MECHANISTIC INSIGHTS INTO THE EFFECTS OF EXERCISE ON INFLAMMATORY RESPONSES.

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ABSTRACT

Despite the importance of the inflammatory response in fighting infections, prolonged or unwanted inflammation is the root cause for serious human diseases including atherosclerosis and cancer. Therefore, understanding how inflammation is regulated is crucial for preventing these diseases. The objectives of this study include gaining an in-depth understanding of the molecular pathways and the regulatory elements of the inflammatory response that are altered following long-term exercise in mice. C57/Bl6 mice were randomly assigned into a control group and an exercise group. Various modes of exercise training programs were performed by the exercise group. BMDMs were isolated and were then stimulated with several inflammatory inducers. Our data from qPCR and Western Blotting demonstrate that NF-κB mediated genes have been downregulated following moderate exercise and up-regulated following high intensity exercise. In addition, IRF3 mediated genes are differentially regulated following both modes of exercise. This indicates that moderate exercise reduces inflammation and high intensity exercise ug-regulates it, which might lead to improved immune responses to certain infections or faster resolving of inflammation in prolonged inflammatory conditions.

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LIST OF ABBREVIATIONS

BMDM – Bone Marrow Derived Macrophages

BSA – Bovine Serum Albumin

CARD – Caspase Recruitment Domains

cGAMP – Cyclic Guanosine Monophosphate–Adenosine Monophosphate

cGAS – Cyclic Guanosine Monophosphate–Adenosine Monophosphate Synthase

CDN – Cyclic Dinucleotides

cDNA – Complementary Deoxyribonucleic Acid

DC – Dendritic Cells

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribonucleic Acid

dsDNA – Double-Stranded Deoxyribonucleic Acid

dsRNA – Double-Stranded Ribonucleic Acid

DTT – Dithiothreitol

EDTA– Ethylenediaminetetraacetic Acid

ER – Endoplasmic Reticulum

FBS – Fetal Bovine Serum

GPPS – Glutamine, sodium Pyruvate (NaPy), Penicillin and Streptomycin

HCL – Hydrochloric Acid

IFN – Interferon

IκB – Inhibitor of Nuclear Factor Kappa-B

IKK – Inhibitory Kappa-B Kinase

IL-1 – Interleukin-1

IL-1β – Interleukin-1-Beta

IL-4 – Interleukin-4

IL-6 – Interleukin-6

IL-8 – Interleukin-8

IL-12 – Interleukin-12

IL-18 – Interleukin-18

iNOS – Inducible Nitric Oxide Synthase

IRAK – IL-1R-Associated Kinase

LCM – L929 Conditioned Media

LPS – Lipopolysaccharides

LRR – Leucine-Rich Repeat-containing Proteins

MAPK – Mitogen-Activated Protein Kinase

MAVS – Mitochondrial Antiviral Signaling Protein

MDP – Bacterial Muramyl Dipeptide

M-MuLV – Moloney Murine Leukemia Virus

MyD88 – Myeloid Differentiation Primary Response Protein-88

NaCl – Sodium Chloride

NEAA – Non-Essential Amino Acid

NF-κB – Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B-Cells

NK – Natural Killer Cells

NLR – NOD-like Receptors

NOD – Nucleotide-binding Oligomerization Domain

NP – Nonionic Polyoxyethylene

PAMP – Pathogen-Associated Molecular Patterns

PBS – Phosphate Buffered Saline

PCR – Polymerase Chain Reaction

Poly dA:dT – Poly (deoxyAdenylic-deoxyThymidylic)

Poly I:C – PolyInosinic–polyCytidylic

PRR – Pattern Recognition Receptors

RHD – Rel Homology Domain

RICK – Receptor-Interacting Serine/Threonine Kinase

RIG-I – Retinoic acid-Inducible Gene I

RIP2 – Receptor Interacting Protein-2

RLR – RIG-I-Like Receptors

RNA – Ribonucleic Acid

RPM – Revolutions Per Minute

SDS-PAGE – Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

STING – Stimulator of Interferon Genes

TAK1 – TGF-β Activated Kinase

TBK1 – TANK-Binding Kinase-1

TBST – Tris-Buffered Saline and Tween

TGFβ – Transforming Growth Factor Beta

TIR – Toll-IL-1-Receptor

TLR – Toll-like Receptors

TNFα – Tumor Necrosis Factor-Alpha

TRAF6 – TNF Receptor-Associated Factor 6

URTI – Upper Respiratory Tract Infections

WT – Wild Type

CHAPTER 1

INTRODUCTION

1.1) Innate immunity and inflammation

The prevalence of cancer and autoimmune diseases such as rheumatoid arthritis continues to grow worldwide. Such conditions are significantly correlated with chronic inflammation, which among others, can be caused by biochemical and metabolic inducers, and the effects include further complications such as insulin resistance (such as diabetes), cardiovascular disease (responsible for 31% of all deaths globally), autoimmune disorders, allergies and dementia. Inflammation is a key factor in protecting hosts from invading pathogens. However, over-activation or unwanted activation of the innate immune system can lead to chronic and aberrant inflammation. This can be indicated by an elevation in pro-inflammatory cytokines like tumor necrosis factor-alpha (TNF-alpha), interleukin-1 beta (IL-1beta), interleukin-6 (IL-6), and interleukin-8 (IL-8) (Gleeson et al., 2011).

Innate immunity is a non-specific sub-system of the overall immune system that is responsible for protecting the host from disease causing-organisms and neoplasms (Iwasaki & Medzhitov, 2010). This system involves transient and immediate defensive mechanisms against pathogens by various means including physical barriers to pathogens, inflammation, and the complement system (Iwasaki & Medzhitov, 2010). The immune cells involved in innate immunity include circulating monocytes that will differentiate into resident macrophages in tissues, which are responsible for digesting the invading organisms; phagocytes that also consume and destroy bacteria; neutrophils; basophils; natural killer cells (NKs); and dendritic cells (DCs) (Iwasaki & Medzhitov, 2010). These immune cells produce distinct types of chemical mediators called

cytokines, which are small signalling molecules in charge of communication between cells and recruitment of other immune cells to the site of infection (Iwasaki & Medzhitov, 2010).

1.2) Intracellular innate immune responses

The innate immune response relies on recognition of certain molecular signatures or patterns from pathogens, termed pathogen-associated molecular patterns (PAMPs), through a limited number of germ line-encoded pattern recognition receptors (PRRs) that include Toll-like receptors (TLRs), NOD-like receptors (NLR), RIG-I-like receptors (RLR) and Stimulator of Interferon Genes (STING) (Mogensen, 2009).

1.2.1) Toll-like receptors (TLRs)

The extracellular domain of TLR pathway, leucine-rich repeat (LRR) is able to sense PAMPs and the intracellular toll/interleukin receptor (TIR) relays the signal by interacting with myeloid differentiation primary response protein (MyD88), which initiates an intracellular signal through Nuclear Factor kappa-light-chain-enhancer of activated B cells (NF-**κ**B). Typically, NF-κB is structured as a p50-p65 dimer. The formation of these protein dimers is essential for their translocation to the nucleus and DNA binding (Krawczyk, C. M. et al., 2010). The NF-κB complex is composed of 5 proteins including p65/RelA, RelB, c-Rel, p105/p50, and p100/p52, which are also called NF-κB/Rel proteins due to their shared structural homology. NF-**κ**B is mostly inactive and stay in cytoplasm when normal body cells are at resting state. NF-**κ**B activation in the cells requires the separation of its proteins from their inhibitors, which are called I**κ**B proteins and include I**κ**Bα, I**κ**Bβ and I**κ**Bγ. The binding between the I**κ**B proteins and the Rel homology domain (RHD) of NF-**κ**B will result in an interference with the nuclear localization sequence (NLS) of this nuclear transcriptional factor, which causes the Rel proteins to be retained in the

cytoplasm (Pandey, Kawai, & Akira, 2015). As mentioned, the activation of NF-**κ**B requires an intracellular signal that comes from the binding between the TLRs and their molecular ligands such as LPS. This signal will activate I**κ**B kinase (IKK) complex (heterodimer of IKKα and IKKβ and IKK), the IL-1R-associated kinases 1, 2 and 4 (IRAK1, 2 and 4), TNF receptor-associated factor 6 (TRAF6), and the TGF-β activated kinase 1 (TAK1). The activation of IKK complex will catalyze the phosphorylation and subsequent degradation of I**κ**Bs, which leads to the translocation of the NF-**κ**B dimers to the nucleus, DNA binding and activation of certain down-stream gene transcription, which include among others, what is known as pro- and anti-inflammatory cytokines, such as TNFα, IL-1β, IL-10, iNOS and TGFβ (Kondo, Kawai, & Akira, 2012). It is important to note that TLR4 is a unique member of these receptors in its ability to induce not only NF-**κ**B, but also another important nuclear transcriptional factor called Interferon Regulatory Factor 3 (IRF3). Interestingly, the activation of endosomal TLR3 can initiate a signal though IRF3 to promote the production of type I IFNs (Fig. 1.1).

1.2.2) Nod-like receptors (NLRs)

NLRs are a family of pattern recognition receptors that include NODs (NOD1 and NOD2), and other NLRPs. NLR pathway can be activated upon stimulation with bacterial muramyl dipeptide (MDP), which interacts with the leucine-rich repeat-containing proteins (LRRs) of NOD1 and NOD2, which leads to the interaction with the adaptor molecule RICK (RIP2) via Caspase recruitment domains (CARD-CARD) (Abdul-Sater, Koo, Häcker, & Ojcius, 2009). This will generate an intracellular signal though other components of NF-kappa -B and MAPK pathway, which will also promote the production of pro- and anti-inflammatory cytokines (Girardin et al., 2003) (Fig. 1.1).

1.2.3) Retinoic acid-inducible gene-I-like receptors (RIG-I)

RIG-I pathway can be stimulated by a viral double-stranded RNA (dsRNA) or a double-stranded DNA (dsDNA), which interacts with the serine/threonine-protein kinase (TBK1) to initiate an intracellular signal that phosphorylates interferon regulatory factor-3 (IRF3), which then can translocate to the nucleus and induce the expression of type I IFNs (e.g. IFN-β and IFN-αs) (Yoneyama & Fujita, 2007) (Fig. 1.1).

1.2.4) cGAS–STING pathway

One of the most important signalling molecules associated with the Endoplasmic Reticulum (ER) is STING, which also plays an essential role in regulating the transcription of certain host defence genes, such as IFNs (Ishikawa & Barber, 2008). The transmembrane homodimer of STING that is connected to the ER will bind cyclic dinucleotides (CDNs), including 3'3'-cGAMP, which subsequently induce TBK1-IRF3-dependent production of IFN-β and other bacterial secondary messengers (Burdette et al., 2011). This interaction will initiate the translocation of STING to the Golgi Apparatus, which will activate TANK-binding kinase 1 (TBK1) and IRF3 through a series of intracellular mechanism that are phosphorylation-dependent (Liu et al., 2015). Interestingly, signalling molecule, STING also plays a role in the activation of NF-**κ**B, which induces the transcription of numerous pro- and anti-inflammatory cytokines (Abdul-Sater, A.A. et al. 2013) (Fig. 1.1).

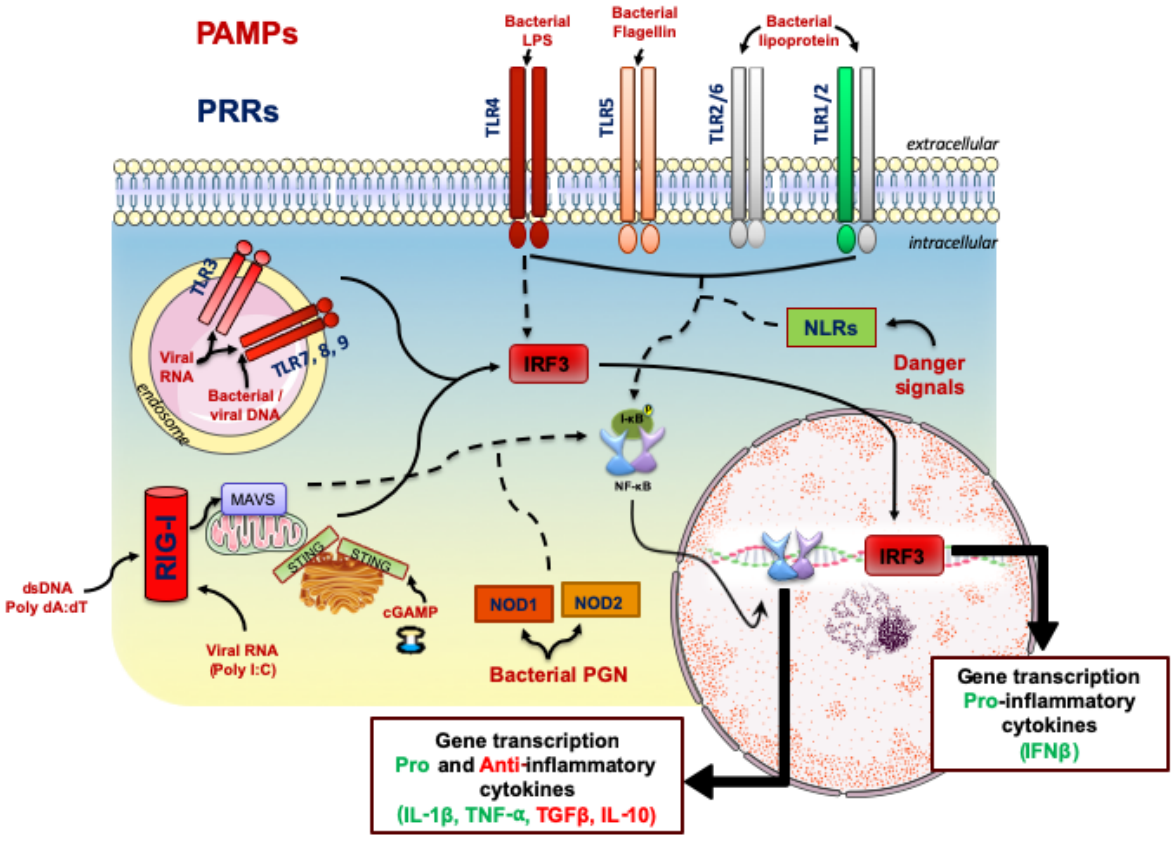

Figure 1.1- Summary of Intracellular Innate Immune Responses. The innate immune system employs a variety of germ-line encoded receptors termed PRRs, which can be membrane bound, such as the toll like receptors or TLRs, or intracellular such as the RIG-like, the NOD-like and STING receptors. These PRRs, as their name indicate recognize certain molecular signatures or patterns from pathogens, called PAMPs. Upon stimulation of these PRRs, a series of intracellular signaling events are initiated which leads to the transcription of a large number of genes, which include among others, what is known as pro- and anti-inflammatory cytokines that help in the clearance of infections (*Modified from A. Abdul-Sater*).

1.3) The effect of exercise on innate immunity and inflammation

The association between exercise and innate immunity has been receiving a lot of well-deserved attention in the past decade due to its increasingly clear implications for health and wellness (Mathis & Shoelson, 2011). A great body of research has established that physical activity can attenuate the cytokines that are involved in disease-promoting inflammation (Lira et al., 2014). Pro-inflammatory cytokines, such as IL-8, TNF-α and IL-1, initiate the immune response, so these

cytokines are increased in the initial step of inflammation (Hahn et al., 2014). However, the anti-inflammatory cytokines (IL-4 and IL-10) are produced at a later time for down-regulation of inflammation (Steensberg, Fischer, Keller, Møller, & Pedersen, 2003). Low-grade inflammation disrupts this response, and the pro-inflammatory mediators keep increasing, which leads to serious human diseases including diabetes, cardiovascular diseases, autoimmune diseases and cancer (Jager, Grémeaux, Cormont, Le Marchand-Brustel, & Tanti, 2007; Spranger et al., 2003). Exercise training, such as aerobic and resistance exercise has been shown to be an effective intervention for reducing this low-grade inflammation as it produces an anti-inflammatory effect by increasing the circulating levels of the steroid hormone cortisol, and the anti-inflammatory interleukins (such as IL-6, IL-10, and IL-1ra) (Spranger et al., 2003). Together, these exercise-stimulated cytokines inhibit IL-8, TNF-α and IL-1β, thereby down-regulating the inflammatory response (Petersen & Pedersen, 2005). However, how exercise affects the intracellular pathways that initiate the inflammatory response has not been explored.

A number of mechanisms through which exercise training reduces chronic inflammation have been identified. The muscle tissue activity stimulates release of muscle-derived anti-inflammatory cytokines ("myokines"), which in turn have an effect on favorable macrophage cytokine production (Pedersen, 2009). Physical activity also reduces the adipose tissue hypoxia that induces macrophage pro-inflammatory gene expression, phenotype, and functional changes (You et al., 2013; Pedersen, 2009). Most significantly, physical activity reduces cytokine production systemically, by lowering the number of pro-inflammatory cells and reducing overall pro-inflammatory cytokine production. (Norata et al., 2015; You et al., 2013; Johnson et al., 2012). Nevertheless, most of these studies investigated the effects on inflammation immediately after

exercise training has ended. Any potential long-term or long-lasting effects of exercise on inflammation has not been properly studied.

The implications of exercise's influence on innate immunity is a relatively new field of study and is still in its infancy. Many areas of study need to be covered before it can begin bearing fruit in terms of clinically significant therapies. This makes it a rich field of study for researchers. Therefore, the purpose of this study is to cover the areas that remains to be investigated, for example, understanding how exercise affects the various intracellular inflammatory pathways and alters infection risk as well as uncovering the long-term and long-lasting effect of exercise on inflammatory responses.

1.3.1) The effect of exercise on infection risk

The interest in this field intensified when researchers found a relationship between exercise training load and infection risk, in particular upper respiratory tract infections (URTI; e.g. coughs and colds, influenza, sinusitis, tonsillitis, other throat infections and middle ear infections). Initial evidence suggested that a "J-shaped" model best outlines the relationship between exercise intensity/duration and infection risk, particularly URTI (Fig. 1.2) (Nieman, 1994). In this model, the average risk and severity of URTI decreases when people participate in regular moderate exercise compared to those with a sedentary lifestyle, but increases with acute bouts of prolonged, intensive training or heavy volumes of training usually endured by elite athletes. Importantly, these effects of exercise intensity on infection risk hold true even after accounting for confounding factors such as weight, age, gender, stress, diet and body mass index. While multiple factors (e.g. stress, nutritional status, sleep) may account for the higher URTI incidences experienced by some athletes and lower number of illnesses in individuals that exercise moderately on a regular basis, changes in immune function has been touted as a main contributor to these effects.

