

Does Caffeine Consumption before High-Intensity Intermittent Exercise Enhance Immunity?

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Abstract

Regular participation in moderately intense physical activity decreases the risk of picking up common colds below that of a sedentary individual. However, performing prolonged, high-intensity, exercise, or sustained periods of strenuous training, is associated with an above average risk of getting infections. However, less is known about how the immune system responds to brief (less than 30 min) bouts of high-intensity intermittent exercise (HIIE), which has become popular due to its efficacy in enhancing health and fitness in general and clinical populations. Furthermore, nutrition and exercise have powerful influences on the body's immune system and therefore dietary factors and exercise could be coupled to help improve immune function. One potential dietary substance is caffeine, which is now a common element in most people's diet, due to its alertness-enhancing effects. Though research is limited, caffeine has been shown to enhance the activation of both natural state and antigen-stimulated T ($CD4^+$ and $CD8^+$) and NK cells following strenuous endurance exercise. However, there is no research investigating the interaction between HIIE and caffeine ingestion on the lymphocytes of innate and adaptive immune functions. Therefore, the aim of this study was to determine the effects of ingesting caffeine before HIIE on innate and adaptive immune functions following brief (20 min) HIIE.

A double-blind cross-over design was adopted, during which 10 healthy active men participated in two exercise trials following acute (60 min pre-exercise) consumption of 6 mg.kg^{-1} caffeine or placebo. Each trial required participants to perform a 20 min HIIE protocol (10 x 1 min at $\sim 90\% \text{ HR}_{\text{max}}$; 1 min active recovery, 50W) in the laboratory on a cycle ergometer. Venous blood samples were collected pre-supplement, pre-exercise, immediately post-exercise and 1 h post-exercise. Samples were analysed for numbers of natural stage and antigen-stimulated T ($CD4^+$ and $CD8^+$) and NK cells expressing CD69 markers, as well as the GMFI of the expressed CD69. Serum caffeine and, plasma cortisol and adrenaline concentration were also determined.

Consuming caffeine one hour before HIIE increased the number of circulating NK cells by 56% at the pre-exercise ($P < 0.01$) stage. Although not significant, caffeine also increased the circulating NK cells number by 13% measured immediately post-exercise. However, caffeine had minimal effect on the number of circulating $CD4^+$ and $CD8^+$ T cells. Caffeine also increased the number of unstimulated and antigen-stimulated NK cells expressing CD69 (unstimulated: 71%, $P < 0.01$; stimulated: 51%, $P < 0.05$) at pre-

exercise stage, but had little effect on T cells. Although not statistically significant, caffeine increased the number of NK cells expressing CD69 by 7% for unstimulated and 13% for stimulated cells at the post-exercise stage. However, caffeine had minimal effect on GMFI expression of CD69 on T and NK cells. Compared with pre-supplement, HIIE induced the main time effect of CD69 GMFI expression of antigen-stimulated NK cells ($P<0.01$) at 1 h post-exercise.

Overall, the thesis findings suggest that caffeine ingestion one hour before HIIE may increase the innate immune function, as NK cell numbers and activation were increased. However, caffeine prior to HIIE does not appear to alter the circulating number and activation of adaptive immune cells ($CD4^+$ and $CD8^+$ T cells). While the observed acute innate immune response to caffeine consumption appears desirable, it remains to be determined if acutely improved innate immune function will actually result in reduction of an individual's susceptibility to infection following HIIE.

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Attestation of Authorship

I hereby declare that this submission is my own work and that, to the best of my knowledge and belief, it contains no material previously published or written by another person (except where explicitly defined in the acknowledgements), nor material which to a substantial extent has been submitted for the award of any other degree or diploma of a university or other institution of higher learning.

A handwritten signature in black ink, appearing to read 'KHINE THIDA', with a stylized flourish above the text.

Signed _____ Date ____30.09.16____

Khine Thida

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List of Abbreviations

ANOVA	analysis of variance
AMP	adenosine monophosphate
APC	antigen presenting cell
ARK	adrenergic receptor kinase
ATP	adenosine triphosphate
BM	body mass
BW	body weight
BSA	bovine albumin serum
CAF	caffeine
cAMP	adenosine 3', 5'-cyclic monophosphate (cyclic AMP)
CD	cluster of differentiation
CD3 ⁻ CD16 ⁺ CD56 ⁺	natural killer cell
CD4 ⁺	T helper cell
CD8 ⁺	T cytotoxic cell
CD45RA ⁺	naïve T cell
CD69	early lymphocyte activation antigen
ConA	concanavalin A
CNS	central nervous system
DC	dendritic cells
EDTA	ethylene diamine tetraacetic acid
FITC	fluorescein isothiocyanate
FL-1	FL-1 = 525 nm band pass filter (Green)
FL-2	FL-2 = 575 nm band pass filter (Orange-Red)
FL-3	FL-3 = 620 nm band pass filter (Red)
FoxP3	fork-head box protein 3
GC	glucocorticoid
GM-CSF	granulocyte-macrophage colony-stimulating factor
GMFI	geometric mean fluorescence intensity
HIIE	high-intensity intermittent exercise

HIT	high-intensity training
Hsp	heat shock protein
HSPC	hematopoietic stem and progenitor cell
HPLC	high performance liquid chromatography
HR	heart rate
HR _{max}	maximal heart rate
IFN	interferon
IL	interlukin
IgE	immunoglobulin E
K3EDTA	tripotassium ethylene diamine tetraacetic acid
MAB	monoclonal antibodies
MAMPs	microbial-associated molecular patterns
MHC	major histocompatibility complex
MPS	mononuclear phagocyte system
NK	natural killer
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PE	phycoerythrin
PE-Cy5	R-phycoerythrin-Cy5
PHA	phytohaemagglutinin
PKA	protein kinase A
PLA	placebo
PRRs	pattern recognition receptors
RPE	ratings of perceived exertion
RPMI	Roswell Park Memorial Institute medium
SD	standard deviation
T _C	T cytotoxic
TCR	T cell receptor
TGF	transforming growth factor
T _H	T helper

TNF	tumor necrosis factor
Treg	T regulatory
URTI	upper respiratory tract infection
VO_{2max}	maximum oxygen uptake
$VO_{2\ peak}$	peak oxygen uptake
Wmax	maximal workload

Chapter 1 Introduction

Exercise can impact either negatively or positively on an individual's immune function and susceptibility to infection, particularly upper respiratory tract infections (URTI), depending on a range of factors such as exercise intensity and duration, sports discipline, and the individual's fitness level, nutrition and lifestyle (Gleeson, Bishop, & Walsh, 2013; Nieman, 1994, 1998). Specifically, it has been shown that there is a temporary reduction in the number of lymphocytes (lymphocytopenia), and cytotoxic and phagocytotic activity for 3 to 24 h following both prolonged (>1.5 h) and high intensity ($>70\%$ peak oxygen intake, $VO_{2\text{peak}}$) exercise (Gleeson, Bishop, & Walsh, 2013). This period is commonly known as the “open window period”, which is considered to account for the increased susceptibility to URTI (Ekblom, Ekblom, & Malm, 2006; Gleeson, Bishop, & Walsh, 2013; Nieman, 1994).

The “J-shaped” (Figure 1) model was first proposed by Nieman (1994) to reflect the relationship between exercise intensity, the immune system and susceptibility to infection. The J-shaped curve illustrates that moderate exercise has a positive impact on immune health, thereby reducing the risk of URTI; whereas acute bouts of high-intensity, prolonged exercise negatively affects the immune system, resulting in impairment in a range of immune parameters, thus increasing the risk for URTI, when compared to sedentary persons (Nieman, 1994).

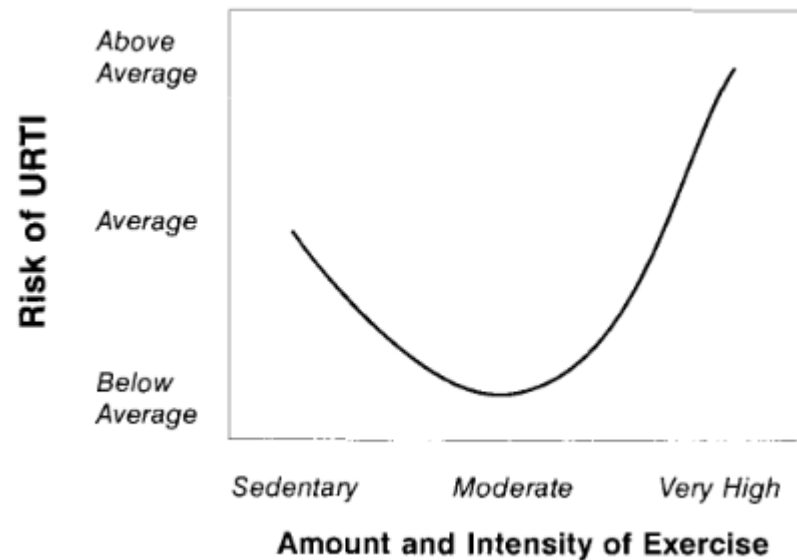


Figure 1. J-shaped relationship between exercise intensity, immune function and infectious risk.

Adapted from “Exercise, Upper Respiratory Tract Infection, and the Immune System,” by D.C. Nieman, 1994, *Medicine and science in sports and exercise*, 26(2), p. 129. Reprinted with permission.

A vast amount of research has been conducted to better understand the risk of infection with opportunistic pathogens during the “open window” period that leads to increased episodes of URTI, especially in people who usually pursue intense exercise (Gleeson, Bishop, & Walsh, 2013; Natale et al., 2003; Neves et al., 2015; Newsholme, 1994; Nieman, 1994; Nieman et al., 2000; B. K Pedersen & Hoffman-Goetz, 2000).

Specifically, research has shown that high intensity training (HIT) increases the secretion of circulating cortisol and adrenaline (Jacks, Sowash, Anning, McGloughlin, & Andres, 2002; Lovallo, Farag, Vincent, Thomas, & Wilson, 2006; Nemet, Mills, & Cooper, 2004; Vincent et al., 2004), both of which are known immunosuppressive stress hormones (Gleeson, Bishop, & Walsh, 2013). Therefore, HIT is believed to result in immune suppression (Krüger & Mooren, 2014). However, there is limited evidence on the effect of brief (less than 30 min) high-intensity intermittent exercise (HIIE) on the immune system (Walsh, Gleeson, et al., 2011). Recently, HIIE protocols have evolved, based on the accumulating evidence that HIT is a time effective way to enhance an individual’s fitness and health in general and clinical populations (Boutcher, 2010). For this reason, HIIE appears to be more prevalent than continuous long duration exercise in the fitness and health industry.

In addition to exercise approaches and their effect on immune function, there is growing interest in the manipulation of diet and nutritional aspects to overcome immune suppression following acute bouts of high intensity or endurance exercise (Claassen et al., 2005; Gleeson, Bishop, & Walsh, 2013; J. B. Mitchell et al., 1998; Zydek, Michalczyk, Zajac, & Latosik, 2014). This has included altering the consumption of carbohydrates, protein and fat, as well as supplementing the diet with vitamins and antioxidants (Andrew et al., 2001; Erlenbusch, Haub, Munoz, MacConnie, & Stillwell, 2005; Zydek et al., 2014). In this regard, caffeine has become one of the emerging dietary supplements in exercise immunology research, as it is a legal substance and widely consumed in the diet in different forms such as food, drink, supplements, and drugs, to enhance an individual's alertness (Andrews et al., 2007).

Caffeine is classed as a methylxanthine, which stimulates the central nervous system. The main mechanism of caffeine is inhibition of adenosine (Wesselink et al., 2016). The secondary mechanism of caffeine is the stimulation of adrenaline (epinephrine) release, which in turn induces a series of secondary metabolic changes, resulting in psychoactive effects (Graham, 2001; Kamimori et al., 2000). Caffeine intake may also account for immunomodulation, either directly via adenosine receptor antagonism, or indirectly via stimulation of adrenaline release. In addition, caffeine also increases the secretion of cortisol, which may also result in immune dysfunction (Krüger & Mooren, 2014; Lovallo et al., 2006). Despite the prevalence of use of caffeine and potential influences of caffeine on the immune system, a limited amount of research has focused on how caffeine may affect integral immune markers such as T lymphocytes and Natural Killer (NK) cells (Fletcher & Bishop, 2011a).

Despite the number of studies on the effect of caffeine intake on the immune system and manipulation of caffeine intake to prevent the "open window" period after exercise, there are several limitations and gaps in the literature that need to be addressed. Firstly, most studies that address the interaction of caffeine and the immune system are based on prolonged high intensity or endurance exercise (Doherty & Smith, 2004; Fletcher & Bishop, 2012; Gleeson, Bishop, & Walsh, 2013; Graham, 2001). Of the research studies that have been conducted, most have chosen endurance exercise (70-75% $\text{VO}_{2\text{max}}$ for 60-90 min) for their experimental trials (Bishop, Fitzgerald, Porter, Scanlon, & Smith, 2005; Desbrow et al., 2012; Fletcher & Bishop, 2011a). Bishop, Fitzgerald, et al. (2005) and Fletcher and Bishop (2012) have found that taking a 6 mg.kg^{-1} dose of caffeine 1 h before prolonged strenuous exercise enhances the activation of T and NK cells.

However, to the author's knowledge, no study to date has investigated the effect of short duration (20 min) HIIE on T and NK cells. Secondly, exercise immunology research often emphasises only one aspect of immune function, despite the immune system having two defence arms; innate and adaptive (Fletcher & Bishop, 2012; Rohde, MacLean, Richter, Kiens, & Pedersen, 1997). Thirdly, to my knowledge, no study to date has reported the interaction between caffeine ingestion and antigen-stimulated lymphocyte activation following short duration HIIE. As lymphocyte activation occurs before proliferation and not all activated cells go through to proliferation stages (Elgert, 2009), this study investigated the effects of caffeine ingestion combined with 20 min HIIE on antigen-stimulated lymphocyte activation. Lastly, most caffeine research has been conducted using *in vitro* investigations with rodent models, with the concentrations of caffeine used in these studies tending to be more pharmacological (500 to 5000 $\mu\text{mol/L}$) than "normal" physiological concentrations (70 $\mu\text{mol/L}$ or less) (Graham, 2001). Accordingly, the current study set out to investigate the effect of the ingestion of a physiological dose of caffeine on both innate and adaptive immune function following short duration HIIE.

1.1 Aims

The objective of this study was to extend current knowledge regarding the responses of the innate (NK cells, CD3-CD56+) and adaptive (T cells, CD4+ and CD8+) arms of the immune system in humans following HIIE and a 6 mg.kg^{-1} BW dose of caffeine consumption. The specific aims of the thesis were therefore to determine the effects of caffeine on the:

- number of circulating T and NK cells following HIIE
- activation of T and NK cells, both in their natural and antigen-stimulated stages, which are measured in term of the cells expressing early activation markers CD69⁺
- geometric mean fluorescence intensity (GMFI) expression of expressed CD69⁺ on activated T and NK cells.

1.2 Hypothesis

Theoretically, 6 mg.kg^{-1} BW of caffeine ingestion is expected to increase the T and NK cell activation following antigen sensitization after the high intensity exercise. However, it is worthwhile to note that T cell activation was suppressed and NK cell activation was

stimulated in the caffeine study conducted by Fletcher and Bishop (2012). As this study possessed similar protocols to Fletcher and Bishop (2012), but differed in exercise mode and participant fitness levels, it was hypothesised that:

- Administration of a single 6 mg.kg^{-1} BW dose of caffeine would have immunosuppressive effects on T cells, but immunostimulatory effects on NK cells, in response to antigen stimulation following 20 min of HIIE.

Chapter 2 Influences of Caffeine Consumption on Immune System following Strenuous Exercise

An extensive amount of literature demonstrates that regular participation in moderate exercise not only reduces the relative risk of infection, but also is beneficial for the treatment and prevention of different diseases. For example, Gleeson, Bishop, Oliveira, and Tauler (2013) report that there is ~20-45% decrease in the risk of URTI in moderately active individuals compared with inactive individuals, due to the positive effect regular moderate exercise has on both the innate and adaptive immune systems. However, in contrast, Gleeson, Bishop, and Walsh (2013) and Walsh, Hoffman-Goetz, et al. (2011) report that prolonged bouts of strenuous exercise suppress various immune parameters, with suppression usually lasting for about 3 to 24 h after exercise. This might account for the increased susceptibility to URTI (Gleeson, Bishop, & Walsh, 2013). This immune dysfunction appears to be most pronounced in continuous, prolonged (>1.5 h), moderate to high intensity (55% to 75% maximum oxygen intake, $\text{VO}_{2\text{peak}}$) exercise (Gleeson, Bishop, & Walsh, 2013). This notion is supported by Nieman et al. (2006), who demonstrated that nearly one in four runners out of 155 ultra-marathoners reported an URTI episode during the two week period following a 160-km race. Nieman et al. (2006) suggest that this could have been due to a significant decrease in cell-mediated immunity. Stelzer et al. (2015) also seem to lend support to this notion that ultra-endurance exercise induces the functional impairment of both mature hematopoietic cells and immature hematopoietic stem and progenitor cells (HSPC), which are essential for immune system function and regeneration (Alaiti, Ishikawa, & Costa, 2010; De Lisio & Parise, 2013).

Although most of the exercise immunology research focuses mainly on the immunosuppressive effects of prolonged moderate intensity training, some high intensity short duration training (cycling at 90% $\text{VO}_{2\text{peak}}$ for 5 min) studies have also reported immunosuppressive changes in leukocyte counts (Natale et al., 2003) similar to that of endurance training (cycling 60% $\text{VO}_{2\text{max}}$ for 2 h) in healthy active individuals. It is interesting to note that T lymphocyte counts fell below baseline, even after 3 h post-exercise with short duration high intensity exercise (90% $\text{VO}_{2\text{peak}}$ for 5 min), but had returned to baseline levels at the same time-point with prolonged endurance (60-65% $\text{VO}_{2\text{peak}}$ for 2 h) exercise. In agreement with Natale et al. (2003), Tuan et al. (2008) suggest that short term high intensity exercise (85% $\text{VO}_{2\text{peak}}$ for 30 min) leads to deleterious effects on the major immune cells, the leukocytes. Similar to Natale et al.

(2003) and Tuan et al. (2008), other investigators have reported the changes in leukocyte and subset counts following high intensity exercise in both short and prolonged duration, and they are considered to be potential causes of URTI (Nemet et al., 2004; Radom-Aizik, Zaldivar, Leu, Galassetti, & Cooper, 2008; Sand, Flatebo, Andersen, & Maghazachi, 2013). Nevertheless, it is interesting to note that the majority of reported URTI episodes were not clinically confirmed (Pacque, Booth, Ball, & Dwyer, 2007; Tiollier et al., 2005). Therefore, it has been highlighted that there is a possibility that the self-reported URTI were simply airway inflammation (i.e., sore throats) rather than URTI (Peters, Shaik, & Kleinvelde, 2010). Indeed, increased incidence of sore throats may be due to the increase in respiratory rates during exercise, which possibly leads to the excessive inhalation of polluted dry air, triggering airway inflammation (Gleeson, Bishop, & Walsh, 2013; Peters et al., 2010; Tiollier et al., 2005).

Despite the well-established exercise immunology research, the majority of studies use either animal models or *in vitro* examination of immune cells (Harper Smith et al., 2011; Walsh, Hoffman-Goetz, et al., 2011). Additionally, only a handful of studies examine the effects of short duration (<30 min) HIIE on the whole immune system (i.e., on both the innate and adaptive systems) and *in vivo* immune responses in humans (Harper Smith et al., 2011; A. Smith et al., 2004; Walsh, Hoffman-Goetz, et al., 2011). Furthermore, there has been considerable interest in preventing the immune suppression following strenuous exercise by manipulating an individual's diet. As an example, J. B. Mitchell et al. (1998) looked at the impact of carbohydrate status on the immune system before and after endurance exercise. Caffeine is one of the growing elements in most people's diet, and researchers have been looking at how consuming caffeine may enhance the immune system after strenuous exercise (Bishop, Fitzgerald, et al., 2005; Fletcher & Bishop, 2012).

Therefore, the aim of this literature review was to understand the following four main topics:

- the innate and adaptive immune system
- the effects of acute bouts of exercise on lymphocyte mobilisation
- the effects of acute bouts of exercise on lymphocyte activation
- the impacts of dietary intake on the immune system.

2.1 The Immune System: Functions and Immune Cells

The complete immune system consists of two important immune defense arms: the innate and the adaptive. The innate arm of the immune system is the first defence against invading pathogens (Elgert, 2009). The adaptive arm of the immune system initiates when pathogens break through the innate arm and delivers specific responses to the pathogens (Elgert, 2009).

2.1.1 The Innate Immune System

When pathogens attempt to enter the body, the body's immune system attacks the pathogens immediately by initiating the innate immune system. As its name suggests, the innate immune cells are primitively present in healthy individuals (Abbas, Lichtman, Pillai, & Clinical, 2014). The innate immune system has a number of major functions. Firstly, it is available to act as the earliest non-specific barrier to entry of foreign bodies, rapidly killing the invaded microorganisms and eliminating the dead cells (Abbas et al., 2014). Secondly, it plays a role in the initiation and instruction of adaptive immune responses to mount specific and long-term defence (Kumar, Kawai, & Akira, 2011). Thirdly, it occupies important roles in the detection and killing of tumour cells (Abbas et al., 2014).

The innate immune defence consists of cellular and humoral mechanisms, including anatomical and chemical barriers, cellular components and the mononuclear phagocyte system (MPS) (Gleeson, Bishop, & Walsh, 2013; Tosi, 2005). Epithelial layers of skin, mucosal membranes, mucus, ciliary and contraction of muscles act as the anatomical barrier, preventing the entry of microorganisms into the body (Gleeson, Bishop, & Walsh, 2013; Tosi, 2005). Chemical barriers include cytokines, and acute phase and complement proteins, which not only stimulate the activation and proliferation of various immune cells but also facilitate the killing of pathogens (Tosi, 2005). When pathogens invade the body, one of the first innate responses to the pathogens is inflammation initiated by the microbial-associated molecular patterns (MAMPs), which are unique molecular structures broadly shared by groups of microbes, but different from host molecules (Akiko & Ruslan, 2010; Koenderman, Buurman, & Daha, 2014). MAMPs are detected by the sensorial proteins found on the innate immune cells, known as pattern recognition receptors (PRRs) (Akiko & Ruslan, 2010; Koenderman et al., 2014; Kumar et al., 2011). The complement cascade facilitates the immune system to identify pathogens for destruction by the other immune cells, recruitment of phagocytes

and elimination of the pathogens or viral-infected host cells (Tosi, 2005). The leukocytes are essential cellular components of immune function and are derived from multipotent hematopoietic stem cells found in the bone marrow (Abbas et al., 2014; Elgert, 2009; Tosi, 2005). Leukocytes are made up of 60-70% granulocytes, 5-15% monocytes and 15-25% lymphocytes (Gleeson, Bishop, & Walsh, 2013).

