EXERCISE INDUCED ENDOCANNABINOID AND IMMUNE SYSTEM ALTERATIONS

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____

Prof. Dr. Sencer Ayata
Director

I certify that this thesis satisfies all the requirements as a thesis for the degree of Doctor of Philosophy.

____

Assoc. Prof.Dr. Settar Koçak
Head of Department

This is to certify that we have read this thesis and that in our opinion it is fully adequate, in scope and quality, as a thesis for the degree of Doctor of Philosophy.

____

Prof. Dr. Feza Korkusuz
Supervisor

Examining Committee Members

Prof. Dr. Sevim Ercan	(GAZI, MP)	______________________
Prof. Dr. Feza Korkusuz	(METU, PES)	______________________
Prof. Dr. Duygu Uçkan	(HU,BMT)	______________________
Assoc. Prof. Dr. Petek Korkusuz	(HU,HE)	______________________
Assist. Prof. Dr. Sadettin Kirazcı	(METU, PES)	______________________

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Name, Surname: Rabia Hürrem Özdurak

Date:

Signature:

ABSTRACT

EXERCISE INDUCED ENDOCANNABINOID AND IMMUNE SYSTEM ALTERATIONS

Özdurak, Rabia Hürrem

Ph.D., Department of Physical Education and Sports

Supervisor : Prof. Dr. Feza Korkusuz

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Endocannabinoid and immune system alterations at moderate (18 m/min) and endurance (32 m/min) exercise intensities were assessed and compared to controls. Rats were exercised for 60 minutes/day, 5 days/week for 16 weeks. Immune effector cell proportions (T cell subtypes, B cells, NK cells, and neutrophils) and endocannabinoid serum levels were determined. Anandamide (ANA) and 2 arachidonyl-glycerol (2-AG) serum levels increased with endurance type of exercise. mRNA expression of the CB1 receptor increased together with ANA in the same group. Apoptotic index increased while immune effector cells responded divergently. B lymphocyte percentage decreased while T lymphocyte and NK cell percentage increased in blood. $CD8^+$ subtypes increased whereas $CD11b^+$ cell and $CD25^+$ cell numbers decreased in the spleen in the endurance type of exercise group.

Rats were grouped as the control, the endurance type of exercise, the AM281 (CB1 receptor antagonist) and the AM281+AM630 (CB2 receptor antagonist) groups in the second part of the study. Flow cytometry and microarray analyses of the spleen and the thymus were conducted. Endurance type of exercise associated significantly to immunological changes particularly to that of the T lymphocytes. T lymphocytes increased whereas cytolytic T lymphocytes decreased in blood. T cell and double positive T cell percentages significantly increased in the spleen. Activated T cells and NK like T cells

furthermore decreased in the spleen. AM281 and/or AM630 could partially reverse the effect of exercise in blood but not in the spleen. Alterations in the thymus were not observed. Exercise altered 302 genes, some of them related with the immune system. Up-regulation of heat-shock protein coding genes was the most significant ones.

Keywords: Anandamide, 2-arachidonylglycerol, T lymphocytes, exercise intensity, spleen

ÖZ

FİZİKSEL EGZERSİZE DAYALI ENDOKANNABİNOİD VE İMMÜN SİSTEM DEĞİŞİKLİKLERİ

Rabia Hürrem Özdurak
Doktora, Beden Eğitimi ve Spor Bölümü
Tez Yöneticisi: Prof. Dr. Feza Korkusuz
Kasım 2009, 186 sayfa

Endokannabinoid ve imün sistemdeki değişiklikler orta şiddet (18 m/min) ve endurans (32 m/min) egzersizde ölçülerek control grubu ile karşılaştırılmıştır. Sıçanlar günde 60 dakika ve haftada 5 gün olacak şekilde 16 hafta boyunca egzersiz yaptırılmışlardır. Imün efektör hücre oranları (T hücre alt tipleri, B hücreler, NK hücreler ve nötrofiller) ve endokannabinoid serum düzeyleri belirlenmiştir. Anandamid (ANA) ve 2 araşidonilgliserol (2-AG) serum düzeyleri endurans tipi egzersiz ile artmıştır. CB1 reseptörünün mRNA ekspresyonu ANA ile aynı grupta artmıştır. Kanda imün efektör hücreler değişken tepkiler verirken apoptotik indeks artmıştır. B lenfosit yüzdesi azalırken T lenfosit ve NK hücre yüzdesi artmıştır. Endurans tipi egzersiz sonrasında $CD8^+$ alt tipi artarken $CD11b^+$ hücre ve $CD25^+$ hücre sayıları dalakta azalmıştır.

Sıçanlar çalışmanın ikinci bölümünde control, endurans tipi egzersiz, AM281 (CB1 reseptör antagonisti) ve AM281+AM630 (CB1 ve CB2 reseptör antagonisti) olacak şekilde gruplandırılmışlardır. Dalak ve timus dokularında akışkan hücre tekniği ve microarray analizleri yapılmıştır. Endurans tipi egzersiz T hücreleri başta olmak üzere imünolojik değişiklikler ile ilişkilendirilmiştir. Kanda T lenfositler artarken sitolitik T lenfositler azalmıştır. Double pozitif T hücreler dalakta anlamlı şekilde artmıştır. Dalakta aktive T

hücreler ile NK benzeri T hücrelerde azalmıştır. AM281 ve/veya AM630 kanda egzersizin etkisini kısmen ortadan kaldırabilirken dalakta etkili olamamıştır. Timusta değişiklik gözlenmemiştir. Egzersiz 302 genin ekspresyonunda değişikliğe sebep olmustur. İçerisinde bağışıklık sistemi ile ilişkili olan genlerinde bulunduğu grupta en çok değişikliği heat shock proteinlerini kodlayan genler oluşturmuştur.