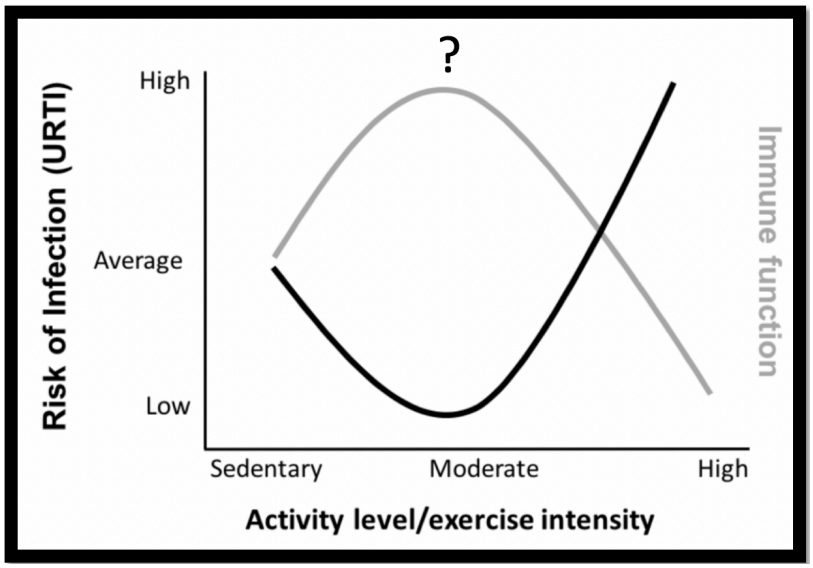

Figure 1.2- "J-shaped" Model. Relationship between exercise intensity/duration and infection risk, particularly URTI (*Modified from Nieman*).

Exercise have an effect on both the innate and adaptive arms of the immune response. While prolonged or intensive training regimens generally cause a depression in the immune response, the effect of moderate exercise intensity on immunity and inflammation is more complex (Walsh et al., 2011). Some studies showed that following moderate exercise training, there is an increase in the number of circulating immune cells, increase in natural killer (NK) cytolytic activity, enhancement in neutrophil respiratory burst, and an increase in salivary IgA (sIgA) levels. On the other hand, studies demonstrated that regular bouts of moderate exercise induce an anti-inflammatory state (Gleeson, Bishop, & Walsh, 2013). Some studies showed that this might be attributed to increased levels of anti-inflammatory cytokines from skeletal muscles, reduced expression of TLRs, decrease in monocyte and macrophage infiltration and potential changes in transcription factors (Gleeson et al., 2011). Therefore, an optimized exercise training program could potentially strike a fine balance between providing protection from URTI but, without the risk of exuberant inflammation and the diseases that are associated with it. In order to do so, one

needs to improve their anti-viral responses by improving the intracellular signalling pathway associated with IRF3 nuclear transcriptional factor and IFN-I gene expression levels, while lowering NF-**κ**B mediated inflammation.

1.4) Research Objectives

The objectives of this thesis were four-fold:

1. Identifying long-term and long-lasting effects of moderate and vigorous intensity exercise on inflammatory responses
2. Revealing the molecular mechanisms through which distinct modes of exercise affect various intracellular inflammatory pathways
3. Designing an optimized exercise that might lower infection risk while preventing excessive inflammation.
4. Determining the cumulative effects of physical activity on the relevant cytokine levels. This will include the pro-inflammatory cytokines, and the anti-inflammatory cytokines.

1.5) Hypotheses

The identification of the immunological effects of physical activity has intensified academic interest in its long-known therapeutic effects. Therefore, dedicating further studies to how the frequency, intensity, duration, and mode of physical activity affects the activation of immune system and the resulting dysfunctional inflammation is significant (Norata et al., 2015; You et al., 2013; Johnson, Milner, & Makowski, 2012). One of the targets of research on the association between exercise and inflammation and one that has not yet been established – is to identify how exercise reduces this inflammation, and how to measure its effects.

It was hypothesized that various forms of exercise will have distinct long-term and long-lasting effects on different inflammatory responses, and that these inflammatory responses will be increased against viral PAMPs, while such responses will be down regulated against other PAMPs.

The research literature is virtually devoid of studies examining the dose-effect of physical activity on dysfunctional inflammation, despite the clear implications for clinical use of this information. This study will contribute to filling that gap.

CHAPTER 2

Experimental Procedures

2.1) *Animals and Housing*

5-week-old wild-type (C57BL/6) female mice were obtained from the Charles Rivers Laboratory**.** The mice were maintained in York University Animal Care facility, which is approved by the Canadian Council on Animal Care (CCAC) and are kept in polycarbonate microisolator cages and were given food and water on daily basis.

2.2) *Moderate Intensity Animal Training Protocol*

For the first *in-vivo* experiment, 18 mice were randomly assigned to one of the following two groups following an initial one-week acclimatization period: 1) a control group where no exercise was performed, and 2) a group of 7-week old mice, where a single bout of moderate exercise (i.e. 20 m/min-30 m/min for 60-min on a treadmill) was carried out once a day for 5 days per week for a total of 8 weeks. The intensity of the exercise was increased by 10% on weekly basis and was set at the maximum of 30 m/min on week 5. It is important to note that this speed corresponds to a moderate intensity exercise (65-70% of VO_{2max}) (Fig. 2.1)

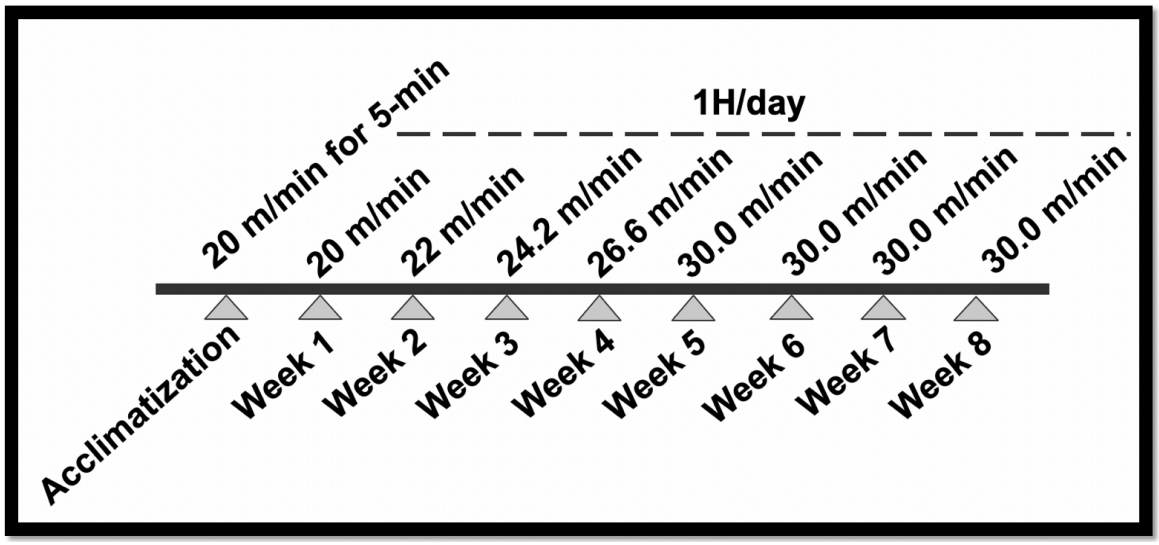

Figure 2.1- Moderate Intensity Animal Training Protocol. This is the standard moderate intensity training protocol implemented in Dr. Abdul-Sater's lab using a standardized duration and intensity of exercise after an initial one-week acclimatization period.

2.2.1) *High Intensity Animal Training Protocol*

For the second *in-vivo* experiment, 8 mice were randomly assigned to one of the following two groups: 1) a control group where no exercise was performed, and 2) a group of 7-week old mice, where a single bout of intensive exercise (i.e. 20 m/min-36.3 m/min for 60 min on a treadmill) was carried out once a day for 5 days per week for a total of 7 weeks. The intensity of the exercise was increased by 10% on weekly basis and was set at the maximum of 36.3 m/min. It is important to note that this speed corresponds to a high intensity exercise (85-90% VO_{2max}) (Fig. 2.2).

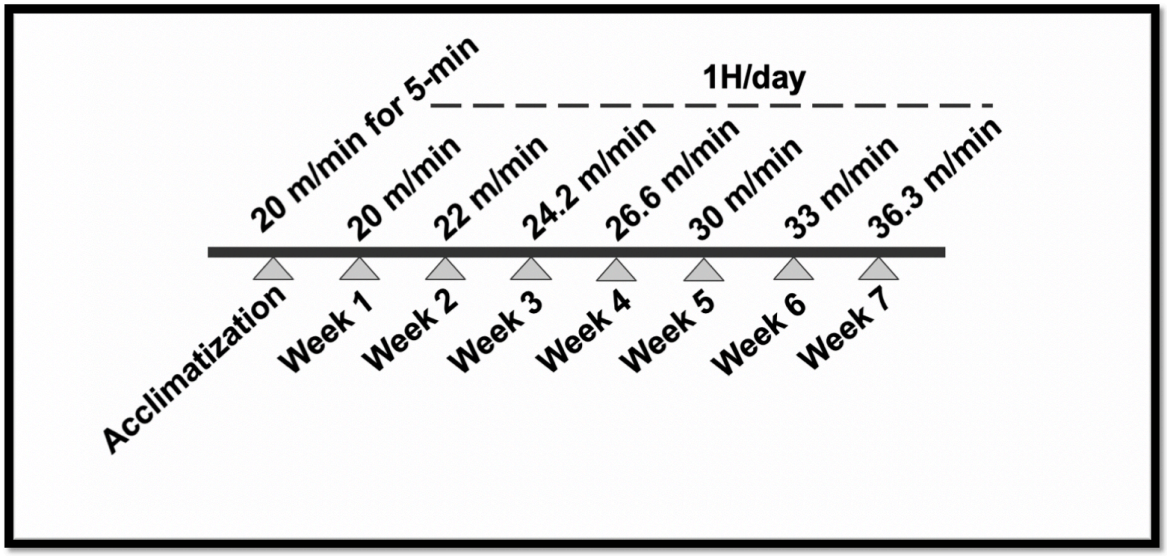

Figure 2.2- High Intensity Animal Training Protocol. This is the standard high intensity training protocol implemented in Dr. Abdul-Sater's lab using a standardized duration and intensity of exercise after an initial one-week acclimatization period.

2.3) *Preparation of Growth Media*

The culture medium that was used for the growth and differentiation of all the bone marrow derived macrophages (BMDM) was made using 500 ml 1x Roswell Park Memorial Institute (RPMI) 1640 media, 50 ml 10% Fetal Bovine Serum (FBS), 5 ml 100x Non-Essential Amino Acids (NEAA), 5 ml 100x Glutamine, sodium Pyruvate (NaPy), Penicillin and Streptomycin (GPPS), and 550 µl 1000x 2-Mercaptoethanol to a final volume of 560.55 ml. The medium was sterilized by the manufacturer.

2.3.1) *Preparation of L929 Conditioned Media*

The conditioned medium used for the growth of the BMDMs, was supplemented with 25% of L929 Condition Media (LCM). LCM contains the growth factor M-CSF, which is required for the differentiation of bone marrow macrophages. In order to prepare this media, L929 cells (mouse

fibroblast stromal cell lines) were grown to confluency in 10 cm tissue culture dishes containing DMEM and 10% FBS. Media was then changed, and cells were grown for 7 days after which supernatant was collected, span down at 1500 RPM for 5-min and then filtered through 0.2 Microns.

2.3.2) ***Preparation of NP-40 Lysis Buffer***

1x NP-40 lysis buffer that was used for the homogenization of the bone marrow derived macrophages (BMDM) was made using 2x NP-40 lysis buffer (100 mM Tris-HCL, 300 mM NaCl, 2% NP-40, and 10 mM EDTA), 5N NaCl, 1M DTT, Protease Inhibitor Cocktails (EDTA Free), 100x Phosphatase Inhibitor Cocktail and ddH_2O.

2.4) ***Harvest of Bones from Mice***

Upon completion of the exercise training program, mice were euthanized using Isoflurane overdose followed by cervical dislocation and were sprayed by 70% ethanol. Euthanized mice were then placed on a dissection board and a small incision was made on the abdomen skin using sterile dissecting scissors. Lower abdomen and the legs were exposed after the skin was peeled back towards the feet. In order to remove the feet, a cut was made below the ankle joints and femur head was separated from the hipbone. Muscles and tendons from tibias, femurs and iliac bones were removed and bones (femur and tibia) were placed in culture media on ice to be transferred to a sterile culture hood for the remaining steps of isolation.

2.4.1) ***Bone-Marrow Macrophages Isolation and Cell-Culture***

10 mL syringes containing culture media were prepared prior to bone-marrow flushing. 27-gauge needles were fixed on the syringes and 2.5 mL of media per bone was used to flush the bone-marrow into the labeled conical tubes for each mouse. These tubes were centrifuged at 1,500 RPM for 5 m at 4 °C, supernatants were removed and cells in each tube were incubated with 2 mL

of RBC lysis buffer Hybri-Max for 5-min at room temperature. Immediately after the incubation, 5 mL of culture media was added to each tube before centrifuging them for the second time at 1500 RPM for 5-min at 4 °C. Following the removal of the supernatant, the white pellet was suspended in 4 mL of culture media. 8 x 100 mm bacterial culture petri dishes per mouse were prepared and 500 µL of each sample, 4 mL of culture media and 1 mL of L929 conditioned media were then added to each dish. The dishes now containing bone marrow macrophages were then incubated and cultured for 3 days at 37 °C in an incubator with 5% CO_2. On day 3, bone-marrow macrophages were scraped from the plates and the number of cells were calculated. 0.75 x 10^6 cells for western blotting treatments and 1.0 x 10^6 cells for gene expression treatments were plated for the second time on 6-well tissue culture dishes along with the addition of culture media and L929 conditioned media prior to the incubation for another 3-4 days.

2.5) ***Cell Stimulation (Gene Expression)***

On day 6, bone-marrow macrophages were induced with 100 ng/ml Lipopolysaccharides (LPS) for 1, 3, 6 and 24 hrs, 100 ng/ml 3'3'-cGAMP for 1, 3 and 6 hrs, 10 µg/ml transfected Poly (I:C) for 3, 6 and 24 hrs and lastly 10 µg/ml transfected Poly (dA: dT) for 3 and 6 hrs. Lipofectamine 3000 with the addition of P3000 reagent were used for transfection. Then, BMMs were washed with 1x Phosphate Buffered Saline (PBS) and homogenised in TRIzol reagent. It is important to note that in this study, Bacterial and Viral PAMPs were used instead of infectious diseases, due to the fact that infections are able to activate a large number of inflammatory

pathways at the same time, therefore we have used certain PAMPs, which could only target and activate specific inflammatory pathways.

2.5.1) *Cell Stimulation (Western Blotting)*

On day 7, bone-marrow macrophages were stimulated with 100 ng/ml LPS for 15, 30, 60 and 120 min, 10 µg/ml Naked Poly (I:C) for 30, 60 and 120 min, 10 µg/ml transfected Poly (I:C) for 1, 3 and 6 hrs and lastly10 µg/ml transfected Poly (dA:dT) for 2, 4 and 6 hrs. Lipofectamine 3000 with the addition of P3000 reagent were used for transfection. Then, BMDMs were washed once with 1x Phosphate Buffered Saline (PBS) and homogenised in NP-40 lysis buffer.

2.6) *RNA Isolation and cDNA Synthesis*

Bone-marrow derived macrophages were isolated from the leg bones (femur and tibia) and were homogenised in 1 ml TRIzol (Invitrogen) before incubation at room temperature for 10 min. Then, 200 µl of 1-Bromo-3-chloropropane per 1 ml TRIzol was added to the samples, and samples were centrifuged at 14,800 RPM for 15 min at 4 °C. The aqueous part of each sample, which contained RNA, was transferred into a new Eppendorf tube. Then, 500 µl Isopropanol was added to each tube to precipitate RNA, and samples were again centrifuged at 12,000 RPM for 10 min at 4 °C. The supernatant of each sample was discarded, and in order to wash the RNA pellet, 1 ml of 70 % Ethanol was added to each tube and centrifuged at 1000 RPM for 5 min at 4 °C. The RNA pellet was resuspended in Molecular Biology Water. NanoDrop 1000 Spectrophotometer (Thermo Scientific) was used to measure the concentration of the RNA samples.

2.6.1) *Quantitative Real-Time Polymerase Chain Reaction (Q-PCR)*

cDNA was transcribed from 1 µg RNA using standard reverse transcription with M-MuLV Reverse Transcriptase (New England Bio Labs Inc.) and oligo dT primers (Qiagen). Genes of interest were detected using SYBR Green Master mix (Bio-Rad) (Table 2.1), primers and pre-

diluted cDNA (1:20). The quality of these PCR primers in terms of specificity and amplification efficiency was tested. Cq values of technical duplicates from each sample were obtained and normalized to a house-keeping gene (RPLP0) based on a reference gene panel from Bio-Rad laboratories (Fig. 2.3) and relative transcript levels for each gene were calculated using the ΔΔCq method. It is important to note that the real-time PCR Cq threshold for each gene was set by the detection system and software.

Real time PCR included initial denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 10 s and 60 °C for 30 s, and lastly melt curve data was analyzed at 65-95 °C in 0.5 °C increments at 2-5 sec/step (Bio-Rad CFX384 Touch Real-Time PCR Detection System).

Table 2.1- SYBR green primer sequences used for QPCR analysis

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
RPLP0	GGACCCGAGAAGACCTCCTT	GCACATCACTCAGAATTTCAATGG
iNOS	AACGCTTCACTTCCAATG	CAACTCGCTCCAAGATTC
IL-10	ATAACTGCACCCACTTCCCA	GGGCATCACTTCTACCAGGT
IFN-β	TCCAAGAAAGACGAACATTCG	TGAGGACATCTCCCACGTCAA
IL-1β	GCAGCACATCAACAAGAG	CAGCAGGTTATCATC- ATCATC
TNF-α	AGAATGAGGCTGGATAAGAT	GAGGCAACAAGGTAGAGA
TGF-β	CCTGCAAGACCATCGACATG	TGTTGTACAAAGCGAGCACC

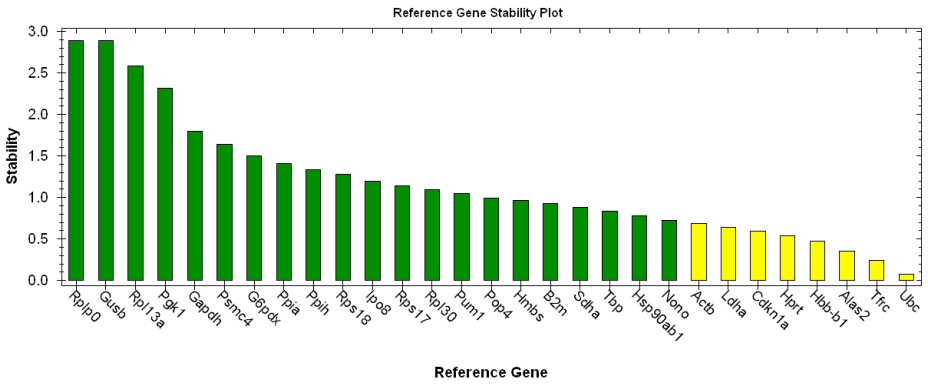

Figure 2.3-Real-time PCR reference gene panel. Reference gene panel was used in this study for the analysis of relative gene expression. Samples were obtained and normalized for their variation in the mRNA amount. The purpose of this analysis was to identify one or more reference genes that are stable and expressed no change in gene expression as a result of treatments. Our data indicates that RPLP0 was the most stable reference gene in real-time PCR analysis.