Granulocytes include neutrophils, basophils and eosinophils. Granulocytes facilitate the inflammation reactions, recruiting more phagocytes to the site of infection, killing pathogens, removing tumour cells and promoting recovery processes (Gleeson, Bishop, & Walsh, 2013). Macrophages and dendritic cells (DC) originate from the monocytes. Monocytes accomplish phagocytosis, intracellular killing, the presenting of antigens to lymphocytes and release of cytokines (Gleeson, Bishop, & Walsh, 2013). Lymphocytes consist of natural killer (NK) cells, T cells (derived from the thymus glands) and B cells (derived from the bone marrow). NK cells are a major innate immune cells (Gleeson, Bishop, & Walsh, 2013).

Natural Killer Cells

NK cells are large granular lymphocytes developed in bone marrow and classically a major cellular component of the innate immune response (Timmons & Cieslak, 2008). NK cells have effective defence mechanisms such as cytokine induction and cytotoxicity, and delivery of anti-microbial, anti-viral and anti-malignancy reactions (Paust & von Andrian, 2011). In addition to its traditional role in innate immunity, recently there has been a paradigm shift in the role of NK cells, with cumulative evidence suggesting that NK cell-mediated adaptive immunity and memory NK cells undergo degranulation and cytokine production rapidly upon the reencounter with the same antigens (Gasteiger & Rudensky, 2014; Netea, Latz, Mills, & O'Neill, 2015; Paust & von Andrian, 2011; J. C. Sun, Beilke, & Lanier, 2009). According to Paust and von Andrian (2011) and Reeves et al. (2015), memory NK cell studies are mostly limited to the animal models, as researchers must rely on the memory NK cells generated *in vivo* in the liver tissue, and it is difficult to obtain fresh human liver tissue. *In vitro* induction of memory NK cells is currently unfeasible due to the limitations in current molecular biology technology (Paust & von Andrian, 2011). Furthermore, another adaptive immunity attribute of NK cells that has been recently discovered is their capability to adapt their response depending on the environment, rather than producing a uniform reaction (Eric et al., 2011). Prompt investigation of the molecular and cellular mechanisms of the crossover role of NK cells in innate and adaptive immunity needs to

be conducted to more accurately evaluate the impact of NK cells on overall immune function (Gasteiger & Rudensky, 2014).

NK cells make up 5-20% of the peripheral blood lymphocyte population and have a life span of 2 weeks (Gleeson, Bishop, & Walsh, 2013; Vivier, Tomasello, Baratin, Walzer, & Ugolini, 2008). NK cells can be detected using the fluorescent-labelled monoclonal antibodies (MAB) to specific combination of cell surface markers, also known as clusters of differentiation (CD) markers, for NK cells (Gleeson, Bishop, & Walsh, 2013). NK cells express both CD16 and CD56, but lack CD3, hence their CD phenotype as $CD3^-CD16^+CD56^+$ (Timmons & Cieslak, 2008). NK cells are traditionally named “natural killer” because they are capable of killing tumours or viral-infected cells lacking MHC class I molecules in the absence of prior immunisation (Gleeson, Bishop, & Walsh, 2013).

Human NK cells are classed into two functional distant subsets, based on their surface expression intensity of CD56; $CD56^{bright}$ and $CD56^{dim}$ (Farag & Caligiuri, 2006). $CD56^{dim}$ cells are the primary population (approximately 90%) of the NK cells and can be found in peripheral blood and spleen (Vivier et al., 2008). $CD56^{dim}$ cells express a low degree of CD56, but a high density of CD16 (Timmons & Cieslak, 2008). $CD56^{bright}$ cells comprise approximately 10% of total NK cells and are mostly found in the lymph nodes and tonsils (Vivier et al., 2008). $CD56^{bright}$ cells express a high density of CD56 but low or no levels of CD16 (Farag & Caligiuri, 2006; Timmons & Cieslak, 2008). $CD56^{bright}$ cells primarily secrete cytokines, whereas $CD56^{dim}$ cells are cytotoxic cells. The difference in the expression of various affinity-level interleukin (IL)-2 receptors differentiates these two subsets’ unique functions (Timmons & Cieslak, 2008). $CD56^{bright}$ cells constitutively express the high- and intermediate- affinity heterotrimeric IL-2 receptors, which promotes the extensive proliferation of $CD56^{bright}$ cells *in vitro* and *in vivo* in response to low concentration IL-2. This leads to the production of substantial amounts of immunoregulatory cytokines and chemokines (Farag & Caligiuri, 2006; Timmons & Cieslak, 2008). In contrast, IL-2 receptors on $CD56^{dim}$ cells only have intermediate affinity with IL-2. This leads to poor cell proliferation and response, even to high concentrations of IL-2, resulting in low cytokine production (Timmons & Cieslak, 2008). Expression of perforin and granzymes in their cytotoxic granules makes $CD56^{dim}$ essential cytotoxic cells (Vivier et al., 2008). Nevertheless, the IL-2 or IL-12 activated $CD56^{bright}$ cells are just as (or more) cytotoxic against NK targets as $CD56^{dim}$ cells (Cooper, Fehniger, Turner, et al., 2001).

2.1.2 The Adaptive Immune System

An adaptive immunity comes into effect when infectious agents manage to survive the innate immune response. The adaptive immune system is also known as specific or acquired immunity, as it is only acquired following the invasion of microbes into the body and it adapts the immunity specific to the invading microbes, mounting the effective antigen specific immune responses (Abbas et al., 2014). In addition to anti-microbial activity, the adaptive immune system also plays a crucial role in the prevention of autoimmune disease through the effective regulatory mechanisms differentiating the “self” from the “non-self” (Gleeson, Bishop, & Walsh, 2013). Another valuable feature of the adaptive immunity is the ability to mount immunologic memory, which enables the immune system to recognise and eliminate persistent and recurrent pathogens more rapidly and effectively. Lymphocytes, which make up 20-25% of leukocytes in peripheral blood, and their products (antibodies), are the major components of the adaptive immune system (Abbas et al., 2014; Gleeson, Bishop, & Walsh, 2013).

The adaptive immune system is comprised of two components; cell-mediated and humoral immunity, targeting the intracellular and extracellular microorganisms respectively (Abbas et al., 2014). T cells are the major element of cell-mediated immunity, facilitating the activation of phagocytes and killing the infected host cells. B cells are the main humoral immune cells, which secrete antibodies to attack the extracellular pathogens and toxins and guard them from entering the host cells (Abbas et al., 2014).

T Cells

T cells are small lymphocytes that primarily mature in the thymus and later on circulate to secondary lymphoid organs such as the spleen, lymph nodes and mucosa-associated lymphoid tissue (Gleeson, Bishop, & Walsh, 2013). They are the major cellular components of the acquired immune response, predominantly responsible for cell-mediated immunity. T cells comprise the majority (60-80%) of peripheral blood lymphocytes (Gleeson, Bishop, & Walsh, 2013). The major function is to provide the body with continuous antigen-specific immunological surveillance (Abbas et al., 2014). T cells attack the “non-self” invaders by releasing cytokines and toxic enzymes upon activation by the antigen presenting cell (APC) (Gleeson, Bishop, & Walsh, 2013). Similar to NK cells, T cells can be identified by using flow cytometry with the use of monoclonal antibodies specific to T cell CD markers. T cells can be classed into T

helper (T_H) cells and T cytotoxic (T_C) cells (Gleeson, Bishop, & Walsh, 2013). T_H cells express CD3 and CD4 markers, hence the phenotype is expressed as $CD3^+CD4^+$. T_C cells express CD3 and CD8 markers, hence the phenotype is expressed as $CD3^+CD8^+$ (Gleeson, Bishop, & Walsh, 2013). T_H cells constitute 60-70% of T cells and promote the acquired immunity by releasing various cytokines to induce T and B cell proliferation and differentiation (Abbas et al., 2014; Gleeson, Bishop, & Walsh, 2013). T_C cells make up 30-40% of the T cell population. As the name suggests, the main function of T_C cells is to eliminate infected cells and tumour cells (Abbas et al., 2014; Gleeson, Bishop, & Walsh, 2013). T_H and T_C cells can be further classified based on their unique cytokine production profiles (Lancaster et al., 2005; Zhu & Paul, 2008). Type 1 (T_{H1} & T_{C1}) and type 2 (T_{H2} & T_{C2}) are the major subtypes (Lancaster et al., 2005).

T Helper Cells

T_H ($CD4^+$) cells are further subdivided into four main subsets, T_{H1} , T_{H2} , T_{H17} and regulatory T (T_{reg}) cells, according to the initial priming signals they received during their first encounter with an antigen (Zhu & Paul, 2008). These subsets are different from each other in their unique cytokine products and distinct functions.

T_{H1} cells play a central role in the protection against intracellular microbes, as well as the development of organ-specific autoimmune disease in the event of abnormal activation (Zhu & Paul, 2008). T_{H1} cells facilitate the cell-mediated immune response through the release of IFN- γ and IL-2. The differentiation into T_{H1} cells from naïve $CD4^+$ T cells is by the stimulation of IL-12 and interferon(IFN)- γ (Zhu & Paul, 2008). IFN- γ upregulates the macrophages' anti-microbial activities. IFN- γ also promotes the proliferation of T_{H1} cells, but has negative effects on the T_{H2} cells (Gleeson, Bishop, & Walsh, 2013).

T_{H2} cells have two major functions: protection of the host from extracellular parasites including helminths, and facilitation of humoral immunity (Liao et al., 2008). Dysfunction in T_{H2} cell activation leads to asthma and allergy (Zhu & Paul, 2008). T_{H2} cells secrete IL-4, IL-5, IL-9, IL-10, IL-13, IL-25, and amphiregulin (B. Sun & Zhang, 2014; Zhu & Paul, 2008). IL-4 serves as a product of activated T_{H2} cells, as well as a positive stimulator of cytokine in the differentiation of T_{H2} cells, with the collaboration of IL-2 (Liao et al., 2008). IL-4 also plays a crucial role in immunoglobulin (Ig) E production by B cells (Zhu & Paul, 2008).

T_{H17} cells protect the host against extracellular microbes and fungi. Malfunctions of T_{H17} cells are associated with several organ-specific autoimmune diseases (Wan, 2010). T_{H17} cells are differentiated by TGF- β , IL-6, IL-21 and IL-23 signals. The major cytokine products of T_{H17} cells are IL-17a, IL-17f, IL-21, and IL-22 (Zhu & Paul, 2008). IL-17a and IL-17f have the analogous functions responsible for immune surveillance against extracellular microorganisms and fungi, as well as being implicated in autoimmunity and allergy (Wan, 2010; Zhu & Paul, 2008). Similar to IFN γ in T_H and IL-4 in T_H2 cells, IL-21 released by activated T_{H17} cells also work as the positive feedback cytokine, to promote T_{H17} cell differentiation. IL-22 promotes the innate immunity of epidermal cells, safeguards the cells against damage and enhances the cells' transformation (Kim et al., 2014; Sabat, Witte, Witte, & Wolk, 2013).

T_{reg} cells play a crucial part in mediating self-tolerance and possess suppressive mechanisms toward autoimmune responses. They secrete TGF- β , IL-10, and IL-35 and comprise 10% of peripheral $CD4^+$ T helper cells (Pandiyani, Zheng, Ishira, Reed, & Lenardo, 2007). T_{reg} cells are CD phenotypically noted as $CD4^+CD25^+$ T cells and express high levels of the specific transcription marker, known as Fork-head box protein 3 (FoxP3) (Beissert, Schwarz, & Schwarz, 2006). Beissert et al. (2006) suggest that T_{reg} cells have to be activated by dendritic cells (DC) to accomplish their suppressive effects on T cells, but the suppression is antigen-independent once activated. TGF- β is a key player in T_{reg} cells immunosuppressive activities *in vivo*.

T Cytotoxic Cells

Similar to T_H ($CD4^+$) cells, T_C ($CD8^+$) cells are also subclassed into T_{C1} and T_{C2} (Lancaster et al., 2005). T_{C1} cells produce IFN- γ and IL-2 and are mainly responsible for cellular immune response to intracellular pathogens. T_{C2} cells produce IL-4, IL-5, IL-10, and IL-13 and mount humoral immune response to extracellular pathogens (Lancaster et al., 2005). T_C ($CD8^+$) cells kill the infected cells directly via two pathways: 1. cytotoxicity of the target cells by releasing perforin and granzymes from lytic granules; and 2. inducing apoptosis through the interaction between Fas ligand on the effector $CD8^+$ T and Fas receptor on the target cell (Wong & Pamer, 2003). $CD8^+$ T lymphocytes also produce cytokines such as TNF- α and IFN- γ to indirectly combat the microbial attack. Additionally, the activated $CD8^+$ T cells recruit inflammatory cells to the site of infection through the release of chemokines (Wong & Pamer, 2003). After $CD8^+$ cells eliminate the primary infection, most of them go through apoptosis. However, some of the $CD8^+$ cells (also known as memory T cells) develop antigen-

specific immunologic memory to effectively and rapidly attack when the same pathogen is reencountered (Finlay & Cantrell, 2011).

Self-Tolerance and Foreign Antigen Recognition Mechanisms of T Cells

T cells acquire the ability to discriminate between “self” and “non-self” during their maturation stage. The T cell receptor (TCR), a unique membrane-bound heterodimer cell surface antigen-binding molecule, is a crucial element that is adapted by the mature T cells, which enables them to distinguish “self” and foreign antigens (Abbas et al., 2014; Andersen, Schrama, Thor Straten, & Becker, 2006). The TCR recognises processed antigen peptides presented by either MHC class I or II molecules. While the TCR on the CD4⁺ T cell binds to the MHC class II molecules expressed by the APC, CD8⁺ T cell TCR interacts with the MHC class I molecule expressed by the nucleated cells (Andersen et al., 2006).

The mature T cell, which has never encountered an antigen, is known as a naïve (CD45RA⁺) T cell. The activation of a naïve T cell and its differentiation into an effector cell requires two signals (see Figure 2); the binding of the TCR to the peptide-presented MHC (primary signal), and the co-stimulatory signal (secondary signal), established by the same APC (Andersen et al., 2006). The requirement for this co-stimulatory signal ensures the immune-competency of the T cells in distinguishing “self” and pathogens, as the APCs that have been exposed to microbes express co-stimulatory molecules strongly. The T cells which establish the primary signal, but no co-stimulatory signal, become anergy (unresponsive) to subsequent exposure to pathogens (see Figure 2) (Abbas et al., 2014). Both CD80 and CD86 are the most common co-stimulators expressed on the APC and they bind to the CD28 receptors on T cells. According to Gleeson, Bishop, and Walsh (2013), the engagement of CD28 on T cells with CD80 on APC leans toward the differentiation into T_{H1} cells; whereas the binding of CD28 with CD86 induces T_{H2} cell differentiation.

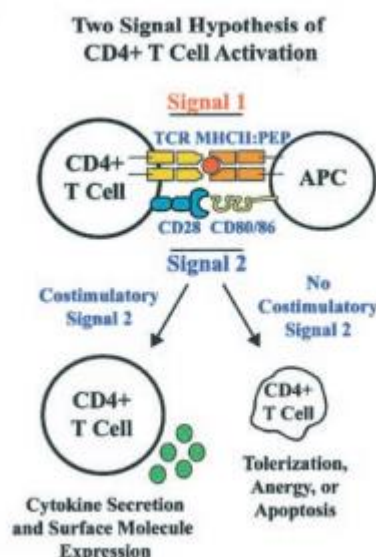


Figure 2. Self-tolerance and activation mechanism of T cells.
APC = Antigen presenting cell.

The binding of TCR on the T cell and MHC molecules on APC deliver the first signal to resting T cell. If the signal 1 is not accomplished with signal 2, T cell undergoes anergy or apoptosis. If signal 2 is delivered, T cell is activated and secret cytokines. Adapted from “Norepinephrine and β_2 -Adrenergic Receptor Stimulation regulate CD4 T and B Lymphocyte Function in Vitro and in Vivo,” by Adam. P. Kohm and Virginia. M. Sanders, 2001, *Pharmacological Reviews*, 53(4), p. 489. Reprinted with permission.

2.2 The Effects of Acute Bouts of Exercise on Lymphocyte Mobilisation

It has been proposed that the alteration of the total lymphocyte count during and after acute exercise follows a biphasic perturbation. The concentration of circulating lymphocytes increases during and immediately following intense exercise (lymphocytosis), but considerably declines below baseline values during the recovery period (lymphocytopenia) (Neves et al., 2015; B. K. Pedersen & Laurie, 2000). However, it is noteworthy that this biphasic pattern alteration only applies to the peripheral lymphocyte count, but does not apply to the function of the individual cell (B. K. Pedersen & Laurie, 2000). This means lymphocytosis does not necessarily result in immune function enhancement. Similarly, lymphocytopenia does not necessarily indicate a suppression in the host immune response when encountering foreign antigens.

Several studies have observed that the change in the lymphocyte count is primarily due to the intensity rather than the duration of exercise (Del Giacco, Scorcu, Argiolas, Firinu, & Del Giacco, 2014; Gleeson, Bishop, & Walsh, 2013; B. K. Pedersen & Laurie, 2000). Navalta, Sedlock, and Park (2007) demonstrated that the lymphocyte apoptosis rate was unchanged with low intensity ($\sim 40\%$ VO_{2max}) exercise, but a substantial

increase in lymphocyte apoptosis started to occur between an exercise intensity threshold of 40% and 60% VO_{2max} . They further illustrated that the rate of lymphocyte apoptosis significantly increases with the increase in exercise intensity (76%, 89% and 100% of VO_{2max}) and returns back to pre-exercise values 60 min after the intense exercise.

2.2.1 Effect of Acute Exercise on NK Cells Number

Many studies suggest that NK cells are the most responsive immune cells to strenuous exercise, showing a remarkable increase in number during and immediately after exercise (50-100% increase rapidly after the exercise), but tending to fall significantly below baseline values 1-2 h after exercise (recovery period) (Del Giacco et al., 2014; Gleeson, Bishop, & Walsh, 2013). Although there are many exercise immunology studies investigating the impact of intense exercise on NK cells, few have investigated the impact of intense exercise on the different NK cell subsets ($CD56^{bright}$ and $CD56^{dim}$). Timmons and Cieslak (2008) propose that the mobilization of NK cell subsets into the circulation is relatively rapid and completed during 30 min of exercise. This proposal is based on the study performed by the same research team in 2006, which found that there was no difference in NK cell counts between blood collected after 30 or 60 min of cycling at 70% VO_{2max} (Timmons, Tarnopolsky, & Bar-Or, 2006). Moreover, B. K. Pedersen and Laurie (2000) suggest that the increase in the concentration of circulating NK cells during exercise is mainly because of the significant increase in the number of the $CD56^{dim}$ NK cell subsets, rather than $CD56^{bright}$ NK cells. This is in agreement with Suzui et al. (2006), that the proportion of $CD56^{dim}$ but not $CD56^{bright}$ NK cells is found to increase significantly following cycling at 70% VO_{2max} for 30 min. However, $CD56^{bright}$ NK cells are dominant in the post-exercise period. As $CD56^{bright}$ NK cells have less cytotoxic activity than the $CD56^{dim}$ NK cells, increases in the $CD56^{bright}$ NK cell count has been proposed as a cause for the increased risk of URTI following intense exercise. Regardless of the decrease in the post-exercise NK cell count, it is interesting to note that NK cell activity on per cell basis does not appear to be suppressed (B. K. Pedersen & Laurie, 2000).

Despite the suggestion from several studies (Gleeson, Bishop, & Walsh, 2013; B. K. Pedersen & Laurie, 2000; Suzui et al., 2006) that there is a relationship between a decrease in the NK cells during recovery from exercise and an increased risk of URTI, one must view this with caution, as some researchers argue that the decrease in NK cell numbers is temporary and, therefore, there is little convincing evidence to link this to

increased risk of URTI (Del Giacco et al., 2014; Nieman, 2000). The study of long term effects of exercise on the immune parameters of 29 soccer players over the whole championship period (~1 year) carried out by Del Giacco et al. (2014) supports this notion that there is no significant link between the reduction in NK cell numbers and an increased risk of URTI. Nieman et al. (2000) also failed to link the changes in NK cell activity with URTI incidence following exercise. Needless to say, the relationship between total NK cell/NK cell subset numbers and acute exercise is debatable.

2.2.2 Effect of Acute Exercise on T Cell Numbers

Evidence suggests that there is a fall in the $CD4^+$ (T_H) to $CD8^+$ (T_C) ratio, indicating a larger increase of $CD8^+$ T lymphocytes than $CD4^+$ T lymphocytes during and immediately after exercise (B. K. Pedersen & Laurie, 2000). However, the trafficking and apoptosis of lymphocytes is higher in $CD8^+$ than $CD4^+$ T cell numbers during the recovery period (up to 24 h after the exercise), resulting in low $CD8^+$ counts (Pereira et al., 2012). Tuan et al. (2008) suggest that this deleterious effect can last up to 72 h post-exercise.

Studies indicate that prolonged exercise (>2 h) also lead to the redistribution of circulating type 1 and 2 T_H and T_C cells, resulting in a major decline in type 1 cells. This concept is demonstrated in the study involving the measurement of lymphocytes after 2.5 h of cycling at 65% VO_{2max} . The finding showed that there was a decrease in the IFN- γ producing T lymphocytes (type 1 cells); whereas there was no change in the IL-4 producing T lymphocytes (type 2 cells) (Lancaster et al., 2005). This concept is also supported by the study carried out by Adam et al. (2001), in which nine endurance-trained male runners were asked to run on a treadmill for 2.5 h at 75% VO_{2max} , and the number of type 1 and 2 T cells were measured. The results showed that there was nearly a 50% decrease in the number of type 1 T cells after exercise and 2 h post-exercise, compared with the baseline values. The results also demonstrate that the percentage of type 2 T cells does not appear to change in response to strenuous exercise. As type 1 T cells are responsible for immunity against intracellular pathogens (Zhu & Paul, 2008), the significant suppression of type 1 T cells may partially account for the proposed increased risk of URTI following acute exercise.

2.2.3 Mechanisms Underlying Exercise-Induced Lymphocyte Mobilisation

Lymphocytosis

Neves et al. (2015) and B. K Pedersen and Hoffman-Goetz (2000) suggest that the lymphocytosis immediately after exercise is due to the mobilisation of lymphocytes from the marginal intravascular and extravascular storage pool such as the spleen, the lymph nodes and the gastrointestinal tract to the vascular compartment, in response to exhaustive bouts of exercise. Although increases in the number of different lymphocyte subpopulations make up the lymphocytosis, NK cells appear to be the main lymphocyte subset contributing the lymphocytosis. B. K Pedersen and Hoffman-Goetz (2000) indicate that the CD56^{bright} NK cells are the major NK cell population recruited to the blood during exercise producing vast numbers of immunoregulatory cytokines.

