Exercise modifies inflammatory responses by changing the epigenetic landscape

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Abstract:

Physical inactivity and low-grade systemic inflammation are associated with chronic metabolic diseases such as atherosclerosis, insulin resistance, obesity etc. It is of great value to study the exercise mediated benefits to alleviate inflammation. Preliminary work in our lab demonstrated that exercise with a particular intensity and duration led to reduced inflammatory responses in bone marrow derived macrophages (BMDMs) by inducing persistent metabolic changes in these cells. In this thesis, we explored how exercise is causing these long-lasting changes in inflammatory responses and hypothesized that it does so by inducing changes in chromatin accessibility leading to changes in gene expression. We employed an unbiased ATAC-seq (Assay for Transposase Accessible Chromatin- sequencing) analysis in BMDMs prepared from sedentary and exercised C57BL/6N mice and obtained a list of differentially accessible chromatin regions. We also demonstrated using real-time PCR that exercise-mediated changes in chromatin accessibility cause a reduction in gene expression of inflammatory genes. Our findings suggested that exercise is protective against inflammatory responses by causing persistent changes at chromatin level.

Keywords: Exercise; Inflammation; chromatin accessibility; Trained Immunity; Immune system.

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List of Abbreviations

AMPK	AMP-activated protein kinase
ATAC-Seq	Assay for transposase accessible chromatin sequencing
ATP	Adenosine triphosphate
BCG	Bacille Calmette-Guerin
BMDMs	Bone marrow derived macrophages
cDNA	complementary deoxyribonucleic acid
CD86	cluster of differentiation-86
CHIP-Seq	Chromatin immunoprecipitation sequencing
DAMP	Danger associated molecular patterns
DCs	Dendritic cells
DGKG	Diacylglycerol kinase gamma
DNA	Deoxyribonucleic acid
FBS	Fetal bovine serum
GSH	Glutathione
HIF	Hypoxia inducible factor
HSC	Haematopoietic stem cell
IFN	Interferon
IL	Interleukin
IRAK3	Interleukin 1 receptor associated kinase 3
IRG1	Immune responsive gene 1
LCM	L929 cells conditioned media
LPS	Lipopolysaccharide
M-MuLV	Molony murine leukemia virus
MPPs	Multipotent progenitors
mTOR	Mammalian target of rapamycin
MYSM1	Myb Like, SWIRM and MPN Domains 1
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NRF2	nuclear factor erythroid 2–related factor 2
oxLDL	oxidized Low-density lipoproteins
PAMP	Pathogen associated molecular pattern
PCR	Polymerase chain reaction
PDK4	Pyruvate Dehydrogenase Kinase 4
PRR	Pathogen recognition receptor
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPM	Revolution Per Minute
SESN3	Sestrin 3
TCA	Tricarboxylic acid
TLR	Toll like receptor
TNF	Tumor necrosis factor

Chapter 1: Introduction

1.1 Immune System: Our body's defense system is comprised of innate and adaptive immunity to prevent us from the infection that can be caused by any foreign objects and or danger signals inside the body. The innate immune system (neutrophils, macrophages, monocytes, mast cells) responds rapidly by recognizing the pathogen associated molecular pattern and also activates the adaptive immune system (T and B lymphocytes) which can take from few hours to days to get activated (Janeway & Medzhitov, 2002). The adaptive immune system has long been known to create a specific memory towards the pathogen which has been named as classical immunological memory (Laird et al., 2000). On the contrary, according to years of published data innate immune system is non-specific and does not create specific memory towards the pathogen. If this is true, then how do plants develop memory towards the pathogen even though they only have innate immune system? Research has suggested that species lacking the adaptive immunity such as invertebrates, fights off the infection using only innate immune system and they can also develop resistant towards the secondary infection (Hoffmann, 2003; Kurtz, 2005). This led the researchers to think that our innate immune system may also have the ability to develop memory towards the pathogen which play an important role in health and diseases. This innate immune memory has been named as "trained immunity" (Netea et al., 2011).

Our immune system not only protects us from the acute infections but also helps to fight off the chronic diseases. Noteworthy, the regulation of the homeostatic activity of the immune system is important because the weakened immune response can increase the susceptibility to infections while the overexpression can lead to autoimmune diseases (Ege, 2017; Kotas & Medzhitov, 2015). Moreover, chronic inflammation has been associated with aggravating the chronic diseases such as atherosclerosis, rheumatoid arthritis, interstitial lung diseases which are

mainly characterized as inflammatory diseases (Pasceri & Yeh, 1999; Ross, 1999). Chronic diseases are becoming the main cause of mortality and morbidity around the world and the risk factors involved are unhealthy lifestyle such as western diet and physical inactivity (Weltgesundheitsorganisation, 2002, 2003) which are the further contributors to the low grade inflammation (Manzel et al., 2014). Literature has suggested that physical inactivity is associated with diseases such as insulin resistance, hypertension, coronary artery disease, and metabolic syndromes (Lee et al., 2012); contributing towards the non-communicable diseases and these diseases cost billions of dollars on the health care system worldwide (Ding et al., 2016). Considering the association between sedentary lifestyle and chronic low grade inflammatory diseases, it has become important to study the biological effects of exercise on immune cells, cytokine release and mechanisms involved at the cellular level.

1.11 Role of innate immunity: Our innate immune system is a first line responder to the invading pathogens such as bacteria and viruses and it protects the host from infection and injury. Although innate immune system responds non-specifically towards the pathogens, however it has a specific system that recognizes the pathogens through pathogen recognition receptors (PRRs) on the cell membrane such as Toll-like receptors (TLRs) (Akira et al., 2001). There are 10 different types of TLRs as of current literature which recognize different microbes (Akira et al., 2001; Chuang & Ulevitch, 2001; Rock et al., 1998). For instance, TLR4 recognizes the lipopolysaccharide (LPS) which is an outer wall component of gram negative bacteria (Hung & Suzuki, 2017), whereas TLR5 recognizes the bacterial flagellin of gram negative as well as of gram positive bacteria (Hayashi et al., 2001). Not only the pathogen recognition, innate immune system also activates and controls the adaptive immune system *via* dendritic cells which directs the signals for the activation of desired immune responses (Waisman et al., 2017).

Macrophages, a type of innate immune cells play an important role in the resolution of tissue repair and injury (Watanabe et al., 2019). Macrophages are the mature form of circulating monocytes and they reside in tissues where they can sense the invasion of foreign objects (Jenkins et al., 2011; Wynn et al., 2013a). Although, macrophages play an important role in clearing the infection; however the continuous insult and activation of them can lead to imbalance which has been found to be associated to the chronic diseases such as atherosclerosis (Cochain & Zernecke, 2017) and cancer etc (Wynn et al., 2013b). Notably, macrophages have heterogenous properties and can release pro-inflammatory and ant-inflammatory cytokines depending on the different stimuli (Gordon, 2003) and hence they are classified as M1 and M2 macrophages respectively. For instance, the stimulation with IFN-γ and LPS produce pro-inflammatory cytokines whereas stimulation with IL4/IL13 leads to anti-inflammatory cytokine production (Martinez, 2008).

Remarkably, research has proven that there is a distinct metabolism between M1 vs M2 macrophages, M1 (proinflammatory macrophages) have increased consumption of glycolysis (Freemerman et al., 2014; Hard, 1970) and decreased oxidative phosphorylation whereas M2 (anti-inflammatory macrophages) have increased activity of oxidative phosphorylation (OXPHOS) and fatty acid oxidation (FAO) (Jha et al., 2015). The LPS induced M1 macrophages lead to increased phagocytic activity, ROS production and pro-inflammatory cytokine production to kill the microbes where the activation of glycolytic pathway is dependent on the redox-sensitive transcription factor HIF-1α (Freemerman et al., 2014; Rodríguez-Prados et al., 2010). Furthermore, the succinate production in the TCA cycle stabilizes HIF-1α and transcriptional activity of pro-inflammatory activity such as IL-1β (Tannahill et al., 2013). The itaconate production from the citrate by the enzyme IRG1 leads to shutdown of pro-inflammatory cytokines in M1 macrophages (Michelucci et al., 2013). Itaconate further activates the NRF2 which

decreases the ROS production by upregulation the enzymes which increases antioxidant pathways such as GSH, haem oxygenase 1, TRX1, and TRXR1 hence achieving the resolution phase. (Muri & Kopf, 2021). While the HIF-1α is associated with M1 polarization, mTOR pathways has been shown to be associated with M2 polarization which indicates that different metabolic pathways are involved in the regulation of macrophage polarization (Kang et al., 2018).

1.12 Trained Immunity: Trained immunity is defined as creating a memory with the primary stimulus in the innate immune cells which leads to mounted or diminished immune response towards the non-specific secondary stimuli as shown in Figure 1 (Netea et al., 2020). Research has suggested that primary stimulus leaves the 'epigenetic scar' at the specific locations on the stimulated genes which could be near the promoter region or distal regulatory elements which may affect the transcriptional activity of that gene leading to the altered functional reprograming (Ghisletti et al., 2010; Netea et al., 2020; Smale et al., 2014).

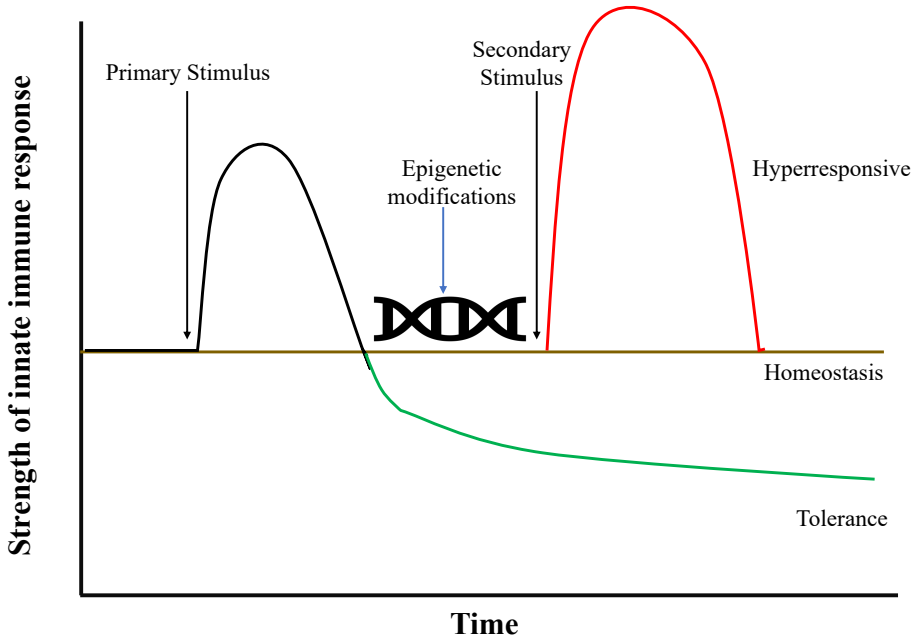


Figure 1**: Trained immunity**: Primary stimulus in innate immune cells makes them either hyperresponsive or tolerogenic after the stimulation with the secondary stimulus.

(Adapted from Netea et al., 2016, 2020a)

Trained immunity can be induced by PAMPs (Pathogen associated molecular patterns)and DAMPs (Damage associated molecular patterns) which involves the fungal activation by dectin-1 pathway, bacterial induction by NOD-2 pathway, and the damage associated pathways induced by oxidized low density lipoproteins (Brown et al., 2003; Moore et al., 2013; Wang et al., 2001). Additionally, trained immunity is non-specific towards the secondary stimulation characterizing the main feature of innate immune system. For instance, an in vivo mice study observed that activation of macrophages with BCG vaccination significantly lowers the yeast infection in spleen, kidney and liver when compared to control mice (Wout et al., 1992). Furthermore, a nude mice model with BCG vaccination showed lower number of pulmonary Schistosomula after 5 days of injecting with Schistosoma mansoni cercaria, indicating the importance and memory of innate immune system (Tribouley et al., 1978). The priming of the hematopoietic stem cells with primary stimulus such as BCG is responsible for creating memory towards the secondary stimuli. This is mainly happening because of the epigenetic changes at the level of HSCs which are being passed on to BMDM and MPPs leading to enhanced protection against the secondary infection (Kaufmann et al., 2018).

The fundamental basis of trained immunity is the underlying epigenetic modifications and functional reprogramming in the myeloid cells. For instance, chromatin remodelling following repeated exposure of LPS in naïve macrophages is responsible for silencing and priming the genes that secrete proinflammatory and anti-microbial cytokines respectively (Foster et al., 2007). Furthermore, an in vivo mouse study demonstrated that dendritic cells also have the capacity to generate memory and observed higher level of cytokine production (Il-2, IFNg, IL-4) in vaccinated mice after they were exposed to cryptococcus. Interestingly, these responses were abrogated in

DCs after they were treated with methyltransferase inhibitor which indicates that histone modification is the critical step in inducing the memory like responses in DCs (Hole et al., 2019). Importantly, metabolism in the innate immune cells is crucial in determining gene expression at the epigenetic level as many energy metabolites act as essential co-factors for epigenetic enzymes which regulates posttranslational histone modification, DNA methylation etc. (Donohoe & Bultman, 2012). One of the mechanisms that play an important role in epigenetic changes is the histone modification such as histone acetyltransferase which leads to chromatin accessibility where the transcription factors can bind DNA; whereas histone deacetylase can make chromatin more compact (Deal et al., 2010; Kouzarides, 2007).

As described earlier, the primary stimulation of macrophages leads to epigenetic adaptations that can make them either hyperresponsive or hyporesponsive towards the secondary stimuli (Natoli & Ostuni, 2019). For instance, chromatin modifications after the acute LPS stimulation makes the cells release antimicrobial particles after they were induced with LPS 24 hours later (Foster et al., 2007; Smale et al., 2014). Importantly, it has been demonstrated that continual repeated exposure of LPS made the macrophages hyporesponsive/tolerogenic towards secondary stimulus which (Seeley & Ghosh, 2017) which could lead to pathological conditions if blunted immune response is unable to clear up the infection (McLane et al., 2019).

It is important to mention here that trained immunity can also have pathological outcomes in diseases that are associated with low grade systemic inflammation due to the hyperresponsive inflammatory reactions (Leentjens et al., 2018). Moreover, studies have observed an association between western diet and hyperresponsive immune response in inflammatory diseases such as atherosclerosis. They observed that while systemic inflammation was decreased after changing to simple chow diet for 4-week, however, bone marrow and immune cell reactivity were still

increased in mice fed with western diet compared to control mice which indicates the diet induced epigenetic and functional reprogramming of innate immune cells (Christ et al., 2018).

Not only this, but innate immune memory also plays important role in shaping the neurological diseases. For instance, an injection of 1xLPS in a mouse model of Alzheimer disease leads to training of the brain immune responses. After analyzing the pathology 6 months later, it was observed that 1xLPS injection led to increased plaque and amyloid-β levels when compared to the control mice group whereas, the IL-10 level was also found to be decreased. (Wendeln et al., 2018).

Apart from this, recent research has elucidated that innate immune system also initiates responses in the allograft rejection (Liu et al., 2012) where the infiltration of macrophages might be responsible for the severe allograft rejection (Hancock et al., 1983). The activation of the innate immune cells with the PRR activates the aerobic glycolytic metabolic pathways and this metabolic switch is associated with underlying epigenetic changes which is a fundamental basis of trained immunity (Netea et al., 2016; Pearce & Pearce, 2013). Interestingly, inhibition of the mTOR pathway using rapamycin has been shown to be effective to prolong allograft survival in an in vivo mice study (Braza et al., 2018). Notably, research has demonstrated that an activation of Akt-mTOR-HIF-1α pathway leads to metabolic switch in the trained monocytes which shifts the metabolism from oxidative phosphorylation to aerobic glycolysis when compared to naïve monocytes (Cheng et al., 2014). This metabolic switch may be responsible for increased phagocytic activity as pharmacological induction of HIF-1a was found to increase the phagocytic capacity against the bacterial infection (Zinkernagel et al., 2008).