2.7) *Western Blot Analysis*

Following protein extraction, a BioRad Protein Assay was used to calculate the total protein concentration. Extracted proteins were incubated in Laemmli sample buffer for 5 min at 100 ° C and were resolved on 10% acrylamide SDS-PAGE gels. Blots were then transferred to polyvinylidene difluoride membranes (Bio-Rad). In order to block the blots, 5% (w/v) non-fat dried milk in TBST was added for 1H. Then, blots were incubated with primary antibodies (Table 2.2) in 5% Bovine Serum Albumin (BSA) in TBST at 4 °C overnight. Membranes were washed three times for 10-min with TBST prior to being incubated with secondary antibodies (diluted in 5% skim milk in TBST) for 60 min at room temperature. Membranes were washed again with TBST and signals were detected using an ECL prime® Western blotting detection kit and visualised with ChemiDoc MP Imaging System (BioRad). Western blot stripping was not required due to the fact that the antibodies used were from different species.

Table 2.2- Primary antibodies used for Western blot analysis:

Primary Antibodies	Origin	Dilution	Company	Catalogue no
IκBα	Mouse	1:1000	Cell Signalling	L35A5
Phospho-IRF-3 (Ser396)	Rabbit	1:1000	Cell Signalling	4D4G
β-Actin	Mouse	1:1000	R&D Systems Inc.	MAB8929

Secondary antibodies used: horseradish peroxide conjugated goat anti-rabbit (1: 10000) and goat anti-mouse (1: 10000) (Jackson Immunolabs).

2.8) *Statistical Analyses*

The statistical analysis was performed using GraphPad Prism software (GraphPad Software Inc, La Jolla, CA) by multiple t-test and results were corrected for multiple comparisons using Holm-Sidak method and considered significant at $p < 0.05$. P-value style used was American Psychological Association (APA): .12 (ns), 0.33 (*), .002 (**), < .001 (***).

CHAPTER 3

Results

3.1.1) *The effect of a moderate chronic exercise training program on body weight and mitochondrial content*

In order to confirm the effectivity of a moderate chronic exercise training program, body weight of the mouse models from both, exercise and sedentary groups was measured. As expected, the mean body weight of the exercise group was on average 18% lower compared to the sedentary group following this mode of exercise (23.42 ± 0.7071 to 19.84 ± 0.7071, p=0.0001, Fig. 3.1.1). To support the validity of our finding that the exercise group has indeed been trained, expression levels of COX-IV protein, which is involved in mitochondrial-cytosolic energy exchange was also measured in both the sedentary and exercise group following this mode of exercise. Previous studies identified that exercise training stimulates mitochondrial biogenesis (Trevellin et al., 2014). Therefore, as anticipated, COX-IV total protein content relative to αß-tubulin in the exercise group increased significantly compared to the sedentary group (p=0.011) (Fig. 3.1.2).

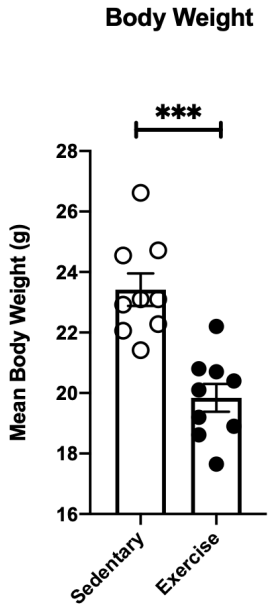

Figure 3.1.1- The effect of moderate chronic exercise on mean body weight. Comparing the body weight of the exercise group to the sedentary group using an unpaired t-test following a moderate chronic exercise training program resulted in a significant effect, t (16) = 5.060, p= 0.0001 with sedentary mice having higher body weight compared to exercise mice.

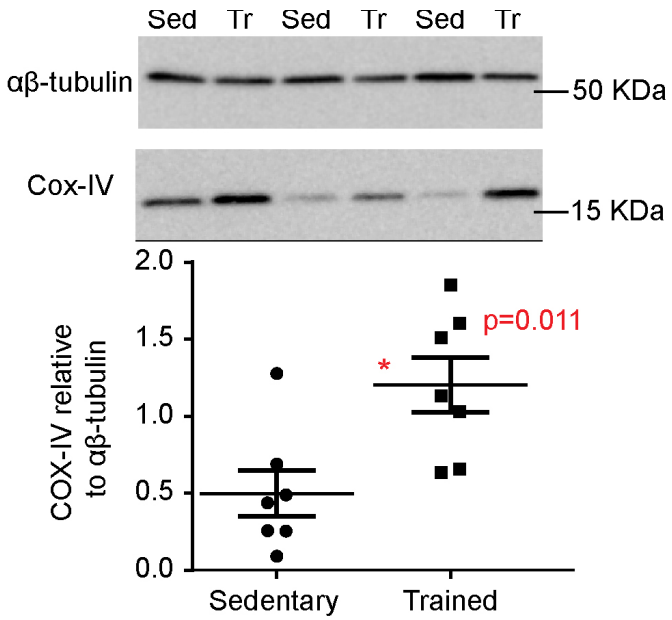

Figure 3.1.2- The effect of moderate chronic exercise on total protein content of complex IV of the electron transport chain. Total content of electron transport chain protein was measured from gastrocnemius muscle upon completion of the exercise training program. Results represent mean ± SEM for 14 subjects. *, Trained > Sedentary, p<0.011. This specific blot was done by Dr. Emilie Roudier group.

Next, stimulation of certain pattern recognition receptors (PRRs) involved in inflammatory responses such as TLRs, RIG-I and STING was carried out. Upon induction of these PRRs, a series of intracellular signaling events are initiated, which leads to the activation of NF-κB or IRF3 transcription factors, followed by induction of gene expression of pro- and anti-inflammatory cytokines that help in the clearance of infections. To characterize the activation of such receptors, we stimulated the bone marrow derived macrophages obtained from mice of both exercise and sedentary groups with distinct inducers of inflammation such as Lipopolysaccharide, Poly (I: C) and Poly (dA: dT) over a considerable number of time points. An in-depth explanation of the effects of a chronic moderate intensity exercise training program on various intracellular innate immune pathways and transcriptional factors including, NFκB and IRF3 can be found in section

3.1.2 to section 3.1.9. Furthermore, the effect of a long-term high intensity exercise on such pathways were also explored (Section 3.2.1 & section 3.2.2).

3.1.2) ***TLR-4 mediated NF-κB induced genes are down-regulated by chronic moderate exercise***

Upon stimulation of BMDMs with LPS, a component of the cell wall of Gram negative bacteria that stimulates TLR4, the exercise group illustrated a significant reduction in the expression levels of NF-κB induced genes when compared to the sedentary group, including IL-1ß (1h Sed: 1.11 ± 0.143 vs. 1h Ex: 0.341 ± 0.143, $p<0.001$. 3h Sed: 1.11 ± 0.132 vs. 3h Ex: 0.190 ± 0.132, $p<0.001$. 6h Sed: 1.03 ± 0.083 vs. 6h Ex: 0.100 ± 0.083, $p<0.001$. 24h Sed: 1.05 ± 0.112 vs. 24h Ex: 0.470 ± 0.112, $p<0.001$; Fig. 3.1.3B), TNFα (1h Sed: 1.01 ± 0.0643 vs. 1h Ex: 0.616 ± 0.0643, $p<0.001$. 6h Sed: 1.09 ± 0.149 vs. 6h Ex: 0.0438 ± 0.149, $p<0.001$; Fig. 3.1.4B), IL-10 (1h Sed: 1.07 ± 0.133 vs. 1h Ex: 0.558 ± 0.133, $p=0.002$. 3h Sed: 1.14 ± 0.0839 vs. 3h Ex: 0.358 ± 0.0839, $p<0.001$. 6h Sed: 1.04 ± 0.0.0917 vs. 6h Ex: 0.105 ± 0.0917, $p<0.001$. 24h Sed: 1.05 ± 0.114 vs. 24h Ex: 0.459 ± 0.114, $p<0.001$; Fig. 3.1.5B), and TGFß (3h Sed: 1.04 ± 0.0891 vs. 3h Ex: 0.433 ± 0.0891, $p<0.001$. 6h Sed: 1.04 ± 0.107 vs. 6h Ex: 0.655 ± 0.107, $p<0.05$. 24h Sed: 1.01 ± 0.122 vs. 24h Ex: 0.593 ± 0.122, $p<0.05$; Fig. 3.1.6B) as measured by real-time PCR. Western blot analysis was also collected and confirmed that while in the sedentary group, LPS stimulation caused the expected activation of NF-κB as evident from the robust degradation of IκB, the exercise group displayed a significantly reduced levels of NF-κB activation due to weaker IκB degradation (Fig. 3.1.7). Interestingly, both pro- and anti-inflammatory NF-κB induced genes were lower in the exercise group when compared to the sedentary group.

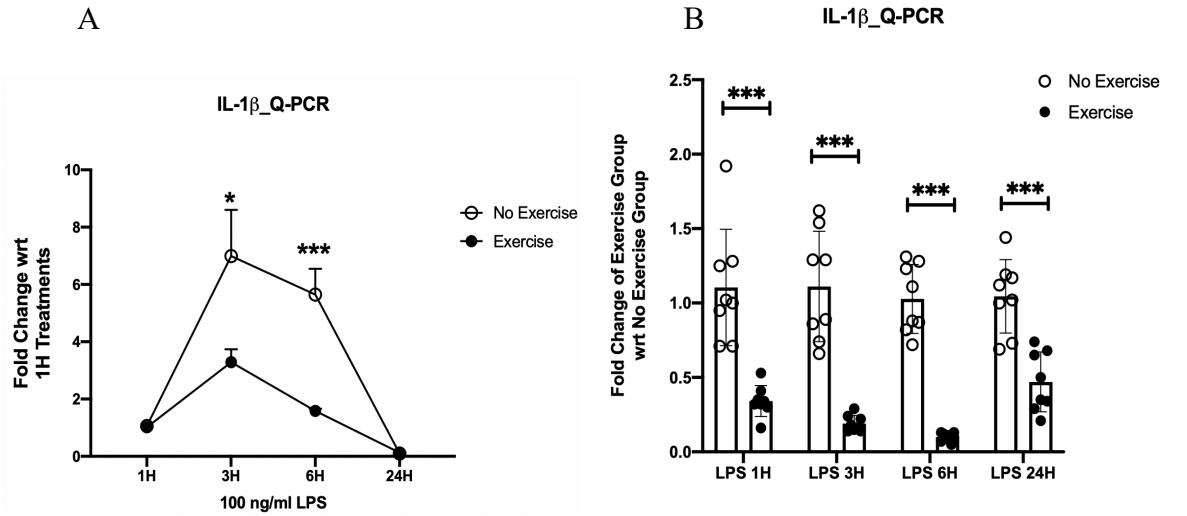

Figure 3.1.3- TLR-4 mediated NF-κB induced pro-inflammatory gene (IL-1β) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and gene expressions of IL-1β were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) *, 3h sedentary > exercise, $p<0.05$. ***, 6h sedentary > exercise, $p<0.001$. (B) ***, 1, 3, 6 and 24h sedentary > exercise, $p<0.001$.

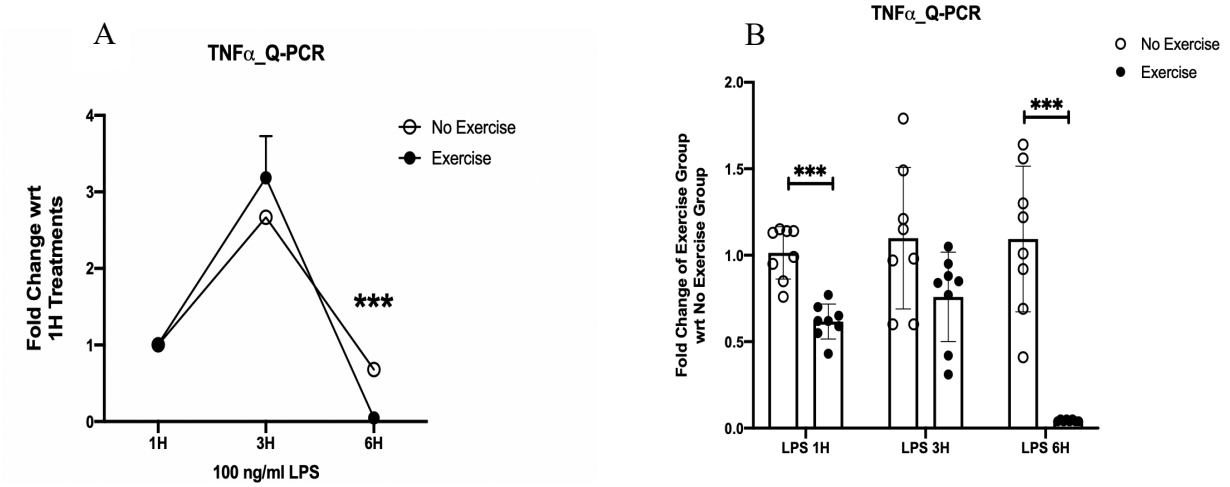

Figure 3.1.4- TLR-4 mediated NF-κB induced pro-inflammatory gene (TNFα) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and gene expressions of TNFα were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A)***, 6h sedentary > exercise, $p<0.001$. (B) ***, 1 and 6h sedentary > exercise, $p<0.001$.

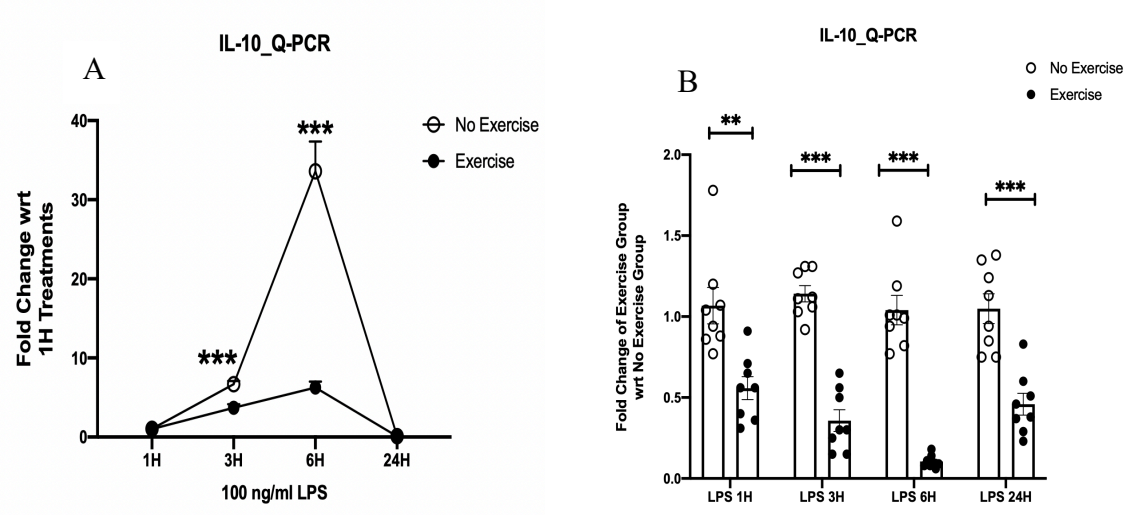

Figure 3.1.5- TLR-4 mediated NF-κB induced anti-inflammatory gene (IL-10) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and gene expressions of IL-10 were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A)***, 3 and 6h sedentary > exercise, p<0.001. (B) **, 1h sedentary > exercise, p=0.002. ***, 3, 6 and 24h sedentary > exercise, p<0.001.

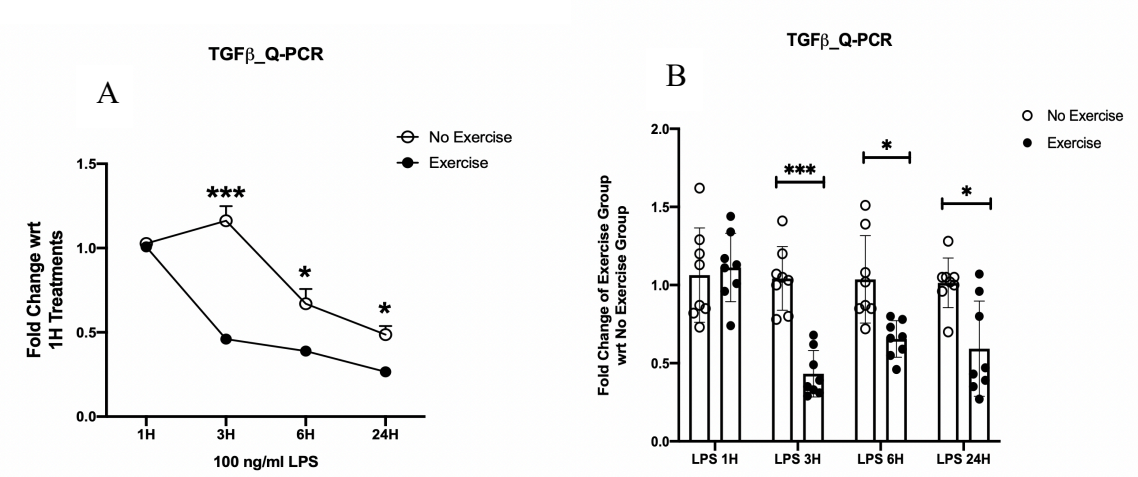

Figure 3.1.6- TLR-4 mediated NF-κB induced anti-inflammatory gene (TGFβ) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and gene expressions of TGFβ were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A)***, 3h sedentary > exercise, p<0.001. *, 6 and 24h sedentary > exercise, p<0.05. (B) ***, 3h sedentary > exercise, p<0.001. *, 6 and 24h sedentary > exercise, p<0.05.