Stress hormones, mainly catecholamines, are thought to play a role in exercise-induced lymphocytosis (Neves et al., 2015; B. K Pedersen & Hoffman-Goetz, 2000; Prestes et al., 2008). Acute bouts of exercise stimulate an increased production of adrenaline by the adrenal medulla, and noradrenaline by the sympathetic nerve terminals. β_2 -adrenoreceptors, present on T and NK cell surfaces, are responsible for the response to catecholamine signalling (B. K Pedersen & Hoffman-Goetz, 2000). Following the stimulating signal from catecholamines, the β_2 -adrenoreceptors on T and NK cells intracellularly connect to the adenylyl cyclase system, which induces the development of cyclic AMP (cAMP). Increases in cAMP transform the surface expression of the adhesion molecules, resulting in the recruitment of lymphocytes into the blood (B. K Pedersen & Hoffman-Goetz, 2000). It is proposed that the degree of lymphocyte demargination induced by the catecholamines is directly proportional to the number of β_2 -adrenoreceptors on the cell surface, and various lymphocytes express a different density of β_2 -adrenoreceptors. In accordance with this, the number of NK cells tends to be more responsive to catecholamines, as they possess the highest number of β_2 -adrenoreceptors. CD8⁺ T cells react intermediately, while CD4⁺ T cells have a poor response, as there is higher density of β_2 -adrenoreceptors on CD8⁺ T cells than CD4⁺ T cells (B. K Pedersen & Hoffman-Goetz, 2000).

Lymphocytopenia

It is proposed that the lymphocytopenia seen during the recovery period from exercise is possibly due to the increase in the lymphocyte apoptosis rate, or a migration of lymphocytes from the circulation to the site where the immune defence is needed, or a combination of both (Pereira et al., 2012).

It is indicated that lymphocyte trafficking and redistribution may play a pivotal role in lymphocytopenia. During an acute bout of exercise, lymphocytes travel to the site of potential immune challenge such as the lymph nodes, Peyer's Patches, bone marrow and lungs, even before the body actually encounters the challenge, in order to enhance immune surveillance and vigilance (Krüger & Mooren, 2007).

Navalta et al. (2007) suggests that the post-exercise apoptosis rate increases with the increase in the exercise intensity. Krüger and Mooren (2014) declare that an increase in the plasma cortisol, a human glucocorticoid (GC), is closely related with the elevated lymphocyte apoptosis rate after exercise. The GC binds to the glucocorticoid receptors (GRs), which express the heat shock protein 90 (HSP 90). It is believed that following activation, the HSP 90 detaches from the GRs, resulting in the migration of the receptor complex to the nucleus. This leads to the alteration of apoptosis regulator proteins in mitochondria such as Bcl-2, Bcl-XL, and Bax, which results in the release of cytochrome-c into the cytosol. This reaction further activates the caspase cascade, inducing apoptosis (Krüger & Mooren, 2014; Phaneuf & Leeuwenburgh, 2001).

Although possible lymphocyte apoptosis mechanisms have been proposed as a mediator for lymphocytopenia, it is important to note that there are also a number of recent studies which have failed to demonstrate the association between apoptosis and the post-exercise lymphocytopenia (Simpson, Florida-James, Whyte, et al., 2007; Steensberg, Morrow, Toft, Bruunsgaard, & Pedersen, 2002). Therefore, further investigations are necessary to elucidate the potential mechanisms responsible for exercise-induced lymphocytopenia.

2.3 The Effects of Acute Bouts of Exercise on Lymphocyte Activation – CD69⁺ Cell Markers

Cell activation is a mandatory prerequisite for lymphocytes to initiate an immune attack against infection. Lymphocyte surface molecules have been intensively studied as a potential indicator to measure immune responsiveness. CD69 is a C-Type lectin cell surface glycoprotein, which has been recognised as an early activation marker (Wieland & Shipkova, 2016). The fact that they are not heavily expressed on resting lymphocytes, and rapidly detectable within one to two h after lymphocyte activation, validates them as an early activation marker. After expression, they remain detectable for at least three days (Shipkova & Wieland, 2012; Wieland & Shipkova, 2016; Ziegler, Ramsdell, & Alderson, 1994). Once expressed, they also have a role in co-stimulation of lymphocyte proliferation and act as a signal-transmitting receptor on T and NK cells (Shipkova & Wieland, 2012; Wieland & Shipkova, 2016).

Several studies have focused on the effect of acute bouts of exercise on the expression of CD69 on T and NK cells. For example, Vider et al. (2001) showed that the number of activated CD4⁺ and CD8⁺ T cells expressing CD69 declines considerably immediately after exhaustive treadmill running in endurance athletes, with a mean of 69.72 ± 8.73 ml/kg/min $\text{VO}_{2\text{max}}$, but returns to pre-exercise baseline levels after 30 min of recovery. The findings of C. Smith and Myburgh (2006) also demonstrate a decrease in *in vitro* mitogen-induced CD69 expression by both CD4⁺ and CD8⁺ T cells following a 4 week high-intensity cycling training intervention. In contrast, Ronsen, Pedersen, Øritsland, Bahr, and Kjeldsen-Kragh (2001) demonstrated that even after both single and repeated bouts of cycling at 75% $\text{VO}_{2\text{max}}$ for 75 min per bout on the same day, the percentage of CD4⁺ and CD8⁺ T cells expressing CD69 marker was unchanged, in both mitogen-stimulated and unstimulated cells. Nonetheless, Ronsen et al. (2001) illustrated a pronounced decrease in the percentage of mitogen-induced CD56⁺ NK cells expressing CD69 as well as the density of this marker on the activated CD56⁺ cells, after two bouts of exercise. It is important to note that no percentage change is observed in the single bout of exercise, in neither CD4⁺ and CD8⁺ T cells, nor CD56⁺ NK cells. This may indicate the carry over of immune impairment consequences from single to repeated bouts of exercises (Ronsen et al., 2001).

No tangible literature review has been found as an explanation for the contradictory findings between Vider et al. (2001), C. Smith and Myburgh (2006), and Ronsen et al. (2001). However, it is assumed that differences in exercise duration; number of bouts of

exercise; intensity, type and nature of exercise challenges; participants' dietary intake; environment factors; and laboratory testing protocols may contribute to these inconsistent findings. A comprehensive evaluation performed by Reddy, Eirikis, Davis, Davis, and Prabhakar (2004) suggests that the type of stimulating agents, time of culture and type of blood samples (whole blood vs peripheral blood mononuclear cell, PBMC) may also account for the conflicting results. For example, Reddy et al. (2004) demonstrated that the stimulation of PBMC with phytohemagglutinin stimulant (PHA-P) exhibited increased density of CD69 in a time-dependant manner within a 3 to 12 h period, which was still detectable at significant levels up to 24 h, but the level dropped thereafter. Additionally, both Reddy et al. (2004) and Green, Rowbottom, and Mackinnon (2003) stress the importance of considering the change in relative composition of lymphocytes after intensive exercise when examining the percentage of CD69 expressing cells, as the lymphocyte numbers increase with exercise. Yet, most of the research findings are calculated based on the constant PBMC. There is also criticism of using the numerically adjusted cell proliferation data in the investigation of variable CD69 induction on different lymphocyte subsets (Green et al., 2003). Moreover, the concentration of the stimulant is also shown to be responsible for the variation in CD69 induction, with the expression of CD69 on T cells increasing directly proportionally to the raised level of the PHA stimulant (DuBose et al., 2003).

2.3.1 Mechanisms Underlying Exercise-Induced Alterations in Lymphocyte Activation

Adenosine

Adenosine is an endogenous molecule which belongs to purine nucleosides and can be found in both intracellular and extracellular compartments. Its major function is to regulate tissue and immune function by acting as an immunoregulatory signal (Haskó, Linden, Cronstein, & Pacher, 2008; Sitkovsky & Ohta, 2005). Although immune cells are vital in combating pathogens via a complex immune defence (refer to section 2.1), over-reactive, prolonged or inappropriately-reactive immune cells can lead to excessive collateral damage and destruction to normal tissues (Sitkovsky & Ohta, 2005). Extracellular adenosine concentration elevates in response to metabolic stress and cell damage in hypoxia, and acts as an immunosuppressive to anti-inflammatory molecules (Haskó et al., 2008; Sitkovsky & Ohta, 2005). The reference range of plasma adenosine concentration in normal healthy individuals ranges roughly from 40-50 nmol.L⁻¹ (Chouker et al., 2005). In general, adenosine is formed as a result of rapid

dephosphorylation of adenosine triphosphate (ATP) by ectoenzymes. The extracellular adenosine generated by this process is usually taken up by the cells from extracellular compartments, which is then metabolised either by adenosine kinase to form AMP, or adenosine deaminase to convert adenosine to inosine (Haskó et al., 2008). This process maintains the plasma adenosine equilibrium in unstressed, healthy human tissues. Nevertheless, in stressful conditions, this equilibrium is disturbed, leading to a significant rise in extracellular adenosine concentration (Haskó et al., 2008). Chouker et al. (2005) demonstrated that the plasma adenosine concentration increases from 40 nmol.L⁻¹ to 80 nmol.L⁻¹ after 4-4.5 h of strenuous hiking at low altitude.

Adenosine interacts with specific cell surface receptors commonly seen on the immune cells. Adenosine receptors are classed as purinergic receptors and are coupled to either Gs or Gi proteins (Figure 3) (Sitkovsky & Ohta, 2005). Adenosine receptors are subdivided into four subsets: A₁, A_{2a}, A_{2b} and A₃ (see Figure 3) (Sitkovsky & Ohta, 2005). The subsets A₁ and A_{2A} have a high affinity for adenosine, compared to A_{2B} and A₃ receptors. This means the A_{2B} and A₃ receptors may only activate under high metabolic or stressful cellular conditions (Evans & Ham, 2012; Fredholm, IJzerman, Jacobson, Klotz, & Linden, 2001). All four receptors execute their effector functions through either stimulation or inhibition of adenylyl cyclase, depending on which receptor is activated (Sitkovsky & Ohta, 2005). Binding of adenosine with A_{2A} and A_{2B} receptors stimulates the adenylyl cyclase, which produces the second messenger intracellular cyclic AMP (cAMP) in high levels, leading to the immunosuppression of immune cells (Figure 3). In contrast, activated A₁ and A₃ receptors inhibits the adenylyl cyclase, resulting in a low level of cAMP (see Figure 3) (Fredholm et al., 2001; Sitkovsky & Ohta, 2005). The cAMP signals primarily through cAMP-dependent protein kinase A (PKA) in mammalian cells (Skalhegg & Tasken, 2000). According to Skalhegg and Tasken (2000), PKA type I (PKAI) is responsible for the diminished proliferation, induced via the T-cell receptor (TCR)/CD3 complex.

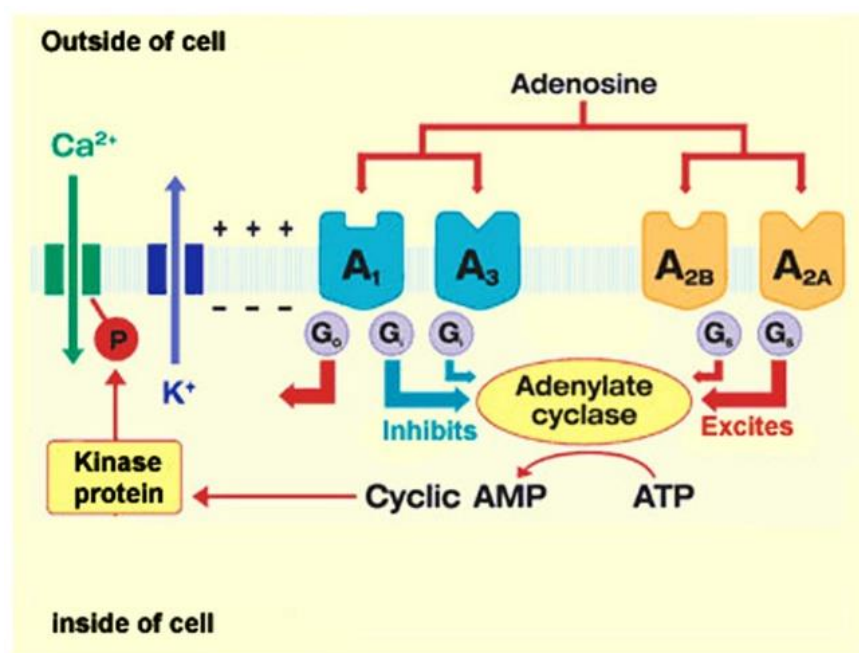


Figure 3. Mechanism of adenosine receptors.

AMP = Adenosine monophosphate; ATP = Adenosine triphosphate.

A1 and A3 receptors inhibit the adenylyl cyclase leading to production of cAMP, whereas A2A and A2B stimulate the adenylyl cyclase leading to decreased levels of cAMP. Adapted from "An Emerging Role for Adenosine and its Receptors in Bone Homeostasis," by B. Evans and J. Ham, 2012, *Frontiers in Endocrinology*, 3(113), p. 3. Retrieved from <http://dx.doi.org/10.3389/fendo.2012.00113>. Reprinted with permission.

The potential effects extracellular adenosine may have on lymphocyte function depends on the subtypes of adenosine receptors on the specific lymphocytes and the relative receptor affinity for adenosine (Hoskin, Mader, Furlong, Conrad, & Blay, 2008).

T lymphocytes are known to express A_{2A}, A_{2B} and A₃ receptors, with A_{2A} being the most expressed receptor (Gessi et al., 2004; Koshiba, Rosin, Hayashi, Linden, & Sitkovsky, 1999; Mirabet et al., 1999). Researchers believe that the expression of these adenosine receptors on T cells can be regulated by TCR signalling, and the expression is upregulated in activated T cells, which may reinforce adenosine-induced effects on immune cells (Gessi et al., 2004; Koshiba et al., 1999; Mirabet et al., 1999). There is limited research about the subtype of adenosine receptors expressed on NK cells. Some investigators propose the presence of A₁, A_{2A} and A_{2B} on murine NK cells, following the study of differential responses to adenosine subtype receptor-selective agonists (Priebe, Platsoucas, & Nelson, 1990; Raskovalova et al., 2005). The A₃ receptor is also thought to be present on murine NK cells, as there is an increase in NK cell activity following oral administration of the A₃ adenosine receptor-selective agonist (Harish, Hohana, Fishman, Arnon, & Bar-Yehuda, 2003). Priebe et al. (1990) suggest that A₁

and A₂ receptors may be present on human NK cells. However, further confirmation on the type of adenosine receptor subtypes on human NK cells at the mRNA or protein level is yet to be determined (Hoskin et al., 2008).

Adenosine is known to downregulate the inflammatory process, and impair the T lymphocyte activation and effectors function (Hoskin et al., 2008). Aandahl et al. (1998) and Kammer, Laxminarayana, and Khan (2004) suggest PKAI as an acute inhibitor of T cell activation. Lappas, Rieger, and Linden (2005) further indicated that TCR-induced CD69 expression was suppressed in the introduction of 100 nmol.L⁻¹ of adenosine. The researchers propose that the immunosuppressive effect of adenosine is likely due to the stimulation of adenylyl cyclase by accumulated extracellular adenosine, via the A_{2A} receptor. This increases the secretion of cAMP, which in turn enhances the PKAI activity, exerting the abolishment in T cell activation and proliferation, leading to a suppression in cytokine production and decreased cytotoxicity by T cells (Apasov & Sitkovsky, 1999; Lappas et al., 2005; Sitkovsky & Ohta, 2005; Skalhegg & Tasken, 2000). Another proposed means of adenosine-mediated T cells immunosuppression is that the interfering of the APCs' function by the A_{2A} receptor, as TCR-driven T cell activation requires the presenting of an antigen peptide to the T cell, and secretion of costimulatory molecules by the APCs (refer to section 2.1.2) (Panther et al., 2001). Hoskin et al. (2008) show that adenosine has a negative impact on the expression of costimulatory molecules by B lymphocytes, which in turn attenuate T cell activation. The A_{2A} receptor is proposed to be a mediator in this process, as B cells are believed to express abundant A_{2A} adenosine receptors (Lukashev et al., 2003). However, it is noteworthy that most of the studies use rodent models and are either *ex vivo* or *in vitro*. Therefore, it may not correctly reflect the condition *in vivo* in human. Despite the well-established knowledge on the mechanisms underlying the immunosuppressive effects of the A_{2A} adenosine receptor on T cell activation and effector function, little is known about the mechanism of A₃ adenosine receptor-mediated T cell inhibition, and further investigation is demanded (Hoskin et al., 2008).

In contrast to the T cells, at present no report is available about the effect of adenosine on NK cell activation and the expression of the early activation marker CD69. However, there are studies that focus on the effects of adenosine on the cytotoxicity of NK cells (Hoskin et al., 2008). Priebe et al. (1990) suggest that adenosine receptor A₁ has stimulatory, whereas A₂ has inhibitory, effects on the cytotoxicity of mouse NK cells. As intracellular cAMP is known to modulate the cytotoxic activity of NK cells

(Haraguchi, Good, & Day, 1995), the opposing effects of A₁ and A₂ adenosine receptors on the cytotoxic activity of NK cells are most probably due to the differential effects of these receptors on the cAMP level (Hoskin et al., 2008). A₁ adenosine receptors inhibit the adenylyl cyclase, leading to a fall in intracellular cAMP concentration, which, in turn, increases the cytotoxicity of NK cells. On the other hand, A₂ adenosine receptors induce adenylyl cyclase, leading to the production of elevated intracellular cAMP, which in turn complex with PKAI to negatively impact the cytotoxic activity of NK cells (Fredholm et al., 2001; Sitkovsky & Ohta, 2005).

Adrenaline

Adrenaline, also known as epinephrine, belongs to the catecholamine group. It is produced in the medulla of the adrenal gland, in which the amino acids phenylalanine and tyrosine are metabolised into adrenaline (Kjaer, 1998). Exercise is one of the major physiological stimuli to adrenaline secretion. Zouhal, Jacob, Delamarche, and Gratas-Delamarche (2008) report that adrenaline concentration increases 1.5 to >20 times during exercise, depending on the exercise intensity and duration. It is noteworthy that the response of adrenaline to exercise is determined by several factors such as exercise nature, training status and sex (Kochanska-Dziurawicz et al., 2013; Zouhal et al., 2008). When compared at an identical absolute submaximal exercise level, the adrenaline response to exercise is less pronounced in well-trained, compared to sedentary, participants (~1 nmol.L⁻¹ for endurance trained men; ~3.5 nmol.L⁻¹ for sedentary men after 20 min of cycling at 210 W) (Kjaer, 1998; Zouhal et al., 2008). This is likely due to a decrease in sympathetic stimulation of the adrenal medulla. However, under identical relative workloads, it is found that well-trained individuals secrete more adrenaline compared to untrained individuals (Kjaer, 1998; Zouhal et al., 2008). This concept is known as “sports adrenal medulla”, meaning the adrenal medulla has adapted to long-term endurance training (Kjaer, 1998). Furthermore, Kjaer (1998) mentions that the rise in the plasma adrenaline concentration is directly proportional to the relative % VO_{2max} of the exercise bout. Indeed, Boutcher (2010) concludes that the catecholamine response is significantly higher for HIIE than moderate intensity aerobic exercise. This is further supported by the greater increase in plasma adrenaline concentration (6.3 fold from baseline) following HIIE consisting of ten sprints with a 30 s recovery between each sprint compared to one 6-s sprint (Bracken, Linnane, & Brooks, 2009).

Adrenaline binds to and stimulates different subtypes of α - and β -adrenoreceptors to execute its effects. It is shown that adrenaline has a stronger interaction with the β - than α - adrenoreceptors (Kochanska-Dziurawicz et al., 2013). The β_2 -adrenoreceptors are primary adrenoreceptors found on the surface of lymphocytes including T, B, and NK cells, macrophages, and neutrophils (Kohm & Sanders, 2001; B. K Pedersen & Hoffman-Goetz, 2000). As mentioned previously in section 2.2.3, the expression density of β_2 -adrenoreceptors of $CD4^+$ & $CD8^+$ T cells, and NK cells vary; NK cells express the highest, $CD8^+$ moderate and $CD4^+$ lowest number of β_2 -adrenoreceptors (B. K Pedersen & Hoffman-Goetz, 2000). It has been shown that the responsiveness of these adrenoreceptors to exercise is closely related to the density of these receptors on specific lymphocytes. In accordance with this theory, NK cells are the most responsive, $CD8^+$ are moderately responsive and $CD4^+$ are the least responsive to exercise (B. K Pedersen & Hoffman-Goetz, 2000). Furthermore, Kohm and Sanders (2001) mention that the degree of β_2 -adrenoreceptor expression on the cell surface is influenced by the activation status of lymphocytes, with activated lymphocytes expressing a larger number of adrenoreceptors. However, it is important to mention that the density of β_2 -adrenoreceptors on T cells reduced due to the protein kinase C (PKC)-dependent mechanisms stimulating the expression of β -adrenergic receptor kinase-1 (β ARK1) following longer than 24 h T cell activation (Kohm & Sanders, 2001). This may be the reason the effect of adrenaline on lymphocytes returns back to normal after 24 h in some cases. Classically, the coupling of adrenaline to the β_2 -adrenoreceptors induces adenylyl cyclase activity, which produces the second messenger intracellular cAMP. As discussed in the adenosine session of section 2.3.1, accumulated cAMP ligates with PKAI to down-regulate the lymphocyte activation (Grader-Beck, van Puijenbroek, Nadler, & Boussiotis, 2003). Shimamiya, Wakabayashi, and Terada (2003) support this notion that administration of adrenaline inhibits the expression of the early activation marker CD69 on mitogen-stimulated T and NK cells.

Cortisol

Paccotti et al. (2005) reported that there is a significant increase in cortisol levels following short duration (<30 min) high-intensity isokinetic exercise. The high cortisol level results in immune system dysfunction (Krüger & Mooren, 2014). Phillips, Burns, and Lord (2007) suggest a number of immune-suppressive effects of cortisol as follow. Cortisol attenuates the secretion of proinflammatory cytokines such as IL-1, TNF, Granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-2, IL-3, IL-4, IL-5,

IL-8, prostaglandins and leukotrienes. Cortisol also diminishes the extravasation of inflammatory cells and suppresses neutrophil function. A high level of cortisol induces apoptosis in lymphocytes. Moreover, increase in cortisol secretion inhibits the binding of neutrophils to the endothelial membrane to infiltrate into the tissue. This leads to the increase in numbers of circulating neutrophils (Steensberg, Fischer, Keller, Møller, & Pedersen, 2003). Therefore, cortisol is believed to be responsible for the neutrocytosis and lymphopenia after strenuous exercise (Krüger & Mooren, 2014; Steensberg et al., 2003).