1.2 Exercise and Inflammation: There are several types of myokines that are released during physical activity, whereas IL-6 (interleukin-6) is the important cytokine that increases transiently with the skeletal muscle contraction and rapidly declines after the exercise period (Ostrowski et al., 1998; Steensberg et al., 2001). IL-6 is an important cytokine marker because it leads to elevation of other inflammatory cytokines in the circulation which includes pro-inflammatory cytokines IL-1β, TNFα; anti-inflammatory cytokine IL-10; and cytokine inhibitors sTNF-r1, sTNF-r2 and IL-1Ra (Ostrowski et al., 1998, 1999). Notably, exercise induced release of IL-6 from skeletal muscles has been shown to have anti-inflammatory effects (Mathur & Pedersen, 2008). This indicates that IL-6 maintains the balance between pro-inflammatory and anti-inflammatory cytokines during the exercise period.

Importantly, IL-6 is known to play an important role in the metabolism during exercise (Bruunsgaard, 2005). An in vitro study in elderly humans observed that infusion of IL-6 leads to increase in lipolysis and fatty acid oxidation (E. W. Petersen et al., 2005). Interestingly, an in vivo study in mice observed that IL-6 knockout mice had decreased phosphorylation of AMPK in skeletal muscles and adipose tissues indicating the association between IL-6 and AMPK (Kelly et al., 2004). AMPK is an energy sensor molecule and one of the main activators of PGC1α which is a transcription factor of mitochondrial biogenesis (Ruderman et al., 2003). This association between IL-6 and AMPK further confirms that IL-6 is involved in oxidative phosphorylation (fatty acid oxidation) which is carried out by the mitochondria. This indicates that release of IL-6 with exercise not only affects the inflammatory cytokines, but also play an important role in the cellular metabolism.

Exercise is a process in which energy expenditure occurs and that energy is provided by ATP (adenosine triphosphate), skeletal muscles depend on phosphocreatine, glucose, and oxidative phosphorylation to consume ATP (Hargreaves & Spriet, 2018). The consumption of ATP via above three providers depend on the exercise intensity and duration (Hargreaves & Spriet, 2020). Previous research has observed that transcriptional activity of several metabolic genes such as PDK4, and UCP3, is upregulated during the resting period after exercise (Pilegaard et al., 2002). PDK4 is a pyruvate dehydrogenase kinase which phosphorylates the PDH (pyruvate dehydrogenase) which is bridge between glycolysis and fatty acid oxidation and is known to convert pyruvate to acetyl-coenzyme A (Gudi et al., 1995; Zhang et al., 2014). UCP3 is a uncoupling protein 3 and is known to promote the fatty acid oxidation (Bezaire et al., 2005). The idea here is that working machinery of these metabolic genes is increased to restore the energy consumption during exercise.

Literature has suggested that exercise intensity plays crucial role to achieve beneficiary effects in accordance with dose-response relationship as explained using J-curve (Nieman, 1994) (Haskell et al., 2007). However, there is still need to understand the relationship between dose and response curve since there could be other potential factors that can influence the exercise outcomes such as type of exercise such as acute vs chronic, cardiorespiratory fitness, and age (Garber et al., 2011). For instance, an acute high intensity exercise might lead to immunosuppression due to the potential over production of anti-inflammatory cytokines (Walsh et al., 2011) which could further increase the risk for upper respiratory tract infections in elite athletes (Nieman, 1994). Nonetheless, a randomized control trial involving 9 month of high intensity (80% VO_2 max) aerobic training has shown an increased insulin sensitivity in untrained older men and women compared to subjects

who performed moderate (65% VO_2 max) or low intensity (50% VO_2 max) exercise (DiPietro et al., 2006).

Physical activity (PA) helps in lowering the progression of many chronic diseases which are caused by low-grade systemic inflammation such as atherosclerosis. Macrophages play an important role in this disease because of their phagocytotic properties as they engulf the oxLDL and other debris, however, macrophages become foam cells when they can no longer process the oxLDL. Foam cells further start secreting the pro-inflammatory cytokines which further increases the inflammation and the progression of the disease (Aqel et al., 1985; Khallou-Laschet et al., 2010). Researchers have observed that physical activity acts as an antioxidant defence system against the oxidative stress as it alleviates the expression and activity of NADPH oxidase and increases expression of superoxide dismutase (SOD) leading to decreased production of reactive oxygen species. PA also increases the expression of endothelin nitric oxide synthase (eNOS) which is mediated by the increased expression and phosphorylation of Akt (Adams et al., 2005; de Moraes et al., 2008; Hambrecht et al., 2003).

The possible mechanisms of anti-inflammatory effects of exercise described by a review article include decrease and increase in the level of pro-inflammatory and anti-inflammatory cytokines by the adipocytes as the visceral fat mass decreases with exercise (Gleeson et al., 2011; A. M. W. Petersen & Pedersen, 2005); and reduced expression level of receptors such as TLRs on monocytes and macrophages (Flynn & McFarlin, 2006). Although adipocytes release about 30% of IL-6 at rest, however, a study in the obese subjects observed that adipocyte resident macrophages are the major contributors to the release of IL-6 (Fried et al., 1998). Even though IL-6 level increases exponentially during exercise, notably this elevation is transient as it returns to

resting level in an hour (Keller et al., 2005; Pedersen & Fischer, 2007). Studies have shown that acute bout of exercise lead to reduced expression level of TLR1, TLR2, TLR4 (Gleeson et al., 2006; Oliveira & Gleeson, 2010); as well as prolong periods of exercise also lead to decreased induction level of pro-inflammatory cytokines following the LPS stimulation of whole blood samples from humans (Lancaster et al., 2005). Notably, to our knowledge, the literature lacks the information on possible factors that could be responsible for the reduced expression level of TLRs in response to exercise such as epigenetic changes, and this still needs to be established.

Research has also suggested that exercise induced release of adrenaline and/or cortisol from the vascular endothelium might be causing transient increase of monocytes in the blood circulation after acute bout of exercise training (Gleeson, 2004; Krüger & Mooren, 2007). Moreover, it has been observed that monocytes switch their phenotypes from pro-inflammatory CD14+/CD16+ form to CD14+/CD16- classical form (Steppich et al., 2000). Noteworthy, several studies have observed that acute strenuous exercise induced reduction in release of pro-inflammatory cytokines such as IL-6, IL-1α, and TNF-α is mainly attributed to the lower expression of TLRs, and not necessarily because of lower circulatory monocytes (Lancaster et al., 2005; Starkie et al., 2001). Although monocytes are important circulatory innate immune cells, however they are premature form of macrophages and short lived cells; therefore laboratory studies involving animals have mainly focused on the tissue resident macrophages to study the effects of exercise (Gleeson, 2004).

Chapter 2: Rationale and Objectives:

2.1 Rationale: Inflammation regulation is important in controlling inflammatory conditions to prevent chronic diseases. Based on the current literature, exercise plays important role in alleviating inflammation by regulating different inflammatory pathways such as TLR. The current literature has mainly focused on the transient changes that occur in immune cells because of exercise yet there is paucity in research investigating whether exercise can induce persistent changes in immune response. There is also a lack of knowledge regarding which specific regions in the genome that may be particularly susceptible to epigenetic changes in response to exercise. Unpublished work from a Ph.D. student in our lab has demonstrated that exercise induces long-lasting changes in the ability of macrophages to respond to inflammatory stimuli by reducing pro-inflammatory signalling and subsequent cytokine production as well as enhancing mitochondrial respiration and membrane potential. This raises the question of how exercise is leading to modifications in immune function.

2.2 Hypothesis: We hypothesized that persistent effects of exercise on inflammatory signalling is due to long-lasting changes in the accessibility of specific genome sites, leading to differentially accessible chromatin regions between macrophages of trained and sedentary mice. We anticipated that these changes would be concentrated in promoter and/or enhancer regions of the chromatin that control the expression of inflammatory or regulatory genes.

2.3 Objectives:

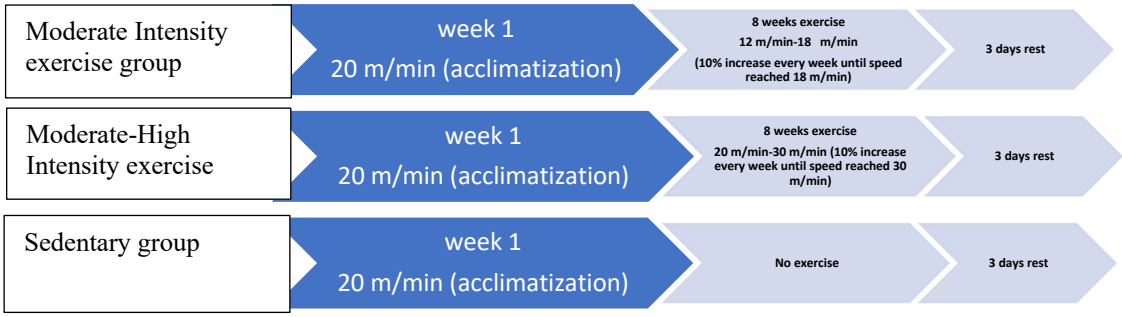
1) To perform genome-wide screening to identify all the chromatin accessibility regions in the chromatin and identify the changes that occur following exercise.

2) To identify the genes that may be altered due to the aforementioned exercise-induced accessibility changes, especially those involved in immune and metabolic pathways that have been altered following exercise.
3) To confirm/validate whether these changes are affecting gene expression.

Chapter 3: Methodology

3.1 Protocol for Treadmill exercise: C57BL/6N, 6 weeks old female mice were chosen because previous studies have observed their immunogenicity effects in immune responses (Song & Hwang, 2017). Mice were acclimatized on the treadmill for 10 mins at 20 m/min for 1 week and then randomly divided into three different sets: sedentary group, moderate to high-intensity group, and moderate-intensity group, with 5 replicates in each group. Following week, moderate-intensity and moderate-high intensity mice group were run at 12 m/min and 20 m/min respectively for one week, and then speed was increased by 10% each week until it reached 18 m/min and 30 m/min respectively which was then continued throughout for 8 weeks. Notably, our moderate intensity and moderate-high intensity exercise groups correspond to 45-50% of VO_2max and 65-70% of VO_2max respectively which were determined based on previous published literature (Høydal et al., 2007; Poole et al., 2020) .

Mice were pushed mildly to run using an air-blower if they sit longer than a few seconds. Mice were run for five days per week where the moderate-intensity and moderate-high intensity group ran for 45 mins and 1 hour respectively. The sedentary mice group was placed near the treadmill during the exercise session, to have them in a similar environment as experimental groups with exercise being the only difference. Throughout the study period, all mice were housed in the York University animal facility at 22 ◦C with a 12-hour day/night cycle. Mice were fed with a simple chow diet and clean H_2O thought the study period.



3.2 Bone marrow derived macrophage preparation and cell culture: After the eight weeks of exercise training, mice from both exercise groups along with sedentary group were given 3 days of rest over the weekend. Then they were euthanized and dissected, femur and tibia bones were harvested and put in S+ media in a 15 ml tube and placed on ice until flushing. S+ media was made in a 500 ml of RPMI media containing 10% Fetal Bovine serum (FBS), 1.1% of 100X non-essential amino acids (NEAA), 1.1% of L-Glutamine-Penicillin-Pyruvate-Streptomycin (GPPS), and 550 µl of 1000X β-mercaptoethanol. Bones were then flushed with S+ media under the biosafety cabinet using the 10 ml syringe and 27G needle. The bone marrow cells were flushed and collected in 50 ml conical tubes, followed by centrifugation at 1500 rpm, 4◦C for 5 mins. Supernatant was decanted, and pellet cells were lysed with 2 ml of RBC lysis buffer for 5 mins. After 5 mins, 5 ml of S+ media was added to neutralize the lysis buffer, followed by centrifugation at 1500 rpm, 4◦C for 5 mins. Again, the supernatant was decanted, and the cells were resuspended in 25% LCM (L929 cells conditioned media) made in S+ media and plated in 10 cm Petri dishes. The plates were incubated at 37 ◦C for 3 days in an incubator containing 5% CO_2. The media of the cells was changed on day 3, and then cells were scraped and replated in 6 well tissue culture plates on day 4, incubated at 37 ◦C. On day 6, old media was removed, and cells were washed with 1 ml of 1X PBS (Phosphate Buffered Saline) modified without calcium chloride and magnesium chloride, and then 1 ml of S+ media was added to the wells, and cells were treated with (100 ng/ml) LPS at 3 different time points: control, 24hr, 6hr, 3hr. At the end of treatments, media was

removed, and cells were washed with ice cold 1X PBS modified with calcium chloride and magnesium chloride and then 1 ml of TRIzol reagent was added to the each well and cells were scraped. Plates were sealed and stored at -80 ◦C until RNA extraction was performed. On day 7, the plates with untreated cells were scraped and cells were collected in a 15 ml tubes and sent for an ATACseq analysis.

3.3 ATACseq and Bioinformatic data analysis: On day 7, cells were counted and approximately 1 million cells were sent to the Princesses Margaret Genomic Centre where they performed bulk ATACseq analysis. ATAC seq is an Assay for Transposase-Accessible Chromatin sequencing. The main purpose of this method is to study the chromatin accessibility in the genome regions (Buenrostro et al., 2013). Cells were harvested and then libraries were prepared using the Tn5 transposase as a tagmented adaptor which binds to the open chromatin regions. These libraries were then amplified by PCR followed by sequencing as described by the original protocol (Buenrostro et al., 2015); ATACseq PE50 on the Novaseq was performed (Figure 3.31). Once the sequencing was done, we paid the bioinformatic team to perform the pre-alignment quality control (QC) and read alignment steps on FastQC generation files to confirm if the data quality looks good. This was then followed by the post alignment processing and QC to ensure the removal of duplicated, low quality and mt DNA. Then core analysis (which includes the peak calling) was performed on the MACS2 (Model-based Analysis of Chip-seq) files to obtain the BigWig, bam and narrow-peak files. Then, with the help of Dr. Abdul-Sater, an advanced analysis was performed which includes peak differential analysis, peak annotation, motif enrichment analysis etc. An open-source web-based platform usegalaxy.org (Afgan et al., 2016) was used to perform DiffBind (Differential Binding) analysis using bam and narrow-peak files. The first step performed by the DiffBind was to read the peaksets obtained from the MACS2, and then overlapped regions

were examined between experimental replicates which is known as consensus peaksets. Then read count was performed by the DiffBind where peaks were re-centred to provide systemized peak intervals for the overlapped reads between the experimental replicates (Ross-Innes et al., 2012). This provided us the differential binding matrix which was used to obtain the list of differentially accessible chromosome regions using the DEbrowser (Kucukural et al., 2019), where false discovery rate (FDR) was set up for 0.1 and relative change between study groups was set for ≥1.5 as is routinely used for ATAC-seq differential analysis (Johnston et al., 2021; Love et al., 2014).

Then the data were visualized using the IGV browser (Robinson et al., 2011) to visualize the peak intensities at each differentially accessible chromatin region between the exercise vs sedentary groups and mapped them to nearby genes to obtain a list of the downregulated and upregulated gene, where the Mouse genome assembly GRCm38 (mm10) was used as a reference. Although vast majority of genes occurred ≤3kb of either upstream or downstream of gene coding region. However, we also wanted to map enhancers to genes near the differentially accessible chromatin regions because enhancers can serve as a platform for transcription factor binding and thereby change the gene expression (Shlyueva et al., 2014). Therefore, we narrowed down the list by considering the differentially accessible regions that were located ≤ 30 kb either upstream or downstream of gene coding region. To further narrow down the list, we focused our attention on the genes that are known to be involved in immunoregulatory and metabolic pathways.

Furthermore, functional enrichment analysis was performed on the genes that were located near the differentially accessible chromatin regions using Gene Ontology (GO) (Ashburner et al., 2000), ShinyGo (Ge et al., 2020).