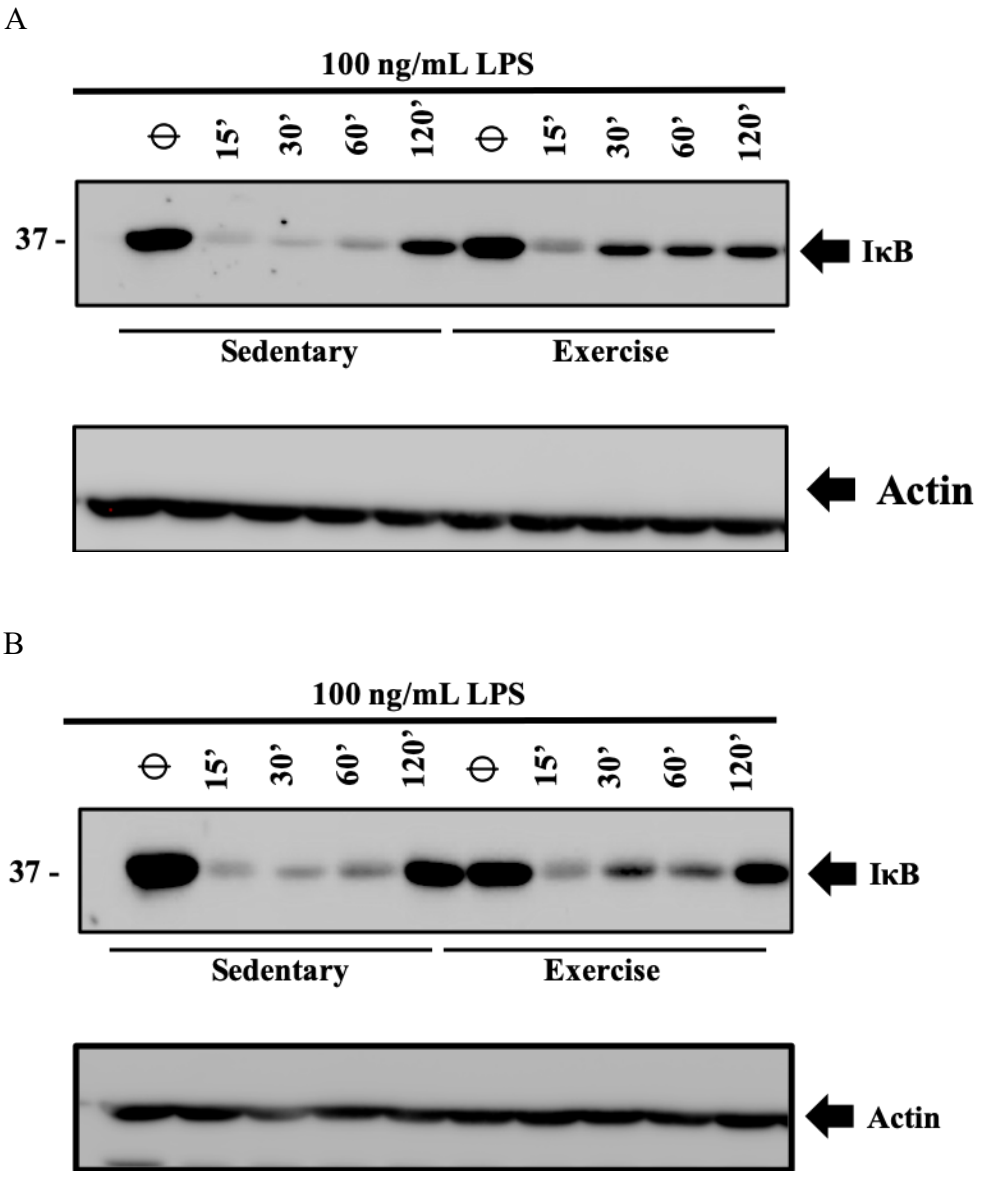

Figure 3.1.7- Effect of moderate chronic exercise on IκB protein content (LPS/TRL4). BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and total content of cellular protein IκB levels were measured following the completion of the exercise training program. 2 representative western blots of 16 mouse models (8-exercise and 8-sedentary) reflecting lower degradation of IκB protein in the exercise group (Refer to Appendix A (1 & 3) for blot images).

3.1.3) *The effect of moderate chronic exercise on endosomal TLR-3.*

To determine whether moderate exercise intensity affects endosomal TLRs the same way it did with the membrane bound TLR4, BMDMs from both groups were stimulated with Poly (I: C) to activate TLR3. Western blotting demonstrated consistent results and showed lower levels of IκB degradation in the exercise group when compared to the sedentary group (Fig. 3.1.8).

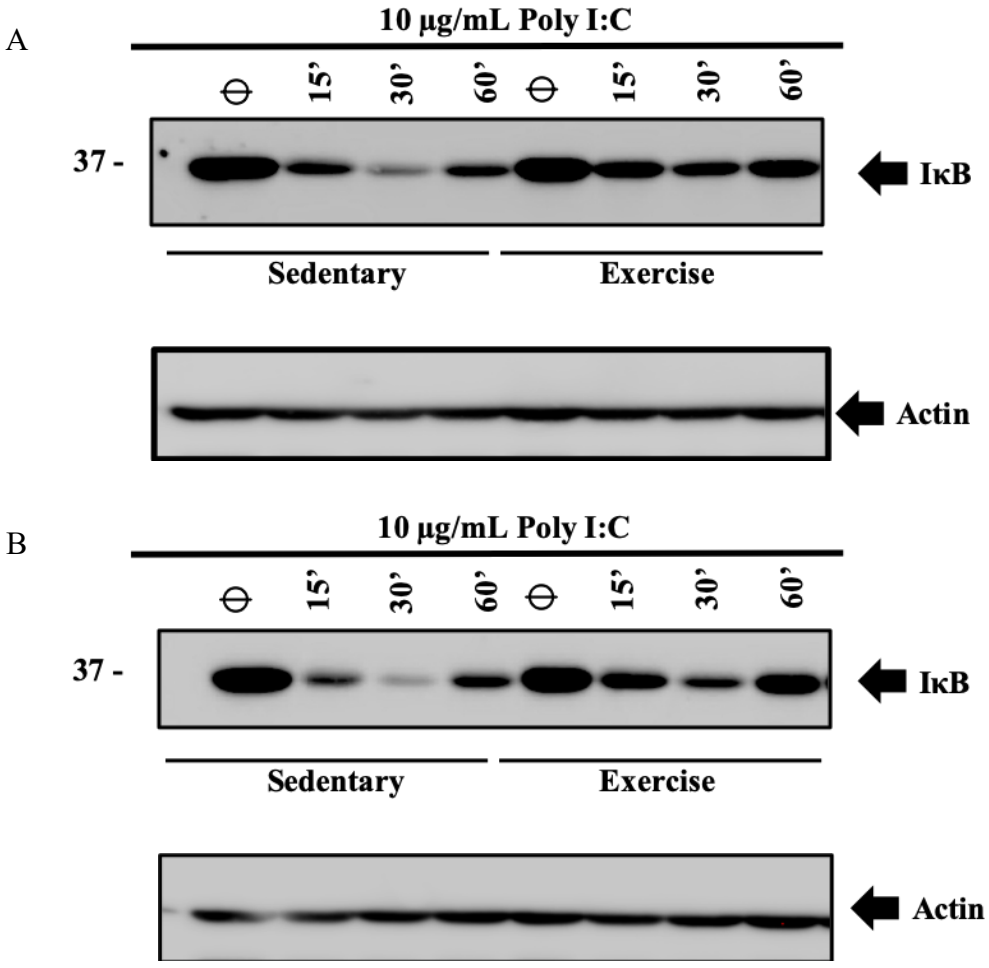

Figure 3.1.8- Effect of moderate chronic exercise on IκB protein content (Poly (I: C)/TLR3). BMDM from C57 BL/6 (WT) mice were stimulated with 10 µg/ml Poly (I:C) for the indicated times and total content of cellular protein IκB levels were measured following the completion of the exercise training program. 2 representative western blots of 16 mouse models (8-exercise and 8-sedentary) reflecting lower degradation of IκB protein in the exercise group (Refer to Appendix A (2 & 4) for blot images).

3.1.4) *STING mediated NF-κB induced genes are down-regulated by chronic moderate exercise*

Next, we wanted to test the effect of moderate exercise on STING mediated inflammation. To this end, BMDMs stimulated with the STING ligand, 3'3'-cGAMP, showed that moderate exercise intensity causes a significant reduction in the expression levels of NF-κB induced genes versus the sedentary group, including IL-1ß (3h Sed: 1.12 ± 0.185 vs. 3h Ex: 0.444 ± 0.185, $p<0.05$; Fig. 3.1.9B), TNFα (1h Sed: 1.08 ± 0.152 vs. 1h Ex: 0.458 ± 0.152, $p=0.002$. 3h Sed: 1.02 ± 0.0784 vs. 3h Ex: 0.548 ± 0.0784, $p<0.001$. 6h Sed: 1.12 ± 0.126 vs 6h Ex: 0.256 ± 0.126, $p<0.001$; Fig. 3.1.10B), IL-10 (3h Sed: 1.16 ± 0.190 vs. 3h Ex: 0.420 ± 0.190, $p=0.002$. 6h Sed: 1.11 ± 0.175 vs. 6h Ex: 0.506 ± 0.175, $p<0.05$; Fig. 3.1.11B), and TGFß (1h Sed: 1.02 ± 0.0942 vs. 1h Ex: 0.734 ± 0.0942, $p<0.05$. 3h Sed: 1.11 ± 0.191 vs. 3h Ex: 0.515 ± 0.191, $p<0.05$. 6h Sed: 1.04 ± 0.105 vs. 6h Ex: 0.539 ± 0.105, $p<0.001$; Fig. 3.1.12B) as measured by real-time PCR. Our results indicated that inflammation caused by this inducer was down-regulated following moderate training.

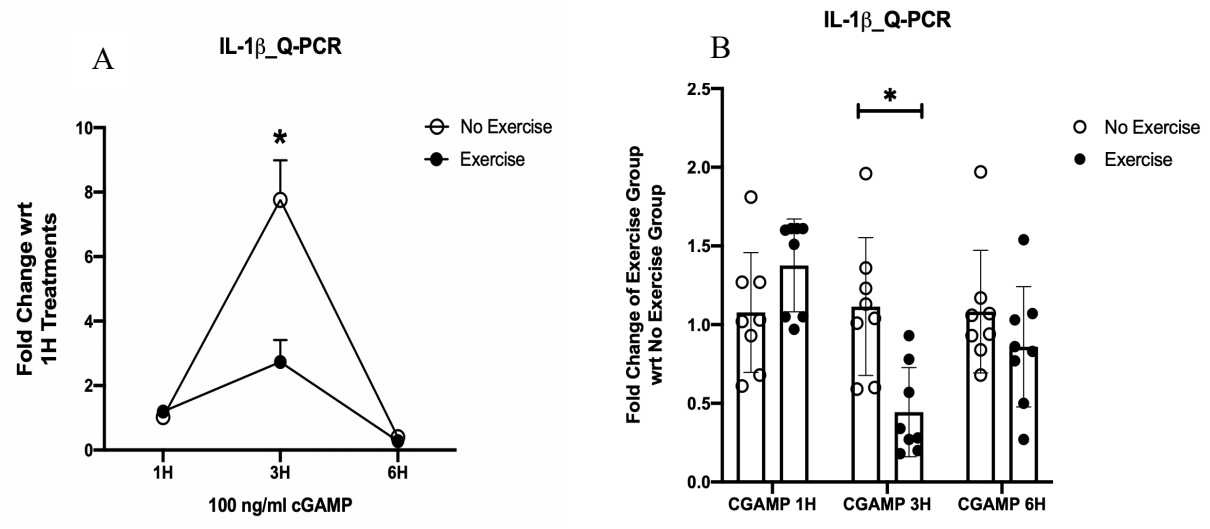

Figure 3.1.9- STING mediated NF-κB induced pro-inflammatory gene (IL-1β) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml cGAMP for the indicated times and gene expressions of IL-1β were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A)*, 3h sedentary > exercise, $p<0.05$. (B) *, 3h sedentary > exercise, $p<0.05$.

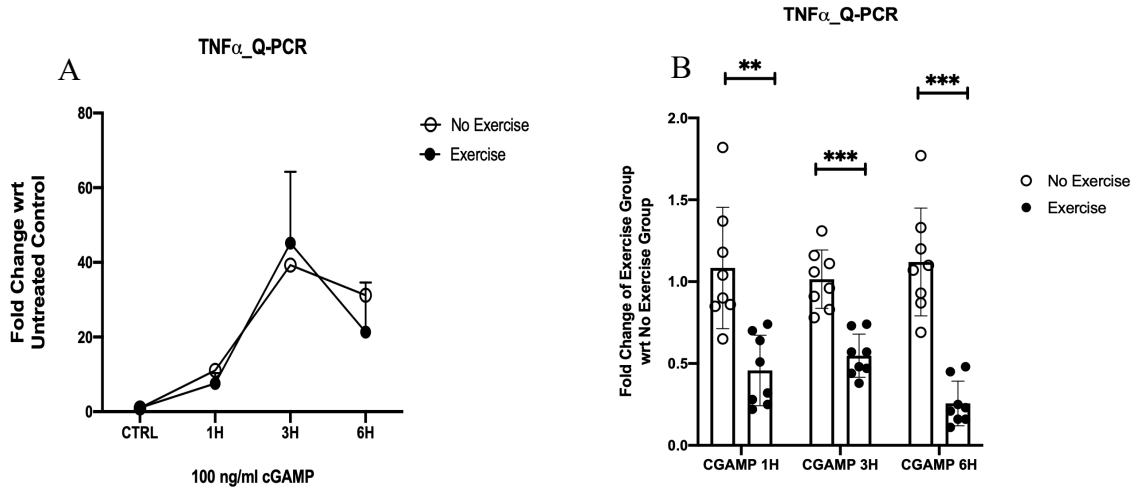

Figure 3.1.10- STING mediated NF-κB induced pro-inflammatory gene (TNFα) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml cGAMP for the indicated times and gene expressions of TNFα were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t untreated controls. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (B) **, 1h sedentary > exercise, $p=0.002$. ***, 3 and 6h sedentary > exercise, $p<0.001$.

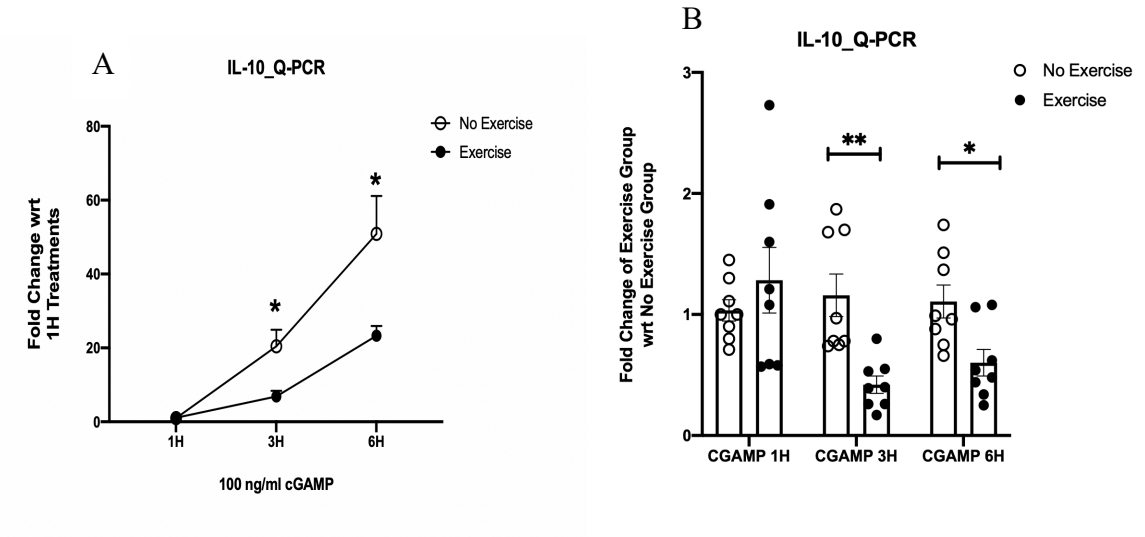

Figure 3.1.11- STING mediated NF-κB induced anti-inflammatory gene (IL-10) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml cGAMP for the indicated times and gene expressions of IL-10 were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) *, 3 and 6h sedentary > exercise, $p<0.05$ (B) **, 3h sedentary > exercise, $p=0.002$. *, 6h sedentary > exercise, $p<0.05$.

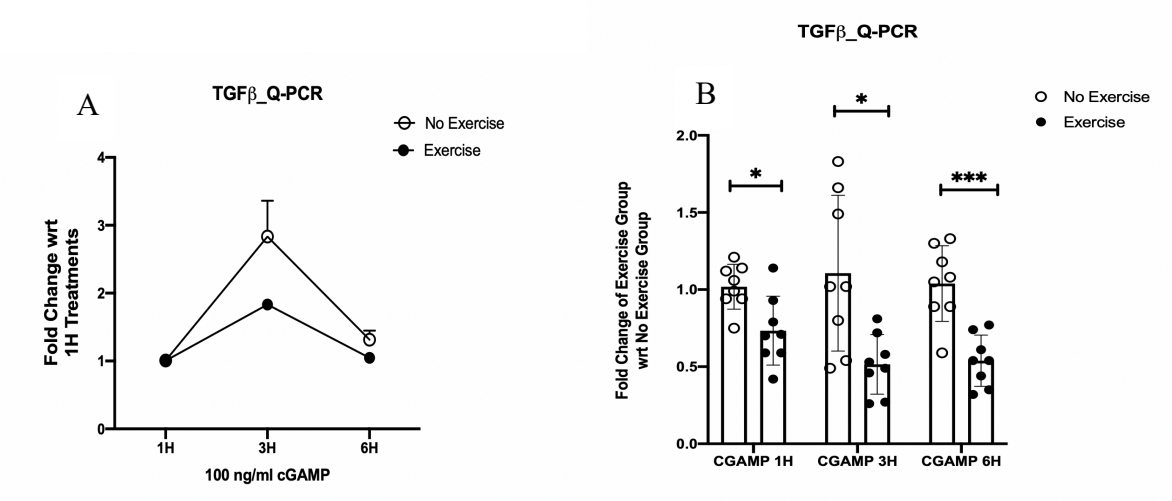

Figure 3.1.12- STING mediated NF-κB induced anti-inflammatory gene (TGFβ) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml cGAMP for the indicated times and gene expressions of TGFβ were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (B) *, 1 and 3h sedentary > exercise, $p<0.05$. ***, 6h sedentary > exercise, $p<0.001$.