Keywords: Anandamide, 2-arachidonylglycerol, exercise intensity, T lymphocytes, spleen

To My Dad

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CHAPTER 1

INTRODUCTION

The relationship between exercise and the immune system has become an interdisciplinary and popular research area for many exercise physiologists, clinicians, immunologists and scientists of behavioral research. Not only the trainers and physiologists, who are concerned about the health of athletes, but also clinicians try to understand the effect of exercise on health since it is well known that exercise has a positive effect on diseases such as osteoporosis, hypertension, obesity, non-insulin dependent type of diabetes, cardiovascular diseases, Alzheimer's disease and depressive illness in terms of prevention or reducing the treatment time and thereby reducing the high cost values during the treatment of these diseases (Cara and Chaiken 2006; Cayley, 2007; Cotman and Berchtold, 2007; Midtgaard et al., 2006; Mora et al., 2006; Pedersen, 2006; Pedersen and Saltin, 2006; Stevens and Killeen, 2006; Tremblay and Therrien, 2006). Moreover, recent research showed that there is a close relationship between exercise and illnesses like cancer and AIDS which are strongly dependent on life style (Hunt-Shanks et al., 2006; O'Brien et al., 2004). Latest research in the area of psychoneuroimmunology and/or behavioral immunology has focused on the effect of stress on the immune system (Demark-Wahnefried et al., 2006; Midtgaard et al., 2006; Polo et al., 2006; Rosenberg et al., 2006; Sax, 2006).

Literature showed that regular exercise has a positive effect on health by decreasing the risk of coronary artery disease and osteoporosis, increasing the resistance to psychological stress, delaying aging and extending someone's life period (Cooper 2006; Oguma and Shinoda-Tagawa, 2004; Pedersen 2006; Raso et al., 2007; Shea et al., 2004; Strasser et al., 2006). Although research in cardio-vascular area showed the positive effects of regular exercise, research at the beginning of 1970's showed that student participating the sport teams are more susceptible to hepatitis B, polio, meningitis, gastrointestinal system and

respiratory system infections when compared to their sedentary schoolmates. It has been accepted that exercise has not only immunological, but also endocrinological consequences (Asgeirsson and Bellanti, 1987; Baron et al., 1982; Day et al., 2006; Droste et al., 2007; Fediuc et al., 2006; Fleshner, 2005; Griesbach, 2007; Morse 1972; Kohut et.at., 2004; Sasse et.at., 2008; Weinstein 1973) and scientists started to search whether exercise has a stimulatory or inhibitory effect on the immune system (Figure 1.1).

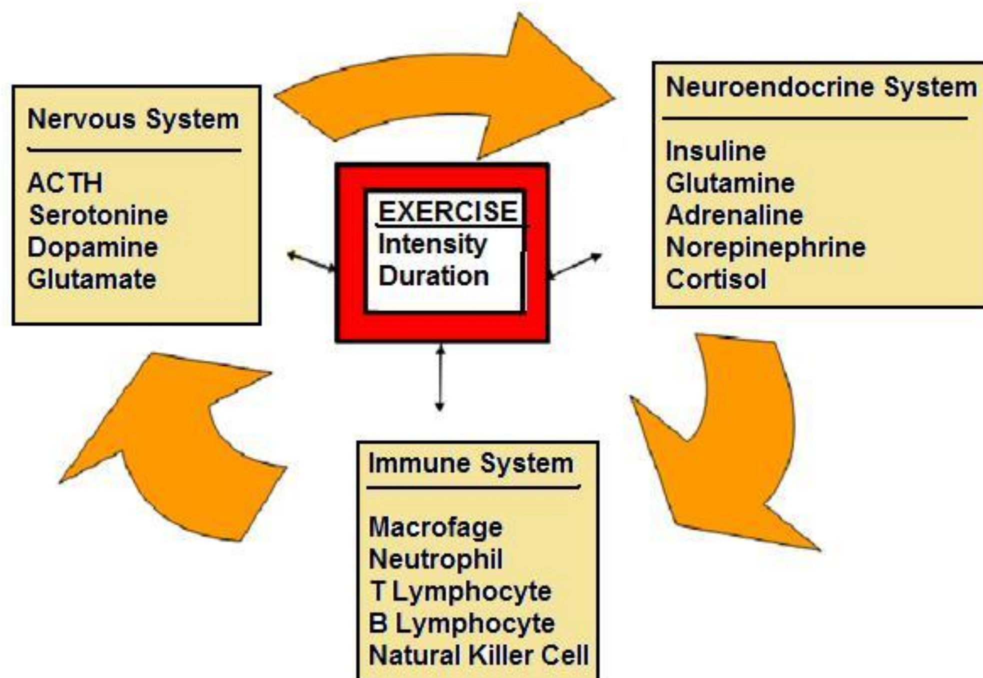


Figure 1.1 The relationship between exercise and other systems of the body

The effect of regular exercise on the immune system has been argued for a long time. Some research showed that chronic exercise decreases the risk of infections (Davis et al., 2004; Gleenson et al., 2006; Lowder et al., 2006; Nieman and Pedersen, 1999; Vanitallie, 2002) and inhibits tumors (Davis et al.,1998; Kruk and Aboul-Enein, 2007). Another research proved that exercise at moderate intensity increases not only the number of natural killer (NK) cells, but also their cytolytic activity (Brahmi et al., 1985; McFarlin et al., 2005;