The study of B. K. Pedersen et al. (1990) supports the above notions of cortisol-induced lymphopenia that the NK cell activity measured 2-4 h post-exercise is well below the pre-exercise values when 15 young, healthy volunteers are asked to cycle at 75% $\text{VO}_{2\text{max}}$ for 60 min. A study by Hansen, Wilsgård, and Østerud (1991) also indicates that the number of lymphocytes after exercise is 32%–39% lower than the pre-exercise values in seven healthy runners running at their maximum speed in short- (1.7 km), middle- (4.8 km) and long- (10.5 km) term runs. This decrease in lymphocyte numbers is correlated to the increase in the cortisol levels. A recent study of immunoendocrine responses to strenuous exercises also shows that a high level of cortisol is found after a 5,000 m running race (Li, Hsu, Suzuki, Ko, & Fang, 2015), which is believed to be responsible for the leukocyte apoptosis (Krüger & Mooren, 2014).

2.4 Impacts of Dietary Intake on the Immune System

There has been growing interest in the manipulation of dietary intake to prevent immune impairment after strenuous exercise (Gleeson, Bishop, & Walsh, 2013; Rodriguez, DiMarco, & Langley, 2009). Caffeine is one of the food elements of interest, as it is widely consumed among people who actively exercise as well as non-active people.

2.4.1 Caffeine

Caffeine is consumed worldwide in various forms, due to its alertness-enhancing or ergogenic effects (Chester & Wojek, 2008; Graham, 2001). As the commercial sale and marketing of caffeine-containing beverages and food expands, caffeine is available and consumed by people of almost all age groups, although it is neither a typical dietary constituent nor an essential nutrient for health (Graham, 2001; Harris & Munsell, 2015). According to Burke (2008), approximately 90% of adults consume caffeine in their routine diet. Caffeine is commonly found in coffee, tea, cocoa beverages, cola,

chocolate, carbonated sodas, energy drinks, some bottled water, alcoholic beverages, weight loss products, over-the-counter medications and dietary supplements (see Table 1) (Andrews et al., 2007; Graham, 2001)

Table 1. Common Name and Label Terms of Caffeine-Containing Beverages and Food

Common name, ingredient	Label terms identified
Coffee	Coffee, coffea, caffeine
Cocoa, cacao	Cocoa, <i>Theobroma cacao</i> , chocolate
Guarana	Guarana, <i>Paullina cupana</i> , Brazilian cocoa
Kola nut	Kola nut, cola seeds, <i>Cola nitida</i>
Green tea, black tea	Green tea, black tea, <i>Camellia sinensis</i> , <i>Theo sinensis</i> , <i>Camellia</i> sp.
Yerba mate	Yerba mate, mate, <i>Ilex paraguariensis</i>

Adapted from “The Caffeine Contents of Dietary Supplements commonly purchased in the US: Analysis of 53 products with Caffeine-Containing Ingredients,” by K. W. Andrews, A. Schweitzer, C. Zhao, J. M. Holden, J. M. Roseland, M. Brandt, . . . K. D. Fisher, 2007, *Analytical and bioanalytical chemistry*, 389(1), p. 232. Reprinted with permission.

*This table is a brief representation of some of the main caffeine-containing foods and beverages.

The United States (U.S) Food and Drug Administration (FDA) has set the maximum allowable limits of caffeine concentration per serving as 32.4 mg of caffeine per 6-oz (~177 ml), or 65 mg of caffeine per 12 oz (~355 ml) of fluid (McCusker, Goldberger, & Cone, 2006). Depending on the regulations of the country, the amount of caffeine contained in products varies. In New Zealand, the maximum allowable limits of caffeine in cola-type drinks is 145 mg l⁻¹ (mg kg⁻¹) and 145-320 mg l⁻¹ for other formulated caffeinated beverages such as energy drinks/shots (Food Standards Australia New Zealand, 2016; New Zealand Food Safety Authority, 2013). Table 2 shows the caffeine concentration of commonly consumed beverages.

Table 2. Caffeine Content of Commonly Consumed Beverages

Food or drink	Serving	Caffeine, mg*
Instant coffee	250 mL (8 oz) cup	60 (12–169)
Brewed coffee	250 mL (8 oz) cup	80 (40–110)
Short black coffee or espresso	1 standard serving	107 (25–214)
Starbucks Breakfast Blend brewed coffee (Venti size)	600 mL (20 oz)	415 (300–564)
Iced coffee (commercial brands)	500 mL bottle (16 oz)	30–200
Frappuccino	375 mL (12 oz) cup	90
Tea	250 mL (8 oz) cup	27 (9–51)
Iced tea	600 mL (20 oz) bottle	20–40
Hot chocolate	250 mL (8 oz) cup	5–10
Chocolate milk	60 g	5–15
Dark chocolate	60 g	10–50
Viking chocolate bar	60 g	58
Coca-Cola	375 mL (12 oz) can	49
Pepsi cola	1375 mL (12 oz) can	40
Jolt soft drink	1375 mL (12 oz) can	75
Red Bull energy drink	250 mL (8 oz) can	80
Red Eye Power energy drink	250 mL (8 oz) can	50
V Energy drink	250 mL (8 oz) can	50
Smart Drink - Brain fuel	250 mL (8 oz) can	80
Lift Plus energy drink	250 mL (8 oz) can	36
Lipovitan energy drink	250 mL (8 oz) can	50
Black Stallion energy drink	250 mL (8 oz) can	80
AMP Energy tallboy	500 mL (16 oz) can	143
Spike Shotgun energy drink	500 mL (16 oz) can	350
Fixx energy drink	600 mL (20 oz) can	500
Ammo energy shot	30 g (1 oz)	170
Jolt endurance shot	60 g (2 oz)	150
PowerBar caffeinated sports gel	40 g sachet	25
PowerBar double caffeinated sports gel	40 g sachet	50
GU caffeinated sport gel	32 g sachet	20
Carboshotz caffeinated sports gel	50 g sachet	80
PB Speed sports gel	35 g sachet	40
PowerBar ActicaF Performance bar	65 g bar	50
Jolt caffeinated gum	1 stick	33
No-Doz (Australia)	1 tablet	100
No-Doz (U.S.)	1 tablet	200
Extra Etrength Excedrin	1 tablet	65

Adapted from “Caffeine and Sports Performance,” by L. M. Burke, 2008, *Applied Physiology, Nutrition, and Metabolism*, 33(6), p. 1320. Reprinted with permission.

Note: There are large variations in the caffeine content of tea and coffee. This is due to the difference in brand, method of preparation of the beverage, and the size of cup/mug.

A large scale caffeine consumption survey carried out by D. C. Mitchell, Knight, Hockenberry, Teplansky, and Hartman (2014) between 2010 and 2011 indicates that 85% of the U.S. population consumes a minimum of one caffeine-containing beverage per day, and 96% of those drinks are in the form of coffee, tea and soft drink. On average, the mean daily intake of caffeine in the U.S. is 165 ± 1 mg for all ages. Of caffeine consumers, people aged between 50–64 years old consume the highest levels of caffeine (226 ± 2 mg per day). The average caffeine intake for consumers at the 90th percentile is 380 mg per day among all ages. Despite the fact that coffee is the most commonly consumed beverage in New Zealand, it is interesting to note that there is no caffeine consumption data available for New Zealand. This is because there are varieties

of caffeine containing drinks on the market and new drinks are constantly being developed and released (Ministry for Primary Industries, 2010).

2.4.2 Caffeine Habituation and Abstinence

An individual can experience a variety of caffeine withdrawal symptoms depending on whether they are a low, moderate or high caffeine consumer (Fredholm, Bättig, Holmén, Nehlig, & Zwartau, 1999; Sigmon, Herning, Better, Cadet, & Griffiths, 2009). Increased sleepiness, weariness, weakness, drowsiness, headaches, heart rate, and a decrease in performance and mental alertness, are common physiological symptoms of caffeine withdrawal (Fredholm et al., 1999; James & Rogers, 2005; Rogers, Heatherley, Mullings, & Smith, 2013). However, habitual caffeine users develop tolerance to some of these effects of caffeine quickly (Carrillo & Benitez, 2000; Fredholm et al., 1999; Sigmon et al., 2009). Nevertheless, the re-sensitisation to caffeine usually occurs within 48-72 h of abstention (Benowitz, Jacob, Mayan, & Denaro, 1995).

Dodd, Brooks, Powers, and Tulley (1991) showed that while habitual caffeine users have higher heart rate, expired ventilation volume and VO_2 at rest compare to non-caffeine users, there is no significant difference during incremental graded exercise. A study by Van Soeren, Sathasivam, Spriet, and Graham (1993) illustrate the notable increase in plasma adrenaline following the caffeine ingestion during exercise in the habitual caffeine users with 4 days of abstinence from caffeine. However, there is very little plasma adrenaline response to caffeine without the withdrawal period in habitual caffeine users. Bangsbo, Jacobsen, Nordberg, Christensen, and Graham (1992) also demonstrate that the adrenaline response to caffeine is blunted after 6 weeks of caffeine supplementation. The findings from Bangsbo et al. (1992) and Van Soeren et al. (1993) appear to suggest the habituation of adrenaline response to caffeine usage. It is interesting to note that there is no significant difference in adrenaline response to caffeine between two and four days of caffeine withdrawal among habitual caffeine users (Van Soeren & Graham, 1998). Graham (2001) indicates that the circulated caffeine level is reduced to barely detectable levels after 48 h of withdrawal from caffeine containing substances. A study by Rogers, Heatherley, Mullings, and Smith (2013) illustrates that the mental alertness of medium-high caffeine users (caffeine intake of < 40 and ≥ 40 mg/day, respectively) who have had overnight caffeine abstinence are similar to that of non-caffeine user after consuming 100-150 mg of caffeine. This finding represents the effect of the caffeine withdrawal reversal process. Therefore, although habitual caffeine users may experience caffeine withdrawal

symptoms, these adverse effects can be reversed by re-introducing caffeine (James & Rogers, 2005; Rogers et al., 2013).

2.4.3 Mode of Action and Metabolism

Caffeine, the plant alkaloid, belongs to the methylxanthine family classed as 1, 3, 7-trimethylxanthine. It is naturally found in the leaves, seeds and fruit of more than 60 plants including tea, coffee, cacao and kola tree (Andrews et al., 2007). The physiological concentration of caffeine is normally between 20-70 $\mu\text{mol/L}$ (Graham, 2001). Upon administration, caffeine stimulates the central nervous system (CNS), and elicits an elevated heart rate and blood pressure (Hartley, Lovallo, & Whitsett, 2004; Rauh, Burkert, Siepmann, & Mueck-Weymann, 2006).

The primary mode of action for caffeine is via inhibition of the adenosine receptors (Davis et al., 2003; Graham, 2001). Caffeine has a structure similar to adenosine, and hence it binds to the adenosine receptors on the cell surface, resulting in inhibition of adenosine function (Davis et al., 2003; Graham, 2001). Adenosine is known to be closely associated with alertness. It is believed to inhibit the release of excitatory transmitters in the central nervous system (CNS) which lead to decreased arousal and increased sleepiness (Davis et al., 2003; Porkka-Heiskanen, 1999). As described previously in section 2.3.1, adenosine executes its effects via binding with its receptors to regulate cAMP. Blocking of adenosine receptors with caffeine results in a compromise of the effects caused by adenosine (Davis et al., 2003; Graham, 2001). It is also known that caffeine increases the plasma adrenaline level, which may induce a series of secondary metabolic changes to provoke ergogenic effects (Graham, 2001). Considering the above caffeine mechanisms, caffeine also has the potential to regulate lymphocyte activation via either acting as an adenosine antagonist, or the stimulation of adrenaline release (Section 2.4.4) (Bishop, Fitzgerald, et al., 2005).

According to Fredholm et al. (1999), when caffeine is injected directly into the blood stream, it can be absorbed rapidly and completely by the gastrointestinal tract within 45 min. Following oral administration, the plasma caffeine concentration peaks within 15-120 min (Fredholm et al., 1999). For doses lower than 10 $\text{mg} \cdot \text{kg}^{-1}$, the half-life of plasma caffeine is 2.5-10 h in healthy adults, with a 30-50% reduced rate in smokers, but doubled in women taking the oral contraceptive pill (Ali, O'Donnell, Starck, & Rutherford-Markwick, 2015; Fredholm et al., 1999; Magkos & Kavouras, 2005). These data suggest that there is a large variation between individuals in caffeine metabolism.

Caffeine metabolism occurs in the liver and is mediated by the cytochrome P450 oxidase enzyme system (Ali et al., 2015). The caffeine is mainly eliminated through demethylation of trimethylxanthine into three different dimethylxanthines: paraxanthine (80%), theobromine (11%) and theophylline (5%). The remaining caffeine (~4%) is thought to clear via the pathways leading to trimethyluric acid and trimethyluracil, as well as through the urination of unchanged caffeine (Magkos & Kavouras, 2005). It is known that paraxanthine and theophylline are also a potent adenosine antagonist and elicit similar immunomodulatory effects as caffeine (Horrigan, Kelly, & Connor, 2006). The dimethylxanthines are then further catabolised into other forms of metabolites. The human polycyclic aromatic hydrocarbon (PAH)-inducible cytochrome P450 family CYP_{1A} subtype A₂ (CYP_{1A2}) is the major catalysts of caffeine (Carrillo & Benitez, 2000). CYP_{1A2} also plays a major role in metabolism of numbers of clinically important drugs such as paracetamol, cimetidine, fluvoxamine and aspirin, etc (Carrillo & Benitez, 2000). As caffeine has low affinity for CYP_{1A2}, taking caffeine together with drugs that are substrates for CYP_{1A2} tends to impair the caffeine clearance in the body (Carrillo & Benitez, 2000). For example, taking fluvoxamine together with caffeine can decrease the rate of caffeine clearance by 80% and increase the caffeine half-life by 500%.

Another factor influencing the caffeine pharmacokinetics is the form of caffeine consumed (Graham, 2001; Graham, Hibbert, & Sathasivam, 1998). A study by Graham et al. (1998) suggests that ingestion of pure caffeine (4.45 mg. kg⁻¹) enhance the endurance performance of running time to exhaustion (85% VO_{2max}) by 31% but no endurance enhancement is seen with consumption of same amount of caffeine in regular coffee. Graham et al. (1998) speculates the finding of pure caffeine is better performance enhancer than regular coffee to the antagonised properties of chlorogenic acids found in the coffee. However, the findings from Hodgson, Randell, and Jeukendrup (2013) do not support the Graham et al. (1998) finding. Hodgson et al. (2013) illustrate that consumption of high dose of caffeine (5 mg. kg⁻¹) in coffee form is no inferior to pure caffeine ingestion at improving endurance exercise performance. Hodgson et al. (2013) suggest that the discrepancy in findings may be due to the intensity and type of exercise. In the study of Graham et al. (1998), eight actively trained endurance runners ran at 85% VO_{2max} whereas in Hodgson et al. (2013) study, eight trained cyclists cycle at 55% VO_{2max}. Clearly, more comparative studies are

needed to fully understand the influence of the form of caffeine intake on the performance.

Additionally, the serum caffeine concentrations and time to peak concentration are found to be significantly slower with consumption of high carbohydrate meal prior to caffeine ingestion (Skinner et al., 2013). It is noteworthy that caffeine pharmacokinetics are similar for both men and women (Magkos & Kavouras, 2005; McLean & Graham, 2002). The study by McLean and Graham (2002) suggests that gender, exercise, and thermal stress have no significant impact on caffeine pharmacokinetics in men and women. In addition, caffeine pharmacokinetics are independent of exercise and hydration status (Graham, 2001).

2.4.4 Effect of Caffeine on Lymphocytes

A vast number of studies have demonstrated that caffeine has modulatory effects on various aspects of both innate and adaptive immunity (Horrigan et al., 2006). Studies have demonstrated that caffeine suppresses cytokine secretion, lymphocyte proliferation, leucocyte chemotaxis and the cytotoxicity of NK cells, but it increases the leukocyte counts (Horrigan et al., 2006).

Concentration of Caffeine Employed and Nature of Studies

It is important to note that most of the studies are *in vitro* investigations and use rodent models. Additionally, most *in vitro* studies with rodents administer one single high dose of caffeine, whereas most humans habitually consume the caffeine over multiple times per day. This may lead to having residual levels of caffeine and/or metabolites in their plasma, prior to an experiment with human participants. Differences in half-life, plus the variation in caffeine consumption patterns between individuals and countries, make it very difficult to correctly examine the effects of caffeine consumption on individuals, especially during *in vitro* investigations. This may contribute to the inconsistent findings between studies to date.

Fredholm et al. (1999) suggest that a peak plasma concentration of 1-10 μM is observed following the consumption of one cup of coffee. Varani et al. (2005) also demonstrate that consumption of 600 mg of caffeine every day for a 7 day period results in a mean peak plasma caffeine concentration of 63 μM in 11 participants. When trying to extrapolate data from rodents to humans, it is important to consider the dose equivalence between animals and humans by using a molecular body weight correction

factor (Fredholm et al., 1999). Fredholm et al. (1999) suggest that 10 mg. kg⁻¹ in a rat represents about 250 mg of caffeine in a human weighing 70 kg (3.5 mg. kg⁻¹), which is the equivalent of approximately two to three cups of coffee. Based on these estimated plasma caffeine concentrations, studies adapting 100 µM of caffeine or less can be considered as relevant to human caffeine consumption (Horrigan et al., 2006).

Effects of Caffeine on NK Cell Function

Kantamala, Vongsakul, and Satayavivad (1990) illustrate that at the human physiological caffeine concentration, caffeine has no effect on NK cell cytotoxicity *in vitro* in rats. Nevertheless, Kantamala et al. (1990) show that caffeine's effect on NK cells is dependent on the dose. At a caffeine dose of 2 and 18 mg.kg⁻¹ per day, there is no effect on NK cell cytotoxicity, whereas a dose of 6 mg. kg⁻¹ per day suppresses the NK cell cytotoxicity *in vivo* in mouse. In contradiction of this, a study by Horrigan, Kelly, and Connor (2004) with human participants demonstrates that, at a caffeine dose of 100 µM, it consistently downregulates the secretion of TNF-α in lipopolysaccharide-stimulated human whole blood in all participants via the cAMP/PKA pathway. However, Fletcher and Bishop (2011a) show that a caffeine dose of 2 and 6 mg.kg⁻¹ BW increases the activation of both unstimulated and antigen-stimulated NK cells.

The discrepancy in the findings may be due to the difficulties in comparing studies between rodents and humans, as well as the different consumption patterns across individuals, as discussed above.

Effects of Caffeine on T cell Function

Studies have indicated that caffeine suppresses the T cell functions, including cytokine production and lymphocyte proliferation (Horrigan et al., 2006). It showed that, at a caffeine dose relevant to normal human consumption, the secretion of IL-2 and IL-4 by the T cells is inhibited in *in vitro* administration in mice (Ritter et al., 2005). Moreover, Horrigan, Kelly, and Connor (2005) illustrate that caffeine diminishes the production of IL-5 and IFN-γ in concanavalin A (Con A) stimulated human whole blood. In addition, T cell proliferation is also suppressed by caffeine (Horrigan et al., 2006). This is supported by the fact that in both *in vivo* and *in vitro* stimulation of immune cells with T-cell mitogens, Con A and phytohaemagglutinin (PHA), T cell proliferation is attenuated (Horrigan et al., 2005; Kantamala et al., 1990; Rosenthal, Taub, Moors, & Blank, 1992). Additionally, similar to the effect of caffeine on cytokine production in NK cells, it also has a dose-dependent effect on T cell proliferation. A caffeine dose of 2

and 6 mg.kg⁻¹ per day has no effect, but 18 mg.kg⁻¹ per day elevates T cell proliferation in PHA-P-stimulated cells (Kantamala et al., 1990).

On the other hand, Bishop, Fitzgerald, et al. (2005) demonstrate that *in vivo* administration of 6 mg.kg⁻¹ BW of caffeine increases the total lymphocyte count and CD8⁺ T cell count in 1 h following consumption prior to exercise. It also enhances the activation of CD4⁺ and CD8⁺ T cells, as measured by the percentage of cells expressing CD69⁺ both before and after 90 min of cycling at 70% VO_{2peak}. In contrast, Fletcher and Bishop's (2012) *in vivo* human study suggests that a 6 mg.kg⁻¹ BW caffeine dose attenuates the CD4⁺ and CD8⁺ T cell activation in antigen-stimulated cells 1 hour post 90 min cycling at 70% VO_{2peak}. It is noteworthy that Bishop, Fitzgerald, et al. (2005) employ unstimulated, but Fletcher and Bishop (2012) use antigen stimulated cells. Observation in antigen-stimulated cells may more closely represent infection in a real-life situation. A study by Fletcher and Bishop (2012) is in agreement with other *in vitro* and rodent *in vivo* studies, where caffeine is shown to be an immunosuppressive agent for T cells (Horrigan et al., 2006).

Mechanism of Immunomodulatory Effects of Caffeine

As previously discussed, the primary mechanism of caffeine is via acting as an adenosine antagonist. According to Fredholm et al. (1999), caffeine can bind to all four adenosine receptors; A₁, A_{2a}, A_{2b} and A₃ at a low concentration. Among them, caffeine preferentially binds to A₁ and A_{2a} receptors (Ferre et al., 2008). Adenosine mediates immune suppressive effects and the extracellular adenosine level increases with metabolic stress during exercise. It is also known that adenosine is activated via interaction with its receptors (Adenosine, Section 2.3.1) (Haskó et al., 2008; Sitkovsky & Ohta, 2005). By caffeine binding with the specific adenosine receptors, it is expected to inhibit the effects of particular adenosine receptors (Ribeiro & Sebastiao, 2010). For example, the binding of caffeine to the A_{2A} receptor may inhibit the immunosuppressive effects of the adenosine A_{2A} receptor, whereas ligation with A₁ may inhibit the immunostimulatory effects of the adenosine A₁ receptor via the cAMP pathway (Adenosine, Section 2.3.1) (Fletcher & Bishop, 2012). As T lymphocytes primarily express A_{2A} (Gessi et al., 2004; Koshiba et al., 1999; Mirabet et al., 1999), the adenosine antagonism activity of caffeine inhibits the action of A_{2A}, leading to stimulation of T cell function (Fletcher & Bishop, 2012). As NK cells express both A₁ and A_{2A} (Priebe et al., 1990), ingestion of caffeine may inhibit the effect of A₁ and A_{2A}

receptors, resulting in both immunosuppression and immunostimulation of NK cells (Fletcher & Bishop, 2012).

Another mechanism of caffeine is the stimulation of adrenaline production (Graham, 2001). Adrenaline binds to the adrenoreceptors on the surface of the cells to execute its effects (Adrenaline, Section 2.3.1). β_2 -adrenoreceptors are the major receptors on the surface of T and NK cells (Kohm & Sanders, 2001; B. K Pedersen & Hoffman-Goetz, 2000). As discussed previously in section 2.3.1, ligation of adrenaline with β_2 -adrenoreceptors provokes the adenylyl cyclase system, leading to an increase in cAMP. This results in the suppression in T and NK cell activation.