3.1.5) *RIG-I mediated NF-κB induced genes are differentially regulated by chronic moderate exercise*

Finally, the effect of moderate exercise intensity on RIG-I mediated inflammation was studied. RIG-I is an important sensor of viral nucleic acids and is interesting in that it signals through the mitochondrial protein MAVS (Broquet, Hirata, McAllister, & Kagnoff, 2011). To this end, BMDMs were transfected with Poly (I: C), which mimics viral RNA. Surprisingly, different results were seen in the expression levels of NF-κB induced genes of the exercise group. For example, pro-inflammatory genes IL-1ß (3h Sed: 1.04 ± 0.121 vs. 3h Ex: 0.711 ± 0.121, $p<0.05$. 6h Sed: 1.06 ± 0.140 vs. 6h Ex: 0.528 ± 0.140, $p=0.002$; Fig. 3.1.13B) and TNFα (. 6h Sed: 1.12 ± 0.172 vs. 6h Ex: 0.316 ± 0.172, $p<0.001$; Fig. 3.1.14B) were down-regulated, while anti-inflammatory genes IL-10 (6h Sed: 1.09 ± 0.414 vs. 6h Ex: 3.28 ± 0.414, $p<0.001$. 24h Sed: 1.08 ± 0.954 vs. 24h Ex: 7.17 ± 0.954, $p<0.001$; Fig. 3.1.15B) and TGFß (24h Sed: 1.07 ± 0.233 vs. 24h Ex: 4.60 ± 0.233, $p<0.001$; Fig. 3.1.16B) were up-regulated as measured by real-time PCR. Interestingly, western blot analysis for the same stimulator demonstrated lower levels of IκB degradation in the exercise group, which also explains that there is a reduction in NFκB activation, therefore, inflammation was down-regulated (Fig. 3.1.17).

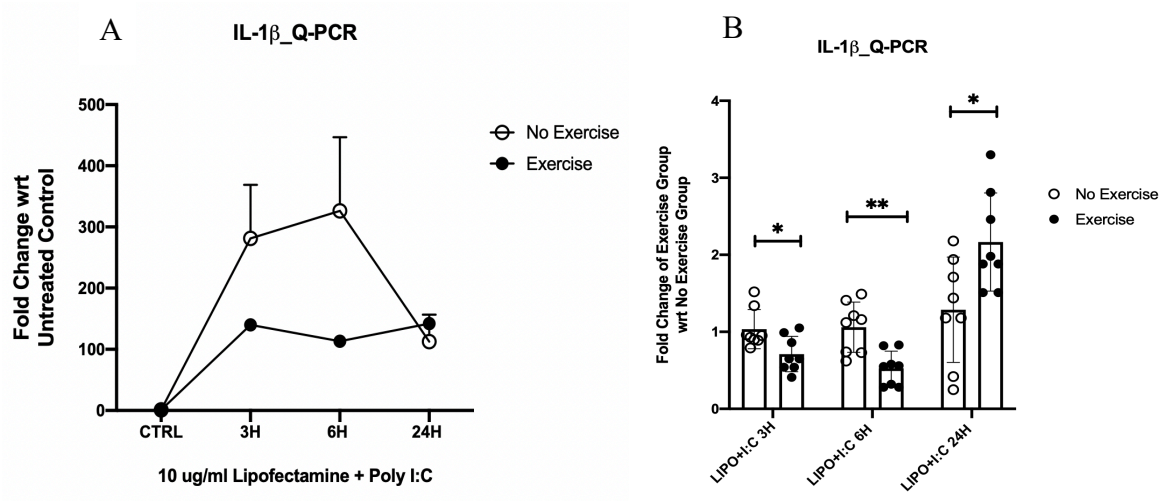

Figure 3.1.13- RIG-I mediated NF-κB induced pro-inflammatory gene (IL-1β) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 μg/ml Poly (I:C) for the indicated times and gene expressions of IL-1β were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t untreated controls. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (B) *, 3 and 24h sedentary > exercise, $p<0.05$. **, 6h sedentary > exercise, $p=0.002$.

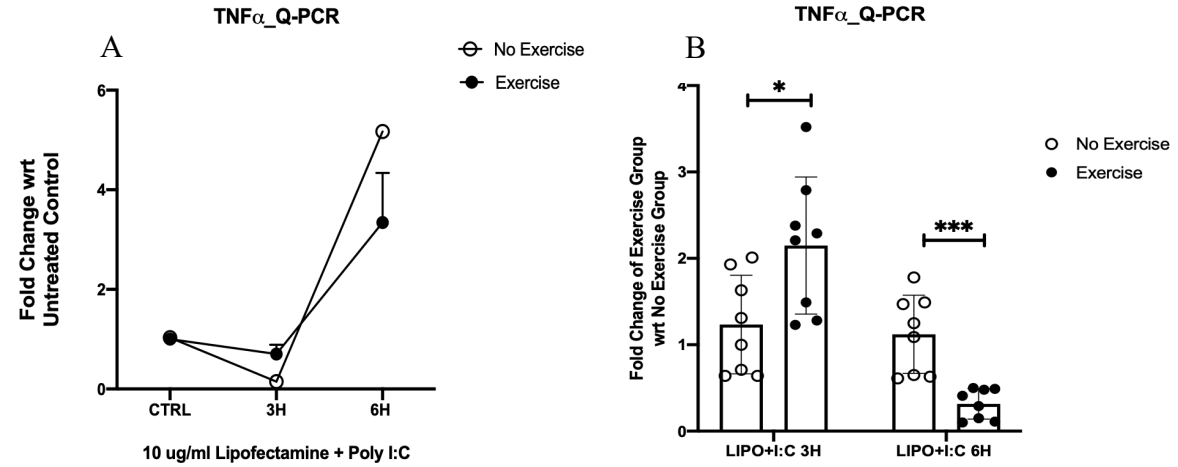

Figure 3.1.14- RIG-I mediated NF-κB induced pro-inflammatory gene (TNFα) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 μg/ml Poly (I:C) for the indicated times and gene expressions of TNFα were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t untreated controls. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (B) *, 3h sedentary < exercise, $p<0.05$. ***, 6h sedentary > exercise, $p<0.001$.

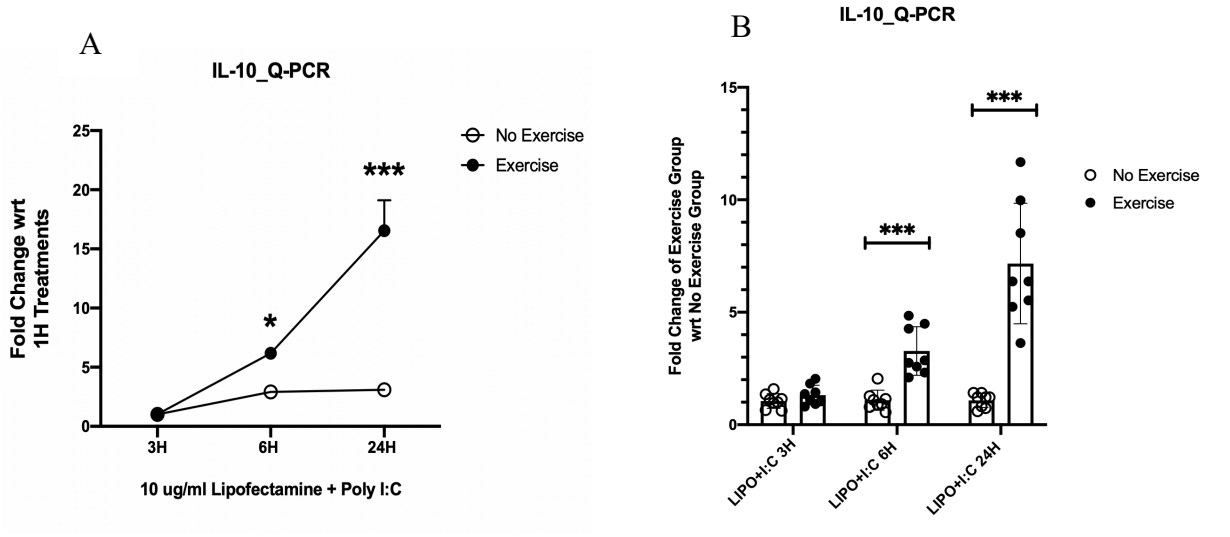

Figure 3.1.15- RIG-I mediated NF-κB induced anti-inflammatory gene (IL-10) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (I:C) for the indicated times and gene expressions of IL-10 were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 3h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) *, 6h, sedentary < exercise, $p<0.05$. ***, 24h sedentary < exercise, $p<0.001$. (B) ***, 6 and 24h sedentary < exercise, $p<0.001$.

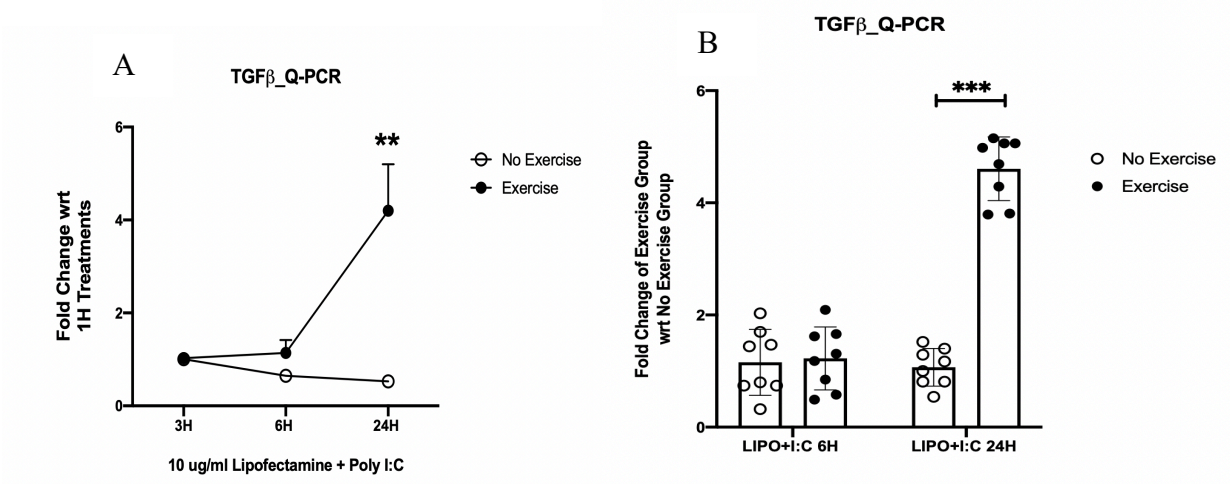

Figure 3.1.16- RIG-I mediated NF-κB induced anti-inflammatory gene (TGFβ) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (I:C) for the indicated times and gene expressions of TGFβ were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) **, 24h sedentary < exercise, $p=0.002$. (B) ***, 24h sedentary < exercise, $p<0.001$.

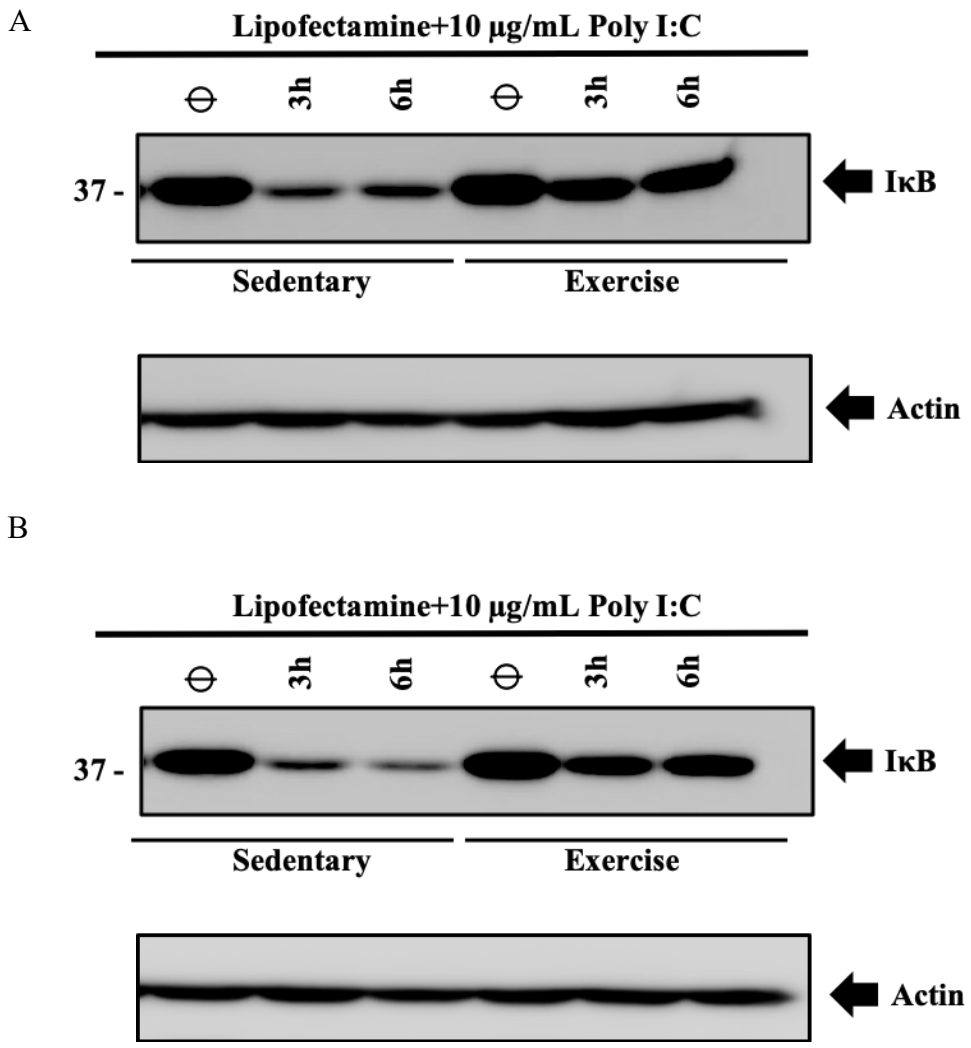

Figure 3.1.17- Effect of moderate chronic exercise on IκB protein content (Transfected Poly (I: C)/RIG-I). BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (I:C) for the indicated times and total content of cellular protein IκB levels were measured following the completion of the exercise training program. 2 representative western blots of 16 mouse models (8-exercise and 8-sedentary) reflecting lower degradation of IκB protein in the exercise group (Refer to Appendix A (2 & 4) for blot images).

3.1.6) *Poly (dA: dT) stimulated BMMs exhibit higher NF-κB induced genes following moderate exercise training.*

Poly (dA: dT) is known to be a synthetic analog of B-DNA. Upon transfection, RNA polymerase III transcribes Poly (dA: dT) into dsRNA, which is a molecular ligand for RIG-I receptors. Once stimulated, RIG-I will induce a series of intracellular signals through the mitochondrial protein MAVS, which triggers the transcription of certain NF-**κ**B genes (Abe & Barber, 2014). BMDMs of the exercise group treated with transfected Poly (dA: dT) showed a significant increase in the expression levels of NF-κB induced genes, including IL-1ß (6h Sed: 1.16 ± 0.412 vs. 6h Ex: 3.8 ± 0.412, $p<0.001$; Fig. 3.1.18B), TNFα (6h Sed: 1.28 ± 0.353 vs. 6h Ex: 2.26 ± 0.353, $p<0.05$; Fig. 3.1.19B), IL-10 (3h Sed: 1.22 ± 0.222 vs. 3h Ex: 1.76 ± 0.222, $p<0.05$. 6h Sed: 1.05 ± 0.337 vs. 6h Ex: 4.25 ± 0.337, $p<0.001$. 24h Sed: 1.10 ± 0.242 vs. 24h Ex: 2.79 ± 0.242; Fig. 3.1.20B) and TGFß (6h Sed: 0.802 ± 0.173 vs. 6h Ex: 1.64 ± 0.173, $p<0.001$. 24h Sed: 1.08 ± 0.138 vs. 24h Ex: 1.42 ± 0.138, $p<0.05$; Fig. 3.1.21B) as measured by real-time PCR. Western blot analysis was also collected and confirmed that while in the sedentary group, transfected Poly (dA: dT) stimulation caused the expected activation of NF-κB as evident from the robust degradation of IκB, the exercise group displayed a significantly increased levels of NF-κB activation due to stronger IκB degradation (Fig. 3.1.22). Interestingly, both pro- and anti-inflammatory NF-κB induced genes were higher in the exercise group when compared to the sedentary group.

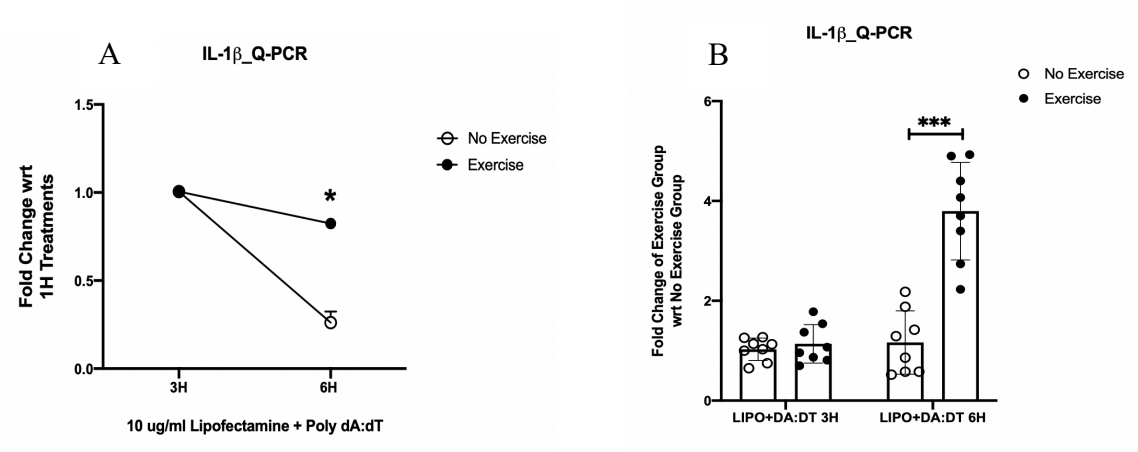

Figure 3.1.18- RIG-I mediated NF-κB induced pro-inflammatory gene (IL-1β) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (dA: dT) for the indicated times and gene expressions of IL-1β were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 3h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) *, 6h sedentary < exercise, $p<0.05$. (B) ***, 6h sedentary < exercise, $p<0.001$.

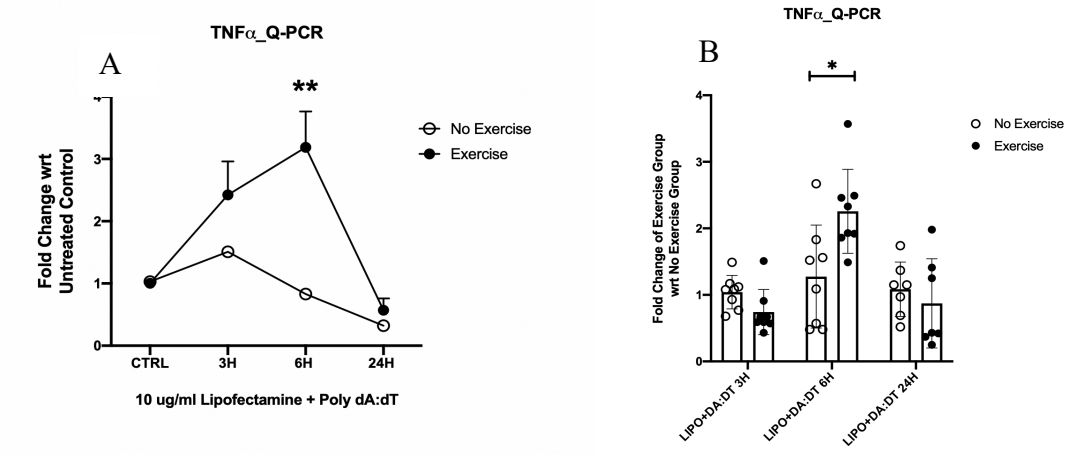

Figure 3.1.19- RIG-I mediated NF-κB induced pro-inflammatory gene (TNFα) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (dA: dT) for the indicated times and gene expressions of TNFα were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t untreated controls. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) **, 6h sedentary < exercise, $p=0.002$. (B) *, 6h sedentary < exercise, $p<0.05$.