Suzui et al., 2004; Timmons and Bar-Or, 2007). However, it has been widely accepted that exhaustion as a result of exercise at high intensities for a long time causes immune system suppression and thereby increases the risk of infections (Fitzgerald, 1991; Nieman, 2003; Pyne et al., 2000, Suzuki et al., 2002; Suzuki et al., 2003).

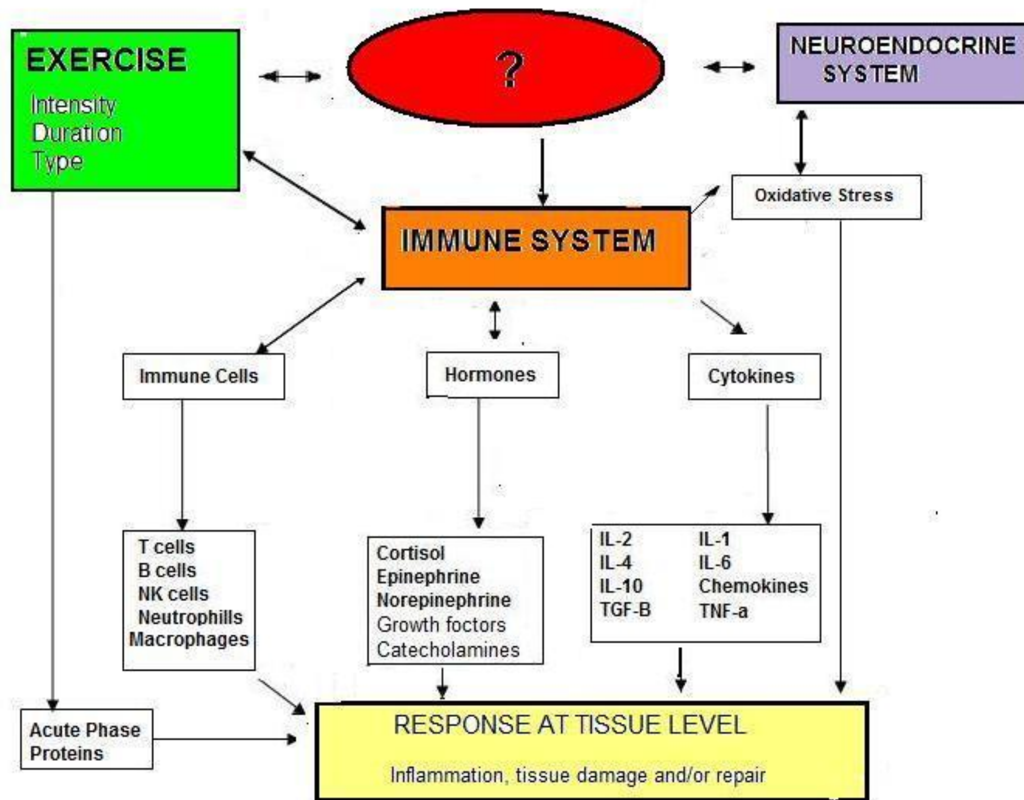


Figure 1.2 The complex and bidirectional response of the immune system stimulated by different components of the neuroendocrine system during exercise.

The reason of these contradictive results are the complexity of the immune system, the unknown relationship of exercise with the neuroendocrine system, the heterogenity of the exercise type and subjects studied so far. Exercise alters many aspects of the immune function, by stimulating some immune effector cells while supressing others. Regardless of the contradictive results, there is a dose-response relationship between exercise amount (duration and/or intensity) and the specific immune response. Today, it is well accepted that regular moderate exercise has a positive effect on the immune system, whereas high intensity exercise for long duration has a suppressive effect on the immune system (Kumae et al., 2009; McFarlin et al., 2004; Murakami et al., 2009; Pyne et al., 2000). Some studies showed that endurance type of exercise may cause immune system suppression and alterations in immune system parameters (Table 1.1).

The molecular mechanism of the bidirectional response of the immune system depending on the exercise intensity is still unknown. Recent literature showed that all immune effector cells respond differently to a certain type of exercise intensity. Moreover, each immune effector cell type may react in a different way with diverse exercise intensities (Table 1.2). In the light of these results, it has been thought that there may be other mechanisms and/or immune modulator molecules involving in the exercise intensity and duration dependent bidirectional response of the immune system.

