents normalized mitochondrial mass, proliferation, or percent dead of unstimulated cells. Error bars represent standard deviation. (D) At 7, 24, 31, 48 and 55 hours post-stimulation, samples were stained with 100 nM MitoTracker Green. Data shown from n=5 experiments for 48 hours, n=3 for all other time points. (E) BL41 NGFR.LMP1 cell concentration was determined by Trypan blue exclusion. Data is shown from n=3 experiments. (F) At 48 hours post-stimulation, samples were stained with 50 µg/mL 7-AAD for 5 minutes. Mean fold change is shown from n=3 experiments. Mean and SDs are shown, and statistical significance was determined by Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

signaling domain of LMP1 (Figure 5A). LMP1 is a constitutively active molecule and therefore it is difficult to study the affects of LMP1 signaling. NGFR.LMP1 acts as an inducible signaling molecule that creates a signal indistinguishable from full length LMP1.[36] Addition of mouse anti-NGFR antibodies and cross-linking goat anti-mouse antibodies cluster these chimeric molecules to initiate LMP1 signaling (Figure 5A). At 24 hours, NGFR expression was examined to demonstrate stable expression of the chimeric protein (Figure 5B). Similar to CD40 activation, ICAM upregulation signified LMP1 activation (Figure 5C). Cells were stained with MitoTracker Green at 7, 24, 31, 48 and 55 hours post stimulation to determine the most appropriate staining time for mitochondrial mass. No increase in mitochondrial mass was observed at any time point except 31 hours (Figure 5D). Similarly, no increase in mitochondrial mass was observed in BL41 following CD40 stimulation (Figure 3A). At 31 hours, mitochondrial mass increased only 1.1-fold over unstimulated cells from stimulation of the CD40 mimic, LMP1. In contrast, when CD40 stimulation increased mitochondrial mass, it increased 1.4-fold over unstimulated cells in Ramos. Any increases observed were not a result of increased proliferation as there was a significant decrease in proliferation as the time course progressed (Figure 5E). Cells showed no significant change in cell viability (Figure 5F). Together, these results indicate that the LMP1 signal in BL41 cells does not increase mitochondrial mass as CD40 signaling did in Ramos cells.

Discussion

Following stimulation of the BCR and CD40, B cells undergo the energetically demanding processes of differentiation and proliferation. It is unknown how human B cells modify their metabolic profile to accommodate these changes, but differences in naïve versus activated murine B cell mitochondrial mass lead us to hypothesize that mitochondrial mass would increase following B cell activation.[27] Together, our results show that mitochondrial mass increases in Ramos cells following CD40 stimulation, but not in BL41 cells. It was confirmed that Ramos cells increased mitochondrial mass following CD40 stimulation, and that there was a synergistic relationship when activating both receptors. Additionally, our findings suggest that 10 μg/mL of anti-IgM may produce a larger increase in mitochondrial mass in BL41 cells than 5 μg/mL, which was the concentration used for all Ramos stimulations. In a preliminary trial, the 10 μg/mL concentration resulted in a 3-fold mitochondrial mass increase, rather than the approximately 1.5- fold resulting from 5 μg/mL anti-IgM, but more studies are required to determine if this is significant data. The higher concentration of anti-IgM was not used in Ramos cells because of the significant decrease in proliferation following anti-IgM stimulation at the current concentration. All mitochondrial mass experiments on Ramos and BL41 cells were performed at the 48-hour timepoint, which was previously determined to show the biggest change in mitochondrial mass following stimulation by timecourse experiments.

We know that mitochondrial mass plays an important role regulating cell function in cell types related to human B cells. For example, $CD8^+$ memory T cells have more mitochondrial mass than naïve $CD8^+$ T cells that promotes quick proliferation and

glycolytic capacity.[21] In murine B cells that were shown to increase mitochondrial mass following LPS stimulation, suppression of the glycolytic pathways was sufficient to disrupt antibody production.[27] Additionally in murine B cells, stimulation through the BCR directly modulates glucose metabolism and promotes proliferation.[17] Like murine B cells, human B cells are activated through the BCR and CD40 and therefore Ramos cells may be increasing mitochondrial mass to gain memory function, proliferate and produce antibodies. Confirming this could provide insight into how cells accommodate clonal expansion and lead to targeted methods for improved vaccination strategies.