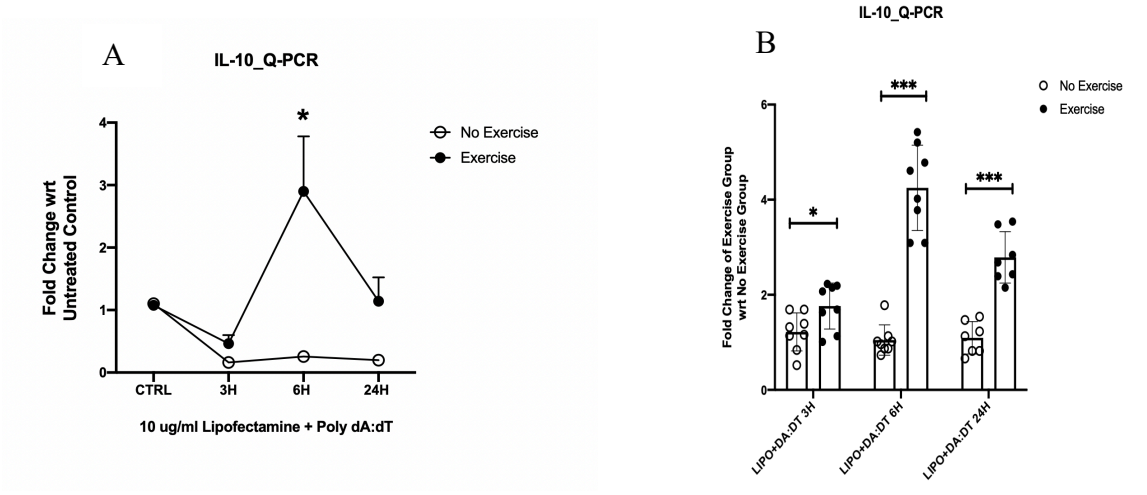

Figure 3.1.20- RIG-I mediated NF-κB induced anti-inflammatory gene (IL-10) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (dA: dT) for the indicated times and gene expressions of IL-10 were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t untreated controls. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) *, 6h sedentary < exercise, $p<0.05$. (B) *, 3h sedentary < exercise, $p<0.05$. ***, 6 and 24h sedentary < exercise, $p<0.001$.

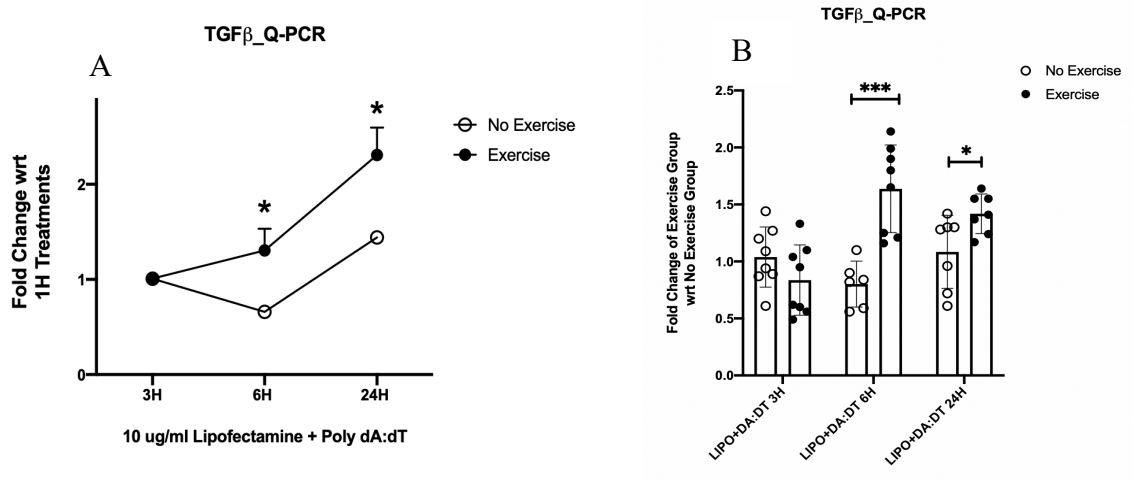

Figure 3.1.21- RIG-I mediated NF-κB induced anti-inflammatory gene (TGFβ) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (dA: dT) for the indicated times and gene expressions of TGFβ were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 3h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) *, 3 and 6h sedentary < exercise, $p<0.05$. (B) ***, 6h sedentary < exercise, $p<0.001$. *, 24h sedentary < exercise, $p<0.05$.

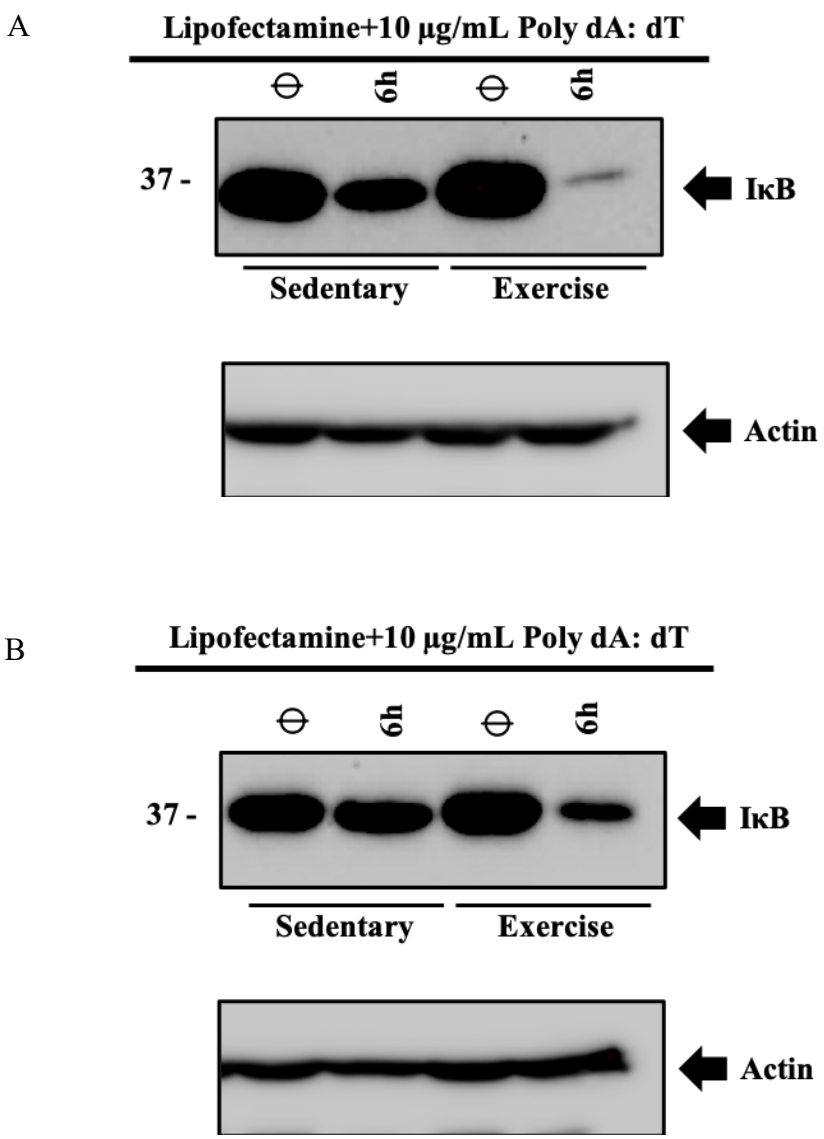

Figure 3.1.22- Effect of moderate chronic exercise on IκB protein content (Transfected Poly (dA: dT)/RIG-I). BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (dA: dT) for the indicated times and total content of cellular protein IκB levels were measured following the completion of the exercise training program. 2 representative western blots of 16 mouse models (8-exercise and 8-sedentary) reflecting lower degradation of IκB protein in the exercise group (Refer to Appendix A (1 & 3) for blot images).

3.1.7) *TLR-4 mediated IRF-3 induced genes are up-regulated by chronic moderate exercise*

Surprisingly, upon stimulation of BMDMs with LPS, a component of the cell wall of Gram negative bacteria that stimulates TLR4, the exercise group illustrated a significant increase in the expression levels of IRF-3 induced genes such as IFNß (3h Sed: 1.20 ± 0.380 vs. 3h Ex: 3.58 ± 0.380, $p<0.001$; Fig. 3.1.23B) when compared to the sedentary group, as measured by real-time PCR. Similar results were also observed in the exercise group when BMDMs were stimulated with 3'3'-cGAMP as there was also a significant increase in the expression of IRF-3 induced gene, IFNß (1h Sed: 1.08 ± 0.406 vs. 1h Ex: 4.05 ± 0.406, $p<0.001$. 6h Sed: 1.11 ± 0.390 vs. 6h Ex: 2.60 ± 0.390, $p=0.002$; Fig. 3.1.25B) as measured by real-time PCR. Western blot analysis was also collected and confirmed that while in the sedentary group, LPS stimulation caused no activation of IRF-3 as evident from the robust degradation of P-IRF3, the exercise group displayed slightly higher levels of IRF-3 activation due to stronger P-IRF3 protein expression (Fig. 3.1.24). This interesting finding is completely different from what we have seen earlier in the expression levels of NFκB induced genes.

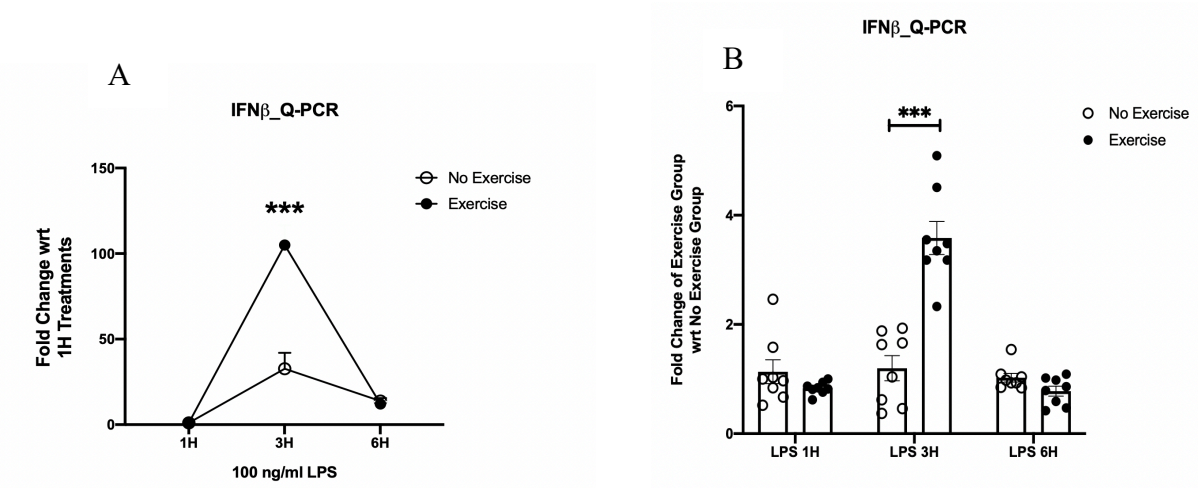

Figure 3.1.23- TLR-4 mediated IRF-3 induced pro-inflammatory gene (IFNβ) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and gene expressions of IFNβ were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t 1h treatments. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) ***, 3h sedentary < exercise, $p<0.001$. (B) ***, 3h sedentary < exercise, $p<0.001$.

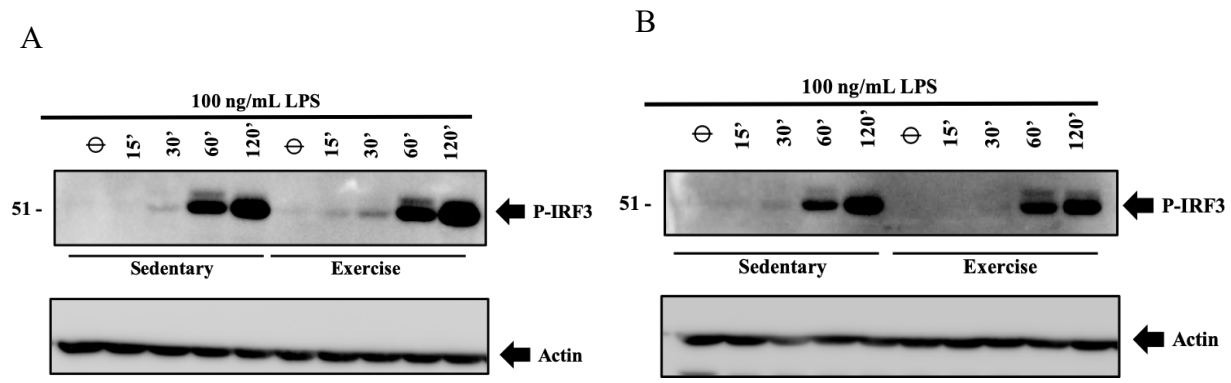

Figure 3.1.24- Effect of moderate chronic exercise on P-IRF-3 protein content (LPS/TLR4). BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and total content of cellular protein P-IRF3 levels were measured following the completion of the exercise training program. 2 representative western blots of 16 mouse models (8-exercise and 8-sedentary) slightly reflecting higher expression of P-IRF3 protein in the exercise group (Refer to Appendix A (5 & 7) for blot images). Note: same blots as Fig. 3.1.7 A and B were used for this experiment.

3.1.8) *STING mediated IRF-3 induced genes are up-regulated by chronic moderate exercise*

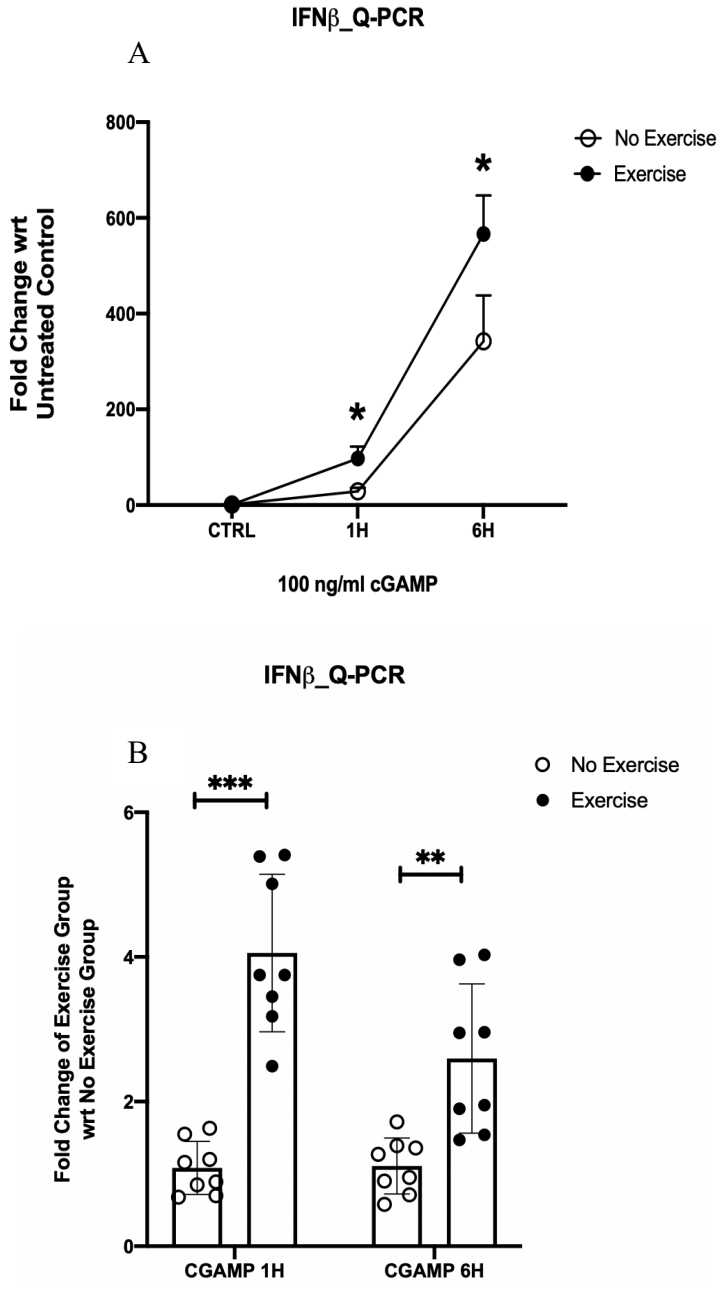

Figure 3.1.25- STING mediated IRF-3 induced pro-inflammatory gene (IFNβ) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml cGAMP for the indicated times and gene expressions of IFNβ were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t untreated controls. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (A) *, 1 and 3h sedentary < exercise, $p<0.05$. (B) ***, 1h sedentary < exercise, $p<0.001$. **, 6h sedentary < exercise, $p=0.002$.

3.1.9) *RIG-I mediated IRF-3 induced genes are down-regulated by chronic moderate exercise*

Finally, the effect of moderate exercise intensity on RIG-I mediated IRF-3 inflammatory genes was studied. Transfected Poly (I: C) stimulates RIG-I receptors, which triggers the transcription of type I IFNs expression levels through the activation of IRF-3 nuclear transcriptional factor (Wang et al., 2016). The expression levels of IRF-3 induced gene, IFNß (3h Sed: 1.09 ± 0.144 vs. 3h Ex: 0.579 ± 0.144, $p<0.05$; Fig. 3.1.26B) were down-regulated as measured by real-time PCR. However, western blot analysis showed no difference in the levels of P-IRF3 protein in the exercise group. (Fig. 3.1.27). Interestingly, similar results were seen earlier in the NF-κB induced pro-inflammatory genes, when the BMDMs were stimulated with the same inducer. Therefore, it is clear that both NF-κB and IRF-3 induced pro-inflammatory genes were lower in the exercise group when compared to the sedentary group.