It has been known that endurance athletes experience not only immune suppression, but also psychological changes associated with prolonged physical activity. A label often applied to these exercise induced changes is the ‘‘runner’s high’’ which parallels the emotional status of people who describe cannabinol usage, drug administration or trance states. The runner’s high has been described subjectively as pure happiness, endless peacefulness, unlimited energy, inner harmony, reduction in pain sensation and addiction to exercise (Bartholomew et al., 1996; Cook and Koltyn, 2000; Kenney et al., 2008).

Table 1.1 Endurance exercise induced immune system alterations

Parameters	Findings	References
Lymphocyte number and function	• Decrease to clinically low levels f • Altered CD4+/CD8+ T-cell ratio • Decrease in CD4+ T cell • stimulated increase of CD25+/CD8+ T-cells • Imbalance in T helper 1/T helper 2 (Th1/Th2) cells • Decreased NK like T cell populations • Decreased IL-4 secretion • Decreased lymphocyte responsiveness to antigens • Inhibited IL-2 production • Altered IL-2 kinetics • Inhibited IFN-γ secretion	Kohut et al., 2002; Lehmann et al., 1996; Fu et al., 2003; Ronsen et al., 2001; Pool et al., 2004; Ru and Peijie, 2009; Northoff et al., 1998; Kohut et al., 2004
Monocyte number and function	• Decreased number • Decreased IL-6 secretion	Flynn et al., 2003; Northoff et al., 1998
Neutrophill count and function	• Lower resting and post exercise values in cyclists • A progressive decrease parallel to the increase of the exercise intensity during the training season of swimmers • Diminished responsiveness to stimulation by lipopolysaccharide • Reduced oxidative burst and • Diminished degranulation responses • Alteration in neutrophil gene expression	Smith et al., 1990; Pyne et al., 1995; Pyne et al., 2000; Robson et al., 1999; Radom-Aizik et al., 2008
NK cell count and activity	• Progressive decrease in the NK cell count over 7 month training season in swimmers • Decrease in NK cell count • Decreased NK cell cytolytic activity	Fry et al., 1994; Gleeson et al., 1995; Shephard and Shek , 1999.
Serum Ig	• Clinically low levels during a 7 month long training season in swimmers	Gleeson et al., 1995

Before the discovery of the opioids, exercise scientists tried to explain these changes in the analgesic and mental states with alterations in the catecholamines adrenalin and noradrenalin (Howley, 1976). A study presented an elevation in endorphin levels and commented that exercise promotes psychological well-being by increasing the secretion of endorphins (Hoffman, 1997). Alterations in catecholamines, glutamine, growth hormone, cortisol, adrenaline and noradrenaline with exercise were described (Droste et al., 2007; Gleeson, 2008; Kohut et al., 2004; Kraemer and Ratamess, 2005; Krüger and Mooren, 2007; Malm et al., 2004; Pedersen et al., 1997; Sasse et al., 2008;). The remarkable change of endorphin levels and their biphasic effects on the immune system is assumed to be duration and intensity dependent. This however cannot be explained with the endorphin hypothesis. Although increase in β endorphin level reduces pain sensation and produces a state of euphoria by binding of to the μ opioid receptor, minimal activation of the same endogenous opioid system is also responsible for the severe respiratory depression and inhibition of gastrointestinal motility, which are not seen in runners. Moreover, peripheral activation in the systemic circulation cannot be taken as indicative of central effects of endorphins since endorphins are too large to cross the blood-brain barrier (Droste et al., 2007; Farell et al.,1986; Farell et al.,1987; Fediuc et al., 2006; Harbach et al., 2000; Howley, 1976; Kolata, 2002).

To date, two cannabinoid receptors, CB1 and CB2, and their endogenous ligands, anandamide (ANA) and 2-arachidonylglycerol (2-AG), have been identified (Devane et al., 1988; Howlett et al., 2004; Munro et al., 1993; Sugiura et al., 2002). Both CB1 and CB2 receptors were found in the central nervous system (CNS) and peripheral organs (Aguado et al., 2006; Aso et al., 2008; Herkenham, 1995; Molina et al., 2007; Van Sickle et al., 2005), especially on the immune effector cells (Bouaboula et al., 1993; Galieque et al., 1995; Kleinet al., 2003). With the discovery of these receptors, the missing link between CNS and both endocrine and immune systems seems to be found and the molecular basis for cannabinoid action on the central nervous and immune systems was established (Devane et al., 1988; Munro et al., 1993).

Table 1.2 The effect of exercise intensity on the immune system

Exercise	Subjects	Findings	Reference
Running	Elderly male	**Immediately after exercise**: Increased CD16/56 expressing NK cells in active subjects compared to sedentary ones	Yan et al., 2001
Strenuous exercise	Mice	**Immediately after exercise**:decrease of CD4 expressing cells, stimulated increase of CD25/CD8 expressing T cells	Fu et al., 2003
Running	Elite swimmer	**Immediately after exercise**: Increase in CD3, CD4, CD5, CD8, CD19, CD57, CD18, CD16; CD22 expressing cells and decrease in proliferative response **Post-24 hours**: All parameters at basal level, no change in cytokines	Espersen et al., 1996
Walking	Elderly subjects	Increased number of CD8 expressing cells producing IL-2 in active than sedentary subjects	Ogawa et al., 2003
5 km running	runners	**Immediately after exercise**: decrease in IL-2 and CD4/CD8 cell ratio, increase in TNF, HLA-DR+ and NK cells **Post 24 hours**: Increase in IL-2, normal TNF level	Espersen et al., 1990
Endurance running ($V0_{max}$ 95)	Endurance runners	**Immediately after exercise**: Increase in NK cells and CD8 expressing T cells, decrease in proliferative response, no change in B and T cell function	Hinton et al., 1997
30 minutes moderate intensity exercise	Male runners	**Immediately after exercise**: Increase in T cells, decrease in CD4/CD8 T cell ratio and proliferation in response to phytohemagglutinin-A (PHA)	Smith et al., 1993
Resistance + endurance	Elderly active subjects	Increase in salivary IgA levels	Akimoto et al., 2003
Moderate exercise	Elderly male	No effect on the T cell subsets, CD28+ or IL-2R	Raso et al., 2007