These studies were performed on Burkitt's lymphoma cancer cell lines, which may not be the best model for studying metabolism because metabolic changes are a common feature of cancerous tissue. For example, the Warburg effect is a well-characterized cancerous metabolic phenotype in which ATP production is produced primarily through glycolysis instead of oxidative phosphorylation independent of oxygen levels.[37] Ramos and BL41 cells are easy to culture and grow a rapid rate that makes them convenient for experimentation. However, their proliferation rate could introduce error in the analysis of cell metabolism as we attempt to generalize our findings to healthy B cell activation. Additionally, each DNA replication cycle risks new mutations and therefore rapid expansion results in increased incidence of such mutations, which spread through the tumor population by genetic drift.[38] Previous studies have also found physiological differences between Ramos and BL41 cells, as we have here with mitochondrial mass. For instance, when stimulated with anti-μ antibody, Ramos cells activate a caspase-independent apoptosis while BL41 cells activate caspase-8-dependent apoptosis.[39, 40] To truly generalize our results these studies must be

performed in primary B lymphocytes. It may also be of interest to repeat the studies in B cell lines that are in different phases of the B cell life cycle, since outcomes of activation are different at different points.[2] For example, lymphomas vary in identity based on the point in the life cycle during which they occurred, expression and signaling of the BCR, and their interaction with cells in their environment.[41] The ability of the B cell life cycle to dictate cellular processes could drive future studies in B cells at different stages.

Future studies may seek to investigate the potential pathways that are activated in Ramos cells to increase mitochondrial mass. Mitochondria are dynamic organelles that use three processes to implement changes of mass, shape and cellular localization. These include mitochondrial biogenesis, mitochondrial-selective autophagy, and dynamic fission and fusion events. Mitochondrial biogenesis increases mitochondrial mass by creating new mitochondria. This process is centrally regulated by PCG1α, a transcriptional coactivator that initiates transcription of genes necessary for mitochondrial DNA replication, such as ESRRA and CYCS.[42] Mitochondrial-selective autophagy, or the elimination of damaged mitochondria, is initiated by expression of proteins NIX, FUNDC1 or PINK1 on the outer mitochondrial membrane that mark it for degradation.[43-45] Inhibition of this pathway may also lead to increased mitochondrial mass. Finally, mitochondrial fission events are regulated by DRP1 and fusion events are regulated by MFN1/2 and OPA1.[46, 47] Increase in mitochondrial fusion events can reduce mitochondrial-selective autophagy and therefore increase mitochondrial mass.[26] All three of these events are conducted by particular known proteins, and therefore the presence of these proteins could indicate a pathway for mitochondrial mass increase. Additionally, strength of signaling and activation could play a role in mitochondrial

dynamics as our preliminary data suggests. We found that 10 μg/mL anti-IgM increased mitochondrial mass almost 3-fold, while stimulation with 5 μg/mL did not increase it. If this is not the case, future studies could use BL41 as a negative control for Ramos experiments. Additionally, differences between these cell types may help identify genetic factors involved in future experiments.

It is hypothesized that EBV is able to establish chronic infection by signaling through its two viral receptors that mimic the BCR and CD40.[7] Therefore, we aimed to determine if the CD40 mimic, LMP1, would increase mitochondrial mass. Our data show only a slight increase in mitochondrial mass after stimulating the LMP1 in BL41 cells. This may be a result of only having this construct expressed in BL41 cells, which did not increase mitochondrial mass following stimulation through CD40 according to our data. Future studies should aim to successfully insert the construct into Ramos cells to determine if cell line is the only determinant in this discrepancy. If the construct is stably express in Ramos cells and still no mitochondrial mass increase is observed, differences in the CD40 and LMP1 signaling pathways could point toward the mechanism or pathway for regulating mitochondrial mass. Additionally, to gain a more complete understanding of the EBV infection mechanism it will be necessary to stably express the EBV BCR mimic, LMP2a, in both cells lines to begin studying its role in mitochondrial mass regulation. Inflammation and cytokine production are also often associated with EBV cancers, which are indications of BCR activation in healthy B cells and therefore could be related to LMP2a signaling.[7]

This work begins to help classify the metabolic profiles of subsets of human B cells. Understanding the metabolic changes through which B cells undergo could

provide insight into all human B cell function, including antibody production as a result of vaccination. Avenues for improved vaccination techniques rely not only on an understanding of the pathogen but also of the immune cells they hope to invoke. Additionally, this study aimed to better understand pathways through which cells alter metabolism that may play a role in tumorigenesis. Cancer cells are able to divide uncontrollably in a manner that mirrors the phenotype of clonally proliferating B cells following activation. In fact, low levels of constituent engagement of CD40 has been suggested to help sustain proliferation in a variety of cancers, and therefore could be related to the same pathways of metabolic change investigated in this study.[48] There are many different associated blood and B cell cancers, such as multiple myeloma, that are more closely related to this work. Understanding how the cell is able to enter and then exit a state of rapid division could be critical in developing methods to treat these diseases.