Additionally, caffeine is known to have a positive impact on the lymphocyte mobilisation (B. K Pedersen & Hoffman-Goetz, 2000). A high dose of caffeine induces the increased secretion of adrenaline (Kamimori et al., 2000). Caffeine combined with high intensity exercise is shown to significantly enhance adrenaline secretion (Fletcher & Bishop, 2012; Kamimori et al., 2000; Zouhal et al., 2008). Adrenaline stimulates the β -adrenoreceptors on the surface of the lymphocytes, which in turn induces the trafficking of lymphocytes into the circulation, causing an increase in the number of circulating lymphocytes (Benschop, Oostveen, Heijnen, & Ballieux, 1993). The rate of lymphocyte mobilisation is closely related to the degree of β -adrenoreceptor expression on the cell surface. Therefore, NK cells are considered to be the most mobilised cells with the highest β -adrenoreceptor expression; $CD8^+$ are the most mobilised cells and $CD4^+$ are the least mobilised cells, with the lowest density of β -adrenoreceptors (B. K Pedersen & Hoffman-Goetz, 2000). It is noteworthy that these two mechanisms may occur concurrently, and the overall effect on the lymphocytes' function may be the absolute sum of stimulatory and inhibitory consequences of caffeine (Fletcher & Bishop, 2012).

2.5 Summary of the Literature Review

To summarise, continuous bouts of strenuous exercise can result in immune impairment in innate (NK cells) and adaptive (T cells) immune arms up to 3 to 24 h after exercise, which may account for the increased risk of URTI. Generally, the number of T and NK cells increases immediately after the exercise, but declines below baseline value during the recovery period. It is believed that the alteration in lymphocyte numbers is due to the intensity rather than the duration of the exercise (B. K. Pedersen & Laurie, 2000). Therefore, it is believed that performing HIIE, which has become popular among active

people recently, may also result in immune dysfunction after exercise. Recently, a considerable amount of research has focused on how diets can be modified to lessen the degree of immune suppression after exercise. Caffeine is one of the prominent dietary elements of interest in such strategies. Administration of caffeine before exercise has been found to affect T and NK cells. However, the findings are inconsistent, as some studies have reported caffeine as an immunosuppressive agent for T cells (Horrigan et al., 2006; Ritter et al., 2005), but others have found that it enhances the T cell function (Bishop, Fitzgerald, et al., 2005). Additionally, most of the caffeine research performed has been based on animal models rather than using human participants (Horrigan et al., 2006; Ritter et al., 2005). Furthermore, one of the caffeine studies which used human participants (Bishop, Fitzgerald, et al., 2005) only examined the unstimulated T lymphocytes, which may not mimic the actual situation of when a body encounters antigens, and T cells only represent the adaptive immune arm of the immune system. To the author's knowledge, there is only one research study looking at the impact of caffeine consumption on antigen-stimulated T and NK cells following strenuous exercise (Fletcher & Bishop, 2012). However, Fletcher and Bishop's study was based on prolonged exercise, rather than HIIE. There is a need for further research to determine the effects of caffeine ingestion prior to HIIE on innate and adaptive immune function in humans.

Chapter 3 Methods

3.1 Ethical Approval

All study protocols presented in this thesis were approved by Auckland University of Technology Ethics Committee (AUTEC). All study participants were given a study participant information sheet, which fully informed them of the following information:

- Concept and nature of the study
- Research questions with academic rationale
- Background of the researchers
- The methodology used and the demand of the exercise
- The required time investment in the study
- The potential benefits of this research to the participants, the researchers and the wider community
- The intended dissemination of study
- The potential risk and discomforts likely to experience along with their rights prior to and throughout the study.

Participants were provided enough time to consult with the researchers, friends and family prior to enrolment in the study and before providing written informed consent (Appendix A).

3.2 Experimental Overview

This study was a randomised, repeated-measures, double-blind, crossover study. Participants acted as their own control. Supplement capsules were prepared by a lab technician and capsules were only marked as either number one or two to ensure blinding from all researchers and participants. All participants performed two experimental trials (either placebo or caffeine) in a randomised order, separated by approximately seven days (Figure 4). The protocol for the HIIE experimental trials are detailed in section 3.6.

Venous blood samples were taken at four time points during each experimental trial as follows (Figure 4):

1. Pre-supplement (T1) samples after 10 min of resting quietly in the lab

2. Pre-exercise (T2) samples taken one h after ingesting either caffeine or placebo capsules (immediately before starting exercise)
3. Post-exercise (T3) taken immediately after exercise
4. One h post-exercise (T4), taken after one h of resting following cessation of exercise.

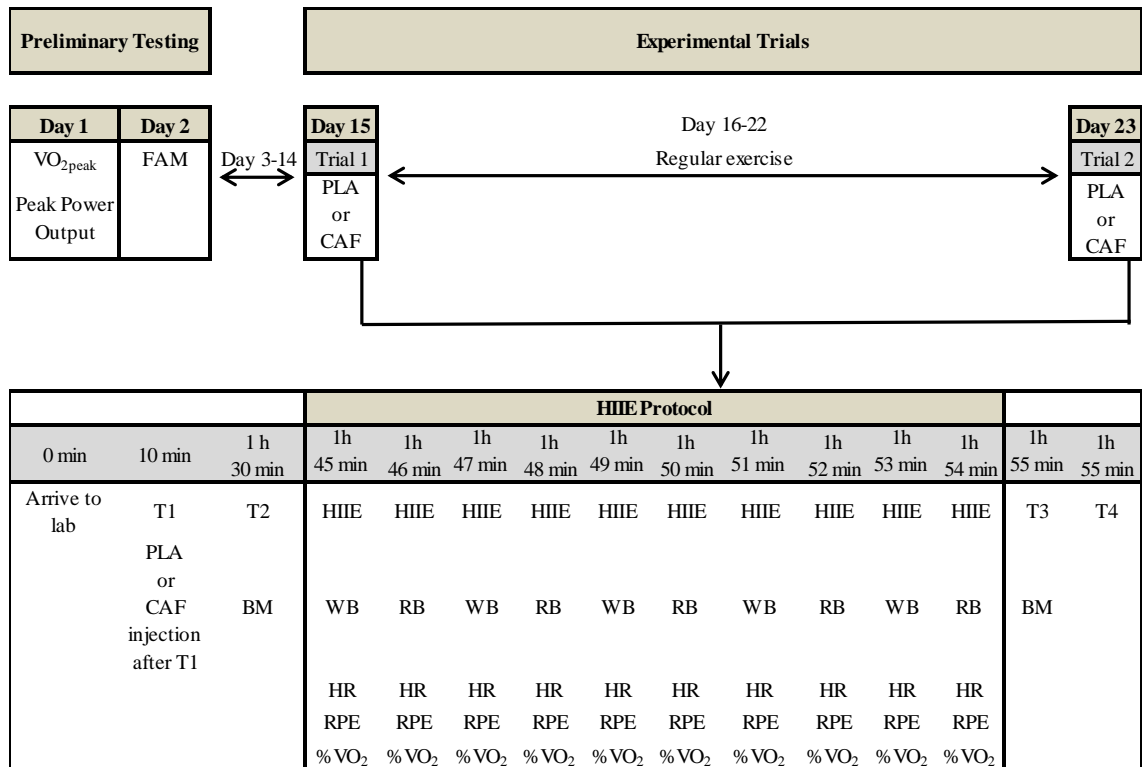


Figure 4. Schematic representation of study design.

PLA = Placebo; CAF = Caffeine; VO_{2peak} = Peak oxygen uptake;

FAM = Familiarisation session; HIE = High intensity intermittent exercise;

WB = Work bout that was equivalent to the participant's peak power output that elicited ~90% HR_{max}; RB = Recovery bout at 50W; HR = Heart rate; RPE = ratings of perceived exertion; BM = Body mass; T1 = Pre-supplement blood sample; T2 = Pre-exercise blood sample; T3 = Immediately post-exercise blood sample; T4 = 1 h post-exercise blood sample.

3.3 Participants

Ten healthy active men aged between 18 - 35 years were recruited through advertising in local gyms and AUT University. Participants were required to meet the following health, physical and dietary criteria to take part in the studies. Participants completed a pre-test health questionnaire (Appendix B) and physical activity questionnaire (Appendix C) during their initial visit to the laboratory to ascertain if they were eligible

for the study. Any participants who smoked or were currently on any medication or had experienced a URTI in the four weeks prior to the start of the study were excluded. Participants needed to be currently engaged in some form of regular moderate-high intensity exercise ($\text{VO}_{2\text{peak}} \geq 40 \text{ ml. kg. min}^{-1}$). Participants who met the pre-test health and physical activity criteria then completed a caffeine consumption questionnaire (Appendix D) to confirm that caffeine was consumed as part of their normal diet and to identify participants as low, moderate or high caffeine users. There was no minimum caffeine consumption requirement as long as the participants were consuming some amount of caffeine.

On the day of each experimental trial, participants were again asked to complete a subsequent health-screening questionnaire (Appendix E) to confirm they were not currently experiencing any symptoms of URTI and their willingness to honour the consent form that they signed.

3.4 Exploratory/Pilot Methods

Before the main experimental trials began, preliminary/pilot laboratory tests were performed to ensure both the protocol and methodology for the study was sound. The laboratory protocols chosen for this study were previously used in a study by Fletcher and Bishop (2012), and as such have been validated for feasibility and reliability.

The Infanrix[®] hexa vaccine (GlaxoSmithKline Biologicals s.a, Rixensart, Belgium) containing a combination of diphtheria-tetanus-acellular pertussis, hepatitis B, enhanced inactivated polio and Haemophilus influenzae type b vaccine was used as the stimulant for this study. This vaccine was chosen because people were generally given this vaccine as a primary and booster vaccination when they were infants. As such, stimulating the cells with the same vaccine helps to produce a secondary recall immune response, thereby mounting a rapid optimal response. The vaccine was titrated to elicit a maximal lymphocyte response, which was shown to occur at a concentration of 1:1000 (Appendix G).

A resting blood sample was drawn into a sodium heparin and an EDTA tube. The EDTA sample was used to test for complete blood count (CBC), and sodium heparin was used to examine the CD4^+ , CD8^+ and CD69^+ markers using a flow cytometer (BD FACSCalibur[™]). This was to familiarise the researcher with the laboratory procedures and analysers, and the requirements for the preparation of reagents and consumables,

and to achieve better time management in actual study trials. All the preliminary pilot testing was done using the same procedures described in section 3.8. As such, this work helped to ensure that the procedures used in the study by Fletcher and Bishop (2012) were achievable in the Sports Performance Research Institute New Zealand labs. A resting caffeine trial was not undertaken in the current study, as previous work done by Fletcher and Bishop (2012) has demonstrated that caffeine has little to no effect on T and NK cell activation at rest, using similar times of day as the current study.

3.5 Preliminary Testing

3.5.1 Maximal Oxygen Uptake ($\dot{V}O_{2\text{peak}}$) Test

Approximately 14 days before participating in the main experimental trials, each participant was required to complete a graded incremental test to exhaustion to determine $\dot{V}O_{2\text{peak}}$ and peak power output values using an electronically braked cycle ergometer (Lode Sports Excalibur, Groningen, Netherlands) (Figure 4). The testing protocol was adopted from (Fletcher & Bishop, 2011b) and consisted of an initial load of 95W for the first 3 min followed by 35W increments every 3 min until exhaustion, or the participant was not able to maintain pedaling cadence of more than 60 revolutions per min. Maximal work rate (W_{max}) was determined using the following formula mentioned by Jeukendrup, Saris, Brouns, and Kester (1996):

$$W_{\text{max}} = W_{\text{out}} + (t/180) * 35$$

W_{out} is the last completed stage and t is the time in seconds (s) in the final stage.

Oxygen uptake ($\dot{V}O_2$) and heart rate (HR) were measured throughout the test. Expired gases were collected using a calibrated metabolic cart (TrueOne 2400, ParvoMedics, Sandy, UT, USA). HR was measured using heart rate monitors (Polar RS800, Polar Electro, Finland). Collected data was averaged over 30 s intervals, and $\dot{V}O_{2\text{peak}}$ was considered the highest value achieved in a 30 s period.

3.5.2 Familiarisation Session

Participants were required to visit the lab for a familiarisation session to ensure they were able to complete the 20 min high intensity intermittent exercise (HIIE) protocol at the prescribed wattage, and that the session elicited an average HR_{max} of 90%. (see section 3.7) During the familiarisation session, participants were introduced to all

equipment and procedures necessary for the following experimental trials. The test protocol is explained in detail in the following HIIE test section (see section 3.7).

3.6 Standardisation of Pre-Trial Conditions

Participants maintained their habitual caffeine intake until 60 h prior to each main trial. They were then given a list of caffeine-containing foods and beverages, and asked to abstain from any form of caffeine in the 60 h preceding the trials. This caffeine withdrawal duration was chosen to ensure that all the immunological markers and hormones of interest were returned to baseline at the start of experimental trials regardless of if the participant was a high, moderate or low caffeine user. According to (Graham, 2001), the caffeine levels in the circulation drop to barely detectable levels after 48 h of withdrawal. Participants were also asked to refrain from alcohol consumption and any form of exercise for 24 h prior to each experimental trial. Participants were also required to fast overnight (12 h) preceding the main trials. Participants were also required not to be taking any medication and this was ensured through the pre-test health questionnaire (Appendix B).

To standardise their hydration status, participants were asked to drink approximately 300 ml of water upon waking up in the morning on the day of the experimental trials. To standardise their dietary intake, participants were also required to keep a 24 h food diary the day before the first main trial and then asked to follow the same food intake in the 24 h prior to the second trial. The compliance to all the above pre-trial requirements was confirmed by the diet diary (Appendix F).

3.7 High Intensity Intermittent Exercise (HIIE)

Preceding the overnight fast and 60 h of caffeine abstention, participants were asked to arrive at the laboratory at 08:30 am. After 10 min of resting quietly in the lab, an initial venous blood pre-supplement sample (T1) was drawn from the antecubital vein. Following the T1 blood sample collection, participants were given either 6 mg.kg⁻¹ body weight (BW) of caffeine powder (CAF trial) (Acros Organics, Belgium) in the form of cellulose capsules (kybright NZ Ltd., Christchurch, NZ) or corn flour (PLA trial) (Edmonds, Auckland, NZ) prepared in the same cellulose capsules, taken with 5ml.kg⁻¹ BW of plain water. After that, participants rested quietly for one h followed by the pre-exercise (T2) blood sample collection. The participants' pre-exercise body mass (in shorts only) were recorded, just before the exercise trial began. Immediately

following this, participants started the HIIE on a cycling ergometer (Lode Sports Excalibur, Groningen, Netherlands) for 20 min. The Lode bike was set up for each participant and the bike position was replicated in the subsequent trial. The HIIE protocol was adapted from Little et al. (2011). It consisted of 10 x 1 min work bouts interspersed with 10 x 1 min recovery bouts. During work bouts, participants were required to cycle at a wattage that was equivalent to their peak power output from the incremental VO_2 test that elicited $\sim 90\%$ HR_{max} . During recovery bouts, participants cycled slowly at a workload of 50W.

Expired gases were collected every minute throughout the trial using a calibrated metabolic cart (TrueOne 2400, ParvoMedics, Sandy, UT, USA). Rating of perceived exertion (RPE) as defined by Borg (1982) and heart rate using Polar RS800CX, (Kempele, Finland), were recorded every minute for each work and recovery bout. HR and respiratory parameters were measured to ensure that participants were exercising at the required intensity. Immediately upon completion of 20 min of exercise, a further venous blood sample was taken (post-exercise, T3), before post-exercise body mass (in shorts only) was recorded. Participants were then given 5 ml.kg^{-1} body mass of plain water to consume and after resting in the lab for a further hour, a final blood sample was taken (T4, one hour post-exercise sample). During this time, no additional food or fluid was administered to participants.

Standard laboratory conditions were kept during testing (18-20°C, 40-60% humidity), and a fan was used to cool participants during exercise.

3.8 Blood Sampling and Analysis

All the venous blood samples in this study were collected from an antecubital vein by venepuncture, with the participant in an upright seated position. For all samples, approximately 20 ml of blood was collected into four vacutainers (BD Vacutainer® Plus blood tube), one containing ethylenediaminetetraacetic acid (EDTA), one containing sodium heparin, one containing lithium heparin and one containing no additive to obtain serum.

3.8.1 Haematological Analysis

Whole blood in the K_2EDTA tube was used to analyse total and differential leukocyte counts, haematocrit and haemoglobin contents, using a haematology analyser ($\text{A}^{\text{C}}.\text{T}^{\text{TM}}$ 5diff analyser, Beckman Coulter, USA). All cell counts were adjusted for the change in

plasma volume in reference to pre-supplement blood samples based on haemoglobin and haematocrit values, according to Dill and Costill (1974).

3.8.2 Examination for CD4⁺, CD8⁺ and CD69⁺

Whole Blood Culture

Immediately after the blood was withdrawn, the sodium heparin tubes were placed on ice and then mixed using the digital orbital shaker (SHO-1D, Daihan Scientific) at 120 rpm for 20 min prior to setting up the cultures.

A total of 16 tubes, representing two different stimulant conditions, unstimulated and optimal antigen-stimulated, were set up in Falcon 12 x 75 mm-polystyrene round-bottom tubes with caps (Becton Dickinson Biosciences, UK) for the blood collected at each time frame for assessment of both T and NK cells for each participant.

Either 20 µl of RPMI for unstimulated cultures, or 20 µl of Infanrix hexa working strength vaccine (1:100) for stimulated cultures, was added to 200 µl of heparinised whole blood, to yield the final stimulant concentration of 0 (unstimulated) or 1:1000 (stimulated). The tubes were incubated at 37°C, 5% CO₂ for 20 h.

Flow Cytometry Analysis

Staining

Following 20 h of incubation, the peripheral blood cells were stained with a cocktail of Pharmingen monoclonal antibodies (Becton Dickinson Biosciences, UK) against specific human lymphocyte cell surface markers, as shown in Table 3.

Table 3. Staining of T and NK Cell Tubes

	Filter and Fluorochrome		
	FITC (FL-1)*	PE (FL-2)**	PE-Cy5 (FL-3)***
T cell tubes	CD4 ⁺ (5 µl)	CD69 ⁺ (10 µl)	CD8 ⁺ (5µl)
NK cell tubes	CD3 ⁺ (5 µl)	CD69 ⁺ (10 µl)	CD56 ⁺ (20 µl)

*FITC = Fluorescein Isothiocyanate (FITC), FL-1 = 525 nm band pass filter (Green)

**PE = Phycoerythrin, FL-2 = 575 nm band pass filter (Orange-Red)

***PE-Cy5 = R-phycoerythrin-Cy5, FL-3 = 620 nm band pass filter (Red).

All stained and unstained samples were then vortexed using the vortex mixer (, Zx3, Velp Scientifica) thoroughly at 40 Hz and placed on ice for 20 min. After 20 min, 3 ml of FACS lysis buffer (Becton Dickinson, UK) was added to each of the tubes and rigorously vortexed to lyse the erythrocytes and fix the leukocytes. All the tubes were

then incubated in the dark for 10 min. After the 10 min incubation, all the tubes were washed twice using ice cold PBS/BSA/EDTA. The first wash used 1 ml while second wash used 3 ml of PBS/BSA/EDTA. After the two washes, the cells were resuspended in 400µl of ice cold PBS/BSA/EDTA.

Acquisition

Lymphocytes phenotype data were acquired by performing three-colour flow cytometry analysis on a FACS Calibur flow cytometer using Cell Quest analysis software (Becton Dickinson, Oxford, UK). For each sample analysed, 30,000 lymphocyte events were acquired. The lymphocyte population was electronically gated using side and forward light-scatter mode.

Gated lymphocytes were identified using a two parameter quadrant dot plot. Three quadrant dot plots were constructed for each cell type (Q1, Q2, Q3). Unstained unstimulated samples were used as an acquisition baseline as a control for setting of quadrant dot plots boundaries for each of the corresponded analyses.

T cell acquisition

Q1: FL1 (CD4⁺, FITC) versus FL3 (CD8⁺, PE-Cy5)

Q2: FL1 (CD4⁺, FITC) versus FL2 (CD69⁺, PE)

Q3: FL3 (CD8⁺, PE-Cy5) and FL2 (CD69⁺, PE)

The data collected were used to generate histogram plots. The percentages of CD4⁺ and CD8⁺ cells were determined. Only CD8⁺ cells were included in the analysis, as all of them express CD3, whereas only very small number of CD8^{dim} cells express CD3 (Campbell, Guy, Cosgrove, Florida-James, & Simpson, 2008; Simpson, Florida-James, Cosgrove, et al., 2007). Total numbers of CD4⁺ and CD8⁺ lymphocytes were determined by multiplying the percentage values of the cells with the corresponding total lymphocyte count recorded from the haematology analyser.

NK cell acquisition

Q1: FL1 (CD3⁺, FITC) versus FL3 (CD56⁺, PE-Cy5)

Q2: FL1 (CD3⁺, FITC) versus FL2 (CD69⁺, PE)

Q3: FL3 (CD56⁺, PE-Cy5) versus FL2 (CD69⁺, PE)

The data collected were also plotted into histograms. The percentage of CD3⁺ CD56⁺ cells were obtained by subtracting the CD3⁺ region from the total lymphocyte

region. From this, total numbers of CD3⁺CD56⁺ lymphocytes were determined by multiplying the percentage values of CD3⁺CD56⁺ with their total cell count.

CD69⁺ cell acquisition

CD4⁺, CD8⁺^{bright} and CD3⁺CD56⁺ cells were then gated into individual regions.

Histogram plots were constructed for CD69⁺ of each CD4⁺, CD8⁺^{bright} and CD3⁺CD56⁺ cells within each cell region. It was then used to calculate the percentage and geometric mean fluorescence intensity (GMFI) expression of CD69 of CD4⁺, CD8⁺^{bright} and CD3⁺CD56⁺ cells.

Absolute Cells Count

The absolute number of lymphocytes expressing CD4⁺, CD8⁺^{bright} and CD3⁺CD56⁺ cells was calculated by multiplying the percentage of the cell expressing cell surface antigen of interest with the corresponding total lymphocyte count. The CD69⁺ cell count was determined by multiplying the percentage of CD69⁺ cells by the total number of CD4⁺, CD8⁺^{bright} and CD3⁺CD56⁺ cells.

3.8.3 Serum Caffeine, Plasma Cortisol and Plasma Adrenaline Concentration

Serum was obtained from whole blood collected into a serum monovette (5.5 ml), which was left to clot for 1 h before being centrifuged at 1500 x g for 10 min in a refrigerated centrifuge at 4 °C. 1000 µl of serum was then evenly distributed into labelled Eppendorf tubes and frozen at -80 °C. Serum caffeine concentration was measured using high performance liquid chromatography (HPLC) with UV detection, as previously described by Perera, Gross, and McLachlan (2010). Plasma was obtained from blood collected into a K₂EDTA vacutainer. Samples were spun at 1500 x g for 10 min in a refrigerated centrifuge at 4 °C within 5 min of collection. Plasma cortisol concentration was analysed using a commercially available ELISA kit (DRG, New Jersey, USA). The intra assay coefficient of variation for both serum caffeine concentration and plasma cortisol concentration was < 5%. The plasma adrenaline concentration was also tested using a commercially available ELISA kit (BI-CAT[®] ELISA, DLD Diagnostika GMBH) with an intra assay CV < 5%.