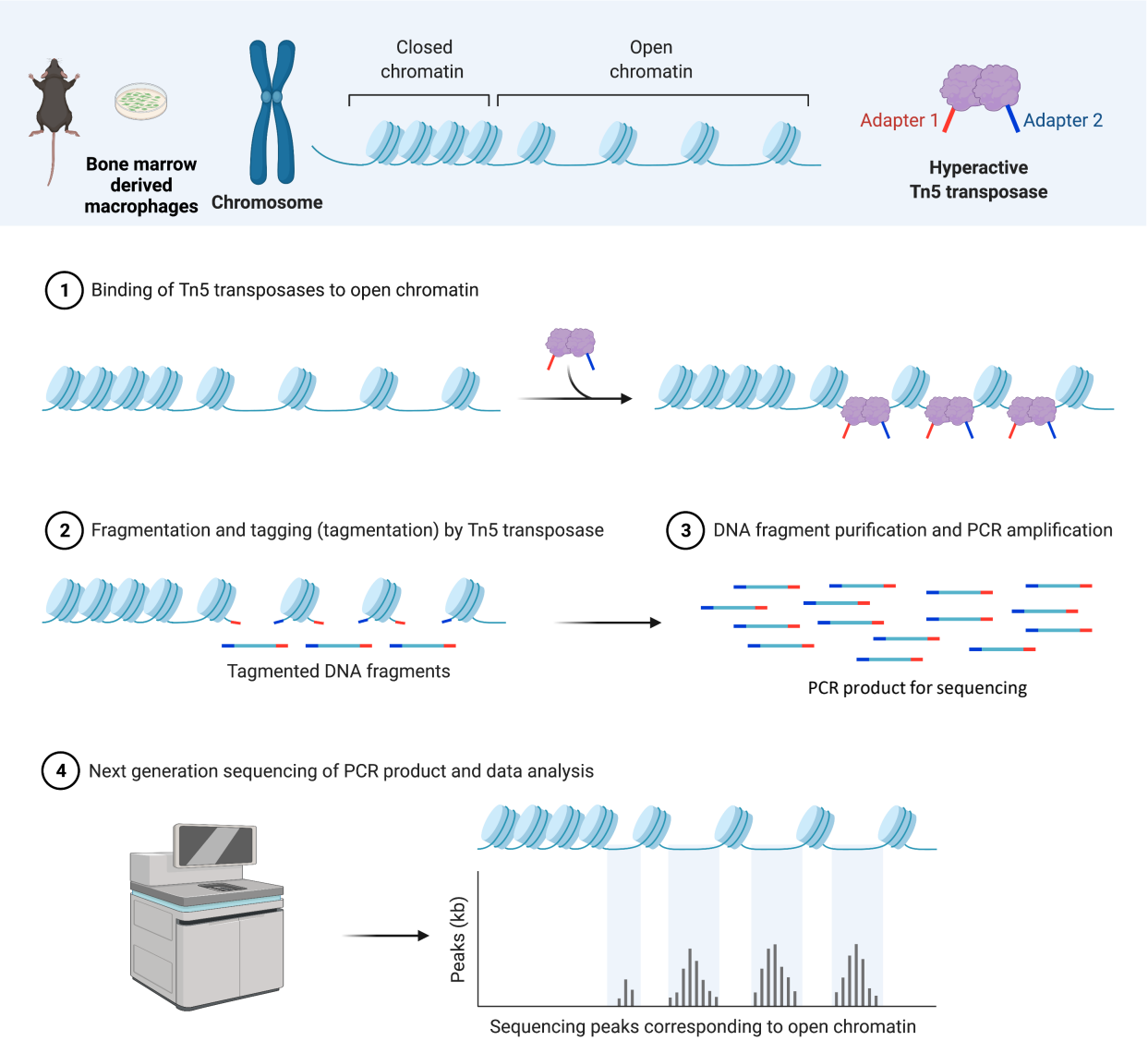


Figure 3.31: A schematic representation of library preparation of BMDMs (Bone marrow derived macrophages) obtained from mouse. Hyperactive Tn5 transposase is loaded with sequencing adapters which binds to open chromatin regions followed by DNA fragment purification and PCR amplification. ATAC-seq libraries are then sequenced with Novaseq using paired-end 50 cycle (PE50) reads.

(Adapted from "ATAC Sequencing", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates)

3.4 RNA extraction: The macrophage samples stored in the TRIzol reagent were taken out of -80◦C and thawed under the fume hood. Then 200 µl of chloroform was added to the sample tubes. These tubes were vortexed for 15 seconds followed by centrifugation at 12500 rpm for 15 mins at 4 ◦C. The top aqueous layer was carefully removed into new Eppendorf tubes with a total volume of 400 µl. Then 500 µl of isopropanol was added to these RNA sample tubes and incubated at room temperature for 10 mins followed by centrifugation at 12000 rpm for 10 mins at 4 ◦C. After the centrifugation, supernatant was carefully aspirated out leaving the pellets behind. Then 1 ml of 70% ethanol was added to these RNA sample tubes followed by centrifugation at 10000 rpm for 5 mins at 4 ◦C. Then supernatant was aspirated out, leaving around 50 µl in the tubes which was removed using 200 µl pipette. The sample tubes were then placed under the fume hood until all the ethanol evaporated, followed by resuspending the pellet with 25 µl of ultrapure H_20. Then using the Thermo scientific Varioskan LUX Multimode Microplate reader, the concentration and purity of the RNA samples was measured at 260 and280 nm and concentration was calculated using Beer-Lambert law.

3.5 cDNA library synthesis: After obtaining the RNA, reverse transcription was performed to obtain cDNA using reverse transcriptase enzyme (M-MuLV) and Deoxynucleotides (dNTPs) via a two-step protocol. cDNA synthesis reaction master mix was made to the final volume make of 30 µl to obtain 750 ng of RNA. To make reaction master mix1, Oligo dT (1.5 ul per sample), 10mM dNTP (1.5 ul per sample), RNA, ultrapure H_2O was added in small PCR tubes and step1 PCR reaction was run in a Bio-Rad PCR instrument for 5 mins at 65 ◦C. After that, 10X M-MuLV reverse transcriptase Buffer (3 ul per sample), RNAse inhibitor murine (0.3 ul per sample), M-MuLV reverse transcriptase (1.5 ul per sample) was added to the sample tubes. In the control NRT (no reverse transcriptase), H_2O was added instead of M-MuLV reverse transcriptase.

Then these control and sample tubes were followed by step 2 reaction in a PCR instrument for 75 minutes at 37 ◦C. The cDNA obtained was then stored at -20 ◦C.

3.6 Real-time PCR: cDNA samples were amplified using Bio-Rad SYBR Green supermix dye via real-time PCR for gene expression. The cDNA samples were diluted with 1:20 and then 1:10 in ultrapure H_2O. The primers for the desired genes obtained from the ATACseq analysis were designed using Beacon Designer8 at a temperature of 60° +/- 5. A list of the primers for the genes demonstrated in this study is shown in Table 1. The desired (targeted) forward and reverse primers were then mixed in an Eppendorf tube followed by the addition of the SYBR green dye. Then 6 µl of primer mix was loaded in duplicates into designated wells in a 384 well plate followed by loading of 4 µl of diluted cDNA into designated wells. In the control NTC, ultrapure H_2O was added instead of cDNA. Then the plate was sealed and pulse centrifugated. Then sample plate was run using the Bio-Rad SYBR protocol in a real-time PCR instrument for 98 mins where the first cycle was run at 95 ◦C for 30 sec, followed by 40 cycles at 95 ◦C for 10 sec, 60 ◦C for 30 sec; then 10 sec cycle at 95 ◦C followed by the last cycle of melt curve between 65 ◦C to 95 ◦C with a heating rate of 0.5 ◦C. After the run was complete, CFX Maestro software was used to analyze the data where Cq values of each sample within each gene of interest were then normalized to the housekeeping gene RPLP0, and then analyzed in an excel file to observe the fold change expression with respect to control and sedentary group in the samples using the equation $2^{-\Delta\Delta Cq}$. GraphPad prism was used to draw the graphs on analyzed data. Notably, samples were excluded from the data analysis with a Cq value of 27 and above for the RPLP0, and/or Cq value for the gene was at least 2 cycles close to the Cq value of NRT.

Table 1: A list of the primers designed by Beacon Designer8 for the genes that showed significant difference at either unstimulated control or at least one treatment time point in the samples obtained from exercise groups compared to sedentary group.

Gene name	Forward Primer	Reverse Primer
DGKG	TCCTCACTACCATACAAG	AGAGAAGACCATCAGAAG
MYSM1	CTGCCTTCTCTTCTCTTG	GTCTCTGTCTCTGTCTCT
CD86	ATACAGGTGACAGCAACAG	CAGGCAGGTGGATTCTTC
IRAK3	CTTGGTATAGGTGAACAGAGAAC	ATTGGCTGCTGGTGACTA
SESN3	CCAGGCGAGGAGAAGAAC	TTGTGTTGTAGGTGAGATTGTAGA

3.7 Statistical Analysis: Data were analyzed using GraphPad Prism 9.0.2. An unpaired t-test was used to make comparison between two different study groups. One-way ANOVA was used to make comparison between three different study groups followed by Dunnett's multiple comparison test, whereas two-way ANOVA using Tukey's multiple comparison test was used to study more than one variable between three different study groups. Data are shown in mean +/- SEM where statistical significance was set at p-value of 0.05.

Table 2: A standard curve data for genes that showed significant difference in the exercise induced trained status.

Gene name	E (Efficiency)	R^2 (Coefficient of determination)	Slope	Y-intercept
DGKG	79.6%	0.977	3.932	16.210
MYSM1	119.3%	0.977	2.933	31.613
CD86	102.1%	0.997	3.273	43.785
IRAK3	109.2%	0.968	3.119	4.979
SESN3	86.9%	0.988	3.682	48.287

We also validated the primers that showed statistically significant difference in real-time PCR data between study groups. This was done to verify the efficiency of real-time PCR assay. A sample with smallest Cq value was selected for each gene and 5-fold serial dilutions were made to obtain a standard curve spanning 6 orders of magnitude. An efficiency (E) and coefficient of determination (R^2) values for each gene are shown in Table 2.

Chapter 4: Results

4.1 Exercise induces sustained changes in chromatin accessibility of BMDMs: Unpublished data obtained by a Ph.D. student in our lab have demonstrated that BMDMs in moderate-high intensity exercise mice group exhibited reduced inflammatory responses when compared to sedentary mice group. The exercise-mediated changes in BMDMs persisted for a week in cell culture and led to enhanced mitochondrial function and increased reliance on oxidative phosphorylation for ATP synthesis. To determine whether these persistent changes are happening because of epigenetic modifications that alter the chromatin accessibility in BMDMs obtained from exercise group, we performed an unbiased genome-wide chromatin accessibility screening, known as ATAC-seq (Assay for Transposase Accessible Chromatin regions). As a result, in this study we observed that there was a significant difference in chromatin accessibility of specific regions between the macrophages obtained from the moderate-high intensity exercise group compared to those obtained from the sedentary group. Notably, there was no statistically significant difference in the chromatin accessibility between moderate intensity and sedentary group. Therefore, we proceeded with analyzing the differentially accessible regions between the moderate-high intensity exercise and sedentary mice group. We discovered that 608 chromatin regions were differentially accessible between moderate-high intensity exercise and sedentary group, 180 of which were downregulated and 428 were upregulated in the moderate-high intensity exercise group compared to sedentary group (Figure 2). The upregulated regions and downregulated regions indicate the increase and decrease in chromatin accessibility respectively.

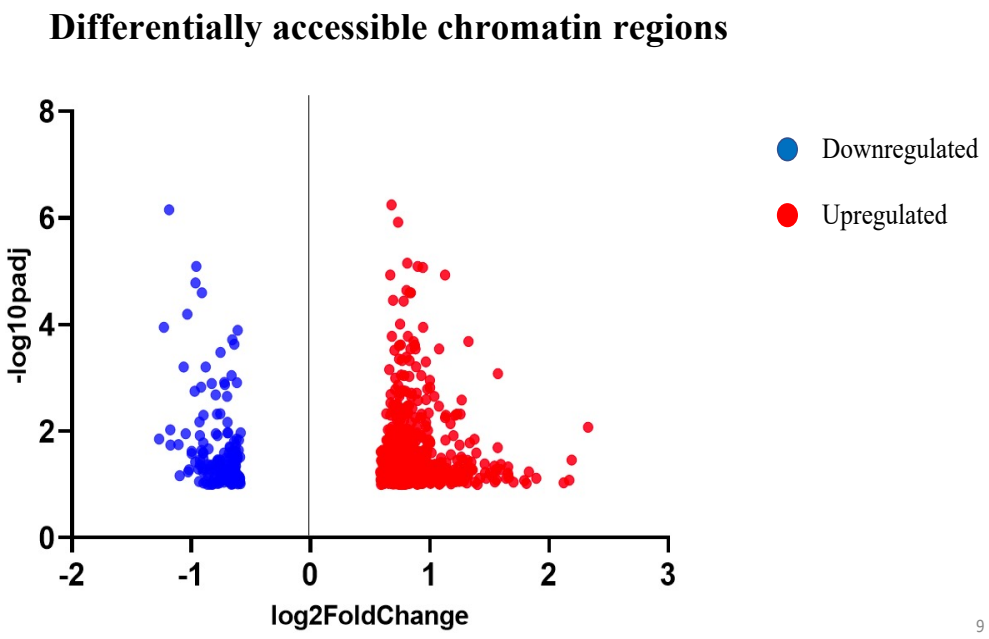


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Figure 2: Differentially accessible chromatin regions obtained from the data analysis of ATAC-seq (Assay for transposase accessible chromatin -sequencing) in the moderate-high intensity exercise group compared to sedentary group in which 30% of the regions were downregulated (shown in blue) and 70% of the regions were upregulated (shown in red). X-axis represents the log2FoldChange which is a log ratio of the differentially expressed peak intensity values between moderate-high intensity exercise and sedentary group and Y-axis represents the significant values where $p<0.05$. Volcano plot was generated using GraphPad prism.

Table 3: A list of the downregulated (green) and upregulated (red) genes that showed significnat difference at specific chromsome region (ID) between sedentary (S5, S3, S1) and moderate-high intensity (HE5, HE3, HE2) exercise group. (FDR=0.1, relative expression between study groups is ≥1.5).