Acknowledgements

I would like to thank Dr. Olivia Hatton for her technical assistance in the experiments and her valuable mentorship. I would also like to thank Dr. Sara Hanson, in addition to Dr. Olivia Hatton, for her critical comments and suggestions in the writing of this thesis. Finally, I am grateful to Nora Watkins, who I worked along side in the lab, for her company and support throughout the research and this thesis. This work was supported by the Alfred W. Alberts Summer Research Prize awarded through the Molecular Biology Department at Colorado College.

References

1. Rajewsky K. Clonal selection and learning in the antibody system. Nature. 1996 Jun;381(6585):751.

2. Küppers R, Klein UL, Hansmann ML, Rajewsky K. Cellular origin of human B-cell lymphomas. New England Journal of Medicine. 1999 Nov 11;341(20):1520-9.

3. Forthal DN. Functions of antibodies. Microbiology spectrum. 2014 Aug 15;2(4):1.

4. Pulendran B, Ahmed R. Immunological mechanisms of vaccination. Nature immunology. 2011 Jun 1;12(6):509-17.

5. Victora GD, Schwickert TA, Fooksman DR, Kamphorst AO, Meyer-Hermann M, Dustin ML, Nussenzweig MC. Germinal center dynamics revealed by multiphoton microscopy with a photoactivatable fluorescent reporter. Cell. 2010 Nov 12;143(4):592-605.

6. Nojima T, Haniuda K, Moutai T, Matsudaira M, Mizokawa S, Shiratori I, Azuma T, Kitamura D. In-vitro derived germinal centre B cells differentially generate memory B or plasma cells in vivo. Nature communications. 2011 Sep 6;2:465.

7. Young LS, Yap LF, Murray PG. Epstein-Barr virus: more than 50 years old and still providing surprises. Nature Reviews Cancer. 2016 Dec 1;16(12):789-802.

8. Caldwell, R. G., Wilson, J. B., Anderson, S. J. & Longnecker, R. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. *Immunity* **9,** 405–411 (1998).

9. Uchida, J. *et al.* Mimicry of CD40 signals by Epstein-Barr virus LMP1 in B lymphocyte responses. *Science* **286,** 300–303 (1999).

10. Rickinson A. Epstein–Barr virus. Virus research. 2001 Dec 28;82(1-2):109-13.

11. Caldwell RG, Wilson JB, Anderson SJ, Longnecker R. Epstein-Barr virus LMP2A drives B cell development and survival in the absence of normal B cell receptor signals. Immunity. 1998 Sep 1;9(3):405-11.

12. Casola S, Otipoby KL, Alimzhanov M, Humme S, Uyttersprot N, Kutok JL, Carroll MC, Rajewsky K. B cell receptor signal strength determines B cell fate. Nature immunology. 2004 Mar;5(3):317.

13. Crawford DH, Rickinson A, Johannessen I. Cancer virus: the story of Epstein-Barr virus. Oxford University Press; 2014.

14. Mehta MM, Weinberg SE, Chandel NS. Mitochondrial control of immunity: beyond ATP. Nature Reviews Immunology. 2017 Jul 3.

15. Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, Wang CR, Schumacker PT, Licht JD, Perlman H, Bryce PJ. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. Immunity. 2013 Feb 21;38(2):225-36.

16. Tyrakis PA, Palazon A, Macias D, Lee KL, Phan AT, Veliça P, You J, Chia GS, Sim J, Doedens A, Abelanet A. S-2-hydroxyglutarate regulates CD8+ T-lymphocyte fate. Nature. 2016 Dec;540(7632):236.

17. Peng M, Yin N, Chhangawala S, Xu K, Leslie CS, Li MO. Aerobic glycolysis promotes T helper 1 cell differentiation through an epigenetic mechanism. Science. 2016 Oct 28;354(6311):481-4.