3.9 Statistical Analysis

SPSS 18.0 software for Windows (SPSS Inc. Chicago IL, USA) was used for statistical analysis. All the data in figures, tables and texts are presented as mean±standard

deviation (\pm SD). All data were analysed using a two-way ANOVA (trial x time). Data that were not normally distributed were recast using logarithmic transformation as described by Valiela (2009). The data were rechecked for normal distribution following log transformation. The assumption of sphericity for the ANOVA was evaluated using Mauchley's method. Adjustment was made using the Huynh-Feldt method if there was violation in assumption. Student's paired *t*-tests with Holm-Bonferroni corrections were used to analyse significant data in multiple comparison. Student's paired *t*-tests were used to assess single comparisons between trials for $\text{VO}_{2\text{max}}$, HR, RPE and, changed in body mass and plasma volume. Significance was accepted at $P < 0.05$. The observed powers of the reported main and interaction effects are all >0.8 .

Chapter 4 Results

4.1 Participant Characteristic

The results reported here are from ten healthy active men [Mean \pm (SD): age 27 (4.3) years; body mass 78 (9.9) kg; $\text{VO}_{2\text{peak}}$ 46 (6) $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; peak power output 308 (57.2) W; HR_{max} 193 (4.3) bpm] who met the physical and health criteria. Participants were classified as medium caffeine users consuming 50-250 mg of caffeine daily.

4.2 Exercise Intensity, Heart Rate and Rated Perceived Exertion

There was no significant difference in mean exercise intensity (% VO_2) between trials during both the work [CAF: 79.4% (5.3), PLA: 75.9% (5.5), $P>0.05$] and recovery bouts [CAF: 67.2% (9.2), PLA: 65.4% (7.4), $P>0.05$]. Similarly, the heart rate (% HR_{max}) during exercise was comparable between the trials during both the work [CAF: 86% (6), PLA: 86% (7), $P>0.05$] and recovery bouts [CAF: 79% (6), PLA: 78% (7), $P>0.05$]. The RPE was also similar between trials during both the work [CAF: 16 scale (2.0), PLA: 16 scale (2.0), $P>0.05$] and recovery [CAF: 13 scale (3.0), PLA: 13 scale (3), $P>0.05$] bouts.

4.3 Changes in Body Mass and Plasma Volume

There was no change in body mass (corrected for fluid intake) following exercise for both of the trials [CAF: -0.4 (0.5) kg, PLA: -0.2 (0.1) kg, $P>0.05$]. There was no significant time x trial interaction effect ($P>0.05$) for changes in plasma volume compared with the pre-supplement blood samples. The percentage of plasma volume change in relation to pre-supplement samples decreased at post-exercise in both of the trials [CAF: 13.7 (3.8) %, PLA: 10.5 (4.0) %, $P<0.01$].

4.4 Serum Caffeine

A significant time x trial interaction was found for serum caffeine concentration ($P<0.01$), with higher concentrations at pre-exercise, post-exercise, and one h post-exercise in the CAF group compared with the PLA group ($P<0.01$; see Figure 5). Within the CAF trial, values on the above time points were significantly higher than for the pre-supplement (pre-supplement Vs pre-exercise: $P<0.01$; pre-supplement Vs post-exercise: $P<0.01$; pre-supplement Vs 1 h post-exercise: $P<0.05$) (see Figure 5).

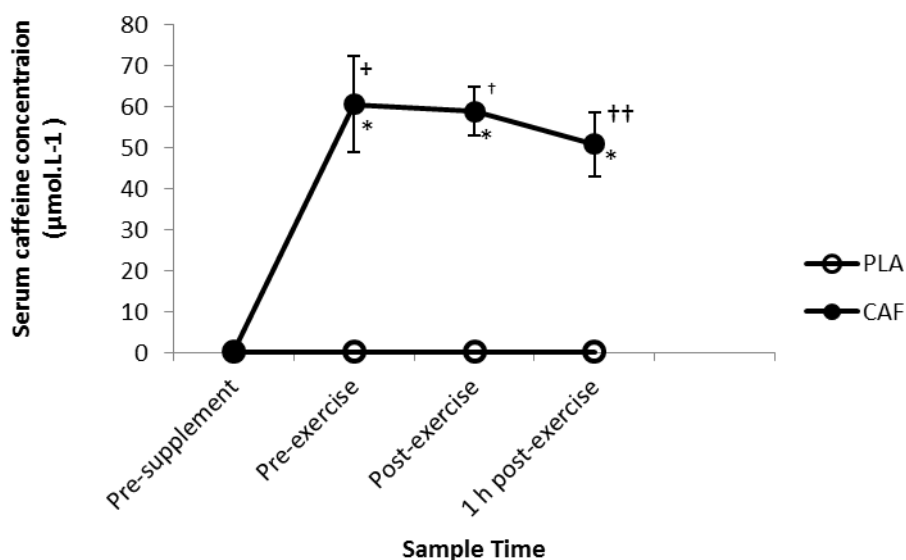


Figure 5. Serum caffeine concentrations during the PLA and CAF trials.

PLA = Placebo; CAF = Caffeine.

Values are means \pm SD.

* Significantly higher in the CAF compared with the PLA trial ($P < 0.01$)

Significantly higher than pre-supplement within the trial $^{\dagger} P < 0.01$, $^{\dagger\dagger} P < 0.05$

4.5 Plasma Cortisol

A significant time \times trial interaction was found for plasma cortisol concentration, with a higher concentration at one h post-exercise in the CAF compared to the PLA trial ($P < 0.05$; see Figure 6). Within both the CAF and PLA trials, the post-exercise concentration was significantly higher than the pre-supplement ($P < 0.01$; see Figure 6). Within the CAF trial, the concentration was also elevated above the pre-supplement level at one h post-exercise ($P < 0.05$; see Figure 6).

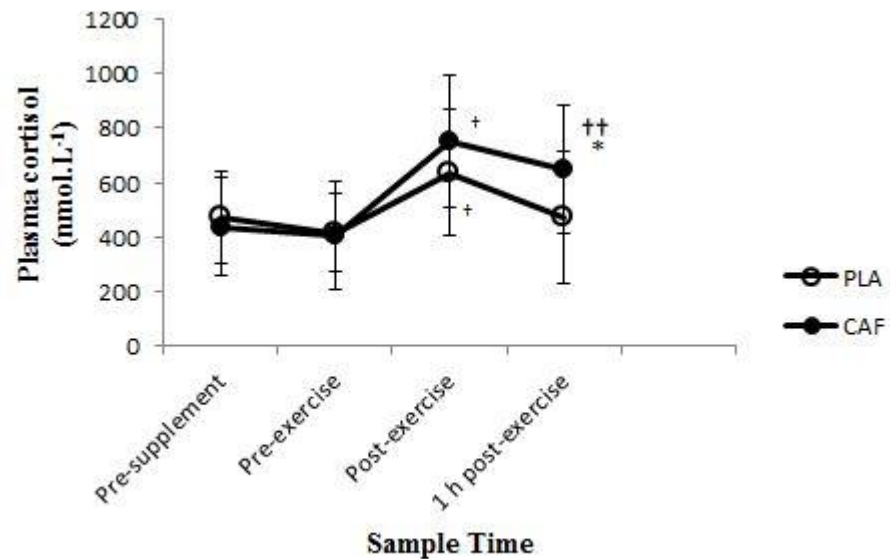


Figure 6. Plasma cortisol concentrations during the PLA and CAF trials.

PLA = Placebo; CAF = Caffeine.

Values are means \pm SD.

* Significantly higher in the CAF compared with the PLA trial ($P < 0.01$)

Significantly higher than pre-supplement level within the trial [†] $P < 0.01$, ^{††} $P < 0.05$

4.6 Plasma Adrenaline

No significant time \times trial interaction ($P > 0.05$) was found for the plasma adrenaline.

However, a significant time effect was seen ($P < 0.01$). The plasma adrenaline concentration was significantly higher at pre-exercise, post-exercise and one-hour post-exercise compared to the pre-supplement stage ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively; see Figure 7).

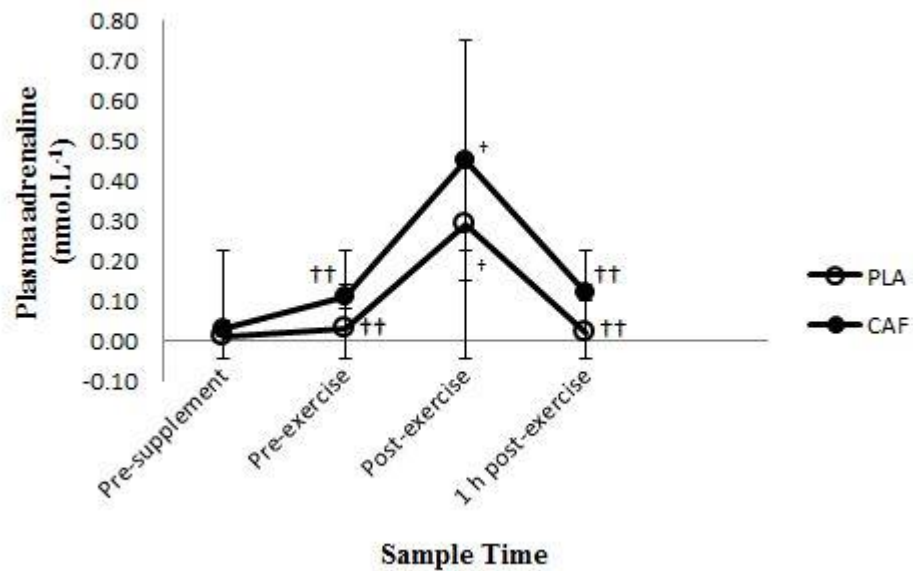


Figure 7. Plasma adrenaline concentrations during the PLA and CAF trials. PLA = Placebo; CAF = Caffeine. Values are means \pm SD. Significantly higher than pre-supplement level within the trial † $P < 0.01$, †† $P < 0.05$

4.7 Total Lymphocyte Counts

A significant time \times trial interaction was found for the total lymphocyte count ($P < 0.01$). The ingestion of caffeine increased the number of circulating lymphocytes at pre-exercise and post-exercise, compared to the PLA trial ($P < 0.05$ and $P < 0.01$, respectively; see Table 4). Within both the CAF and PLA trials, higher circulating lymphocyte counts were found at post-exercise when compared to pre-supplement stage ($P < 0.01$; see Table 4). However, the total lymphocyte count at one h post-exercise fell below the pre-supplement level in both the CAF and PLA trials ($P < 0.01$ and $P < 0.05$, respectively; see Table 4). During the CAF trial, the administration of caffeine also increased the total lymphocyte numbers at pre-exercise, compared to the pre-supplement values ($P < 0.01$; Table 4).

4.8 Numbers of CD4⁺, CD8⁺ and CD3⁺CD56⁺ Cells

No significant time \times trial interaction was found for the number of either CD4⁺ or CD8⁺ cells within the circulating lymphocyte population (see Table 4). However, there was a significant main effect for time ($P < 0.01$; see Table 4), with values elevated at post-exercise when compared to pre-supplement stage ($P < 0.01$; see Table 4). Caffeine ingestion also elevated the number of CD8⁺ cells at the pre-exercise compared to pre-supplement stage ($P < 0.05$; see Table 4). Nevertheless, the CD8⁺ cell numbers fell below

the pre-supplement level following one h post-exercise ($P<0.01$; see Table 4). The number of $CD8^+$ cells also expressed the main effect for the trial, with the value significantly higher in the CAF compared to the PLA trial ($P<0.05$; see Table 4).

Table 4. Serum Caffeine, Plasma Cortisol, Plasma Adrenaline, Total Lymphocyte Counts and Number of Circulating $CD4^+$, $CD8^+$ and $CD3^+CD56^+$ Cells during CAF and PLA Trials

	Pre-supplement	Pre-exercise	Post-exercise	1h post-exercise
Total lymphocyte counts ($\times 10^9$ cells.L⁻¹)				
CAF	1.6 (0.3)	2.1 (0.6) ^{**†}	3.8 (0.8) ^{*†}	1.4 (0.2) [†]
PLA	1.7 (0.3)	1.7 (0.3)	3.2 (0.5) [†]	1.4 (0.3) ^{††}
$CD4^+$ cells ($\times 10^9$ cells.L⁻¹)^a				
CAF	0.69 (0.16)	0.75 (0.23)	1.15 (0.39)	0.65 (0.13)
PLA	0.74 (0.18)	0.73 (0.13)	0.99 (0.19)	0.68 (0.13)
$CD8^+$ cells ($\times 10^9$ cells.L⁻¹)^{a, b, c, d}				
CAF	0.47 (0.15)	0.60 (0.27)	1.93(0.33)	0.41 (0.11)
PLA	0.49 (0.11)	0.49 (0.15)	1.0 (0.18)	0.37 (0.10)
$CD3^+CD56^+$ cells ($\times 10^9$ cells.L⁻¹)				
CAF	0.47 (0.16)	0.75 (0.18) ^{*†}	1.61 (0.47) [†]	0.43 (0.15)
PLA	0.46 (0.11)	0.48 (0.09)	1.43 (0.33) [†]	0.37 (0.12) ^{††}

PLA = Placebo; CAF = Caffeine.

Values are mean (SD).

* $P<0.01$, ** $P<0.05$: significantly higher in the CAF than the PLA.

† $P<0.01$, †† $P<0.05$: significantly different from pre-supplement values within the trial.

^aMain effect for time: significantly higher than pre-supplement values at post-exercise, $P<0.01$.

^bMain effect for time: significantly higher than pre-supplement values at pre-exercise, $P<0.05$.

^cMain effect for time: significantly lower than pre-supplement values at 1 h post-exercise, $P<0.01$.

^dMain effect for trial: significantly higher values on CAF than PLA, $P<0.05$.

A significant time x trial interaction was found for the number of $CD3^+CD56^+$ cells within the total circulating lymphocyte population, with higher numbers at the pre-exercise stage in the CAF trial compared with the PLA ($P<0.01$; see Table 4). The $CD3^+CD56^+$ cell numbers were significantly higher at post-exercise than pre-supplement in both the CAF and PLA trials ($P<0.01$; see Table 4). Similar to the circulating $CD4^+$ and $CD8^+$ cell numbers, caffeine raised the number of $CD3^+CD56^+$ cells following one hour of ingestion (pre-exercise) ($P<0.01$; see Table 4). During the PLA trial, $CD3^+CD56^+$ cell numbers dropped significantly below the pre-supplement levels following one hour post-exercise ($P<0.05$; see Table 4).

4.9 Number of Unstimulated and Antigen-Stimulated CD4⁺, CD8⁺ and CD3⁺CD56⁺ Cells Expressing CD69

There were no time x trial interactions, but a main effect was found for time for both unstimulated and antigen-stimulated CD4⁺ and CD8⁺ cells expressing CD69 (see Table 5). During both the CAF and PLA trials, the number of unstimulated and antigen-stimulated CD4⁺ and CD8⁺ cells expressing CD69 was found to be higher at post-exercise compared to pre-supplement (unstimulated CD4⁺ CD69⁺ cell: $P<0.05$; stimulated CD4⁺ CD69⁺ cells: $P<0.01$; both unstimulated and stimulated CD8⁺ CD69⁺ cells: $P<0.01$; see Table 5). The number of unstimulated CD4⁺ CD69⁺ cells was significantly lower at one h post-exercise than pre-supplement ($P<0.01$; see Table 5). In contrast, the number of unstimulated CD8⁺ CD69⁺ cells increased above the pre-supplement level at one h post-exercise ($P<0.05$; see Table 5).

A significant time x trial interaction was found for both unstimulated and antigen-stimulated CD3⁺CD56⁺ cells expressing CD69 (unstimulated: $P<0.01$; stimulated: $P<0.05$; see Table 5) at pre-exercise stage. During the CAF and PLA trials, the number of both unstimulated and antigen-stimulated CD3⁺CD56⁺ cells expressing CD69 at post-exercise was higher than at pre-supplement ($P<0.01$, Table 5), but fell significantly below pre-supplement one h later ($P<0.01$; see Table 5). Similar to the circulating lymphocytes numbers, one h after the ingestion of caffeine (pre-exercise), the number of CD3⁺CD56⁺ cells expressing CD69 increased compared to pre-supplement levels in both unstimulated and antigen-stimulated cells ($P<0.01$; see Table 5).

Table 5. Number of Unstimulated and Antigen-Stimulated CD4⁺, CD8⁺ and CD3⁺CD56⁺ Cells Expressing CD69 During CAF and PLA Trials

	Pre-supplement	Pre-exercise	Post-exercise	1h post-exercise
CD4⁺ CD69⁺ cells (x10⁶ cells.L⁻¹)^{a, c, e}				
Unstim CAF	185 (49.18)	187 (52.94)	263 (102.09)	155 (46.19)
Unstim PLA	191 (56.65)	193 (42.75)	261 (93.66)	154 (59.63)
1:1000 CAF	267 (55.91)	260 (71.44)	373 (99.58)	250 (52.30)
1: 1000 PLA	301 (76.61)	284 (63.76)	356 (92.72)	263 (77.10)
CD8⁺ CD69⁺ cells (x10⁶ cells.L⁻¹)^{b, d, e}				
Unstim CAF	30 (14.40)	47 (30.45)	150 (83.30)	50 (21.15)
Unstim PLA	41 (25.22)	36 (12.34)	139 (76.53)	45 (28.13)
1:1000 CAF	49 (34.71)	47 (10.66)	170 (89.05)	55 (55.35)
1:1000 PLA	70 (70.39)	66 (90.52)	167 (119.31)	69 (65.19)
CD3⁺CD56⁺ CD69⁺ cells (x10⁶ cells.L⁻¹)				
Unstim CAF	93 (37.83)	148 (46.17) ^{*†}	305 (167.96) [†]	67 (22.92) [†]
Unstim PLA	82 (29.70)	87 (31.75)	284 (124.11) [†]	61 (33.63) [†]
1:1000 CAF	150 (44.71)	230 (65.08) ^{**†}	423 (102.41) [†]	103 (29.83) [†]
1:1000 PLA	149 (50.94)	153 (45.28)	374 (161.99) [†]	104 (46.56) [†]

PLA = Placebo; CAF = Caffeine.

Values are mean (SD).

* P<0.01, significantly higher in the CAF trial compared to the PLA.

* P<0.05, significantly higher in the CAF trial compared to the PLA.

† P<0.01: significantly different from pre-supplement within the trial.

Main effect for time on unstimulated cells: significantly higher than pre-supplement at post-exercise, ^aP<0.05, ^bP<0.01.

^c Main effect for time on unstimulated cells: significantly lower than pre-supplement at 1 h post-exercise, P<0.01.

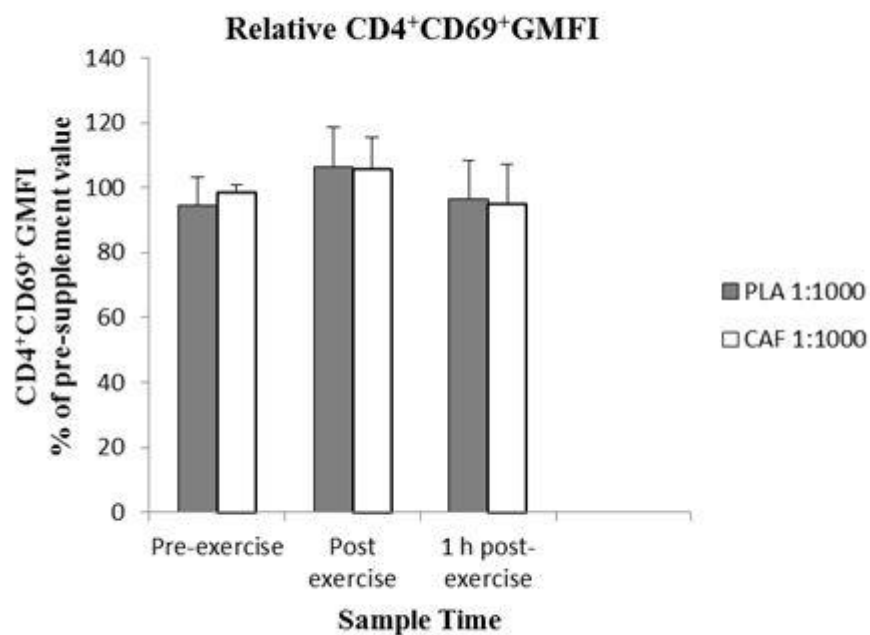
^d Main effect for time on unstimulated cells: significantly higher than pre-supplement at 1 h post-exercise, P<0.05.

^e Main effect for time on stimulated cells: significantly higher than pre-supplement at post-exercise, P<0.01.

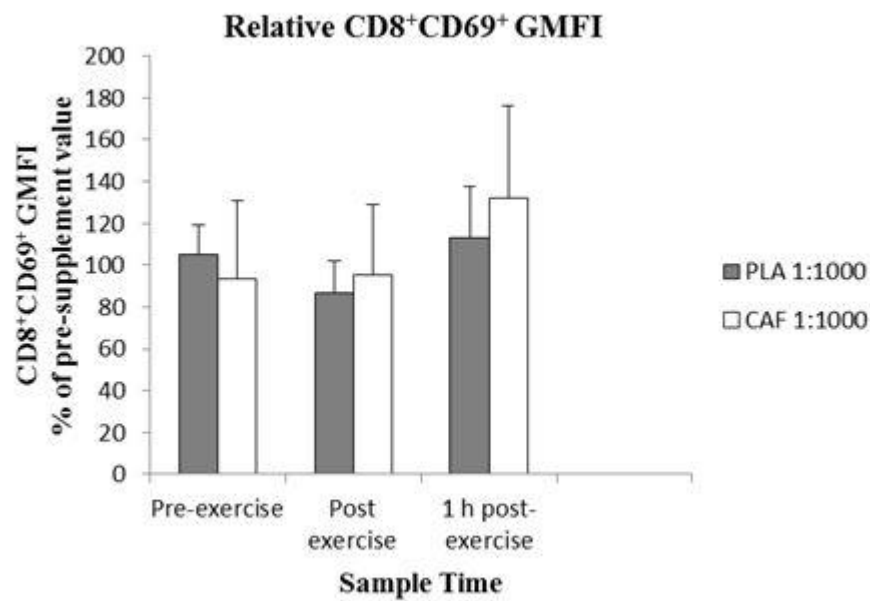
4.10 Geometric Mean Fluorescence Intensity (GMFI) of CD69 Expression on Unstimulated and Antigen-Stimulated CD4⁺, CD8⁺ and CD3⁺CD56⁺ Cells

There was no time x trial interaction for GMFI of either the unstimulated or antigen-stimulated CD4⁺, CD8⁺ and CD3⁺CD56⁺ cells (see Figure 9a, b & c). There was neither time nor trial main effects found on GMFI of both the unstimulated and antigen-stimulated CD4⁺, CD8⁺, as well as the unstimulated CD3⁺CD56⁺ cells (see Figure 9a, b & c). When CD3⁺CD56⁺ cells were stimulated with antigen, the main effect for time was found ($P < 0.01$), with the GMFI levels falling below pre-supplement levels at one hour post-exercise (see Figure 9c).