Recent studies showed that the activation of the endocannabinoid system reduces pain at the central and the peripheral levels (Di Marzo et al., 2000; Rice et al., 2002) as seen in endurance type of exercise. Moreover, cannabinoids have several effects on the immune system. They enhance and/or inhibit B and T cell proliferation and migration (Derocq et al., 1995; Joseph et al., 2004), decrease IL-2 production (Herring and Kaminski, 1999; Rockwell et al., 2006; Rockwell et al., 2008), cause increased immunoglobulin secretion as a response to CD4+/CD8+ ratio imbalance in T lymphocytes (McKallip et al., 2005; Schatz et al., 1993), inhibit cytolytic T cell chemotaxis (Joseph et al., 2004), stimulate apoptosis in the spleen and thymus (McKallip et al., 2002), stimulate NK cell migration (Khishimoto et al., 2005) and inhibit the cytotoxicity of NK cells (Massi et al., 2000).

Other studies showed that endocannabinoids stimulate IL-6 in adipocytes (Hoareau et al., 2006), suppress lipopolysaccharide stimulated TNF-α, IL-4, IL-6 and IL-8 secretion in macrophages (Berdeshev et al., 1997; Cabral et al., 1995). Moreover, literature showed that endocannabinoids stimulates apoptosis in dendritic cells via CB1 and CB2 receptors and thereby suppresses the immune system, causes anti-inflamation and effects dendritic cell maturation (Do et al., 2004). Some peripheral effects are summarized in Table 1.3.

All these variable and contradictory outcomes are the result of the complexcity of the mechanism, the expression of both CB receptors on the immune effector cell surface (Nong et al., 2001), the difference in receptor density of CB1 and CB2 receptors on different immune effector cells (Onaivi et al., 1996), the difference in the binding affinity of endocannabinoids produced at the same time interval having concentration dependent agonistic and/or antagonistic effects (Do et al., 2004; Lee et al., 1995). Although studies showed that endocannabinoid system is closely related to the immune system, the mechanism of this relationship and the stimulatory and/or inhibitory molecules playing role in this mechanism could not be explained so far.

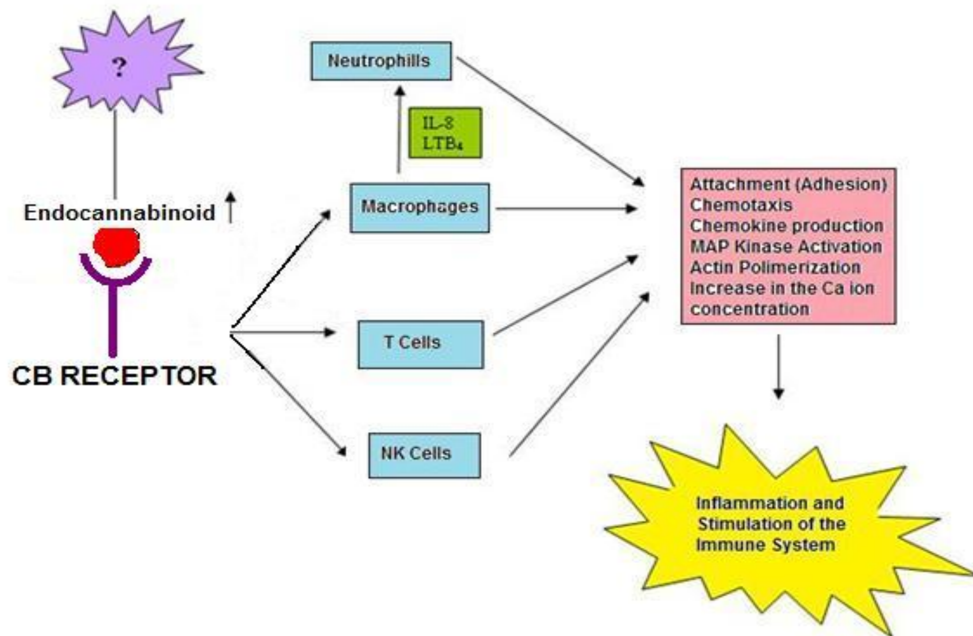


Figure 1.3 The effect of endocannabinoids on the immune system

A recent study showed that acute moderate running or cycling exercise at an intensity of 70-80% of maximal heart rate increases serum concentrations of ANA in college students (Sparling et al., 2003). This was also supported by two other studies Hill and his coworkers stated that showed an increase in the endocannabinoids as a result of voluntary wheel running and this enhances progenitor cell proliferation in the hippocampus (Hill et al., 2009). Keeney and his coworkers proved that endocannabinoid system may play a role in the perception of neurobiological rewards during and after prolonged exercise (Keeney et al., 2008). This might be a possible explanation for exercise induced immune and nervous system alterations. However, these studies did not include different exercise intensities, especially, endurance type of exercise where “runner’s high” and immune suppression is mostly observed.