ID	S5	S3	S1	HE5	HE3	HE2	foldChange	log10padj	Gene
chr1:59271724-59272155	547.69	323.15	353.95	186.14	178.84	203.381	0.46	1.75	Cdk15
chr4:154465107-154465448	326.24	270.77	274.41	148.11	134.88	161.509	0.51	2.75	Prdm16
chr4:105094122-105094417	214.53	228.28	219.73	104.08	113.90	129.606	0.52	2.18	Prkaa2
chr3:126796112-126796421	182.89	158.12	148.14	75.06	95.92	90.724	0.54	1.29	Camk2d
chr3:8888680-8888982	241.22	344.89	321.14	173.13	161.86	150.542	0.54	1.59	Mrps28
chr16:14219190-14219609	236.28	310.30	339.03	167.12	174.85	151.539	0.56	1.35	Myh11
chr2:131076772-131077130	341.07	346.87	395.70	222.16	248.78	209.363	0.63	1.37	Siglec1
chr3:126672969-126673386	399.40	417.03	446.41	234.17	243.79	315.042	0.63	1.12	Camk2d
chr4:66796665-66797098	833.40	834.06	824.22	443.33	504.56	649.026	0.64	1.08	Tlr4
chr7:126463981-126464579	949.06	883.47	986.28	553.41	573.50	732.771	0.66	1.16	Atp2a1
chr2:113820072-113820541	316.35	279.67	309.21	197.15	205.82	195.406	0.66	1.15	Scg5
chr4:143099433-143099873	391.49	327.10	355.93	226.17	242.79	244.257	0.66	1.12	Prdm2
chr2:102900432-102900737	210.57	210.49	221.71	336.25	302.74	330.993	1.51	1.22	Cd44
chr1:55237885-55238081	196.73	233.22	201.83	328.24	325.72	300.087	1.51	1.05	Mars2
chr3:107783262-107783700	1800.25	1984.36	2302.64	3308.45	3195.23	2736.68	1.52	1.41	Csf1
chr1:60912997-60913667	414.23	361.69	370.85	572.42	635.45	547.335	1.53	1.55	Ctla4
chr16:22629911-22630295	188.82	184.80	160.07	274.20	285.75	257.218	1.53	1.03	Dgkg
chr10:20723503-20723831	245.17	278.68	287.33	425.32	454.61	369.875	1.54	1.16	Pde7b
chr12:32001647-32002039	333.16	343.90	309.21	528.39	549.52	447.638	1.55	1.39	Prkar2b
chr17:7176304-7176837	652.48	712.51	625.37	1064.79	1077.07	948.116	1.55	2.33	Rps6ka2
chr17:45581563-45581913	478.49	447.67	484.19	836.62	739.36	617.123	1.56	1.27	Hsp90ab1
chr4:94999228-94999803	285.71	235.20	235.63	412.31	435.62	343.954	1.58	1.06	Mysm1
chr1:39895094-39895790	914.46	890.39	860.01	1530.13	1380.80	1298.05	1.58	3.16	Map4k4
chr8:106005937-106006174	360.84	341.93	392.72	539.40	568.51	625.098	1.58	1.88	Ddx28/Dus2
chr3:135713213-135713888	262.97	285.60	351.96	479.36	500.57	449.632	1.59	1.24	Nfkb1
chr16:36633158-36633378	149.28	168.00	177.97	296.22	274.76	233.29	1.62	1.08	Cd86
chr12:4595145-4595405	409.28	336.98	299.26	625.46	570.51	509.45	1.63	1.26	Itsn2
chr3:107749389-107749717	126.54	132.42	122.29	224.17	184.84	214.348	1.63	1.10	Csf1
chr4:149765237-149765577	127.53	119.58	110.36	203.15	217.81	177.46	1.67	1.12	Slc25a33
chr2:165922189-165922832	647.54	733.26	734.74	1328.99	1391.79	848.419	1.69	1.01	Zmynd8
chr10:44459140-44459721	288.67	274.73	305.23	542.40	550.52	391.808	1.71	1.39	Prdm1
chr14:16240077-16240328	135.44	144.28	155.10	258.19	258.78	230.299	1.72	1.64	Oxsm
chr16:10981791-10981987	133.46	130.45	158.08	255.19	266.77	208.366	1.73	1.24	Litaf
chr8:126509978-126510326	117.64	89.93	105.39	180.13	196.83	166.494	1.74	1.10	Tarbp1
chr5:136212813-136213088	400.39	459.52	487.17	848.63	881.24	613.135	1.74	1.46	Prkrip1
chr19:36579280-36579670	120.61	131.43	139.19	251.19	241.79	188.427	1.74	1.20	Hectd2
chr10:34193646-34194014	256.05	260.89	294.29	557.41	548.52	337.972	1.78	1.02	Dse
chr10:59642715-59643157	107.76	92.89	76.56	159.12	157.86	179.454	1.79	1.08	Mcu
chr10:20723222-20723440	136.43	159.10	117.32	270.20	238.79	237.278	1.81	1.52	Pde7b
chr7:142460524-142461069	649.51	682.86	718.83	1562.16	1350.83	829.477	1.82	1.08	Lsp1
chr17:28697331-28697741	415.21	422.96	512.03	854.63	992.14	638.059	1.84	1.56	Mapk14
chr10:120204683-120204886	124.56	121.55	125.27	244.18	238.79	205.375	1.85	2.07	Irak3
chr14:14351567-14351947	281.75	195.67	152.12	418.31	337.71	451.626	1.92	1.02	il3ra
chr1:152817470-152817756	63.27	77.08	59.65	141.10	135.88	108.669	1.93	1.09	Ncf2
chr1:182954995-182955386	202.66	201.60	194.87	480.36	486.58	264.196	2.05	1.27	Tlr5
chr7:4460510-4461581	223.43	286.59	195.86	429.32	667.42	384.829	2.099	1.323066	Rdh13
chr16:36662729-36662925	75.13	77.08	91.47	179.13	168.85	165.497	2.107	2.469032	Cd86
chr1:138179751-138180460	174.98	164.05	208.79	420.31	508.56	230.299	2.116	1.002043	Ptprc
chr16:10981225-10981500	124.56	145.27	160.07	365.27	373.68	207.37	2.20	1.41	Litaf
chr2:25257648-25257843	149.28	92.89	172.00	360.27	314.73	261.21	2.26	1.56	Tmem203/Ndor1
chr9:14287261-14287603	82.05	63.25	55.68	185.14	187.84	102.69	2.37	1.15	Sesn3
chr4:63853753-63854449	99.85	91.90	118.31	243.18	349.70	144.56	2.38	1.08	Tnfsf8
chr8:22712821-22713346	107.76	111.67	130.24	305.23	329.71	206.37	2.41	2.58	Ikbkb
chr1:127838637-127838965	64.26	59.29	47.72	185.14	157.86	81.75	2.48	1.03	Map3k19
chr1:91240774-91241090	46.46	57.32	68.60	188.14	207.82	82.75	2.78	1.12	Ube2f
chr16:38131191-38131398	44.49	37.55	61.64	148.11	154.87	123.62	2.97	3.08	Gsk3b

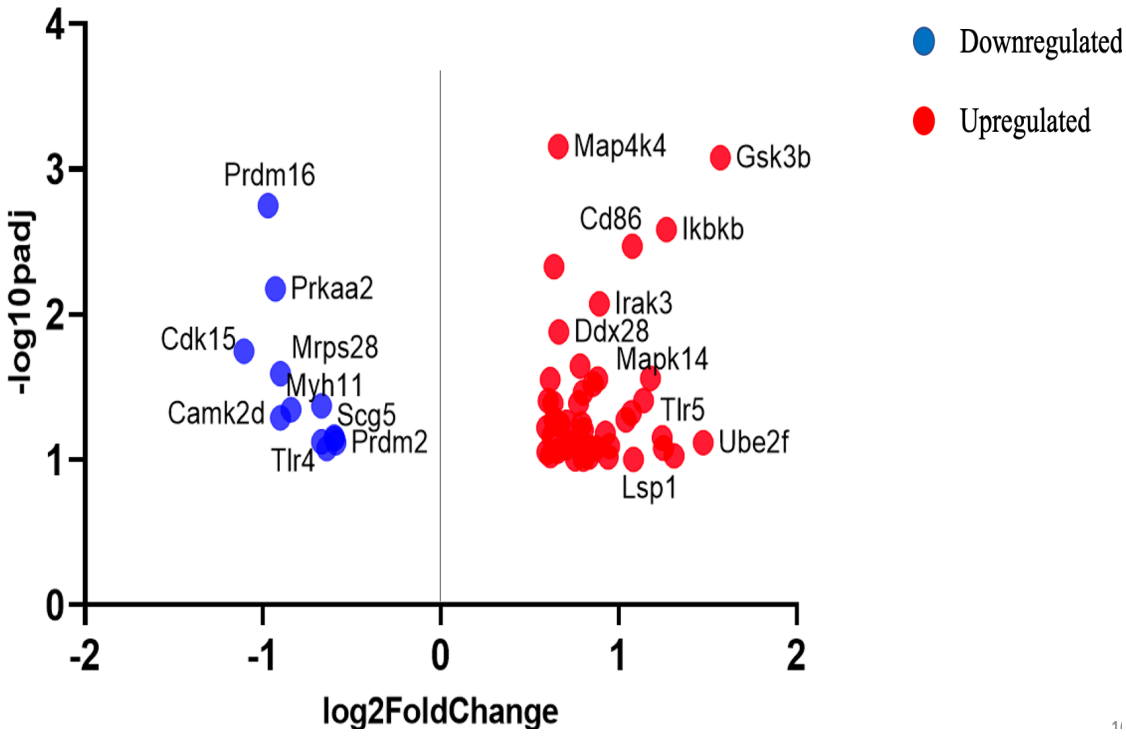

Figure 3: Upregulated (red) and downregulated (blue) genes in the moderate-high intensity exercise group vs sedentary group obtained from analysis of differentially accessible chromatin regions. X-axis represents the log2FoldChange which is a log ratio of the differentially expressed peak intensity values between moderate-high intensity exercise and sedentary group and Y-axis represents the significant values where $p<0.05$. Volcano plot was generated using GraphPad prism.

Next, we wanted to identify which differentially accessible chromatin regions may potentially affect gene expression which could alter the immune responses between sedentary and exercise mice groups. Therefore, we visualized the peak intensities at each differentially accessible chromatin region between the moderate-high intensity exercise vs sedentary group and mapped them to nearby genes to obtain a list of the downregulated and upregulated gene (Table 2, Figure 3). Although, all genes listed in Table 3 above were located near the statistically significant differentially accessible regions between moderate-high intensity exercise and sedentary group. However, here we demonstrated the basal expression level at the chromatin accessibility and peak intensity at those differentially accessible chromatin regions of only those genes that showed statistically significant difference in exercise induced trained status (Figure 4-13).

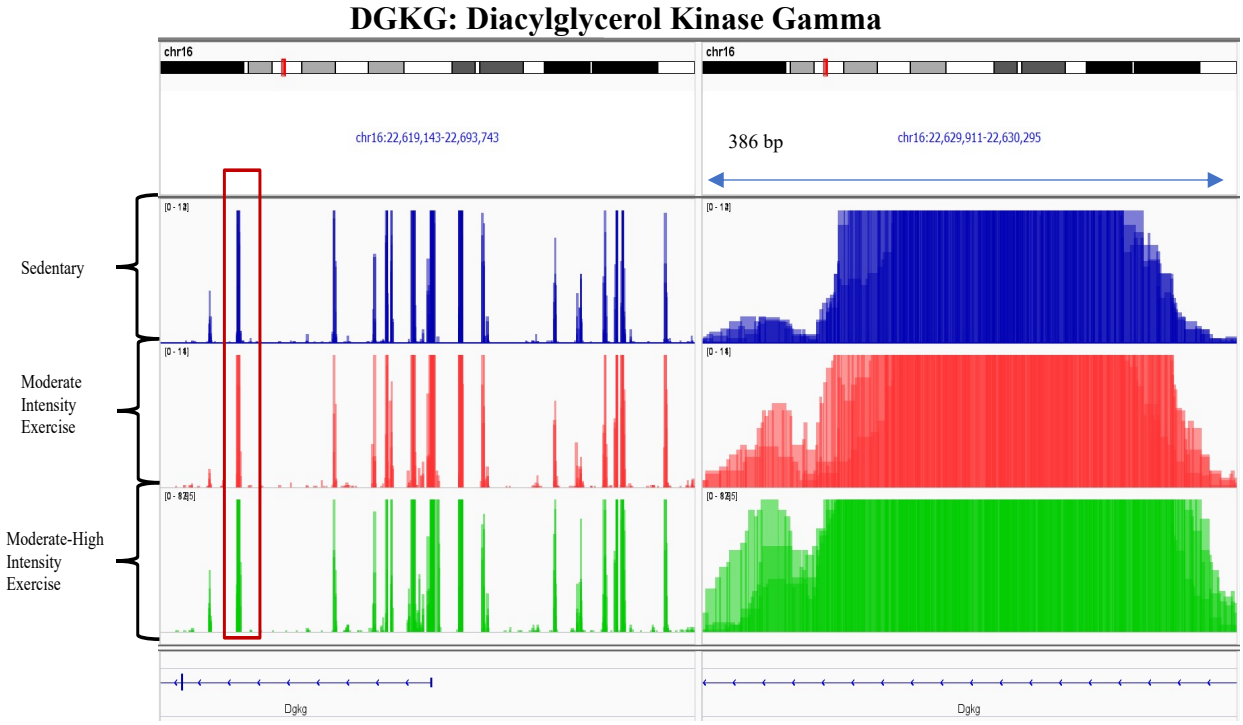

Figure 4: **Chromatin accessibility**: Representative plot of DGKG demonstrating the peaks in the unstimulated bone marrow derived macrophages (BMDM) located 25 kb downstream of promoter region in study groups. Right panel shows the zoomed in peaks of highlighted region on the left panel where moderate-high intensity exercise group is significantly upregulated compared to sedentary group. Peaks in each study group represent the overlay tracks of replicates where n=3.

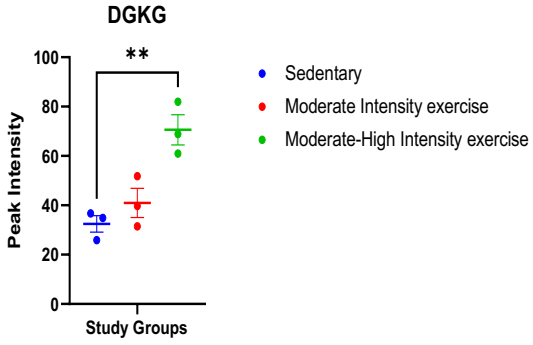

Figure 5: Peak intensity values obtained from the list of the differentially accessible regions between study groups, (one way ANOVA followed by Dunnett's multiple comparison test, **p<0.01). Dots represent the number of mice; n=3.

Mysm1: Myb-like SWIRM and MPN domains 1

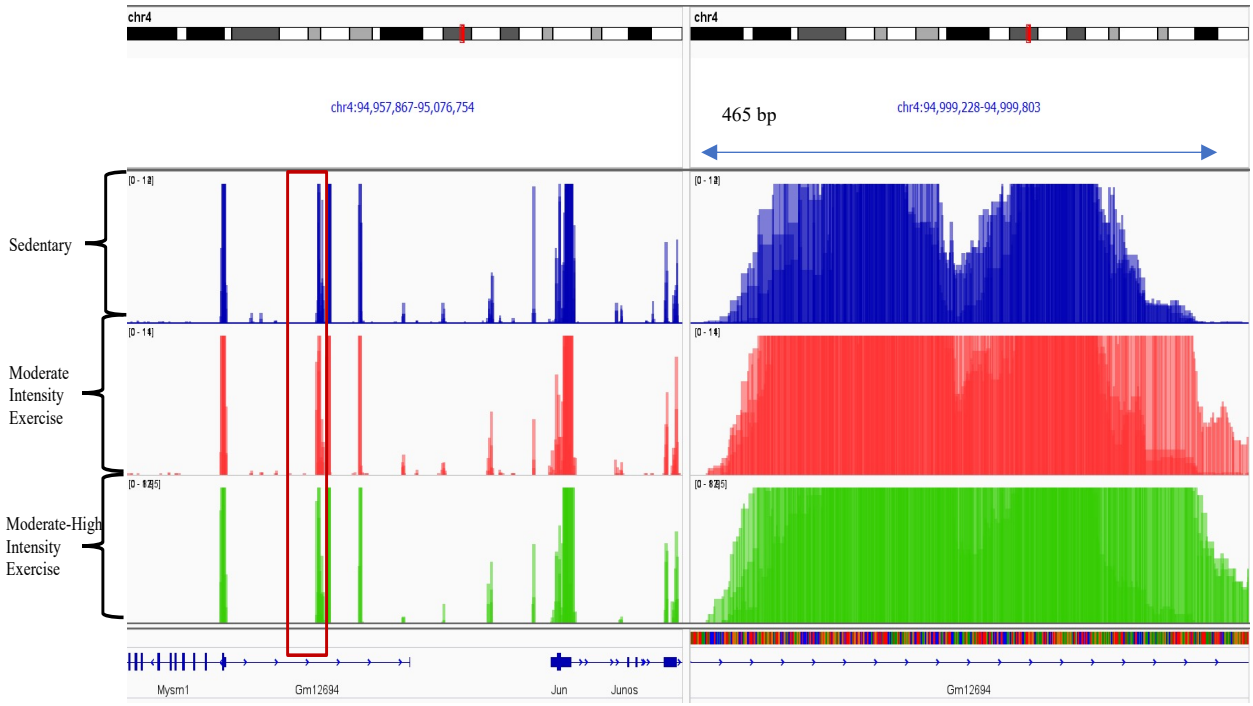

Figure 6: **Chromatin accessibility**: Representative plot of Mysm1 demonstrating the peaks in the unstimulated bone marrow derived macrophages (BMDM) located 20kb upstream of the promoter region in study groups. Right panel shows the zoomed in peaks of highlighted region on the left panel where moderate-high intensity exercise group is significantly upregulated compared to sedentary group. Peaks in each study group represent the overlay tracks of replicates where n=3.

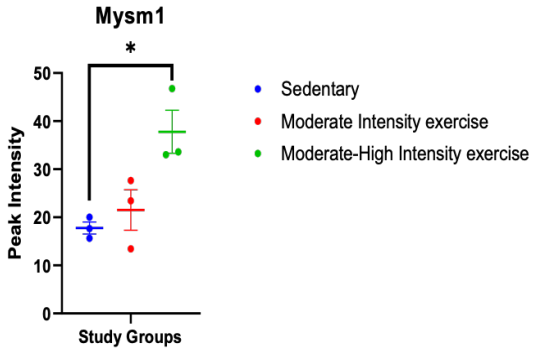

Figure 7: Peak intensity values obtained from the list of the differentially accessible regions between study groups (one way ANOVA followed by Dunnett's multiple comparison test, *p<0.05). Dots represent the number of mice; n=3.