(a)



(b)



(c)

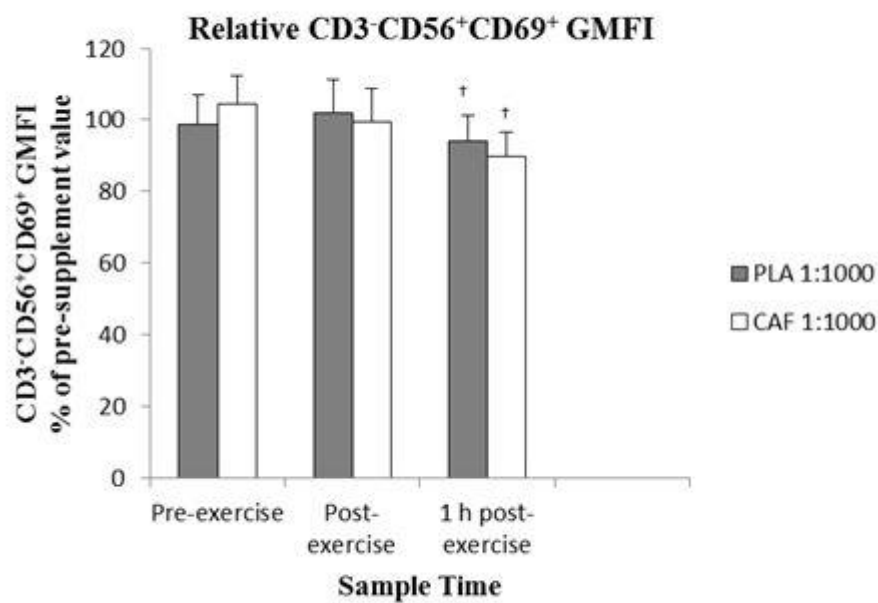


Figure 8. Geometric Mean Fluorescence Intensity (GMFI) of CD69 expression on antigen-stimulated (a) CD4⁺ T cells, (b) CD8⁺ T cells and (c) CD3⁺CD56⁺ NK cells within the circulating lymphocyte population during CAF and PLA trials.

PLA = Placebo; CAF = Caffeine.

[†] Main effect for time on stimulated cells: significantly lower than pre-supplement at 1 h post-exercise, $P < 0.01$.

Chapter 5 Discussion

5.1 Research Outline

This thesis aimed to determine the effects of caffeine on lymphocyte activation following short duration (20 min) high intensity intermittent exercise (HIIE). To examine this, participants were given either placebo (6 mg.kg^{-1} BW of corn flour) or caffeine (6 mg.kg^{-1} BW of caffeine) capsules one h prior to performing HIIE. Bloods samples were taken at four different time points: pre-supplement, pre-exercise, immediately post-exercise, and one h post-exercise. The blood samples were stimulated using the Infanrix[®] hexa vaccine (GlaxoSmithKline Biologicals s.a, Rixensart, Belgium), containing a combination of diphtheria-tetanus-acellular pertussis, hepatitis B, enhanced inactivated polio and Haemophilus influenzae type b vaccine. The blood samples were used to measure the total circulating lymphocyte counts, and number of circulating T and NK cells. Additionally, the number of activated T and NK cells expressing CD69, as well as the level of activation of each cell (CD69 GMFI expression), were examined. This is the first study to consider the effects of 6 mg.kg^{-1} BW of caffeine dose on antigen-stimulated T and NK cell activation following short duration HIIE.

5.2 Main Findings of the Research

This study found that administration of 6 mg.kg^{-1} BW of caffeine one h preceding short duration (20 min) HIIE was associated with the following effects:

- i. Caffeine mobilised a greater number of total lymphocytes into the circulation one h after ingestion (pre-exercise), and immediately following short duration HIIE, compared with the placebo.
- ii. Caffeine had no significant effect on the number of circulating CD4^{+} and CD8^{+} T cells.
- iii. Caffeine significantly mobilised a greater number of NK cells into the circulation one h following ingestion.
- iv. Caffeine preserved the fall in peripheral NK cell numbers at one h post-exercise; that is, the number of circulating NK cells at the one h post-exercise was closer to baseline levels in the CAF trial; whereas in the PLA trial, the circulating NK cell numbers dropped significantly compared to pre-supplement.

- v. Caffeine increased the number of unstimulated and antigen-stimulated NK cells expressing CD69, but had little effect on T cells.
- vi. Caffeine had no effect on the unstimulated or antigen-stimulated CD69 GMFI expression of either T or NK cells.
- vii. The ingestion of caffeine further increased the concentration of cortisol in addition to the exercised-induced cortisol increase seen one h post-exercise.
- viii. Short-duration HIIE increased the number of circulating T and NK cells immediately after exercise, but reduced these numbers below baseline levels one h post-exercise.
- ix. Short-duration HIIE reduced CD69 GMFI expression of antigen-stimulated NK cells at one h post-exercise compared to pre-supplement.

5.3 Effects of Caffeine on Lymphocyte Mobilisation following HIIE

As part of this study, the influence of a 6 mg.kg^{-1} BW dose of caffeine on peripheral lymphocyte mobilisation in response to short duration (20 min) HIIE was examined. It was found that ingestion of 6 mg.kg^{-1} BW dose of caffeine one h prior to HIIE increased the number of circulating total lymphocytes, with NK cells being the most mobilised cells immediately following short duration HIIE. Furthermore, caffeine increased the number of circulating total lymphocytes, and T and NK cells, within one h of ingestion. This finding aligns with the findings of Bassini-Cameron et al. (2007), where 45 min of variable distance running with intermittent recovery increased the total circulating lymphocyte population by 38%, and administration of a 5 mg.kg^{-1} BW dose of caffeine with exercise further increased the total peripheral lymphocyte numbers by an additional 35%. Conversely however, the study by Machado et al. (2009) reported no change in total circulating lymphocyte count following approximately 29 min of intermittent sprints with the ingestion of a 4.5 mg.kg^{-1} BW dose of caffeine 35 min prior to exercise. This may suggest that the length of time between caffeine ingestion and onset of exercise plays a role in caffeine's effects on total lymphocyte counts. Indeed, a number of studies have demonstrated that time to peak plasma caffeine concentration is usually ~60 min (Fredholm et al., 1999; Kamimori et al., 2002). It is important to note that the discrepancy in the finding of total circulation lymphocyte count between the current study and the study by Machado et al. (2009) may be due to the difference in participant's diet. In the study by Machado et al. (2009), the participants consumed a high carbohydrate breakfast 10 min prior to caffeine ingestion. In contrast, overnight

fasting was required for the current study. According to Skinner et al. (2013), peak serum caffeine concentration is significantly delayed by consumption of a high carbohydrate meal prior to caffeine ingestion compared to a fasted condition. In the fasted state, the peak serum caffeine concentration is 60 min and it is increased up to 120-180 min with combined intake of caffeine with carbohydrate (Skinner et al., 2013). This may explain why the total circulating lymphocyte counts were not changed after caffeine ingestion in the study by Machado et al. (2009) but increased in the total circulating lymphocytes number was seen in the current study. Although the current study did not find a statistically significant effect of caffeine on lymphocyte mobilisation one h following exercise, it can be seen from the results that caffeine does appear to minimise the reduction in number of peripheral lymphocytes one h post-exercise (Table 4). It could also be suggested that caffeine minimises the attenuation of the number of circulating lymphocytes one h post-exercise.

In the present study, an increase in number of circulating NK cells was observed in the CAF trial following HIIE (see Table 4) which suggests that the high level of NK cells mobilised into the circulation may have been due to an increase in caffeine-induced adrenaline concentration. This is supported by the fact that the adrenaline level increased with the ingestion of a 6 mg.kg^{-1} BW dose of caffeine during HIIE in the current study, which is consistent with the study of Fletcher and Bishop (2012). Fletcher and Bishop (2012) reported that a high level of plasma adrenaline concentration in the CAF trial at post-exercise resulted in an increased number of peripheral NK cells. Similarly, Walker, Caudwell, Dixon, and Bishop (2006) and Tauler et al. (2016) also support this concept that a 6 mg.kg^{-1} BW of caffeine dose increases the adrenaline level following high intensity exercise. Caffeine elicits its adrenaline-mediated immunomodulatory effects via the β_2 -adrenoreceptors on the surface of lymphocytes. Interaction of adrenaline with β_2 -adrenoreceptors stimulates the production of intracellular cAMP. Elevated levels of cAMP concentrations are responsible for the weakening of the adhesion strength of lymphocytes to endothelial cells, resulting in the mobilisation of lymphocytes into the circulation (Benschop et al., 1993). The density of β_2 -adrenoreceptors determines the level of mobilisation of each lymphocyte subset. It is known that there are different degrees of β_2 -adrenoreceptor expression in CD4^+ and CD8^+ T cells, and $\text{CD3}^-\text{CD56}^+$ NK cells, with $\text{CD3}^-\text{CD56}^+$ NK cells expressing the most, CD8^+ having a moderate expression, and CD4^+ the smallest expression (B. K Pedersen & Hoffman-Goetz, 2000). It is plausible therefore, based on the findings from

this study, to suggest that NK cells are the most mobilised cells. Moreover, it is noteworthy that activated lymphocytes are found to express a higher density of β_2 -adrenoreceptors compared to inactivated cells (Kohm & Sanders, 2001). Therefore, out of the recruited peripheral NK cells, the majority of the cells are likely to be the activated NK cells.

The current finding of an increased number of NK cells recruited into the circulation with the ingestion of caffeine following HIIE concurs with the findings of the study performed by Fletcher and Bishop (2011a). Fletcher and Bishop (2011a) demonstrated that there was an increased mobilisation of NK cells into the circulation immediately following 90 min of cycling at 70% $\text{VO}_{2\text{peak}}$. Exercise-induced stress from HIIE is also likely to be another factor that enhanced the degree of NK cell mobilisation in the current study, as exercise-induced lymphocytosis is one of the most replicated findings in exercise immunology (Gleeson, Bishop, & Walsh, 2013; Krüger & Mooren, 2007). Furthermore, it is worthwhile to mention that there is no significant difference in the degree of mobilisation between prolonged continuous and short term HIIE, regardless of administration of caffeine (Fletcher & Bishop, 2012; Turner et al., 2016). It could be suggested that the lymphocyte mobilisation effects seen in the current study are likely to be the sum effects of caffeine and exercise-induced increases in adrenaline concentration.

Additionally, evidence has demonstrated that there are further subdivisions within each of the T (CD4^+ and CD8^+) and NK ($\text{CD3}^+\text{CD56}^+$) lymphocyte subsets, with unique functional and phenotypical characteristics (Campbell et al., 2009). Studies have further suggested that there is a preferential mobilisation of these distinct subsets into the circulation, which favour subsets with high cytotoxicity, low proliferation capability, and high tissue-migrating potential (Campbell et al., 2009). It is suggested that CD8^+ effector memory T and CD56^{dim} NK lymphocytes are the subsets that share the characteristics outlined above (Campbell et al., 2009; Cooper, Fehniger, & Caligiuri, 2001; Cooper, Fehniger, Turner, et al., 2001). Hence, it is conceivable that the increased number of peripheral NK cells seen immediately following exercise in the current study may be due to an increased recruitment of the CD56^{dim} NK cells with high cytotoxicity into the circulation. However, as the number of CD56^{dim} and $\text{CD56}^{\text{bright}}$ NK cells were not identified separately in the current study, this is only speculation. Furthermore, CD8^+ effector memory T cells and CD56^{dim} NK cells have the greatest ability to migrate to tissues with active inflammation. As such, a fall in these circulating lymphocytes may

simply be the reflection of these cells being trafficked into the injury tissues (Campbell et al., 2009; Dhabhar, Malarkey, Neri, & McEwen, 2012). Therefore, it could be suggested that the reduction in circulating lymphocytes seen at one h post-exercise may not indicate a decline in overall immune strength, but rather be the sign of an effective immune surveillance redistribution of the lymphocytes into the sites of possible injury (Dhabhar et al., 2012). Taken together, it could be proposed that administration of a 6 mg. kg⁻¹ BW dose of caffeine potentially increases the innate immune system by the recruitment of cytotoxic NK cells into the circulation immediately after exercise.

It is acknowledged that the higher number of circulating lymphocytes seen in this study does not quite compare to the results of previously published studies (Bishop, Fitzgerald, et al., 2005; Fletcher & Bishop, 2012). This may be due to the difference in exercise mode (intermittent vs. prolonged), as HIIE may have posed greater physiological stress to individuals. Natale et al. (2003) have indicated that prolonged (2 h of cycling at 60-65% VO_{2max}) and peak aerobic exercise (5 min of cycling at 90-97% VO_{2max}) similarly increased the number of circulating lymphocytes immediately after exercise. In fact, in Natale et al.'s (2003) study, the prolonged exercise tended to show the greater increase in total peripheral lymphocyte counts. Nevertheless, it should be noted that the peak aerobic exercise in the Natale et al. (2003) study only lasted for five min. The HIIE in the current study was 10 repeated 1 min bouts of high intensity workload and, as such, is likely to have elicited a stronger physiological stimulus to the immune system. It could also be possible that the participants' fitness level is one of the factors causing higher circulating numbers of lymphocytes compared to other similar studies (Malm, 2006; Zouhal et al., 2008). The participants used in the current study were healthy active individuals, whereas other studies with lower peripheral lymphocytes have used well-trained athletes who have a lower adrenaline response to exercise due to training effects than those of the normal population, and this may account for the difference in lymphocyte numbers (Kjaer, 1998; Zouhal et al., 2008). It is also important to consider the habituation of adrenaline response to caffeine usage (Graham, 2001). Studies by Van Soeren et al. (1993) and Van Soeren and Graham (1998) illustrate that adrenaline response to caffeine is blunt with continuous usage of caffeine but increase response to caffeine with the 4 days of withdrawal in habitual caffeine user. In current study, 60 h of caffeine withdrawal period was implemented to avoid the habituation of adrenaline response to caffeine ingestion. As mentioned previously, the intake of high carbohydrate meal prior to caffeine ingestion, such as in a

study by Machado et al. (2009), may also play an important role in discrepancy effects of caffeine in total circulating lymphocyte count (Skinner et al., 2013). Consumption of caffeine together with carbohydrate significantly delays the caffeine half-life (Skinner et al., 2013).

5.4 Effects of Caffeine on Antigen-Stimulated Lymphocyte Activation following HIIE

This study demonstrates the multifaceted effects of caffeine on antigen-stimulated T and NK lymphocyte activation following 20 min of HIIE. As previous literature indicates, caffeine influences the activation of T and NK cells via the same potential mechanisms; adenosine receptor antagonism and catecholamine stimulation of β_2 -adrenergic receptors (Davis et al., 2003; Graham, 2001). As such, it is logical to assume that caffeine would have similar impacts on both cells. However, the findings from the current study do not seem to support this assumption. In fact, the current findings indicated that caffeine has no significant effect on unstimulated or antigen-stimulated T cell activation (in terms of CD69 number or GMFI expression of CD69), but has significant immunostimulatory effects on NK cells immediately following 20 min HIIE.

5.4.1 T and NK cell Activation: CD69 Expression

Number of T Cells Expressing CD69

The finding of no significant impact of caffeine on both unstimulated and antigen-stimulated T cells in terms of the number of CD69⁺ T cells is interesting, as it contradicts the findings of a similar caffeine study which showed the immunosuppressive effect of caffeine on antigen-stimulated T cell activation following prolonged high intensity exercise (Fletcher & Bishop, 2012). It is also in disagreement with the findings of Bishop, Fitzgerald, et al. (2005), that caffeine increased unstimulated T cell activation following a bout of intensive prolonged exercise. One of the reasons why the findings from this study do not agree with those of Bishop, Fitzgerald, et al. (2005) may be because the Bishop, Fitzgerald et al. study investigated the *in vivo* effects of caffeine on T cell activation in unstimulated cells, whereas this study looked at the antigen-stimulated lymphocytes. The results of Bishop, Fitzgerald et al. may not represent how the immune system reacts when it encounters antigens, as it focused on unstimulated cells. Another reason why the findings from this study differ from Bishop, Fitzgerald, et al. (2005) and Fletcher and Bishop (2012), may be because they used prolonged cycling, whereas this study used 20 min HIIE. Twenty min HIIE

and prolonged strenuous exercises may have utilised different energy systems which may impose different effects to immune cells. Additionally, caffeine is likely to have either positive or negative effects on T cell activation, depending on the absolute sum consequences of stimulatory or inhibitory pathways (Ferre et al., 2008; Kamimori et al., 2000; B. K Pedersen & Hoffman-Goetz, 2000; Ribeiro & Sebastiao, 2010). Given plasma adrenaline concentration was high during the caffeine trial in the current study, this could suggest that caffeine may have acted via the adrenaline-mediated pathway. If this was the case, T cell activation should be suppressed, as adrenaline stimulates the β_2 -adrenoreceptors on T cells, which increases cAMP secretion, leading to a reduction in lymphocyte activation (Bracken et al., 2009; Graham & Spriet, 1995; Kochanska-Dziurawicz et al., 2013). Substantial increases in the plasma cortisol concentration seen during the caffeine trial in the present study also indicates the possibility of suppression in T cell immune function, as cortisol has been shown to negatively affect a range of immune parameters (Krüger & Mooren, 2014; Laurent et al., 2000; Phillips et al., 2007). As T cell activation is not depressed, it seems to suggest that adrenaline or cortisol is not the major driver of caffeine's effect on T cells.

It is also possible that caffeine has acted primarily via the competitive binding to adenosine A_{2A} receptors on the T cells' surface, which would have led to an enhancement in T cell activation through the interference of the A_{2A} receptor function, resulting in the reduction in cAMP levels (Horrigan et al., 2006; Ribeiro & Sebastiao, 2010). However, the T cell activation enhancement effect of caffeine was not seen in this study either, suggesting that effects of caffeine on T cell activation was balanced out between stimulatory and inhibitory consequences, resulting in no significant net impact on the T cells. These findings also seem to suggest that T cells are not as responsive to adrenaline as NK cells.

Although the reasons for these inconsistent findings compared to that of Fletcher and Bishop (2012) and Bishop, Fitzgerald, et al. (2005) are not apparent, the characteristics of exercise (continuous or intermittent; long or short duration; different intensities), the fitness level of participants (well-trained athletes or physically active individuals), previous exposure to specific infections, other undefined possible mechanisms of caffeine, and caffeine tolerance levels, may have influenced the findings. From a health perspective, it may be a positive outcome that T cells were not depressed by caffeine. This may be of benefit for athletes and healthy active people who consume caffeine. Additionally, it is interesting to note that, regardless of the caffeine or placebo trials, the

numbers of unstimulated CD4⁺CD69⁺ T cells at one h post-exercise were significantly lower than those of pre-supplement. Nevertheless, the unstimulated CD8⁺CD69⁺ T cell numbers increased at one h post-exercise compared to that of pre-supplement. It could be suggested that the increase in unstimulated CD8⁺CD69⁺ T cell numbers at one h post-exercise is simply the immune system's way of compensating for the reduction in the CD4⁺CD69⁺ T cell count.

Number of NK Cells Expressing CD69

The elevation in the number of peripheral NK cells expressing CD69 in the CAF trial post-exercise is consistent with the findings of the study by Fletcher and Bishop (2012). Their study supports the present finding of an increased mobilisation of NK cells into the circulation in response to caffeine, to increase the innate immune defence immediately following exercise. For the peripheral NK cells to react to the encountered foreign antigens, the cells need to become activated first. Therefore, it is conceivable that the increase in the number of circulating NK cells is complemented with a greater number of activated NK cells expressing CD69 following stimulation with antigens.

As discussed previously in section 5.3, caffeine is likely to act on the activation of NK cells via an adrenaline-mediated pathway. The adrenaline pathway is known to reduce the NK cells' activation via interaction with the β_2 -adrenoreceptors on the cell surface, which increases the intracellular cAMP level, leading to the negative impact on the lymphocytes' activation (Graham, 2001; B. K Pedersen & Hoffman-Goetz, 2000). As the number of both unstimulated and antigen-stimulated NK cells expressing CD69 increased rather than decreased in this study, it can be assumed that the adrenaline-mediated mechanism is not the dominant mechanism in the NK cells' activation immediately following exercise. However, an adrenaline-mediated pathway cannot be absolutely excluded, as it does play a role in the enhancement of NK cell mobilisation (B. K Pedersen & Hoffman-Goetz, 2000). We should be reminded that the final influence of caffeine on antigen-stimulated NK cells is the absolute sum effect of a number of inhibitory and stimulatory mechanisms.

Taken together, the current findings seem to suggest that the adenosine A_{2A} receptor antagonism pathway is the principal mechanism to exert the positive impact of caffeine on both unstimulated and antigen-stimulated NK cell activation. This positive influence seems to outweigh the negative consequences of other inhibitory mechanisms of caffeine. Adenosine is well known for the attenuation of NK cell activation by

interaction with A_{2A} adenosine receptors on the cell surface. The binding of adenosine to A_{2A} receptors alters the adenylyl cyclase, resulting in increased intracellular cAMP levels. The cAMP signals through PKAI to suppress the NK cells' activation (Haskó et al., 2008; Sitkovsky & Ohta, 2005). Caffeine is a known non-selective adenosine receptor antagonist (Davis et al., 2003). The blockage of A_{2A} adenosine receptors on the surface of NK cells by caffeine leads to a decrease in the intracellular cAMP level, which in turn amplifies the activation of both unstimulated and antigen-stimulated NK cells (Ribeiro & Sebastiao, 2010). Again, this is only speculation, as the plasma adenosine level was not explicitly measured in this study. However, Conlay, Conant, and Wurtman (1997) indicate that a caffeine dose similar to the current study increases plasma adenosine concentration, due to the blockage of adenosine receptors in rats.

It is also interesting to note that the number of antigen-stimulated T and NK cells expressing CD69 during both the CAF and PLA trials in the current study were much higher than those results reported by Fletcher and Bishop (2012). This may have come down to the different antigen-stimulant used in these two studies (current study: Infanrix hexa; Fletcher and Bishop (2012): Pediacel). Numerous studies tend to use a range of different mitogens/antigens at various different concentrations (Horrigan et al., 2006). This makes the interpretation across studies difficult. As such, it may be the reason for the higher number of activated cells in the current study compared to that of the Fletcher and Bishop (2012) study.