Table 1.3 The peripheral effects of ANA and /or 2-AG on the immune system

Biological Effects	References
• Suppressed transcription and secretion levels of IL-2 • Stimulated IL-6 synthesis • Decreased IL-6 and IL-8 synthesis • Inhibited IL-4 synthesis • Inhibited TNF-α production • Reduced INFγ production • Suppressed rat mast cell activation • Stimulated hematopoietic cell development • Stimulated lymphocyte apoptosis • Stimulated nitric oxide release from immune tissue • Decreased B lymphocyte poliferation • Stimulated Ig production and Ig isotype switching • Inhibited neutrophil activity • Inhibited natural killer cell cytotoxicity • Inhibited cytokine and chemokine production • Inhibited cytolytic T cell chemotaxis • Decreased T lymphocyte proliferation • Stimulated Th2 cytokine secretion and polarization • Suppressed Th1 cytokine secretion against bacteria • Inhibited antibody formation • Decreased antigen presentation in macrophages • Decreased migration and phagocytosis in macrophages • Increased adhesion of macrophages • Induced Cell cycle arrest and apoptosis of cancer cells	Rockwell et al., 2008; Gallily et al., 2000; Molina-Holgado et al., 1998; Sacerdote et al., 2005; Berdyshev et al.,1997; Klein et al., 2000; Bueb et al., 2001; Valk et al., 1997; Derocq et al.,1998; Stefano et al., 1998; Maestroni et al., 2004; Patel et al., 1985; Klein et al., 1987; Specter et al., 1986; Kraft and Kress, 2004; Carayon et al., 1998; Do et al., 2004; Kaminski et al., 1994; Titishov et al., 1989; Massi et al., 2000; Schwarz et al., 1994; Lee et al., 1995; Joseph et al., 2004; Zhu et al., 2000; Cabral et al., 1995; Ross et al., 2000; Chang et al., 2001; Sugamura et al., 2009; Cianchi et al., 2009; DeLago et al., 2006; Endsley et al., 2007; DeMorrow et al., 2007; Caffarel et al., 2006.

Although there are many studies focused on the relationship between endocannabinoids and immune system, there is a lack in literature concentrated on endocannabinoids and endurance type of exercise. With the discovery and subsequent characterization of the cannabinoid receptor network and its relationship to the immune system, it might be possible to determine a mechanistic explanation of endurance exercise induced changes and their biphasic effect on the immune system.

1.1 Hypothesis

It is assumed that exercise intensity has an effect on both (1) endocannabinoid system and (2) immune system. (1.a) Different exercise intensities will have different effects on endocannabinoids; ANA and 2-AG serum levels. Moreover, (1.b) the same exercise intensity will have different effects on ANA and 2-AG, separately. Furthermore, (2) exercise depending immune system alterations will be regulated through these endocannabinoids. (2. a) Both ANA and 2-AG will play role in the mechanism of exercise depending immune system alterations. (2.b) This mechanism is assumed to be mediated by the cannabinoid receptors; CB1 and/or CB2 receptors found on the surface of immune effector cells. (2.c) The specific effects of endocannabinoids through their receptors may also vary among the immune effector cell populations found in spleen, thymus and blood.

1.2 Research Questions

1.2.1 Part 1 of the study

- Does exercise intensity (moderate and endurance) have an effect on the endocannabinoid system in terms of ANA and 2-AG serum levels?
- Does the same exercise intensity affect ANA and 2-AG serum levels in different degrees?
- Do the immune effector cells increase and/or decrease as a response to exercise depending endocannabinoid system changes?
- Is there an adaptation in the endocannabinoid system at cellular level as a response to exercise?
- Is there an adaptation in the immune system at cellular level as a response to exercise?

1.2.2 Part 2 of the study

- Are the exercise depending immune system alterations regulated through CB1 and/or CB2 receptors?
- Does the endurance exercise depending immune system alterations differ among blood, spleen and thymus tissues?
- Is there an exercise induced adaptation in the immune and endocannabinoid system at cellular level in spleen and thymus?