CD86: Cluster of Differentiation 86

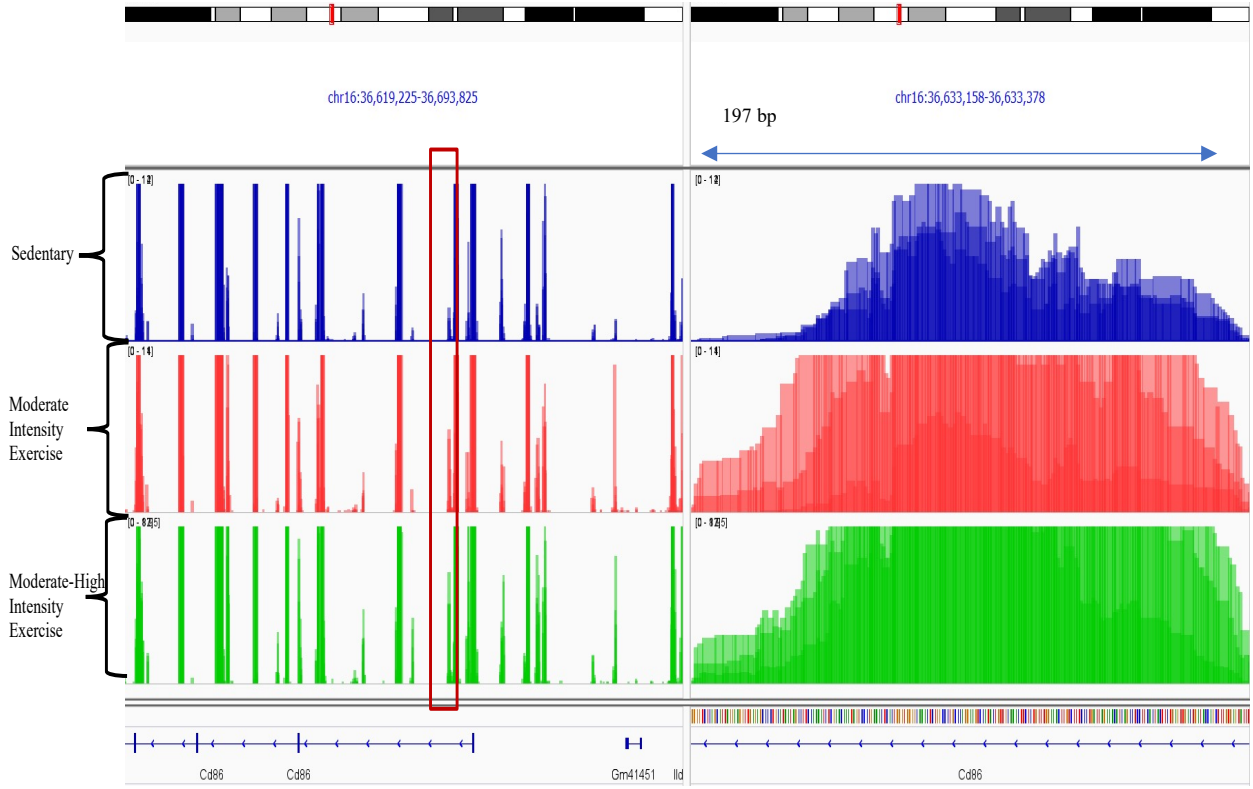

Figure 8: **Chromatin accessibility**: Representative plot of CD86 demonstrating the peaks in the unstimulated bone marrow derived macrophages (BMDM) located 3kb of downstream of promoter region in study groups. Right panel shows the zoomed in peaks of highlighted region on the left panel where moderate-high intensity exercise group is significantly upregulated compared to sedentary group. Peaks in each study group represent the overlay tracks of replicates where n=3.

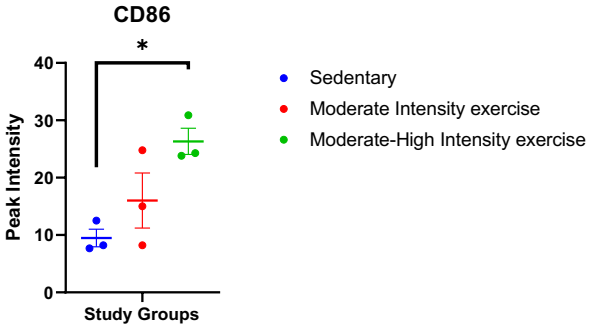

Figure 9: Peak intensity values obtained from the list of the differentially accessible regions between study groups (one way ANOVA followed by Dunnett's multiple comparison test, *p<0.05). Dots represent the number of mice; n=3.

Irak3: Interleukin 1 Receptor Associated Kinase 3

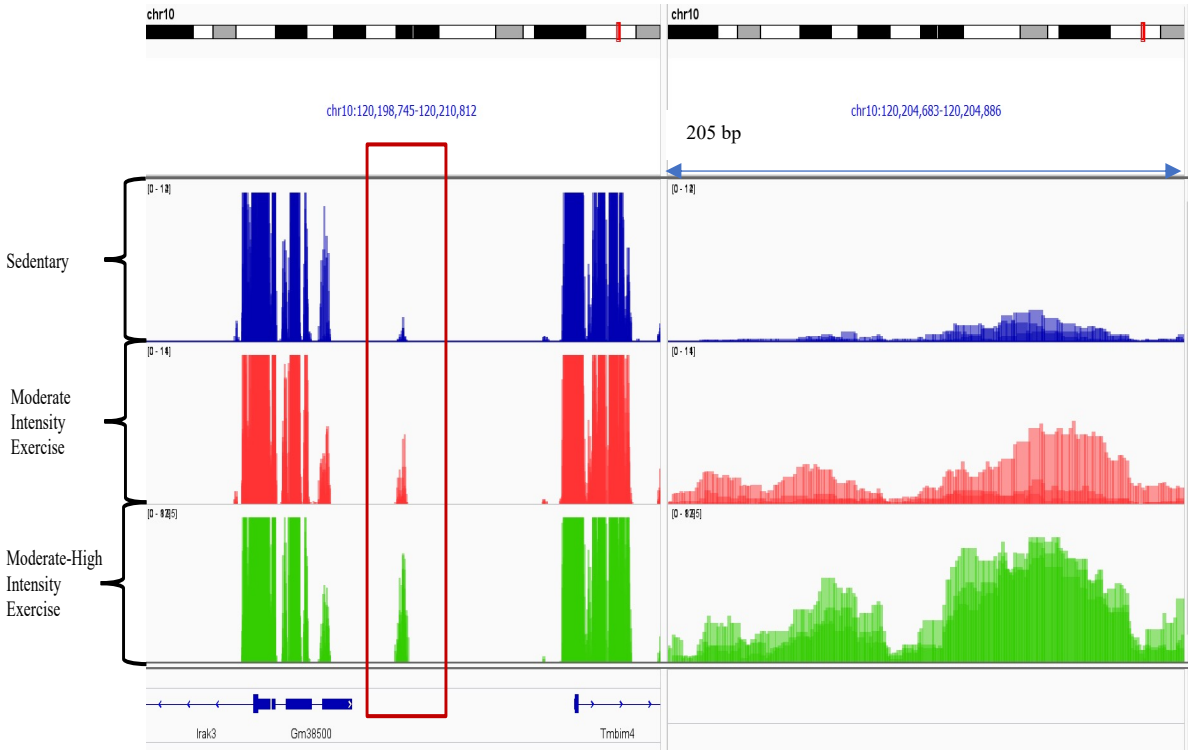

Figure 10: **Chromatin accessibility**: Representative plot of Irak3 demonstrating the peaks in the unstimulated bone marrow derived macrophages (BMDM) located 3 kb upstream of the promoter region in study groups. Right panel shows the zoomed in peaks of highlighted region on the left panel where moderate-high intensity exercise group is significantly upregulated compared to sedentary group. Peaks in each study group represent the overlay tracks of replicates where n=3.

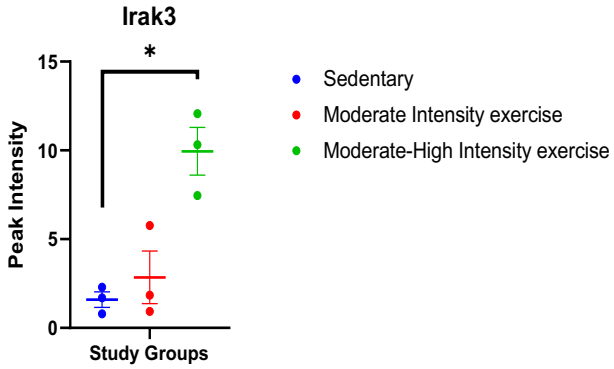

Figure 11: Peak intensity values obtained from the list of the differentially accessible regions between study, (one way ANOVA followed by Dunnett's multiple comparison test, *p<0.05). Dots represent the number of mice; n=3.

Sesn3: Sestrin 3

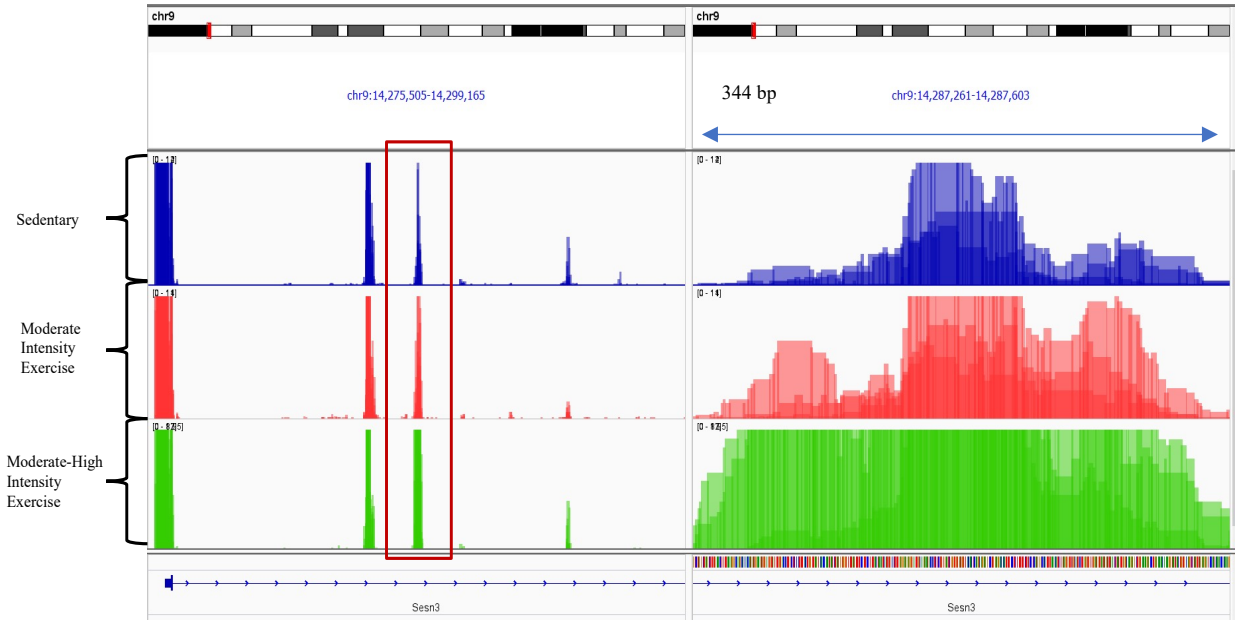

Figure 12: **Chromatin accessibility**: Representative plot of Sesn3 demonstrating the peaks in the unstimulated bone marrow derived macrophages (BMDM) located 13 kb downstream of the promoter region in study groups. Right panel shows the zoomed in peaks of highlighted region on the left panel where moderate-high intensity exercise group is significantly upregulated compared to sedentary group. Peaks in each study group represent the overlay tracks of replicates where n=3.

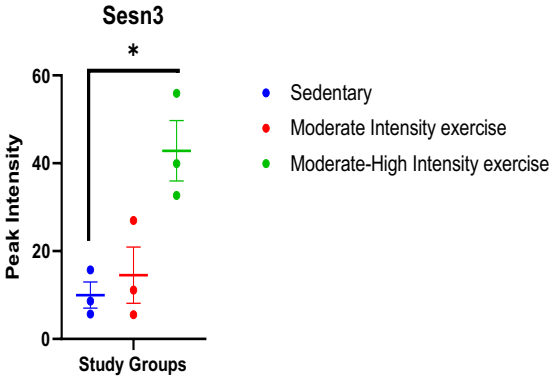

Figure 13: Peak intensity values obtained from the list of the differentially accessible regions between different study groups, (one way ANOVA followed by Dunnett's multiple comparison test, *p<0.05). Dots represent the number of mice; n=3.

After obtaining the list of genes whose expression level might potentially be altered by differentially accessible chromatin regions, shown in Table 3, we performed functional enrichment analysis. Essentially, it is important to understand how genes interact with each other because the cellular functions are regulated by dynamic activities of multiple genes. Knowing the common features of the genes with similar functions will be helpful to understand the biological processes in the cells, since many genes are involved in more than one biological pathways. Therefore, we grouped the genes based on the common functional characteristics (Table 3 and 4), which demonstrated that many genes carry out more than function. For instance, CDK15, PRKAA2, CAMK2D, PRDM2 were found to be involved in nitrogen compound metabolic process, cellular metabolic process, primary metabolic process, and organic substance metabolic process (Table 3). Similarly, IRAK3 from the list of upregulated genes, was found to be involved in the immune system processes, regulation of response to external stimulus, and response to stress (Table 4). Apart from the functional categories and genes mentioned above, there were number of genes that were discovered to be involved in multiple biological processes as shown in Table 3 and 4.

Table 4: A list obtained from the Gene Ontology which shows the downregulated genes grouped into different categories based on the functional characteristics, N=number of genes; $p<0.05$.

N	High level GO category	Genes
4	Nitrogen compound metabolic process	CDK15 PRKAA2 CAMK2D PRDM2
4	Cellular metabolic process	CDK15 PRKAA2 CAMK2D PRDM2
4	Primary metabolic process	CDK15 PRKAA2 CAMK2D PRDM2
4	Organic substance metabolic process	CDK15 PRKAA2 CAMK2D PRDM2
3	Regulation of cellular process	SCG5 TLR4 PRDM2
2	Signaling	SCG5 TLR4
2	Response to stimulus	SCG5 TLR4
2	Cellular response to stimulus	SCG5 TLR4
1	Immune system process	TLR4

1	Immune response	TLR4
1	Biosynthetic process	PRDM2
1	Regulation of metabolic process	PRDM2
1	Localization	ATP2A1
1	Establishment of localization	ATP2A1

Table 5: A list obtained from the Gene Ontology which demonstrates the upregulated genes grouped into different categories based on the functional characteristics, N=number of genes; $p<0.05$.

N	**High level GO category**	**Genes**
17	Organic substance metabolic process	PRKAR2B IRAK3 OXSM GSK3B RPS6KA2 MAP4K4 PTPRC NFKB1 IKBKB PRDM1 ZMYND8 MARS2 MAP3K19 MAPK14 MYSM1 TLR5 TARBP1
16	Cellular metabolic process	PRKAR2B IRAK3 OXSM GSK3B RPS6KA2 MAP4K4 PTPRC NFKB1 IKBKB PRDM1 ZMYND8 MARS2 MAP3K19 MAPK14 MYSM1 TARBP1
16	Primary metabolic process	PRKAR2B IRAK3 OXSM GSK3B RPS6KA2 MAP4K4 PTPRC NFKB1 IKBKB PRDM1 ZMYND8 MARS2 MAP3K19 MAPK14 MYSM1 TARBP1
15	Nitrogen compound metabolic process	PRKAR2B IRAK3 GSK3B RPS6KA2 MAP4K4 PTPRC NFKB1 IKBKB PRDM1 ZMYND8 MARS2 MAP3K19 MAPK14 MYSM1 TARBP1
13	Regulation of cellular process	PRKAR2B LSP1 PDE7B IRAK3 DGKG RPS6KA2 CTLA4 PTPRC NFKB1 PRDM1 ZMYND8 MAPK14 TLR5
12	Response to stimulus	LSP1 PDE7B IRAK3 DGKG RPS6KA2 CTLA4 PTPRC NFKB1 TNFSF8 SESN3 MAPK14 TLR5
9	Signaling	LSP1 PDE7B IRAK3 DGKG RPS6KA2 PTPRC NFKB1 MAPK14 TLR5
9	Cellular response to stimulus	LSP1 PDE7B IRAK3 DGKG RPS6KA2 PTPRC NFKB1 MAPK14 TLR5
5	Immune system process	IRAK3 CTLA4 PTPRC TNFSF8 TLR5
5	Immune response	IRAK3 CTLA4 PTPRC TNFSF8 TLR5
5	Biosynthetic process	OXSM NFKB1 PRDM1 ZMYND8 MARS2
5	Regulation of metabolic process	PRKAR2B NFKB1 PRDM1 ZMYND8 TLR5

4	Response to stress	IRAK3 SESN3 MAPK14 TLR5
3	Regulation of immune system process	IRAK3 CTLA4 PTPRC
3	Regulation of response to stimulus	IRAK3 PTPRC SESN3
2	Response to external stimulus	IRAK3 TLR5
2	Response to biotic stimulus	IRAK3 TLR5
2	Interspecies interaction between organisms	IRAK3 TLR5
2	Positive regulation of biological process	PTPRC TLR5
2	Negative regulation of biological process	IRAK3 PRDM1
2	Localization	ITSN2 NCF2
2	Establishment of localization	ITSN2 NCF2
2	Response to other organism	IRAK3 TLR5
1	Activation of immune response	PTPRC
1	Protein folding	HSP90AB1
1	Cell adhesion	CD44
1	Cell population proliferation	CTLA4
1	Cellular component organization	MYSM1
1	Biological adhesion	CD44
1	Regulation of signaling	IRAK3
1	Multicellular organismal process	TLR5
1	Developmental process	PRDM1
1	Response to chemical	SESN3
1	Leukocyte activation	CTLA4

1	Regulation of multicellular organismal process	TLR5
1	Regulation of biological quality	MCU
1	Cellular component organization or biogenesis	MYSM1

Table 6: A list of the upregulated (red) genes and their functions where relative expression gene expression is ≥1.5. Gene functions were obtained using GeneCards.