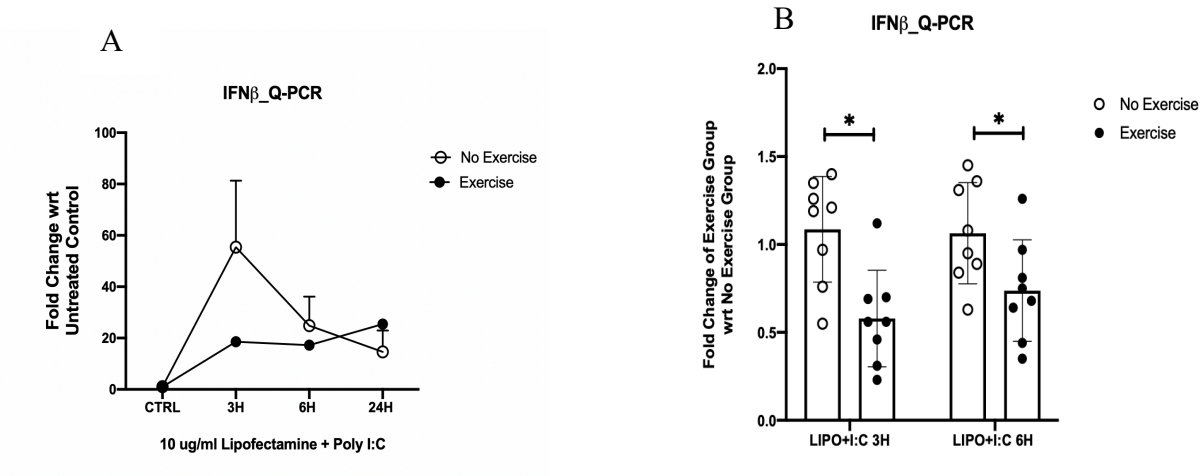

Figure 3.1.26- RIG-I mediated IRF-3 induced pro-inflammatory gene (IFNβ) expression levels following a moderate chronic exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine + 10 µg/ml Poly (I:C) for the indicated times and gene expressions of IFNβ were evaluated by real-time PCR (Q-PCR). (A) Results were normalized to RPLP0 and reported as relative fold change w.r.t untreated controls. (B) Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 16 participants. (B) *, 3 and 6h sedentary > exercise, $p<0.05$.

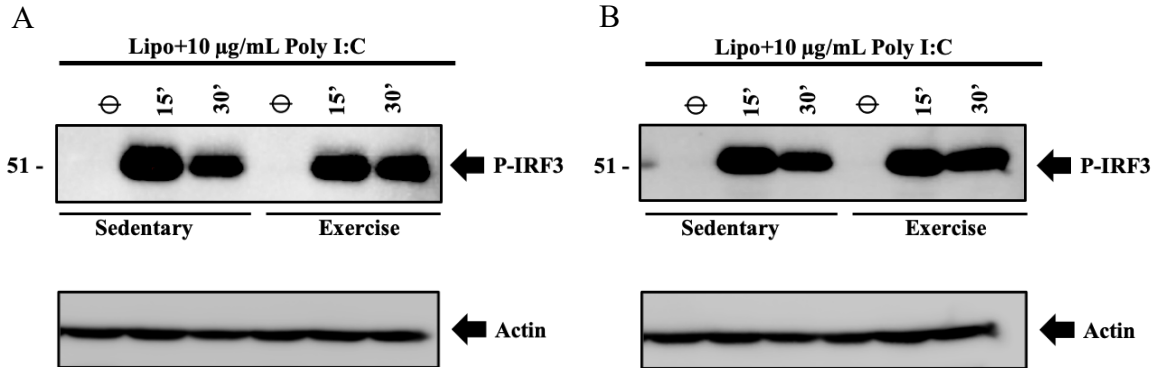

Figure 3.1.27- Effect of moderate chronic exercise on P-IRF-3 protein content (Transfected Poly (I:C)/RIG-I). BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (I:C) for the indicated times and total content of cellular protein P-IRF3 levels were measured following the completion of the exercise training program. 2 representative western blots of 16 mouse models (8-exercise and 8-sedentary) reflecting no difference or slightly lower expression of P-IRF3 protein in the exercise group (Refer to Appendix A (6 & 8) for blot images). Note: same blots as Fig. 3.1.17 A and B were used for this experiment.

3.2.1) *TLR-4 mediated NF-κB induced genes are up-regulated by prolonged high intensity exercise*

In order to identify the difference between the effects of distinct forms of exercise on innate immunity and inflammation, a prolonged high intensity exercise training program was performed by mouse models of exercise. Similar methods and inducers of inflammation were used. Surprisingly, when BMDMs were induced by LPS, a component of the cell wall of Gram negative bacteria that stimulates TLR4, the exercise group illustrated a significant increase in the expression levels of NF-κB pro-inflammatory genes iNOS (4h Sed: 1.07 ± 0.393 vs. 4h Ex: 3.23 ± 0.393, p=0.002; Fig. 3.2.1A), IL-6 (4h Sed: 1.03 ± 0.200 vs. 4h Ex: 1.59 ± 0.200, p<0.05; Fig. 3.2.1B) and TNFα (2h Sed: 1.02 ± 0.0904 vs. 2h Ex: 1.42 ± 0.0904, p<0.05; Fig. 3.2.1C) when compared to the sedentary group, as measured by real-time PCR. Western blot analysis was also collected and confirmed that while in the sedentary group, LPS stimulation caused the expected activation of NF-κB as evident from the robust degradation of IκB, the exercise group displayed a significantly enhanced levels of NF-κB activation due to stronger IκB degradation (Fig. 3.2.2). This indicates that pro-inflammatory NF-κB induced genes were higher in the exercise group when compared to the sedentary group.

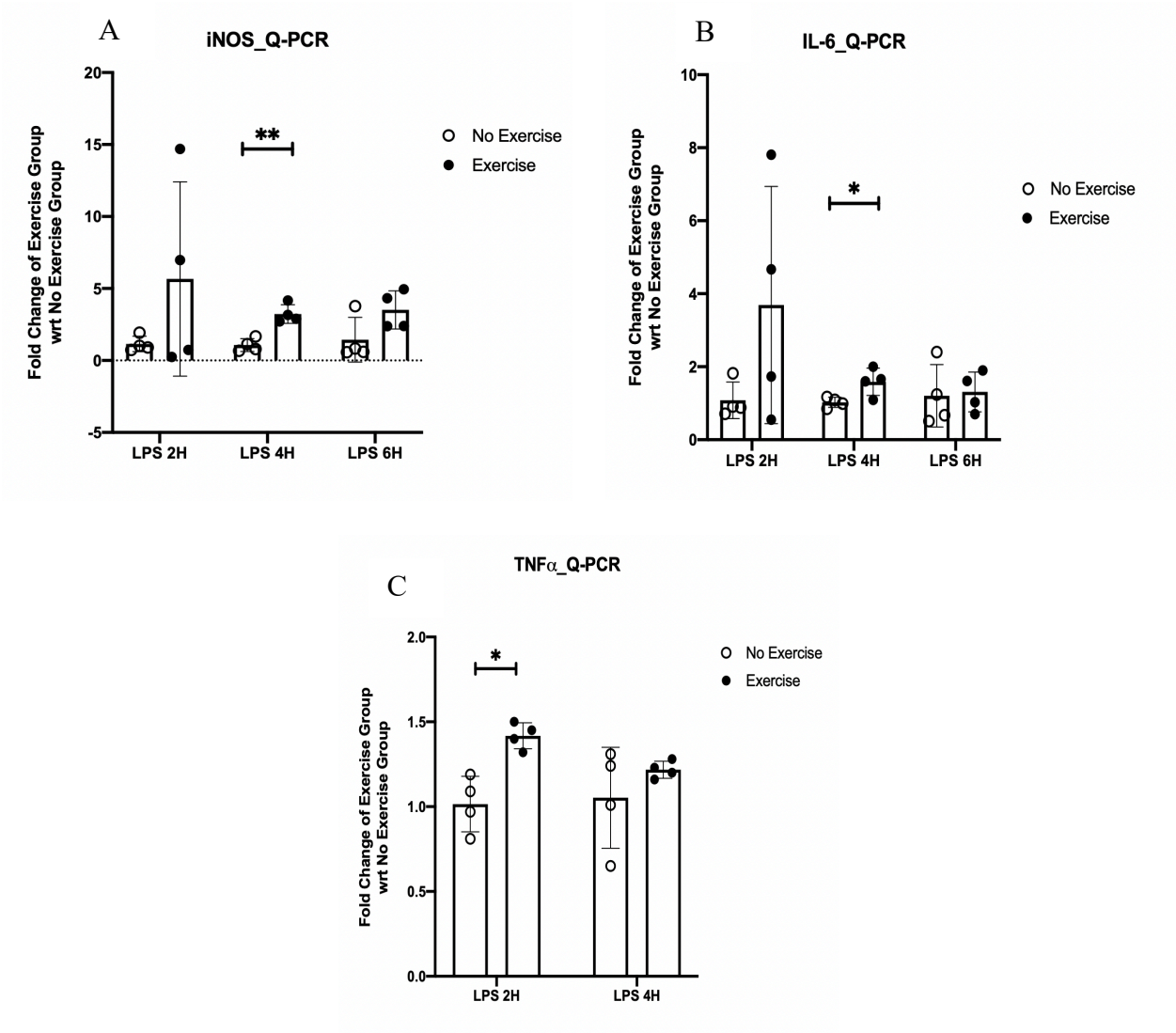

Figure 3.2.1- TLR-4 mediated NF-κB induced pro-inflammatory gene expression levels following a prolonged high intensity exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with 100 ng/ml LPS for the indicated times and gene expressions of iNOS, IL-6 and TNFα were evaluated by real-time PCR (Q-PCR). Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 8 participants. (A) **, 4h sedentary < exercise, $p=0.002$. (B) *, 4h sedentary < exercise, $p<0.05$. (C) *, 2h sedentary < exercise, $p<0.05$.

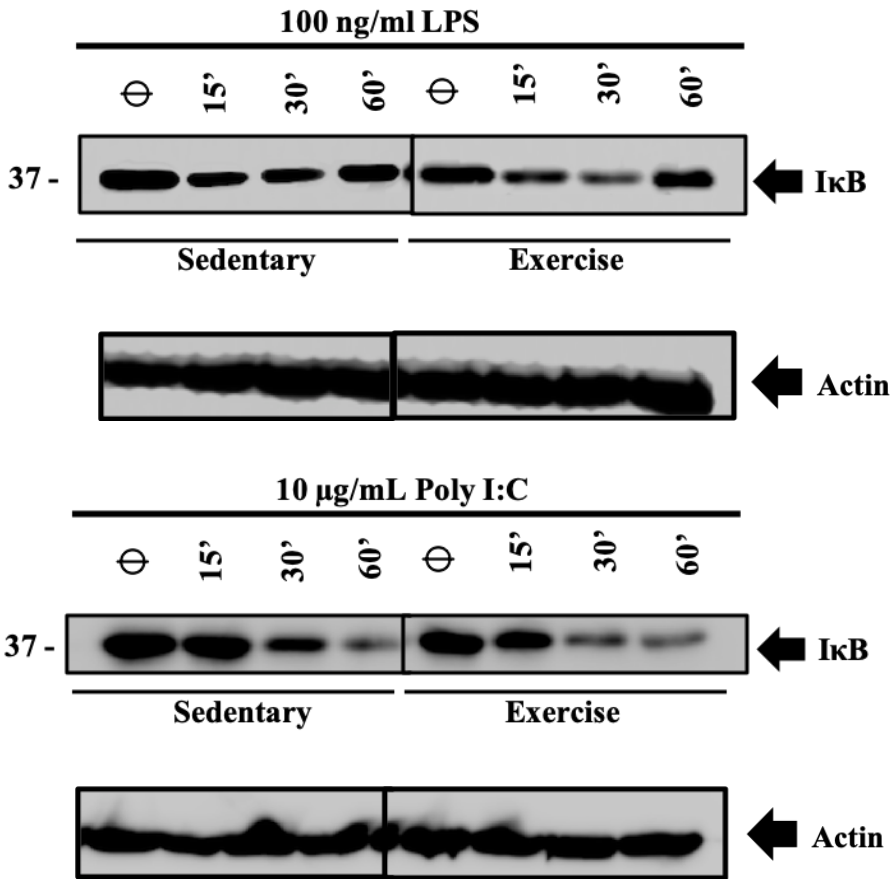

Figure 3.2.2- Effect of prolonged high intensity exercise on IκB protein content (LPS/TLR4). BMDM from C57 BL/6 (WT) mice were stimulated with (A) 100 ng/ml LPS and (B) 10 µg/ml (Poly I:C) for the indicated times and total content of cellular protein IκB levels were measured following the completion of the exercise training program. 1 representative western blot of 8 mouse models (4-exercise and 4-sedentary) from each treatment reflecting higher degradation of IκB protein in the exercise group.

3.2.2) *RIG-I mediated NF-κB induced genes are down-regulated by prolonged high intensity exercise*

Similar to the effect of chronic moderate exercise on RIG-I mediated NF-**κ**B induced genes, a long-term high intensity exercise training program has also showed a reduction in the expression levels of NF-**κ**B induced genes when stimulated with transfected Poly (I: C). For example, the expression of iNOS (6h Sed: 1.05 ± 0.478 vs. 6h Ex: 0.573 ± 0.478, $p<0.05$; Fig. 3.2.3A) and TNFα (3h Sed: 1.02 ± 0.340 vs. 3h Ex: 0.683 ± 0.340, $p<0.05$; Fig. 3.2.3B) were down-regulated as measured by real-time PCR. This demonstrates that pro-inflammatory NF-κB induced genes were lower in the exercise group of both moderate and high intensity modes, when compared to the sedentary group.

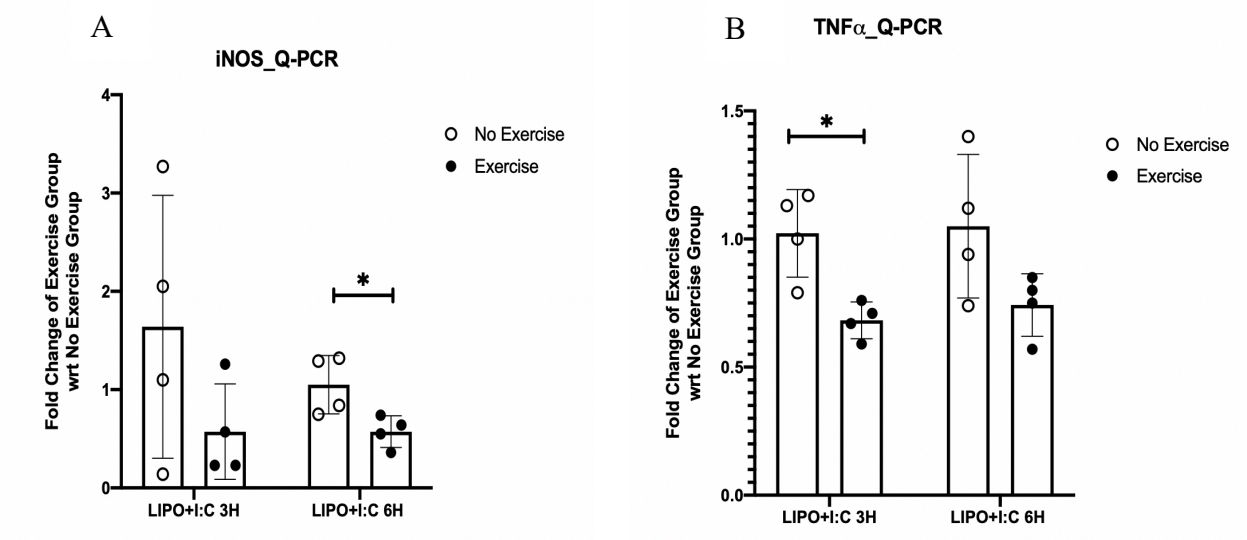

Figure 3.2.3- RIG-I mediated NF-κB induced pro-inflammatory gene expression levels following a prolonged high intensity exercise training program. BMDM from C57 BL/6 (WT) mice were stimulated with Lipofectamine 3000 + 10 µg/ml Poly (I:C) for the indicated times and gene expressions of iNOS and TNFα were evaluated by real-time PCR (Q-PCR). Results were also normalized to RPLP0 and reported as relative fold change of exercise group w.r.t no exercise group. Results represent mean ± SEM for 8 participants. (A) *, 6h sedentary > exercise, p<0.05. (B) *, 3h sedentary > exercise, p<0.05.

CHAPTER 4

Discussion and Conclusion

In this study, we questioned whether the kinetics and molecular mechanisms of intracellular innate immune responses are changed by the alterations in the intensity and duration of physical activity. Most of the previous studies typically target a recreational or moderate level of exercise intensity or various durations of intensive exercise training (continuous or interval). These studies have demonstrated the influence of acute and prolonged exercise on the magnitude and function of the cellular immune response, which has major implications on the inflammatory response and its ability to fight infections, however research that sheds light on the mechanism through which exercise affects cytokine levels and the quantitative and qualitative state of immune cells is lacking. Therefore, understanding how physical activity in the form of exercise induces these changes will lead to the discovery of effective training and enable us to select and optimize training programs that enhance immune function and, reduce the incidence and severity of autoimmune diseases and training-related infections in athletes, while simultaneously enhancing the anti-inflammatory effects of exercise. In this thesis, we have also identified the long-term and long-lasting effects of moderate and vigorous intensity exercise on inflammatory responses. In order to fulfill the objectives of this research study, combinations of *in vivo* and *in vitro* models were used.