1.3 Purpose of the Thesis

There is a long standing gap in the field of exercise immunology focused on the exercise depending immune response that requires coordinated action by several types of cells in a tightly regulated sequence. Studies in literature were performed with parameters such as (a) exercise differing in type, intensity, duration, training period, (b) subjects used in the study differing in age, training condition, gender,(c) complexicity and variety of the response of immune system differing among the immune effector cells to a certain type of stimulus, (d) molecules of interest like adrenaline, noradrenaline, cortisol, growth hormone, glutamine, and (e) measurements of immune effector cells differing in type, source of tissue etc. This broad range in the dependent and in dependent variables in these studies made the comparison of the results almost impossible. Therefore, the mechanistic explanation of the immune response to different types of exercise at different intensities could not be explained with the studies in literature so far. In the present study, immune system response to two different exercise intensities was assessed to explain the endocannabinoid system, exercise and immune system interaction. This is the first study in literature that assessed quantitatively (a) the increase of ANA and 2-AG serum levels and (b) alterations in immune effector cells as a response to different exercise intensities. In the first part of the study, the threshold for endocannabinoid system stimulation and immune system alteration was determined by using moderate and endurance type of exercise protocols. The aim of the first part of the study was to determine the effective exercise intensity that give rise to exercise dependent adaptations in endocannabinoid and immune systems. Then after, this exercise intensity was applied to all exercise protocols where CB1 and CB2 receptor antagonists AM281 and/or AM630 were used in order to identify the mechanism how endocannabinoids may regulate exercise depending immune system alterations. Previous studies were interested in the effect of endocannabinoids on the immune system by applying high concentrations of synthetic endocannabinoids exogenously on different animal models and tissue cultures; however, the concentrations of

these endocannabinoids applied in these studies did not fit the biological ones. This is the first study in literature that reveals endogenously increased ANA serum levels by exercise and shows the exercise depending immune system alterations through the endocannabinoid ANA. Moreoever, response of the immune system was measured in three different tissues at the same time. Blood does not reflect the overall picture since only 3 percentages of the whole lymphocytes are found in blood. Therefore, measurements performed on spleen and thymus tissues and comparing them with blood values gave valuable information.

In the first part of the study, rats were exercised at different intensities of treadmill running. The certain exercise intensity that causes an increase in the endocannabinoid serum levels and alteration in the immune system was determined. This exercise intensity that was used in the exercise protocol determined for the second part of the study. All animals exercised at this determined exercise intensity for the same time. Two CB receptor antagonists were applied to block the effect of endocannabinoids. B lymphocyte, NK cell, T lymphocyte, helper T lymphocyte, cytolytic T lymphocyte and activated T lymphocyte percentages were measured in blood, spleen and thymus. Cellular adaptation at tissue and gene level was questioned in these tissues. This is the first study in literature that measured two types of endocannabinoids (ANA and 2-AG) and several immune system parameters (B lymphocyte, NK cell, T lymphocyte, helper T lymphocyte, cytolytic T lymphocyte and activated T lymphocyte percentages) in three types of tissue (blood, spleen, thymus) at two different exercise intensities (moderate and endurance) in same type of animals at tissue and gene level in the same study.

1.4 Limitations

The limitations of this study were the effect of general anesthesia on the endocannabinoid system and the stress caused by the treadmill. Previous studies showed that general anesthesia causes an increase in the ANA and 2-AG plasma levels (Schelling et al., 2006); however, since all groups have been exposed to ether in our study, we used the endocannabinoid levels of the control group as the reference point for basal level of endocannabinoids ANA and 2-AG in plasma. Moreover, employing activity on a treadmill produces physiological adaptations indicative of chronic stress in Spraque Dawley rats (Moraska et al., 2000) and these changes could potentially increase the ANA plasma levels. Therefore, the control group was placed on the treadmill for one hour without any exercise in order to eliminate the stress caused by the treadmill itself.

Although we planned to sample blood before and after the exercise session at the end of the study of part 1 and part 2, the total rat blood volume allowed only a single measurement of endocannabinoids. Therefore, we were not able to measure the resting values of the dependent variables in highly trained rats. It was a limiting factor also in the immune effector cell assessment. Since only low amount of blood was available for measurements in flow cytometric analysis, commercial available T/B/NK cocktail, T lymphocyte cocktail and activated T lymphocyte cocktail kits were used to determine immune effector cell percentages, T lymphocyte subtypes and activation of these cells. However, the cluster of differentiation markers used in these cocktails was to some extend not tightly cell specific and could be expressed also on some other leukocytes that are not of interest. For instance, T lymphocyte Cocktail was used to identify T lymphocytes, helper T lymphocytes and cytolytic T lymphocytes based on a three-color reagent cocktail by direct immunofluorescence staining with flow cytometric analysis. The OX-35 antibody was used to determine helper T lymphocytes and reacts with the CD4 antigen on most thymocytes which is a subpopulation of mature T lymphocytes (ie, MHC class II-restricted T cells, including most T helper cells). However,

CD4 antigen is also found on monocytes, macrophages, and some dendritic cells which were not of interest in our study.

Another limitation of this study was the method used for assessment of immune system response. Radioactive labeled immunoassays in order to measure proliferation, migration and immune effector cell activities could not be performed in this study since they are also expensive, time consuming and complex measurements with lot of pre-treatments needed to isolate the specific cells of interest.

CHAPTER 2

LITERATURE REVIEW

2.1 Exercise and the immune system

The immune system comprises a range of cells, tissues and chemicals that interact to infection, repair tissue damage and maintain the integrity of the body. The immune response is affected by many factors such as nutrition, environment, stress, genetical & neurological aspects and especially muscular exercise (Calabrese, 1996; Gleeson, 2007).