ID	Fold change	Gene name	Gene function
chr16:22629911-22630295	1.531	DGKG	This gene is involved in lipid metabolism which converts diacylglycerol (DAG) into phosphatidic acid (PA) and maintains the level of bioactive lipids (Kai et al., 1994).
chr4:94999228-94999803	1.575	MYSM1	This gene plays an important role in suppression of innate immunity and autoimmunity (Tian et al., 2020a) and regulates the proper functioning of the haematopoietic stem cells and immune responses (Nandakumar et al., 2013).
chr16:36633158-36633378	1.624	CD86	It is expressed on pro-inflammatory macrophages and antigen-presenting cells. It is known to be involved in the costimulatory signals required for the T-cell proliferation (Lanier et al., 1995).
chr10:120204683-120204886	1.853	IRAK3	This gene is involved in the downstream signalling of immune receptors such as interleukin1 receptor (IL1R) and toll-like receptors (TLRs) (Wesche et al., 1999), (Nechama et al., 2018).
chr9:14287261-14287603	2.366	SESN3	This gene negatively regulates the TORC1 pathways and is also known to play an important role in reducing the oxidative stress (Chantranupong et al., 2014).

4.2: Exercise induces innate memory in BMDMs: Subsequently, we wanted to determine whether the changes observed in the chromatin accessibility between moderate-high intensity exercise and sedentary group could be responsible for the trained status in BMDMs, which could alter their induction level after being challenged by microbial ligands. Therefore, an in-vitro experimental model of exercise induced "trained immunity" was established in our study, where BMDMs obtained from sedentary vs exercised mice (Primary Stimulus) were treated with LPS (Secondary Stimulus) to determine changes in mRNA expression of genes obtained from ATAC-seq analysis. To measure the expression level of genes between study groups, we performed a real-time PCR on cDNA samples obtained from LPS stimulated and unstimulated control samples. We selected genes of interest from the list of genes obtained from bioinformatic analysis (Table 3, Figure 3), which are primarily involved in immunoregulatory and metabolic processes as the functional categories mentioned above for downregulated and upregulated genes in Table 4 and 5 respectively. First, we measured gene expression in the unstimulated control BMDMs between moderate-high intensity exercise and sedentary groups by employing real-time PCR (See Appendix). Next, we measured gene expression in LPS stimulated BMDMs of moderate-high intensity exercise and sedentary groups by employing real-time PCR.

After analyzing the gene expression in our genes of interest from listed genes (Table 3), we found that only DGKG, MYSM1, CD86, IRAK3, SESN3 exhibited significant difference ($P<0.05$) at either unstimulated control or at least one treatment time points in LPS stimulated samples. Then we proceeded with preforming a real-time PCR in the unstimulated control and LPS stimulated BMDMs obtained from moderate-intensity exercise group using the above-mentioned genes that showed statistically significant difference between moderate-high intensity exercise and sedentary group.

A fold change expression values for differentially accessible chromatin regions and the functions of genes that indicated significant difference in the trained status between study groups is listed in Table 6.

After analyzing the data from real-time PCR, we demonstrated that basal gene expression of DGKG was significantly increased BMDMs of moderate-high intensity exercise group when compared to the sedentary group (Figure 14a). Notably, there was a non-significant trend of tolerogenic response to the LPS at 3hr time point in the exercise groups (Figure 14b).

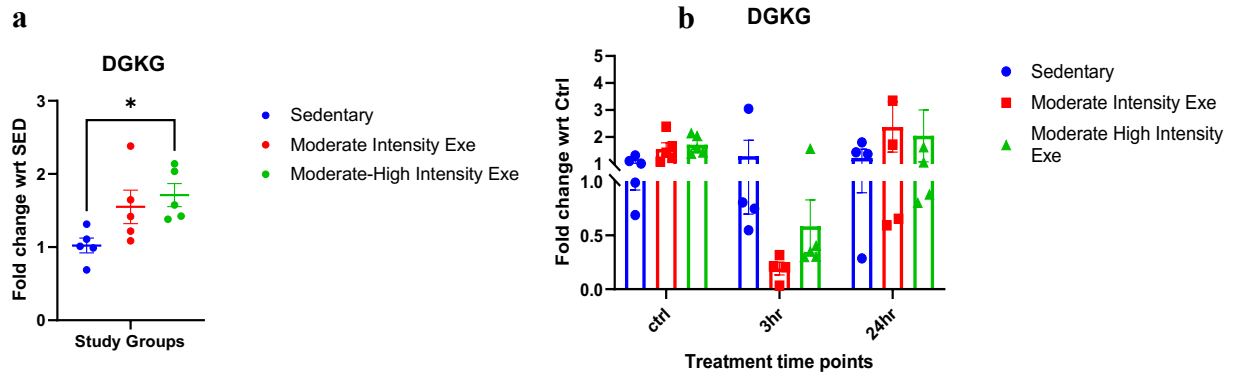

Figure 14: **Trained Immunity in Bone marrow derived macrophages (BMDMs).** real time-qPCR expression of DGKG from primary BMDMs. **a)** Fold change is shown with respect to sedentary (SED) in the unstimulated control where the moderate -high intensity group shows significant difference (*p<0.05) compared to the sedentary group. Dots represent the number of mice; n=5. Comparison between study groups was assessed by one-way ANOVA. **b)** Cells were treated with 100 ng/ml of Lipopolysaccharide (LPS) at 3-hour and 24-hour time points along with unstimulated control (ctrl) in sedentary, moderate intensity exercise and moderate-high intensity exercise groups. Fold change is shown with respect to (wrt) control of each study group; n ≥4. Data are shown in mean +/- SEM. Comparison between groups was assessed by two-way ANOVA and significance testing by Tukey's multiple comparison test.

The difference in gene expression of Mysm1 was non-significant at the basal level between study groups (Figure 15a). However, moderate intensity exercise group showed significant difference at 3 hour when compared to sedentary group (Figure 15b).

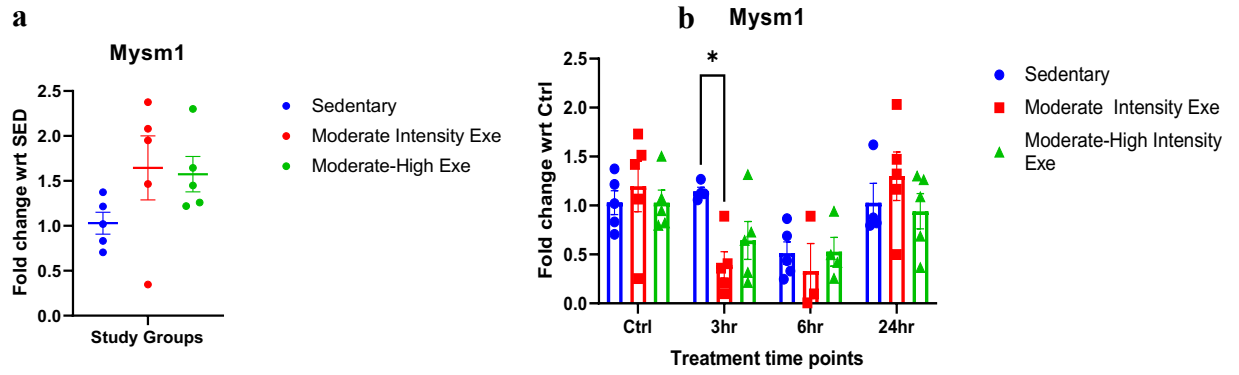

Figure 15: **Trained Immunity in Bone marrow derived macrophages (BMDMs)**. real time-qPCR expression of Mysm1 from primary BMDMs. **a)** Fold change is shown with respect to sedentary (SED) unstimulated control, n=5. Dots represent the number of mice. Comparison between study groups was assessed by one-way ANOVA **b)** Cells were treated with 100 ng/ml of Lipopolysaccharide (LPS) at 3-hour, 6-hour, and 24-hour time points along with unstimulated control (ctrl) in sedentary, moderate intensity exercise and moderate-high intensity exercise groups. A significant difference (*$p<0.05$) was observed between moderate intensity exercise and sedentary group at 3-hour time point. Fold change is shown with respect to (wrt) control of each study group; $n\geq3$. Data are shown in mean +/- SEM. Comparison between groups was assessed by two-way ANOVA and significance testing by Tukey's multiple comparison test.

Furthermore, moderate intensity exercise group showed significant difference at the basal level for CD86 gene (Figure 16a). In response to LPS, moderate intensity exercise group showed tolerogenic response at 3 hour and 6 hour time point when compared to sedentary group. While the moderate-high intensity exercise group was significantly hyporesponsive at 3 hour time point only (Figure 16b).

Next, Irak3 showed non-significant difference at the basal level between the study groups (Figure 17a). However, moderate intensity exercise group was significantly hyporesponsive to LPS at 6-hour time point compared to both sedentary and moderate-high intensity exercise group (Figure 17b).

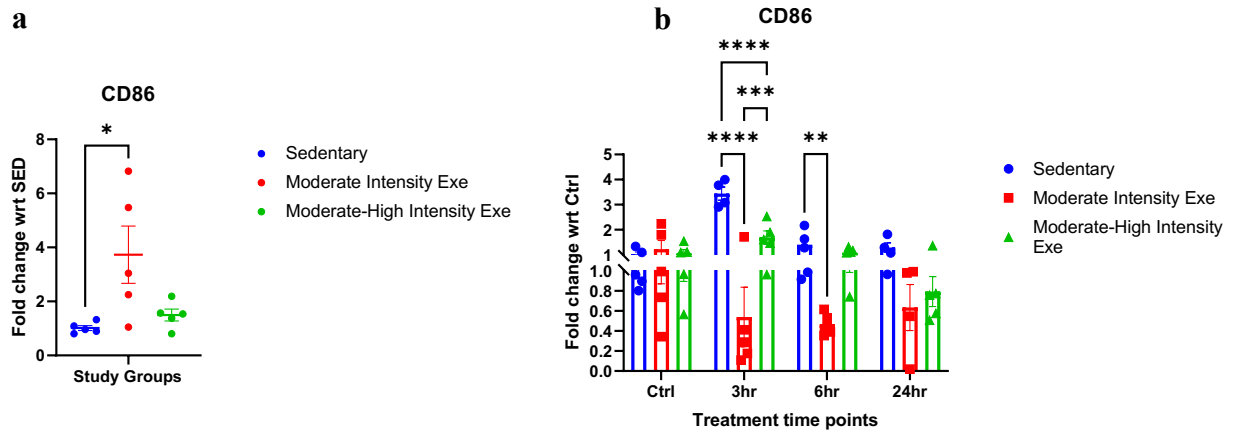

Figure 16: **Trained Immunity in Bone marrow derived macrophages (BMDMs).** real time-qPCR expression of Cd86 from primary BMDMs. **a)** Fold change is shown with respect to sedentary (SED) in the unstimulated control where the moderate intensity group shows significant difference (*p<0.05) compared to the sedentary group. Dots represent the number of mice; n=5. Comparison between study groups was assessed by one-way ANOVA followed by Dunnett's multiple comparison test. **b)** Cells were treated with 100 ng/ml of Lipopolysaccharide (LPS) at 3-hour, 6-hour, and 24-hour time points along with unstimulated control (ctrl) in sedentary, moderate intensity exercise and moderate-high intensity exercise groups. Fold change is shown with respect to (wrt) control of each study group; n≥3. Data are shown in mean +/- SEM. Comparison between groups was assessed by two-way ANOVA and significance testing by Tukey's multiple comparison test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

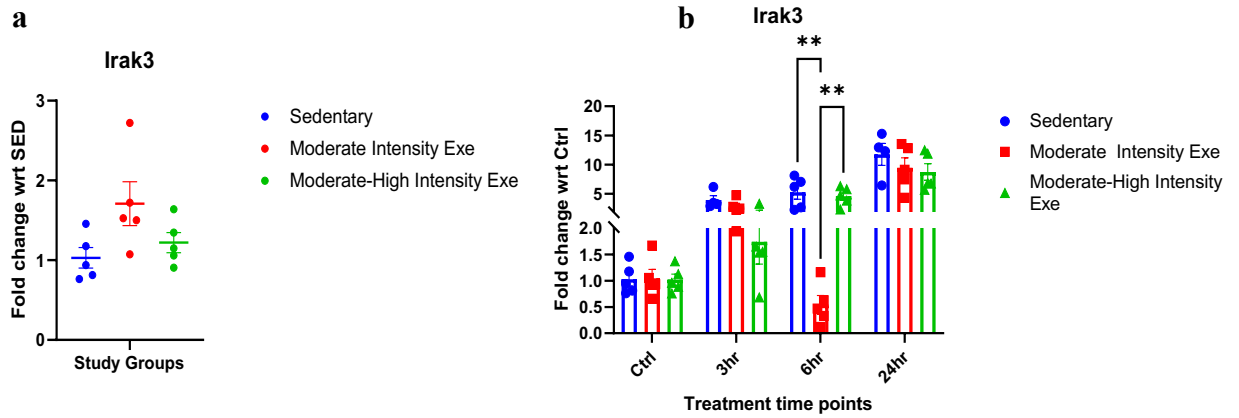

Figure 17: **Trained Immunity in Bone marrow derived macrophages (BMDMs).** real time-qPCR expression of Irak3 from primary BMDMs. **a)** Fold change is shown with respect to sedentary (SED) in the unstimulated control of each study group. Dots represent the number of mice; n=5. Comparison between study groups was assessed by one-way ANOVA **b)** Cells were treated with 100 ng/ml of Lipopolysaccharide (LPS) at 3-hour, 6-hour, and 24-hour time points along with unstimulated control (ctrl) in sedentary, moderate intensity exercise and moderate-high intensity exercise groups. Fold change is shown with respect to (wrt) control of each study group where the moderate intensity exercise group showed significant difference compared to sedentary and moderate-high intensity exercise group at 6-hour time point; n ≥3. Data are shown in mean +/-

SEM. Comparison between groups was assessed by two-way ANOVA and significance testing by Tukey's multiple comparison test, **p<0.01.

Additionally, we determined that basal gene expression of Sesn3 was significantly increased in moderate intensity exercise group (Figure 18a).

Furthermore, moderate intensity exercise group was found to be significantly hyporesponsive to LPS at 3 hour and 6 hour time point when compared to sedentary group; whereas moderate-high intensity exercise group was hyporesponsive to LPS at 6 hour and 24 hour time point when compared to the sedentary group (Figure 18b).