5.4.2 T and NK Cell Activation: GMFI Expression of CD69

It is interesting to note that there was no significant change in the activation degree on a per cell basis (GMFI) for all the lymphocytes of interest (CD4⁺ and CD8⁺ T cells, CD3⁻ CD56⁺ NK cells), either before or following 20 min of HIIE. However, a reduction in the GMFI expression of CD69 on antigen-stimulated NK cells was observed one h post-exercise, compared to pre-supplement baseline levels. However, the reduction was small (CAF: 10.12%, PLA: 6%) and, as such, it may not represent a significant suppression in immune function.

Taking these findings together, the increase in the number of activated CD3⁻ CD56⁺ CD69⁺ NK cells may be an attempt by the immune system to offset the reduction in NK cell activity on a per cell basis (GMFI) to conserve healthy immune functions. However, it is interesting to note that these findings again contradict those of Fletcher and Bishop (2012), which showed an increase in the GMFI expression of CD69⁺ on

antigen-stimulated NK cells one h following exercise. However, it is again noteworthy that the exercise duration and participants' fitness levels differed between this study and that of Fletcher and Bishop. This study deployed short duration HIIE, whereas the Fletcher and Bishop (2012) study used prolonged intensive exercise (cycle for 90 min at 70% $\dot{V}O_{2peak}$). In addition, the participants in the current study were healthy active people, compared to the well-trained athletes in the other study. These exercise and fitness factors may have contributed to the discrepancy in effects of caffeine on NK cell activation on a per cell basis (GMFI) between current study and that of (Fletcher & Bishop, 2012).

5.5 Other Potential Mechanisms of Caffeine

Although adenosine receptor antagonism and the adrenaline-mediated mechanism are the two major mechanisms mostly discussed in the literature (Davis et al., 2003; Graham, 2001; Ribeiro & Sebastiao, 2010), some researchers have also suggested the possibility of other mechanisms, such as heat shock protein 72 (Hsp72) and β -endorphins, playing a role in the effects of caffeine on lymphocyte activation (Bassini-Cameron et al., 2007; B. K Pedersen & Hoffman-Goetz, 2000; Whitham, Walker, & Bishop, 2006). Although research has shown discrepancies in the effects of β -endorphins on T and NK cells, most researchers tend to propose that, while it has a negative correlation with T cell proliferation, it exerts positive feedback on the cytotoxic activity of NK cells (Gein, Baeva, Nebogatikov, & Tendryakova, 2012; Nekrasova, Zolotarev, & Navolotskaya, 2013; B. K Pedersen & Hoffman-Goetz, 2000). This seems to suggest that caffeine may act on NK cells via β -endorphins. However, it is suggested that the β -endorphins pathway may not be a mechanism of an effect of caffeine on T cells in the present study. Furthermore, recent research has been focused on Hsp72 and proposes that it has a positive impact on NK cell activation (Z. Li et al., 2015; Specht et al., 2015). The β -endorphins and Hsp72 concentration have been shown to increase in relation to caffeine administration and high intensity exercise (Laurent et al., 2000; B. K Pedersen & Hoffman-Goetz, 2000; Whitham et al., 2006). In the studies performed by Laurent et al. (2000) and Whitham et al. (2006), the Hsp72 and β -endorphins levels have been shown to considerably increase with the combined stimulus of a 6 mg.kg⁻¹ BW dose of caffeine and high intensity exercise, compared to the impact on those levels of exercise alone. Taken together, the enhancement in the number of NK cells expressing CD69 immediately following HIIE seen in the current study could perhaps be partly influenced by β -endorphins and Hsp72. However, as there was no

alteration seen in T cell activation in the caffeine trial compared to the placebo, this again could indicate that the negative influence of β -endorphins on T cell activation may have been counterbalanced by the other stimulatory mechanisms. Nevertheless, it is noteworthy that most of the studies on β -endorphins and Hsp72 response to caffeine and exercise are based on long duration high intensity exercise. Therefore, the effects of caffeine on β -endorphins and Hsp72 in short duration HIIE, such as in the current study, are not very clear. Since the current study did not measure β -endorphins and Hsp72 concentrations, it cannot be extrapolated as to whether they contributed to lymphocyte activation in response to caffeine following HIIE, given the limited support from other studies.

It is also important to note that this study used whole blood samples to test for the circulating number of lymphocytes and number of activated lymphocytes. This gave the benefit of mimicking *in vivo* conditions in humans. However, using whole blood cultures also means there are other cells and factors in the blood other than T and NK cells. Therefore, this also makes it difficult to differentiate whether the results seen in this study were because of the direct effect of caffeine on the lymphocytes of interest, or they were the side effects of caffeine's effect on other cells, receptors or soluble factors in the whole blood sample (Gleeson & Bishop, 2005).

5.6 Limitations of the Study

The main limitations of this study are as follows:

- This study did not measure cAMP levels. As the two major mechanisms of caffeine are executed through cAMP, measuring cAMP levels would have helped to clarify whether caffeine-induced adenosine- and adrenaline-mediated mechanisms had any influences on the effects seen on T and NK cell activation.
- The sample size of this study was only 10 participants. Therefore, the findings from this study may not represent a larger population with larger diversity. However, all significant results in the current study had power above 0.8.
- Although this study tested CD69 as an early activation marker of lymphocytes, it did not measure downstream immune responses, such as T cell proliferation or cytokines secretion. Therefore, it cannot be extrapolated that the effects of caffeine on CD69 following HIIE may similarly impact on downstream immune functions. As such, it cannot be ascertained whether the effects of caffeine on

CD69 markers seen in this study may actually affect the subsequent lymphocytes function and ultimately alter the immune system.

- Only single dose of caffeine ($6\text{mg} \cdot \text{kg}^{-1}$) was used in this study following 60 h of caffeine abstinence preceding the trials. Comparing the effect of one large bolus dose of caffeine and repeated small doses of caffeine ingested throughout the day may have given an insight into how dosing strategies may affect the lymphocytes activation. This could ascertain whether taking small doses of caffeine throughout the day has similar effects to taking a large dose before exercise as, in real life, it is more possible that people consume caffeine throughout the day.
- This study looked at the effects of caffeine following one HIIE. Looking at cumulative bouts of HIIE may have provided information about whether the consumption of caffeine affects the lymphocytes' function in a similar way when HIIE is performed over a period of days. This may be more useful for exploring practical applications, as in real-life situations, people usually perform HIIE over multiple days.
- A cool down, which is typically recommended after HIIE, was not performed in the present study. Therefore, it is not clear that whether having a cool down procedure has any influence on immune parameters of interest. It may be interesting to perform the same experiments protocol with and without cool down procedures to see if there is any impact on T and NK cells.
- This study was conducted on men only and hence the findings cannot be generalised to women. Comparing the effect of caffeine on immune parameters between men and women would be useful for practical implications in the wider population.

5.7 Future Approaches

This thesis speculates that adrenaline-mediated and A_{2A} adenosine receptor antagonism are the two major mechanisms of caffeine influencing the activation of antigen-stimulated lymphocytes following HIIE. To test these assumptions, future studies examining the alteration in antigen-stimulated lymphocyte activation *in vitro*, in relation to caffeine and adenosine and/or adrenaline following HIIE would be of significant

benefit. Testing of the concentration of cAMP would also help to qualify the effect of caffeine via adrenaline and adenosine pathways via cAMP.

It would also be sensible to test for the effects of caffeine on T and NK cells following exercise using the isolated cells *in vitro* rather than in whole blood. Whole blood contains a variety of cells, and hence it becomes difficult to conclusively attribute the cause of any of the alterations seen in the current study to T and NK specific pathways.

Future studies should also look to determine the influence of caffeine on lymphocyte proliferation and cytokine secretion in response to HIIE, to understand the downstream capabilities of immune function and to have a broader view of the whole immune response, as immune function is complex and regulated by multiple factors.

This study only looked at a single high dose ($6 \text{ mg.kg}^{-1} \text{ BW}$) of caffeine in relation to HIIE. However, other studies have shown the benefits of a low dose ($2 \text{ mg.kg}^{-1} \text{ BW}$) of caffeine over a high dose (Burke, 2008; Desbrow et al., 2012; Graham & Spriet, 1995) on athletes' health, while still enhancing their performance. Future studies comparing the effect of different doses of caffeine, low ($2 \text{ mg.kg}^{-1} \text{ BW}$) and high ($6 \text{ mg.kg}^{-1} \text{ BW}$), on lymphocyte activation in response to HIIE may help us to understand if caffeine exerts similar effects on lymphocyte activation at different doses. It may also be wise to compare a single dose versus repeated doses of caffeine on lymphocyte activation following HIIE to establish whether dosing strategies influence immune function as, in a real-life situation, people usually consume caffeine throughout the day. Moreover, future studies investigating the potential impact of caffeine habituation on the effects of caffeine on antigen-stimulated lymphocyte activation may also help to identify the potential tolerance of caffeine's effect.

Finally, the current study investigated effects of caffeine of T and NK cells following only a single bout of HIIE. It would be interesting to examine effects of caffeine on lymphocytes in response to cumulative bouts (multiple days) of exercise. It would also be interesting to investigate the impact of caffeine on T and NK cells using different exercise durations and intensities, as well as participants with different fitness levels.

5.8 Practical Implications

The primary aim of the study was to examine the effect of a $6 \text{ mg.kg}^{-1} \text{ BW}$ dose of caffeine on T and NK lymphocyte activation following short duration HIIE in men. Hence, the findings from current study are most relevant to men. It was found that a 6

mg.kg⁻¹ BW dose of caffeine increased antigen-stimulated NK cell activation (innate immune function), while it did not significantly increase or decrease T cell activation (adaptive immune function) following short duration HIIE. These findings seem to suggest that caffeine has a beneficial effect on innate immunity following short duration HIIE. Therefore, based on the findings from this study, physically active people in the wider population should aim to consume a 6 mg.kg⁻¹ dose of caffeine one h prior to short duration HIIE to strengthen their innate immune function. The impact of taking smaller repeated doses of caffeine throughout the day is unclear, as it was not measured in this study. Therefore, people should aim to consume a specific 6mg.kg⁻¹ dose of caffeine to experience the enhancement in NK cell activation seen in this study. In this study, the participants abstained from taking caffeine 60 h prior to the trials. This was to minimise the caffeine adaption of habitual caffeine consumers, who may have developed an immunity to caffeine and, as such, may have experienced lesser effects of caffeine on their lymphocytes. Therefore, an individual taking a 6 mg.kg⁻¹ BW dose caffeine to enhance innate immune function should also abstain from taking caffeine 60 h before exercise. The practicality of this approach is potentially limiting but clearly further research is required to identify practical and effective approaches to enhancing immune function.

One potential strategy to enhance T cell activation following HIIE may be the consumption of caffeine together with carbohydrates, as carbohydrate ingestion is found to enhance the antigen-stimulated T cell activation (Bishop, Walker, et al., 2005). As the NK cells' activities are not affected by ingestion of carbohydrates (Nieman et al., 1997), combined administration of caffeine with carbohydrates may help to enhance both innate (NK cells) and adaptive (T cells) immune function.

Despite caffeine's immunomodulatory influence on the activation of NK cells, the clinical significance of this in terms of subsequent infection risk is yet to be determined. The widespread use of caffeine and the popular trend of short duration HIIE amongst athletes and the general population would indicate that there is a need for further research in this area in the future.

Chapter 6 Conclusion

The findings of the current study suggest that the administration of 6 mg.kg⁻¹ BW of caffeine one h prior to HIIE increases the number of total circulating NK cells, as well as the number of unstimulated and antigen stimulated NK cells expressing CD69. Since NK cells are the major innate immune cells, these findings suggest that consuming caffeine before HIIE may enhance the innate immune system. However, caffeine appears not make a significant alteration to the adaptive immune arm since the number of activated T cells or their functional ability (CD69 GMFI expression) was relatively unchanged. The proposed major underlying mechanisms for alteration in lymphocytes are likely to be the adrenaline-induced alteration and A_{2A} adenosine receptor antagonism. Caffeine ingestion is associated with an increased mobilisation of peripheral NK cell numbers via the adrenaline-mediated pathway, and enhancement of NK cell activation via A_{2A} adenosine receptor antagonism. However, T cells seem to be less responsive to adrenaline stimulation, and it is likely that the stimulatory and inhibitory effects of caffeine are balanced out within T cells, resulting in no major alteration in either circulating numbers or activation. From a practical perspective, consuming caffeine prior to intensive exercise may be a worthwhile strategy to improve immune function though further research is required to ascertain if incidence of URTI is reduced following caffeine ingestion prior to exercise over a longer observation period.

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Appendices

Appendix A: Consent Form

<h1 style="margin: 0;">Consent Form</h1>	 <p>AUT UNIVERSITY <small>TE WĀNANGA ARONUI O TAMAKI MAKAU RAU</small></p>
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Project title: Does caffeine consumption before high-intensity intermittent exercise enhance immunity?

Project Supervisor: **Dr Deborah Fletcher**

Researcher: **Khine Thida**

- ☐ I have read and understood the information provided about this research project in the Information Sheet dated 19/10/2015.
- ☐ I have had an opportunity to ask questions and to have them answered.
- ☐ I understand that I may withdraw myself or any information that I have provided for this project at any time prior to completion of data collection, without being disadvantaged in any way.
- ☐ I am not suffering from heart disease, high blood pressure, any respiratory condition (mild asthma excluded), any illness or injury that impairs my physical performance, or any infection.
- ☐ I agree to provide blood samples as outlined in the information sheet.
- ☐ I agree to take part in this research.
- ☐ I wish to receive a copy of the report from the research (please tick one): Yes ☐ No ☐
- ☐ I wish to have my blood samples returned to me in accordance with right 7 (9) of the *Code of Health and Disability Services Consumers' Rights* (please tick one):
Yes ☐ No ☐
- ☐ I agree that you can keep my contact details on file and use them to contact me if another study similar to this one becomes available (please tick one): Yes ☐ No ☐

Participant Signature.....

Participant name

Participant Contact Details (if appropriate):

.....

.....

.....

.....

Date:

Approved by the Auckland University of Technology Ethics Committee on 13.12.2012 AUTEK Reference number 12/331

Appendix B: Pre-test Health Questionnaire

**HEALTH SCREEN FOR STUDY VOLUNTEERS**

Name or Number

It is important that volunteers participating in research studies are currently in good health and have had no significant medical problems in the past. This is to ensure (i) their own continuing well-being and (ii) to avoid the possibility of individual health issues confounding study outcomes.

Please complete this brief questionnaire to confirm fitness to participate:1. **At present**, do you have any health problem for which you are:

- (a) on medication, prescribed or otherwise Yes ☐ No ☐
- (b) attending your general practitioner..... Yes ☐ No ☐
- (c) on a hospital waiting list..... Yes ☐ No ☐

2. **In the past two years**, have you had any illness which require you to:

- (a) consult your GP Yes ☐ No ☐
- (b) attend a hospital outpatient department Yes ☐ No ☐
- (c) be admitted to hospital Yes ☐ No ☐

3. **Have you ever** had any of the following:

- (a) Convulsions/epilepsy..... Yes ☐ No ☐
- (b) Asthma..... Yes ☐ No ☐
- (c) Eczema Yes ☐ No ☐
- (d) Diabetes Yes ☐ No ☐
- (e) A blood disorder Yes ☐ No ☐
- (f) Head injury Yes ☐ No ☐
- (g) Digestive problems..... Yes ☐ No ☐
- (h) Heart problems Yes ☐ No ☐
- (i) Problems with bones or joints Yes ☐ No ☐
- (j) Disturbance of balance/coordination Yes ☐ No ☐
- (k) Numbness in hands or feet Yes ☐ No ☐
- (l) Disturbance of vision..... Yes ☐ No ☐
- (m) Ear / hearing problems..... Yes ☐ No ☐
- (n) Thyroid problems Yes ☐ No ☐
- (o) Kidney or liver problems..... Yes ☐ No ☐
- (p) Allergy to nuts Yes ☐ No ☐

4. **Has any**, otherwise healthy, member of your family under theage of 35 died suddenly during or soon after exercise? Yes ☐ No ☐**If YES to any question, please describe briefly if you wish****(eg to confirm problem was/is short-lived, insignificant or well controlled.)**

.....

.....

5. **Have you** had a cold or feverish illness in the past month? Yes ☐ No ☐6. **Are you** accustomed to vigorous exercise (1-3 hours per week)? Yes ☐ No ☐**Thank you for your cooperation.**

Appendix C: Physical Activity Questionnaire

Physical Activity Questionnaire

The following questions are designed to give us an indication of your current level of physical activity.

Name: _____ Date: ____ / ____ / ____

Are you currently accustomed to regular moderate-high intensity exercise?

YES ☐ NO ☐ .

If Yes, how many days each week do you usually exercise? _____

What intensity do you normally exercise at? _____

How many minutes does each exercise session last? _____

What is your weekly exercise total in hours and minutes? _____

Please break this down according to exercise type. E.g. cycle, swim and run

Are you involved in any of the following training programmes?

Weight training ☐ Interval training ☐ Skills training ☐.

If Yes, how many days each week do you usually train?

How many minutes does each session last?

Appendix D: Caffeine Consumption Questionnaire

**CAFFEINE CONSUMPTION QUESTIONNAIRE**

Please complete the questionnaire concerning your caffeine usage. List the number of times you consume the following substances during a typical day. Please also specify the product you use, E.G. Tea – PG tips.

	Morning	Afternoon	Evening	Night
COFFEE (Regular 5 oz. Mugs)				
Regular Brewed				
Percolated				
Espresso (2 oz. Serving)				
Instant				
Decaffeinated				
TEA (Regular 5 oz mugs)				
Hot Chocolate (Regular 5 oz mugs)				
Chocolate (Regular Bar)				
Chocolate Biscuits / Cakes / Cereals				
Chocolate / Coffee Dairy Products				
SOFT DRINKS (330ml Can Size)				
Coke				
Dr Pepper				
Energy Drinks (Please state which ones)				
OVER THE COUNTER DRUGS				
Pain Killers e.g. Anadin (Please state which ones)				
Pro Plus				

Do you take caffeine prior to exercise? _____

If Yes, How much? _____

What do you take? _____

Why? _____

MANY THANKS

Appendix E: Before Trial Health Screen



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Does caffeine consumption before high-intensity intermittent exercise enhance immunity?

.....

Health Questionnaire

Please complete the following brief questions to confirm your fitness to participate:

1) At present do you have any health problems for which you are:

a) On medication, prescribed or otherwise YES ☐ NO ☐ .

b) Attending your general practitioner YES ☐ NO ☐ .

2) Have you any symptoms of ill health, such as those associated with a cold or other common infection? YES ☐ NO ☐ .

3) Are you presently acting as a subject for any other experiment or research study? YES ☐ NO ☐ .

If you have answered yes to any of the above questions please give more details below:

Do you want to take part in today's experiments? YES ☐ NO ☐ .

Signature: _____

Date: _____

Appendix F: Diet Diary

Khine Thida



Diet Diary

It is important to ensure that conditions are kept similar for all trials, therefore we ask you to keep a record of all food and fluid you consume during the 1 day before the first experimental trial, and ask you to reproduce this in the days prior to the second and third trial. An example is given on the following page.

The following guidelines may help when completing the diary:

1. All foods and beverages including snacks should be recorded (**NOTE: No caffeine should be consumed 60 hours before each trial. No alcohol should be consumed 24 hours before each trial.**)
2. Quantities of foods drinks consumed can be estimated using approximate portion sizes, with amount left over after eating.

e.g.	Breads:	Brown, wholemeal, white
	Milk:	Whole, skimmed, semi-skimmed
	Biscuits:	Shortcake, digestive
	Cheeses:	Kraft processed slices, Scottish cheddar
	Fish	Mackerel, tuna, haddock
	Fruits:	Large apple, tinned fruit in syrup
	Drinks:	Fruit juice, sports drink, decaf coffee
3. Try to describe each item fully, giving type and brand of food.
4. When eating ready-made food please include brand name and description of the food.
5. Please include use of sauces and condiments (tomato ketchup, salad cream etc).
6. Include method of cooking - boiled, fried, grilled etc
7. Indicate whether skins are eaten.
8. Include all food, vitamin and mineral supplements used.
9. Use as many pages as required for each day.
10. Please attempt to record all items immediately after consumption. Do not wait until the end of the day as you may forget some items.

It is important that you are rested prior to the trials, so no exercise should be undertaken in the day before each trial.

If you have any problems completing this diary, or with any aspect of the study, please contact me on 021 162 5823.

Khine Thida

DAY:	EXAMPLE	
TIME	DESCRIPTION OF FOOD OR DRINK CONSUMED	PORTION SIZE
8:30am	Kellogs cornflakes	Small bowl
	Semi-skimmed milk	250 ml
	Toast, Tip Top white bread	2 slices
	Countdown olive margarine	Thin spread
	Tropicana fresh orange juice	1 small glass
11:00am	Coffee, Nescafe decaffeinated	1 mug
	Whole milk	splash
1:15pm	Sandwich:	1 sandwich
	Tip Top White bread	2 slices
	Margarine	Thin spread
	Grated cheddar cheese	20 g
	Tomato	3 slices
4:30pm	Lucozade Sport – orange	500ml bottle
6:00pm	Grilled lean pork chops	2 medium
	Boiled new potatoes with skins	7 small
	Cross & Blackwell peas	2 tablespoons
	Banana	1 medium
7:30pm	Green tea (decaffeinated)	1 mug
	McVities Digestives	4 biscuits

Appendix G: Infanrix® Hexa Vaccine Titration

CD4⁺CD69⁺

Gated on R1 (Total lymphocyte population)

Concentration	Final Concentration	% Gated	GMFI
0	0	4.8	22.79
1:100	1:1000	5.84	26.94
1:200	1:2000	3.81	25.99
1:400	1:4000	5.40	23.84
1:800	1:8000	4.12	24.70
1:1600	1:16000	3.51	26.86
1:3200	1:32000	3.45	24.02
1:6400	1:64000	3.60	25.30

CD8⁺CD69⁺

Gated on R1 (Total lymphocyte population)

Concentration	Final Concentration	% Gated	GMFI
0	0	4.83	27.79
1:100	1:1000	8.86	29.11
1:200	1:2000	8.26	28.89
1:400	1:4000	7.80	28.23
1:800	1:8000	8.17	25.81
1:1600	1:16000	6.96	26.04
1:3200	1:32000	6.62	24.84
1:6400	1:64000	6.83	25.57

CD3⁺CD56⁺CD69⁺Gated on R2 (CD3⁺CD56⁺ lymphocyte population)

Concentration	Final Concentration	% Gated	GMFI
0	0	19.34	61.92
1:100	1:1000	36.77	56.22
1:200	1:2000	31.09	54.14
1:400	1:4000	33.51	55.88
1:800	1:8000	31.92	51.71
1:1600	1:16000	31.29	53.12
1:3200	1:32000	25.11	57.53
1:6400	1:64000	230.03	49.14