It is important to note that effects of exercise on innate immune system are context dependent and exercise will potentially affect the regulators of pro- and anti-inflammatory genes. Our data indicate that the J-shaped relationship between exercise, infection risk and immune function is more complicated due to the fact that inflammatory responses against viral PAMPs were enhanced while such responses against other PAMPs were down-regulated following moderate chronic

physical activity. This mode of exercise potentially modifies inflammatory response by differentially altering intracellular signalling pathways which might lead to improved immune responses to certain infections or resolving inflammation faster in prolonged inflammatory conditions. We found that such exercise intensity, which corresponds to 65-70% of VO_{2max} shows the following results:

The reduction in the TLR mediated NFκB induced genes (e.g. IL-1β, TNFα, IL-10 and TGFβ) and lower degradation of IκB protein levels suggest that inflammation caused by a bacterial molecular ligand, LPS and a synthetic analog of viral infection, Poly (I: C) is indeed down-regulated following moderate chronic exercise. Interestingly, similar results were seen in the STING mediated NFκB induced genes, which indicates that the inflammatory response against a cyclic di-nucleotide (3'3'-cGAMP) is also down-regulated. In order to confirm whether moderate chronic exercise has similar effects on the inflammatory response against viral molecular mimics, we have stimulated the BMDMs with synthetic dsRNA analogue of a viral infection (e.g. Transfected Poly (I: C)). Inflammation caused by such stimulator has shown to be down-regulated as well, however, the molecular mechanism and cytokine levels are different as reflected by the RIG-I mediated NF-**κ**B induced gene expression levels and IκB protein levels. It is evident that the expression levels of pro-inflammatory genes IL-1β and TNFα are lower while, anti-inflammatory genes IL-10 and TGFβ are up-regulated by chronic moderate exercise. Results were confirmed on the mechanistic levels by using western blot analysis, which also demonstrated lower degradation of IκB protein in the exercise group. Furthermore, we characterized innate immune responses of BMDMs to the cytosolic exposure of the dsDNA analogue Poly (dA: dT), which induces a delayed and slow intracellular signal towards NF-κB. We compared the expression of

IL-1β, TNF-α, IL-10 and TGFβ after poly (dA: dT) exposure. Surprisingly, we discovered that Poly (dA: dT) stimulated BMDMs exhibit higher pro- and anti-inflammatory NF-κB induced gene expression following moderate exercise training. This indicates that the activation of RIG-I receptor by transfected Poly (dA: dT) leads to a delayed signal that enhances inflammation, which was also confirmed with western blot analysis, where higher degradation of IκB protein levels were seen in the exercise group. These differences are potentially due to the activation of the innate immune signalling adaptor MAVS and release of substances such as reactive oxygen species (ROS), which signal to generate an anti-inflammatory response by inducing the expression levels of antiviral genes. As discussed earlier, NFκB induced genes are linked to several autoimmune and inflammatory diseases including rheumatoid arthritis (RA), multiple sclerosis, atherosclerosis, type I diabetes and chronic obstructive pulmonary disease. Therefore, our findings might establish the basis of how exercise is able improve the excessive inflammation that is caused by such diseases.

In addition to NF-κB, Interferon Regulatory Factor-3 (IRF-3) is a major transcriptional factor of intracellular inflammatory response. Once activated, this transcription factor is able to induce the expression of pro-inflammatory gene, IFNβ, which is considered as a key cytokine for controlling viral infections and promoting other viral responses. anti-viral innate immunity. In this thesis, we have also evaluated whether the molecular mechanisms of this intracellular innate immune pathway are changed by chronic moderate exercise. The increase in the TLR4 mediated IRF-3 induced gene (e.g. IFNβ) and a slightly higher expression of P-IRF3 protein levels suggest that inflammation caused by a component of gram-negative bacteria (e.g. LPS) is up-regulated following moderate chronic exercise. It is important to note that while the detection of P-IRF3

protein expression appears to be more delayed than the gene expression of IFNβ, this is due to the lack of sensitivity of P-IRF3 detection by western blotting. Notably, similar results were seen in the STING mediated IRF-3 induced genes, which indicates that this inflammatory response against a bacterial cyclic di-nucleotide (3'3'-cGAMP) is also up-regulated. Our results prompted the idea to investigate whether moderate chronic exercise shows identical effects on RIG-I mediated IRF-3 induced genes. Remarkably, inflammation caused by viral molecular mimics (Transfected Poly (I: C) has shown to be down-regulated and it is evident that the expression of pro-inflammatory gene IFNβ is reduced by chronic moderate exercise. Results were also confirmed on the mechanistic levels by using western blot analysis, which indicated no difference or slightly lower P-IRF3 protein levels in the exercise group.

Our surprising findings from the effects of moderate intensity exercise on innate immunity sheds light to further investigate how long-term high intensity exercise, which corresponds to 85-90% of VO_{2max}, affects innate immune response and inflammation. Our data from this experiment indicate that when BMDMs are stimulated with a component of gram-negative bacteria (e.g. LPS), prolonged high intensity physical activity enhances TLR-induced inflammatory gene expression including, iNOS, TNFα and IL-6 (Fig. 3.2.1) as well as higher degradation of IκB protein levels (3.2.2). This indicates that inflammation caused by such inducers, is up-regulated following a long-term high intensity exercise.

Furthermore, the effects of this mode of exercise on viruses were investigated and we found that inflammation caused by viral molecular mimics (e.g. Transfected Poly (I: C)) has shown to be down-regulated and it is evident that TLR-induced inflammatory gene expression levels of iNOS and TNFα are reduced by prolonged high intensity exercise (Fig. 3.2.3).

4.1) *Conclusion*

In the current study, determining the effect of distinct forms of exercise on innate immunity and inflammation found *in vivo* provides a new perspective on the cumulative effects of physical activity on the levels of pro- and anti-inflammatory cytokines. Based on the findings from the present research, and in support of our hypothesis, we found that prolonged moderate intensity exercise training may have a long-term and long-lasting effect on various intracellular innate immune responses and inflammation of mouse models. This will provide the need to find an equivalent exercise protocol, which could be translated into the human population. Given the improvements in understanding the molecular mechanisms in which different modes of exercise affect innate immunity, this study highlights that the exercise routine can be optimized to find the right balance between lowering the risk of URTI, while at the same time preventing excessive inflammation, which could lead to further health complications.

CHAPTER 5

Future Directions

Given that our findings from this study will make a significant difference in the field of exercise and innate immunity, other potential *in vitro* genetic and biochemical studies (gene and protein expression levels) should be employed to confirm the role of the regulators which exhibited a variation in expression levels. Moreover, currently available knockout mice will be used to assess whether the effects of various exercise regimens on cytokine levels and on the number and composition of immune cells are affected. Finally, an unbiased screen such as RNA-seq will be employed to compare gene expression from mRNA obtained from mouse macrophages of various exercise groups. RNA-seq will be performed on an Illumina HiSeq 2500 with 46-52 million reads per sample).

Furthermore, our studies will be directed at investigating how exercise affects the epigenetic control of gene expression in immune cells. For the purposes of chromatin accessibility, a newly emerging, highly sensitive approach called Assay for Transposase Accessible Chromatin using Sequencing (ATAC-seq) can be employed in which a chromatin is probed with hyperactive mutant Tn5 transposase to identify regions of DNA that are accessible and ready to be transcribed. The transposase enzyme will tag the genome with sequencing adapters, which will then be intensified by PCR and used to map the genome of interest (Buenrostro, J. D., et al, 2015).

Additionally, it is of value to conduct further research to determine the pathways and mechanisms responsible for the effects of exercise on immune system in different population groups. As an example, studies could be performed to measure immunologic alterations following exercise training programs of different types, duration and intensities in cancer patients. Results

obtained from such studies could be of importance to the clinicians to optimize patient-specific exercise protocols that would help reduce some of the inflammatory side effects of cancer treatment methods. Such studies could be further optimized by including both pediatric and adult subjects to further appreciate the outcome of training programs in different age groups. Studies focused on different age groups do not only benefit the patients as mentioned above, but also healthy populations in that it can help in gaining insight into the impact of such training on overall health status and risk of infections like URTIs in young vs. elderly population. It is to be noted that benefiting from study designs mentioned above necessitates looking beyond the less predictive animal models and making use of human subjects. This would provide a more clinically reliant platform for studies on effects of exercise on immune function

CHAPTER 6

References

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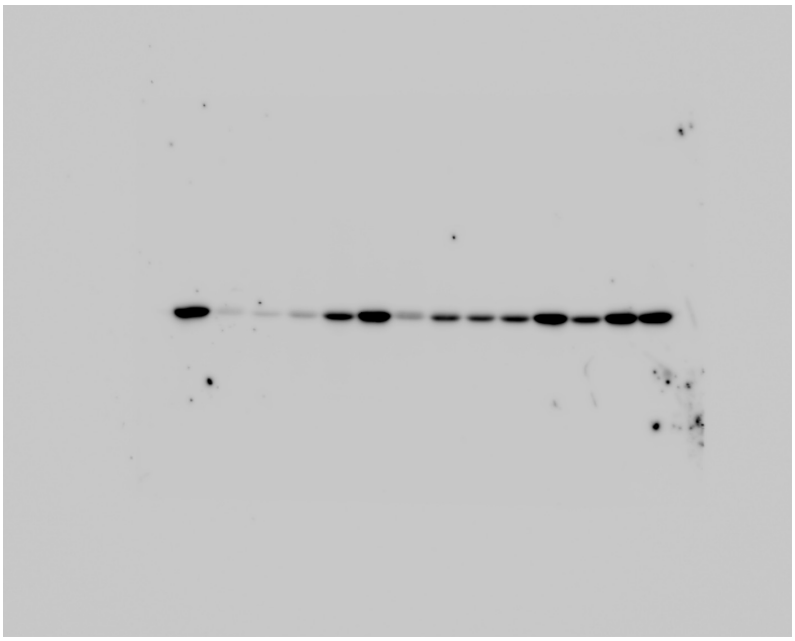
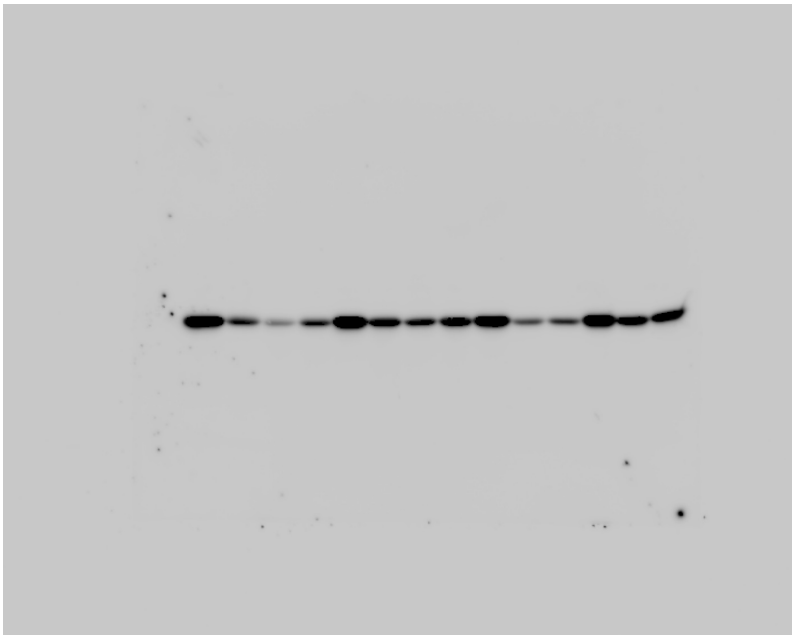
Appendix A

Images of Western Blots

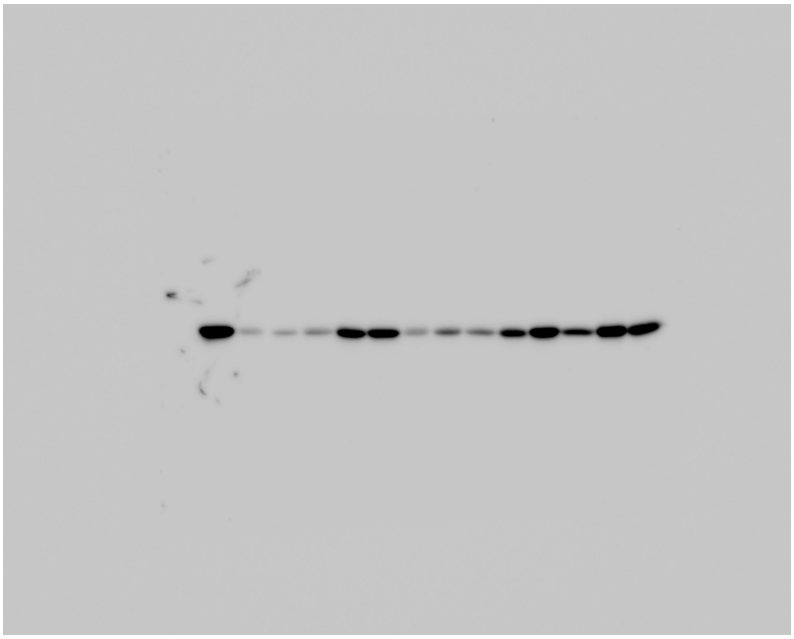
1. I**κ**B (Fig 3.1.7 A and Fig 3.1.22 A)



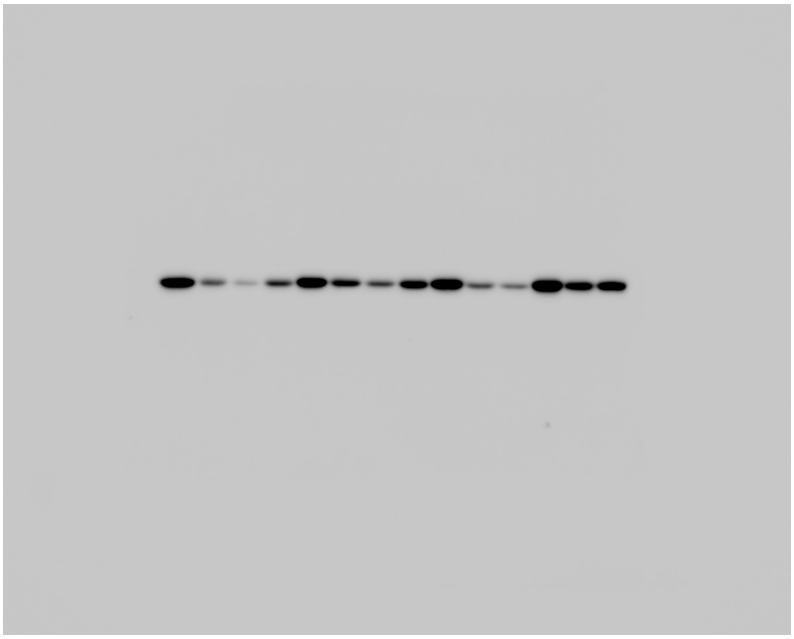
2. IκB (Fig 3.1.8 A and Fig 3.1.17 A)



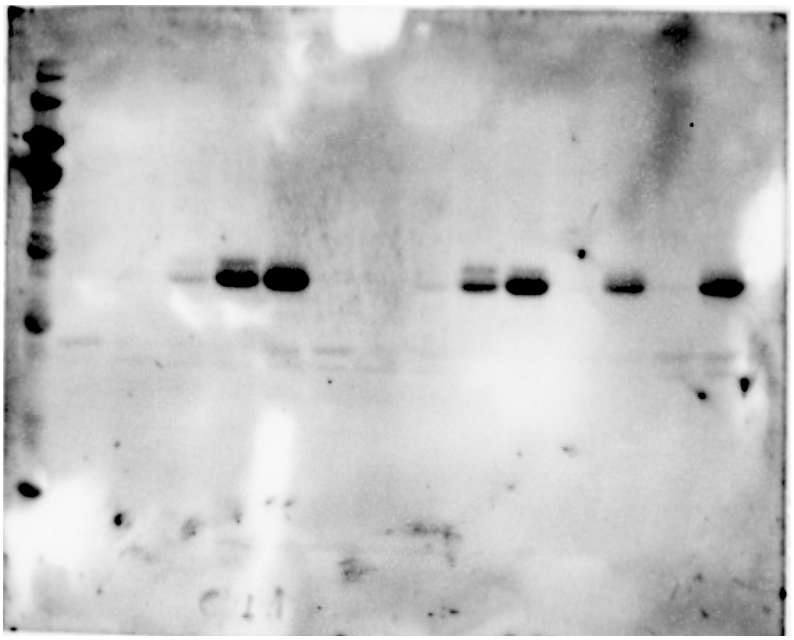
3. IκB (Fig 3.1.7 B and Fig 3.1.22 B)



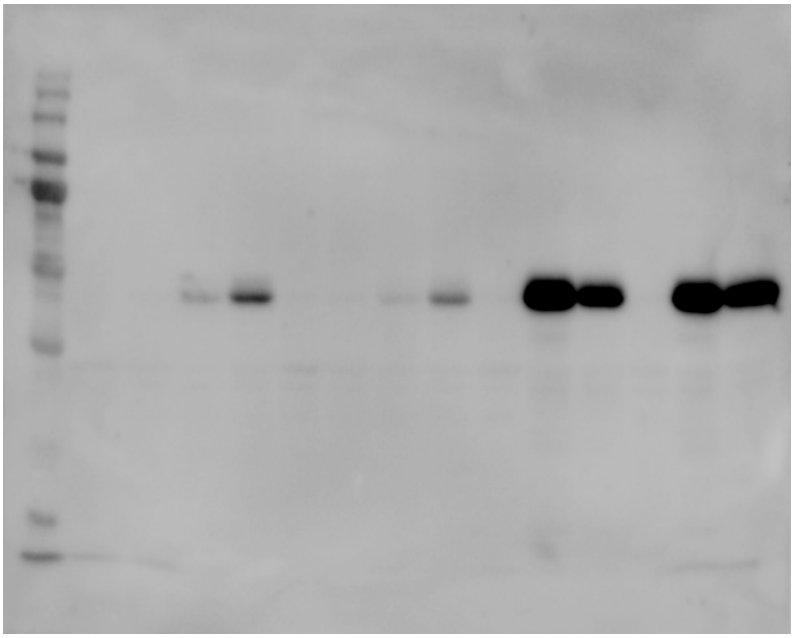
4. IκB (Fig 3.1.8 B and Fig 3.1.17 B)



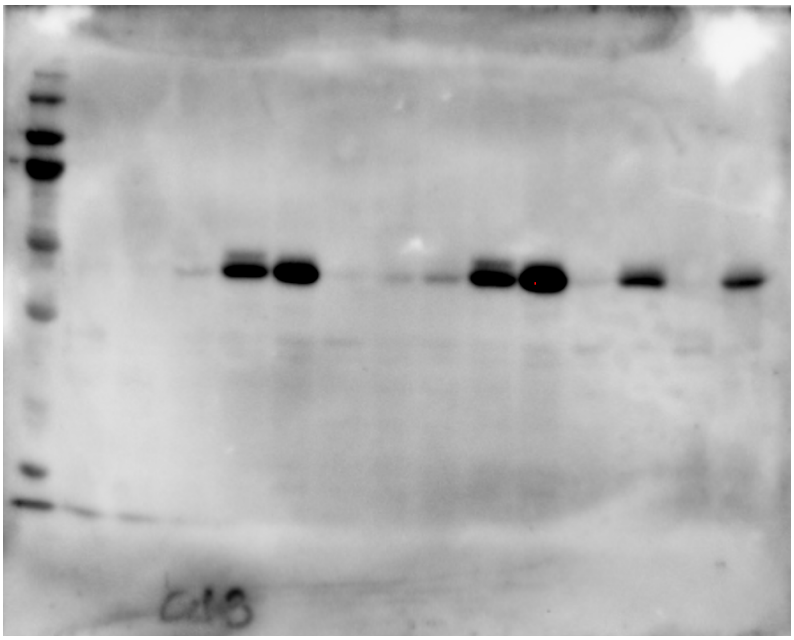
5. P-IRF3 (Fig 3.1.24 A)



6. P-IRF3 (Fig 3.1.27 A)



7. P-IRF3 (Fig 3.1.24 B)



8. P-IRF3 (Fig 3.1.27 B)