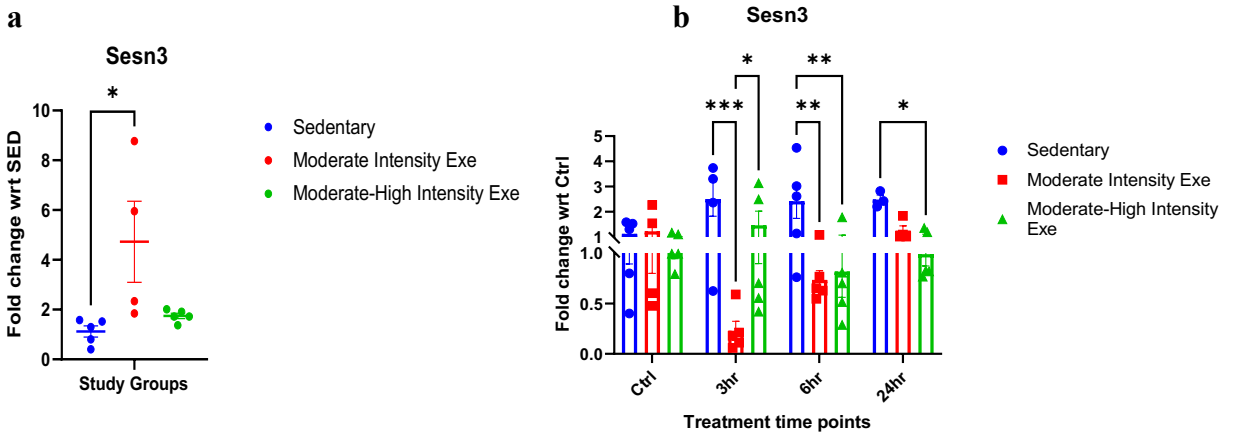

Figure 18: **Trained Immunity in Bone marrow derived macrophages (BMDMs)**. real time-qPCR expression of Sesn3 from primary BMDMs. **a)** Fold change is shown with respect to sedentary (SED) in the unstimulated control of each group where the moderate intensity exercise groups showed significant difference (*p<0.05) when compared to the sedentary group. Dots represent the number of mice; n≥4. Data are shown in mean +/- SEM. Comparison between study groups was assessed by one-way ANOVA. **b)** cells were treated with 100 ng/ml of Lipopolysaccharide (LPS) at 3-hour, 6-hour, and 24-hour time points along with unstimulated control (ctrl) in sedentary, moderate intensity exercise and moderate-high intensity exercise groups. Fold change is shown with respect to (wrt) control of each study group n≥3. Data are shown in mean +/- SEM. Comparison between groups was assessed by two-way ANOVA and significance testing by Tukey's multiple comparison test, *p<0.05, **p<0.01, ***p<0.001.

4.3: Exercise increases the antioxidant enzyme activity: In addition to the genes obtained from the ATAC-seq analysis, we wanted to determine exercise induced changes in genes that are known

to play an important role in inflammation and oxidative stress. Consistent with previous findings in our lab, here we demonstrated that moderate-high intensity exercise modifies the response to LPS in Hmox-1 (Heme oxygenase 1).

We demonstrated that HMOX-1 was significantly tolerogenic in response to LPS at 6hr time point (Figure 19b) in the moderate-high intensity exercise group compared to both moderate intensity exercise and sedentary group.

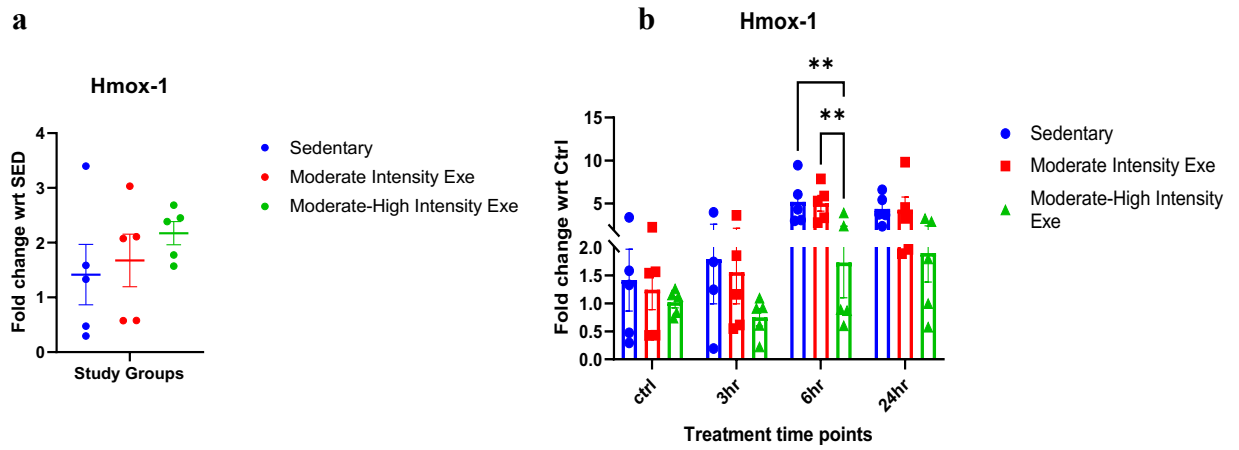

Figure 19: **Trained Immunity in Bone marrow derived macrophages (BMDMs)**. real time-qPCR expression of Hmox-1 from primary BMDMs. **a)** Fold change is shown with respect to sedentary (SED) in the unstimulated control. Dots represent the number of mice; n=5. **b)** cells were treated with 100 ng/ml of Lipopolysaccharide (LPS) at 3-hour, 6-hour, and 24-hour time points along with unstimulated control (ctrl) in sedentary, moderate intensity exercise and moderate-high intensity exercise groups. Fold change (FC) is shown with respect to (wrt) control of each study group; n≥3. Data are shown in mean +/- SEM. Comparison between groups was assessed by two-way ANOVA and significance testing by Tukey's multiple comparison test, **p<0.01.

Chapter 5: Discussion

In this study, we have demonstrated differentially accessible chromatin regions in the BMDMs obtained from moderate-high intensity exercise vs sedentary group by employing ATACseq analysis. Furthermore, we have established that moderate-high intensity exercise (primary stimulus) leads to persistent reduction in inflammatory responses in BMDMs when induced with secondary stimulus such as LPS. This is in an agreement with the previous findings in our lab. As mentioned in the introduction, the trained immune cells can either increase (hyperresponsive) or dampen (tolerance/hyporesponsive) the sensitivity towards the secondary stimulus (Figure 1) (Netea et al., 2020). Correspondingly, to our knowledge, our study is the first to demonstrate the exercise induced trained immunity where we determined that exercise could induce trained immune status where BMDMs were hyporesponsive (tolerogenic) to LPS, which proves that exercise causes trained immunity in bone marrow derived macrophages. Importantly, to answer our research question, here we suggested that genome wide differentially accessible chromatin regions cause changes in gene expression at either basal level or LPS treated BMDMs, which supports our hypothesis. Additionally, our findings of these regions in this study may help us to explain the exercise induced modifications of glycolytic and mitochondrial respiration pathways and immune function which have been demonstrated in the previous unpublished findings in our lab.

5.1 Exercise and Chromatin accessibility: Exercise alleviates the low grade systemic inflammation in a dose dependent manner, although the exact regimen of exercise still needs to be established (Metsios et al., 2020). In our study, we demonstrated that moderate-high intensity exercise is causing statistically significant changes in the accessibility of differentially accessible genome wide chromatin regions leading to lower inflammatory responses. Notably, moderate

intensity exercise also caused changes in the accessibility at the chromatin level, however these changes were not statistically significant and fall in the middle range of sedentary and moderate-high intensity exercise group as shown in the peak intensity graphs (Figure 4-13). This demonstrates that exercise intensity also plays crucial role in causing significant changes in the chromatin accessibility in the macrophages. Additionally, we have demonstrated that chromatin accessibility plays prominent role in changing the gene expression at the basal level. For instance, we found that increase in chromatin accessibility of DGKG in the moderate-high intensity exercise group led to statistically significant increase in mRNA expression of this genes at basal level. Interestingly, moderate intensity exercise group when compared to sedentary group, showed statistically significant difference in mRNA expression at basal level in CD86 and Sesn3.

5.2 Exercise and the inflammatory response: Our study has suggested that both moderate intensity and moderate-high intensity exercise training is significantly protective against the inflammatory responses. For instance, Mysm1 was signifcnalty hyporesponsive to LPS in the moderate intensity exercise group compared to sedentary group. We were interested in this gene because Myms1 is found to be lowering the inflammation. An in-vivo mice model of knockout Mysm1 gene demonstrated strong inflammatory responses in response to viral infections compared to wild type mice (Tian et al., 2020b). Furthermore, both moderate and moderate-high intensity exercise trainings were significantly protective against LPS in CD86 gene. As functions described by previous literatures, CD86 is known to be a costimulatory molecule and is present on the antigen presenting cells (Reincke et al., 2020). Notably, only moderate intensity exercise group compared to sedentary and moderate -high intensity exercise group, showed significantly lower response to LPS in Irak3 gene. Irak3 is involved in downstream signalling of TLR and IL-1R in the innate immune cells. It is known to produce cGMP through which it lowers the production of pro-

inflammatory cytokines (Freihat et al., 2019). Consistently, our study has demonstrated lower inflammatory responses in both CD86 and Irak3.

According to the previous literature, Sesn3 promotes the exercise mediated benefits by upregulating Akt and PGC1α pathway (Kim et al., 2020). Interestingly, our study has shown that gene expression level of Sesn3 is increased in moderate intensity exercise group at basal. Furthermore, both moderate and moderate-high intensity exercise groups showed significantly tolerogenic response to LPS when compared to sedentary group.

Furthermore, this study allowed us to understand that exercise training can cause persistent changes in the macrophages leading to lower inflammatory responses especially in genes associated with immunoregulatory and metabolic pathways. Moreover, genes found in our study are also associated with metabolic diseases which suggests that moderate-high intensity exercise can play an important role in health and diseases by modifying the basal expression level of genes. For instance, previous literature has suggested that muscle disuse and chronic diseases lead to changes in the metabolic pathways, such as PI3K and Akt activity is found to be decreased in insulin resistance which further causes changes in the downstream signalling leading to muscle atrophy (Rudrappa et al., 2016). Noteworthy here, Sesn3 increases the insulin sensitivity by activating the mTORC2-Akt pathways (Tao et al., 2015); and interestingly this gene is found to be upregulated in the moderate-high intensity exercise group in our study (Figure 12). Furthermore, DGKG is found to be downregulated in the colorectal cancer because of the DNA methylation (Kai et al., 2017), whereas moderate-high intensity exercise leads to the upregulation of DGKG (Figure 4) as shown in our study. This suggests that exercise may be protective against the chronic diseases.

5.3 Exercise and Oxidative stress: Exercise modifies the oxidative stress depending on the intensity, such as high intensity leads to greater production of reactive oxygen species (ROS), whereas low intensity leads to lower production of ROS (Clanton et al., 1999). However, long term endurance exercise (e.g., 12 weeks) leads to increase production of antioxidant enzymes due to the muscle adaptations (Vincent et al., 1999). In our study, we observed that HMOX1 was significantly hyporesponsive to LPS in the moderate-high intensity exercise groups. HMOX1 gene is known to play an important role in antioxidant defense system. An in vivo mice study in which endotoxemia was used as stimulus, demonstrated hypersensitivity in response to oxidative stress in the HMOX1 deficient mice (Poss & Tonegawa, 1997).

5.4 Limitations: Although, our findings suggested exercise induced changes in chromatin accessibility in the nearest genes of those accessible regions. However, we did not necessarily confirm the type of exercise induced epigenetic modifications. Furthermore, we did not validate if changes in chromatin accessibility are affecting the protein expression level of the genes.

5.5 Future Directions and recommendations: To further study the epigenetic modifications (such as DNA methylation, histone methylation and acetylation), transcription factor binding sites should be studied which might be responsible for the increase or decrease in gene expression. To study the genome wide interaction between transcription factor and the DNA, ChIP-Seq (Chromatin Immunoprecipitation) is suggested. In addition, western blotting is also recommended to study the protein expression of genes involved in immunoregulatory and metabolic pathways.

Notably, we conducted forced treadmill exercise in this study to examine the exercise mediated benefits in BMDMs. To further study the benefits of exercise, voluntary wheel exercise will be conducted in our lab. The advantage of voluntary wheel running exercise over the forced treadmill exercise will be to avoid any stress related factors that might be caused by forced training.

Additionally, we recommend studying the exercise mediated benefits in diverse array of populations in human beings (such as healthy young and older people; and young and older people with metabolic diseases) by employing the exercise intensities in our study. This will help us further to understand to understand the exercise mediated genome wide epigenome related changes in other species.

5.6 Conclusion: In support of our hypothesis, we have established in the mice model that moderate-high intensity exercise results in differentially accessible chromatin regions in BMDMs. We suggested that exercise caused innate memory in BMDMs which resulted in reduction of inflammatory responses in the macrophages after stimulating with microbial ligand (e.g., LPS).

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Appendix

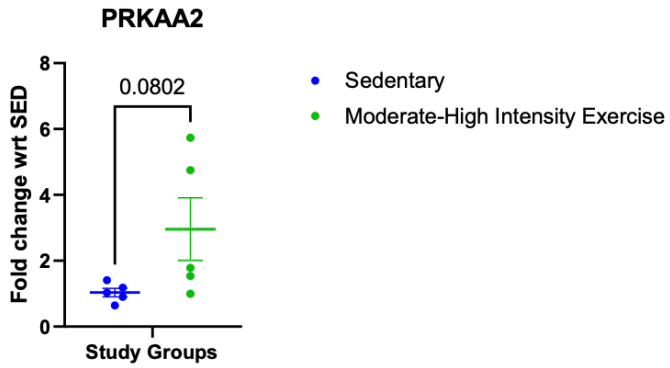

Figure 1: Real-time qPCR expression of **PRKAA2** (Protein Kinase AMP-Activated Catalytic Subunit Alpha 2). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

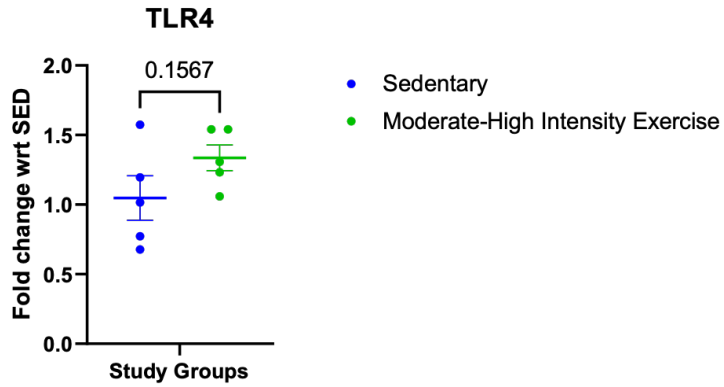

Figure 2: Real-time qPCR expression of **TLR4** (Toll like receptor 4). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

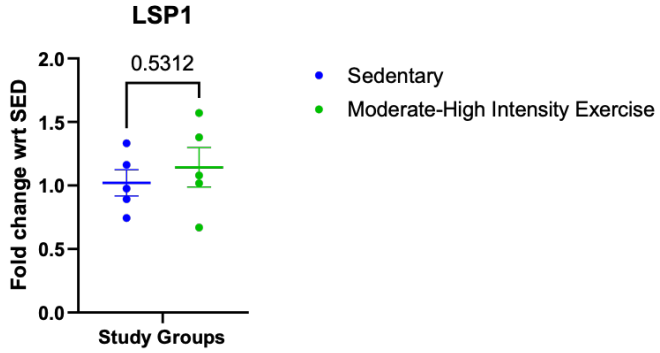

Figure 3: Real-time qPCR expression of **lSP1** (Lymphocyte specific protein 1). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

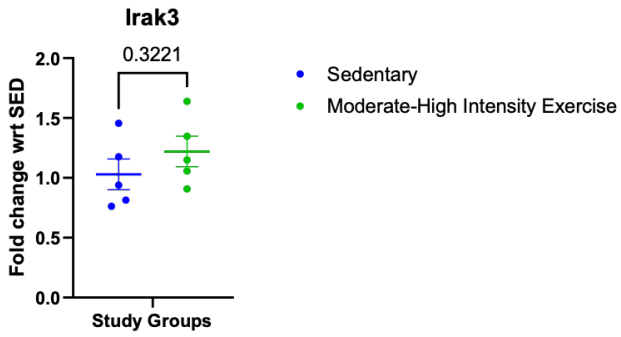

Figure 4: Real-time qPCR expression of **IRAK3** (Interleukin 1 Receptor Associated Kinase 3). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