18. Chang CH, Pearce EL. Emerging concepts of T cell metabolism as a target of immunotherapy. Nature immunology. 2016 Apr 1;17(4):364-8.

19. Sena LA, Li S, Jairaman A, Prakriya M, Ezponda T, Hildeman DA, Wang CR, Schumacker PT, Licht JD, Perlman H, Bryce PJ. Mitochondria are required for antigen-specific T cell activation through reactive oxygen species signaling. Immunity. 2013 Feb 21;38(2):225-36.

20. Quintana A, Schwindling C, Wenning AS, Becherer U, Rettig J, Schwarz EC, Hoth M. T cell activation requires mitochondrial translocation to the immunological synapse. Proceedings of the National Academy of Sciences. 2007 Sep 4;104(36):14418-23.

21. van der Windt GJ, O'Sullivan D, Everts B, Huang SC, Buck MD, Curtis JD, Chang CH, Smith AM, Ai T, Faubert B, Jones RG. CD8 memory T cells have a bioenergetic advantage that underlies their rapid recall ability. Proceedings of the National Academy of Sciences. 2013 Aug 27;110(35):14336-41.

22. Pearce EL, Walsh MC, Cejas PJ, Harms GM, Shen H, Wang LS, Jones RG, Choi Y. Enhancing CD8 T-cell memory by modulating fatty acid metabolism. Nature. 2009 Jul 2;460(7251):103-7.

23. Buck MD, O'Sullivan D, Geltink RI, Curtis JD, Chang CH, Sanin DE, Qiu J, Kretz O, Braas D, van der Windt GJ, Chen Q. Mitochondrial dynamics controls T cell fate through metabolic programming. Cell. 2016 Jun 30;166(1):63-76.

24. Doughty CA, Bleiman BF, Wagner DJ, Dufort FJ, Mataraza JM, Roberts MF, Chiles TC. Antigen receptor–mediated changes in glucose metabolism in B lymphocytes: role of phosphatidylinositol 3-kinase signaling in the glycolytic control of growth. Blood. 2006 Jun 1;107(11):4458-65.

25. Jellusova J, Cato MH, Apgar JR, Ramezani-Rad P, Leung CR, Chen C, Richardson AD, Conner EM, Benschop RJ, Woodgett JR, Rickert RC. Gsk3 is a metabolic checkpoint regulator in B cells. Nature immunology. 2017 Mar;18(3):303.

26. Wheeler ML, DeFranco AL. Prolonged production of reactive oxygen species in response to B cell receptor stimulation promotes B cell activation and proliferation. The Journal of Immunology. 2012 Nov 1;189(9):4405-16.

27. Caro-Maldonado A, Wang R, Nichols AG, Kuraoka M, Milasta S, Sun LD, Gavin AL, Abel ED, Kelsoe G, Green DR, Rathmell JC. Metabolic reprogramming is required for antibody production that is suppressed in anergic but exaggerated in chronically BAFF-exposed B cells. The Journal of Immunology. 2014 Apr 15;192(8):3626-36.

28. Jang KJ, Mano H, Aoki K, Hayashi T, Muto A, Nambu Y, Takahashi K, Itoh K, Taketani S, Nutt SL, Igarashi K. Mitochondrial function provides instructive signals for activation-induced B-cell fates. Nature communications. 2015 Apr 10;6.

29. Lee HH, Dempsey PW, Parks TP, Zhu X, Baltimore D, Cheng G. Specificities of CD40 signaling: involvement of TRAF2 in CD40-induced NF-κB activation and intercellular adhesion molecule-1 up-regulation. Proceedings of the National Academy of Sciences. 1999 Feb 16;96(4):1421-6.

30. Maltzman JS, Carmen JA, Monroe JG. Transcriptional regulation of the Icam-1 gene in antigen receptor-and phorbol ester-stimulated B lymphocytes: role for transcription factor EGR1. Journal of Experimental Medicine. 1996 Apr 1;183(4):1747-59.

31. Honigberg LA, Smith AM, Sirisawad M, Verner E, Loury D, Chang B, Li S, Pan Z, Thamm DH, Miller RA, Buggy JJ. The Bruton tyrosine kinase inhibitor PCI-32765 blocks B-cell activation and is efficacious in models of autoimmune disease and B-cell malignancy. Proceedings of the National Academy of Sciences. 2010 Jul 20;107(29):13075-80.