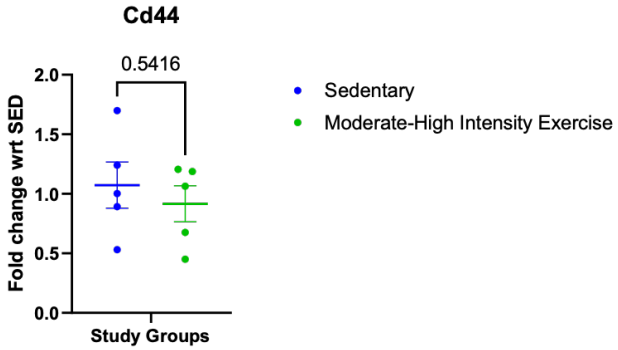

Figure 5: Real-time qPCR expression of **Cd44** (Cluster of Differentiation 44). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

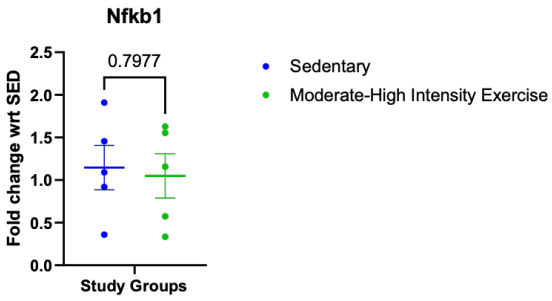

Figure 6: Real-time qPCR expression of **Nfkb1** (Nuclear Factor Kappa B Subunit 1). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

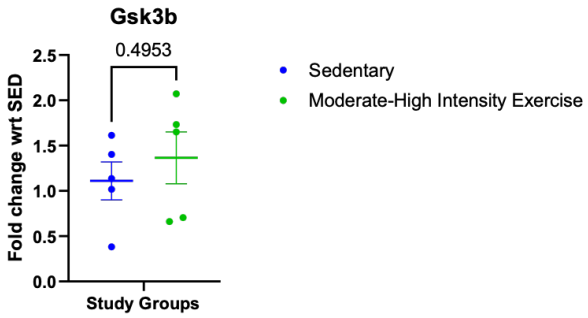

Figure 7: Real-time qPCR expression of **Gsk3b** (Glycogen synthase kinase 3 beta). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

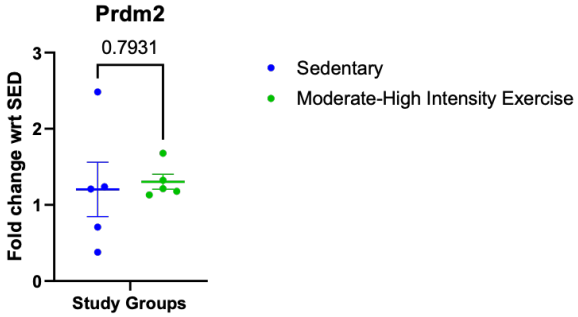

Figure 8: Real-time qPCR expression of **Prdm2** (PR/SET Domain 2). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

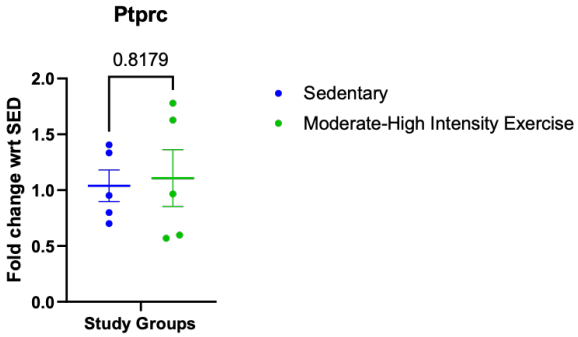

Figure 9: Real-time qPCR expression of **PTPRC** (Protein Tyrosine Phosphatase Receptor Type C). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

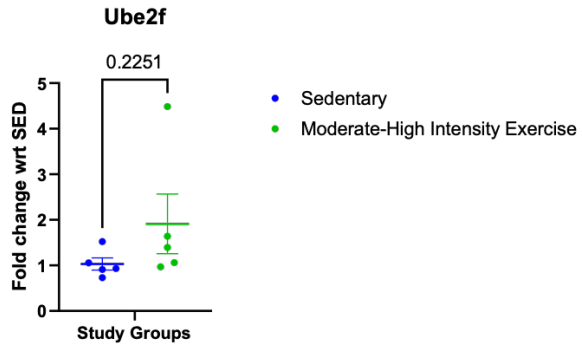

Figure 10: Real-time qPCR expression of **Ube2f** (Ubiquitin Conjugating Enzyme E2 F). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

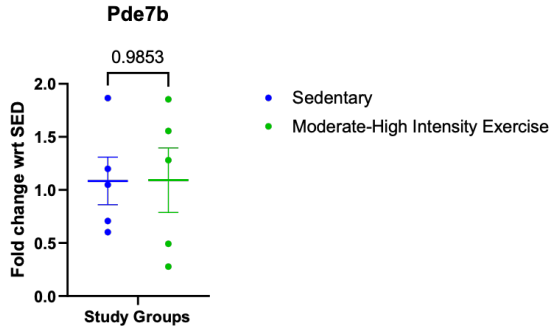

Figure 11: Real-time qPCR expression of ***Pde7b*** (Phosphodiesterase 7b). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

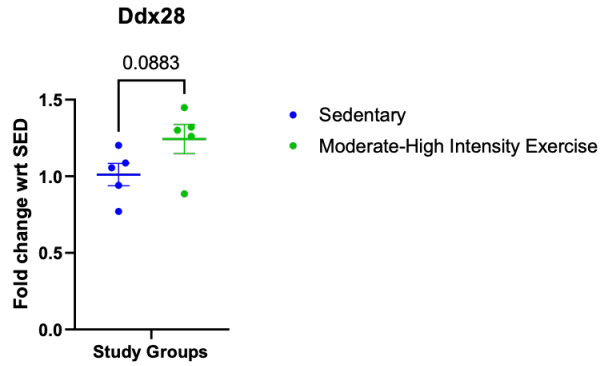

Figure 12 : Real-time qPCR expression of **Ddx28** (DEAD-Box Helicase 28). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

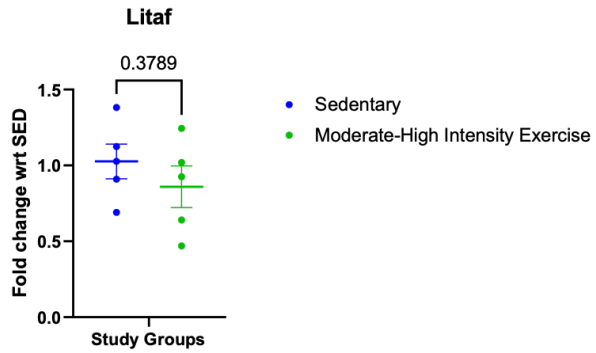

Figure 13: Real-time qPCR expression of **LITAF** (Lipopolysaccharide Induced TNF Factor). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

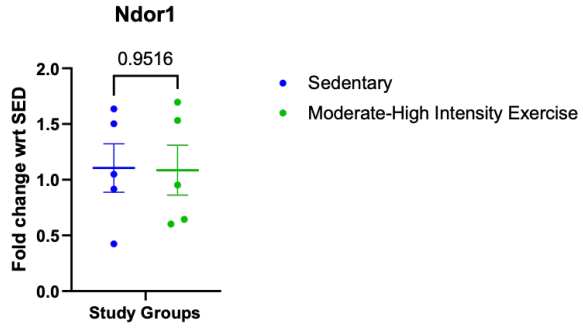

Figure 14: Real-time qPCR expression of **NDOR1** (NADPH Dependent Diflavin Oxidoreductase 1). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

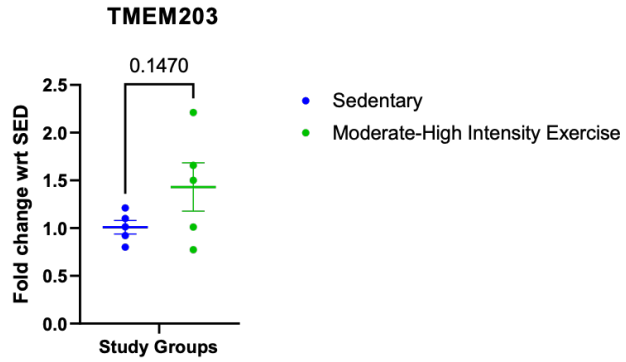

Figure 15: Real-time qPCR expression of **TMEM203** (Transmembrane Protein 203). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

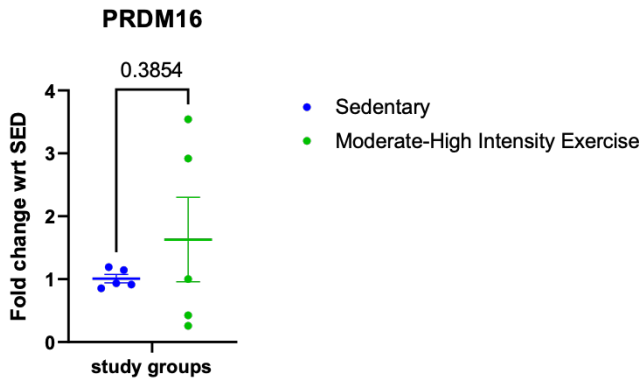

Figure 16: Real-time qPCR expression of **PRDM16** (PR/SET Domain 16). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

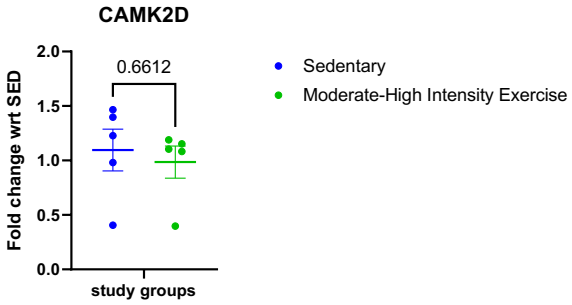

Figure 17: Real-time qPCR expression of **CAMK2D** (Calcium/Calmodulin Dependent Protein Kinase II Delta). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

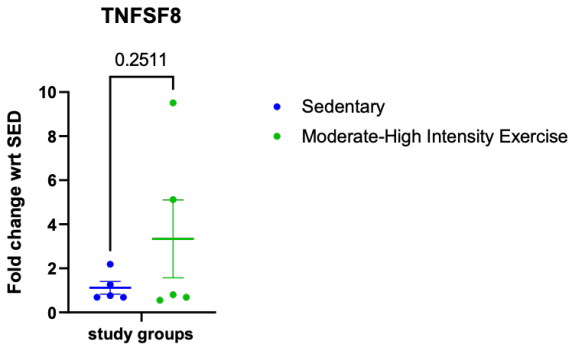

Figure 18: Real-time qPCR expression of **TNFSF8** (TNF Superfamily Member 8). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

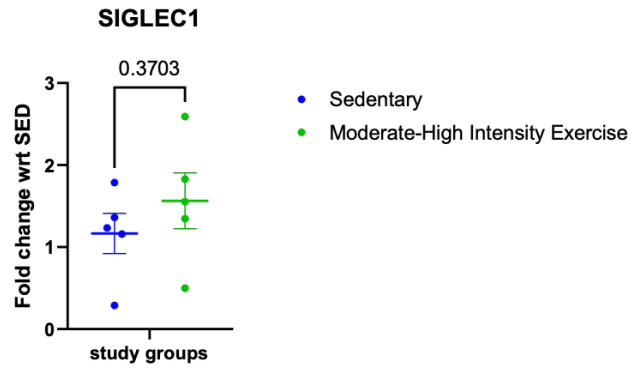

Figure 19: Real-time qPCR expression of **SIGLEC1** (Sialic Acid Binding Ig Like Lectin 1). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

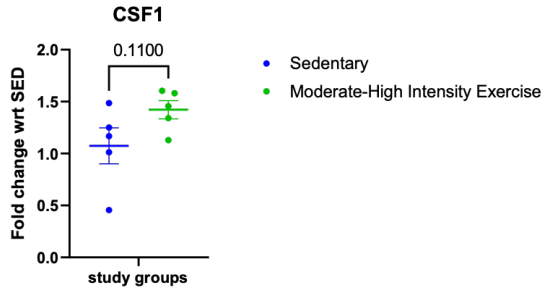

Figure 20: Real-time qPCR expression of **CSF1** (Colony Stimulating Factor 1). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

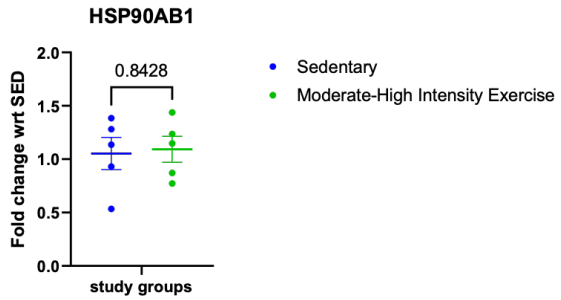

Figure 21: Real-time qPCR expression of **HSP90AB1** (Heat Shock Protein 90 Alpha Family Class B Member 1). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

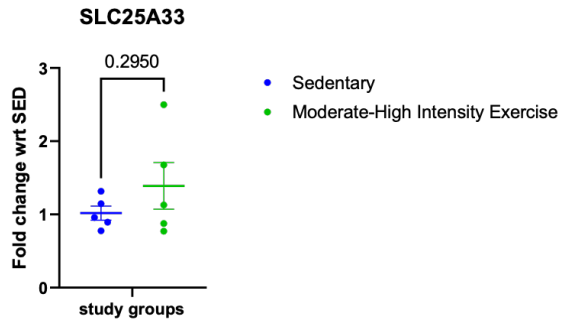

Figure 22: Real-time qPCR expression of **SLC25A3** (Solute Carrier Family 25 Member 3). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

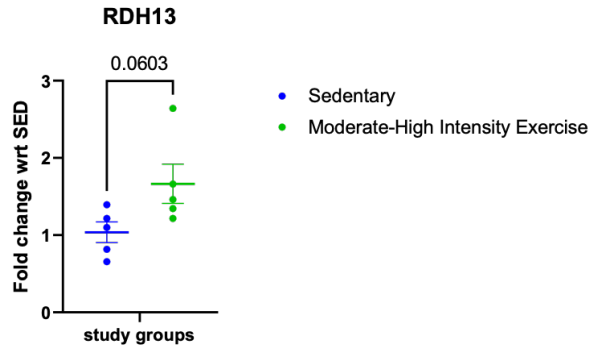

Figure 23: Real-time qPCR expression of **RDH13** (Retinol Dehydrogenase 13). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

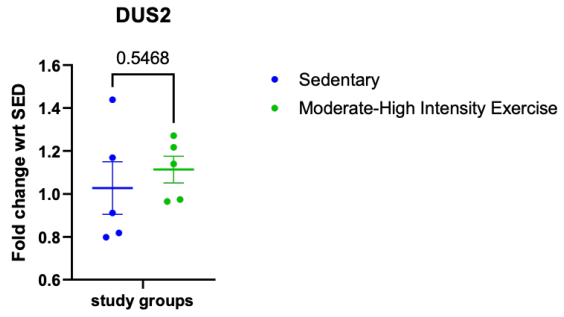

Figure 24: Real-time qPCR expression of **DUS2** (Dihydrouridine Synthase 2). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.

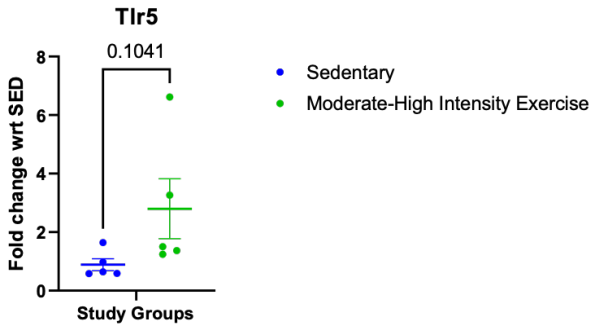

Figure 25: Real-time qPCR expression of **Tlr5** (Toll like receptor 5). Fold change is shown with respect to (wrt) sedentary group in controls of each study group. Dots represent the number of mice; n=5. Data are shown in +/- SEM, comparison between groups was assessed by unpaired t-test where $p<0.05$.