32. Camilleri-Broët S, Vanderwerff H, Caldwell E, Hockenbery D. Distinct alterations in mitochondrial mass and function characterize different models of apoptosis. Experimental cell research. 1998 Mar 15;239(2):277-92.

33. Lee HC, Wei YH. Mitochondrial biogenesis and mitochondrial DNA maintenance of mammalian cells under oxidative stress. The international journal of biochemistry & cell biology. 2005 Apr 1;37(4):822-34.

34. Rustin P. Mitochondria, from cell death to proliferation. Nature genetics. 2002 Apr;30(4):352.

35. Hatzivassiliou E, Miller WE, Raab-Traub N, Kieff E, Mosialos G. A fusion of the EBV latent membrane protein-1 (LMP1) transmembrane domains to the CD40 cytoplasmic domain is similar to LMP1 in constitutive activation of epidermal growth factor receptor expression, nuclear factor-κB, and stress-activated protein kinase. The Journal of Immunology. 1998 Feb 1;160(3):1116-21.

36. Harris‐Arnold A, Arnold CP, Schaffert S, Hatton O, Krams SM, Esquivel CO, Martinez OM. Epstein–Barr Virus Modulates Host Cell MicroRNA‐194 to Promote IL‐10 Production and B Lymphoma Cell Survival. American Journal of Transplantation. 2015 Nov 1;15(11):2814-24.

37. Cairns RA, Harris IS, Mak TW. Regulation of cancer cell metabolism. Nature Reviews Cancer. 2011 Feb 1;11(2):85-95.

38. Sariban-Sohraby S, Magrath IT, Balaban RS. Comparison of energy metabolism in human normal and neoplastic (Burkitt's lymphoma) lymphoid cells. Cancer Research. 1983 Oct 1;43(10):4662-4.

39. Besnault L, Schrantz N, Auffredou MT, Leca G, Bourgeade MF, Vazquez A. B cell receptor cross-linking triggers a caspase-8-dependent apoptotic pathway that is independent of the death effector domain of Fas-associated death domain protein. The Journal of Immunology. 2001 Jul 15;167(2):733-40.

40. Mouhamad S, Besnault L, Auffredou MT, Leprince C, Bourgeade MF, Leca G, Vazquez A. B cell receptor-mediated apoptosis of human lymphocytes is associated with a new regulatory pathway of Bim isoform expression. The Journal of Immunology. 2004 Feb 15;172(4):2084-91.

41. Küppers R. Mechanisms of B-cell lymphoma pathogenesis. Nature Reviews Cancer. 2005 Apr;5(4):251.

42. Wu Z, Puigserver P, Andersson U, Zhang C, Adelmant G, Mootha V, Troy A, Cinti S, Lowell B, Scarpulla RC, Spiegelman BM. Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1. Cell. 1999 Jul 9;98(1):115-24.

43. Novak I, Kirkin V, McEwan DG, Zhang J, Wild P, Rozenknop A, Rogov V, Löhr F, Popovic D, Occhipinti A, Reichert AS. Nix is a selective autophagy receptor for mitochondrial clearance. EMBO reports. 2010 Jan 1;11(1):45-51.

44. Liu L, Feng D, Chen G, Chen M, Zheng Q, Song P, Ma Q, Zhu C, Wang R, Qi W, Huang L. Mitochondrial outer-membrane protein FUNDC1 mediates hypoxia-

induced mitophagy in mammalian cells. Nature cell biology. 2012 Feb 1;14(2):177-85.

45. Lazarou M, Sliter DA, Kane LA, Sarraf SA, Wang C, Burman JL, Sideris DP, Fogel AI, Youle RJ. The ubiquitin kinase PINK1 recruits autophagy receptors to induce mitophagy. Nature. 2015 Aug 20;524(7565):309-14.

46. Mishra P, Chan DC. Mitochondrial dynamics and inheritance during cell division, development and disease. Nature reviews Molecular cell biology. 2014 Oct 1;15(10):634-46.

47. Song Z, Ghochani M, McCaffery JM, Frey TG, Chan DC. Mitofusins and OPA1 mediate sequential steps in mitochondrial membrane fusion. Molecular biology of the cell. 2009 Aug 1;20(15):3525-32.

48. Elgueta R, Benson MJ, De Vries VC, Wasiuk A, Guo Y, Noelle RJ. Molecular mechanism and function of CD40/CD40L engagement in the immune system. Immunological reviews. 2009 May 1;229(1):152-72.