Exercise leads to altered distribution and function of immune effector cells which may be related in part to changes in hormone release, blood flow distribution, and other factors that affect immune system function (Frances et al., 2008). Results of studies in the field of exercise immunology are contradictory, most probably because of the complexity of the immune system and variability of exercise and training protocols in addition to training status of the subjects, nutrition, age, gender, blood sampling time and methods, immune effector cell types and methods used for measurements (Quist et al., 2006; Viru and Viru, 2000; Woods et al., 1997). Contradictory results have been obtained by many studies stating both positive and negative effects of exercise (Cara and Chaiken, 2006; Raso et al., 2007; Sasse et al., 2008). Therefore, it was not possible to compare studies focused on exercise induced immune system alterations and figure out the mechanistic explanation for the bidirectional effects. However, to date, it is well accepted that immune system response is an orchestrated regulation of almost all immune effector cells and is highly depending on the exercise intensity and duration (Raso et al., 2007; Rowbottom and Green, 2000) (Table 2.1).

Table 2.1 Immune effector cells and their main functions studied in exercise depending immune system alterations

Cell type	Marker	Proportion	Major cell function
Neutrophils		70 % of leukocytes	Phagocytosis
Monocytes	CD 14	10-15 % of leukocytes	Phagocytosis, antigen-presentation, cytokine production, cytotoxicity
Lymphocytes	Several	20-25 % of leukocytes	Innate immune response, cytotoxicity, memory, cytokine production
B cells	CD19 CD20	5-15 % of lymphocytes	Memory, antibody production
NK cells	CD16, CD56	5-15 % of lymphocytes	cytotoxicity, cytokine production
T lymphocytes	CD3	60-75 % of leukocytes	Immune regulation, immune cell activation, memory, cytokine production
Helper T lymphocytes	CD4	60-75 % of T lymphocytes	Immune cell activation, cytokine production, antigen recognition
Cytolytic T lymphocytes	CD8	30-45 % of T lymphocytes	Cytotoxicity

During the past few years knowledge has accumulated regarding the effect of physical exercise on different components of the immune system, however, it is still debated, whether physical exercise is beneficial to the immune system or not, and how exercise influences resistance to infectious diseases and tumor growth (Bacurau et al., 2007; Davis et al., 1998; Gleeson, 2007; Kruk and Aboul-Enein, 2007; Lowder et al., 2006; Vanitallie, 2002). In spite of the contradictive results, it is believed that the immune response is enhanced during moderate and severe exercise, but suppressed only after exercise of high intensity and long duration (endurance type of exercise).

Moreover, the exercise-induced immune suppression or immune enhancement is mostly transitory and last differently for different immune effector cell populations (Gleeson, 2005; Kumae et al., 2009; McFarlin et al., 2004; Murakami et al., 2009; Pyne et al., 2000).

2.1.1 Effects of acute and chronic exercise on the immune response

Acute exercise causes a rapid delocalization of various immune cells between lymphoid tissue and circulating blood (Schwindt et al., 2007). The leukocyte number increases during and after a bout of exercise most likely due to detachment from the endothelial lining of bone marrow, lung, spleen, and gastrointestinal tract (Malm, 2004). The increase in the leukocyte count is due primarily to an increase in neutrophils, although lymphocytes and monocytes are increased as well (Schwindt et al., 2007; Shephard, 1997). Exercise intensity, duration and/or fitness level may play a role in the degree of leukocytosis. The more prolonged and high intensity the exercise is the more cell trafficking occurs (McFarlin et al., 2006; Pedersen, 2005). Leukocyte number during exercise can increase four times the resting levels. They return to baseline values after exercise but increase again during the 1-3 post exercise period when the exercise lasted less than 30 minutes (Mackinnon, 1999).

There is conflicting data on the effect of exercise on granulocytes (Lagranha et al., 2004; Pyne et al., 1995). Some investigators report an intensity and duration dependent increase in neutrophil concentration both during and after exercise (Pedersen et al., 1997; Mackinnon, 1999; Murakami et al., 2009). Neutrophils are recruited into the circulation during and after intense exercise and can reach also levels 4 times higher than baseline values. They return to baseline levels within 1 hour after moderate exercise but remain elevated above resting values for 6 hours after endurance exercise and suffered apoptosis (Lagranha et al., 2004; Mackinnon, 1999; Nieman et al., 2005). It has been suggested that neutrophilia is under the control of cortisol, however, this hypothesis was contradicted by studies that showed sustained neutrophil number during increasing cortisol levels (Deutsch et al., 2007; Pedersen and

Hoffman-Goetz 2000). A recent study stated significant alteration in neutrophil gene expression following high intensity exercise, indicating exercise induced immune system adaptations at cellular level (Radom Aizik 2008).

It is well accepted that sampling time, mode of exercise and method of analysis has great effect whether exercise causes an increase or decrease in neutrophil function (Levada et al., 2008; Mackinnon, 1999; Pyne et al., 2000). Although, a study showed that neutrophil oxidative burst activity is reduced following prolonged exercise (Murphy et al., 2007), contradictive results have been obtained in literature that showed a great variability in neutrophil response of subjects exercising at similar exercise intensities but having different training status (Avloniti et al., 2007; Peake, 2002; Smith et al., 1990). To date, it is accepted that alterations in neutrophil number and/or function are affected by illness and/or muscle damage rather than the physical exercise itself (Carrigan et al., 2007; Newburger, 2006; Mc Farlin et al., 2006; Peake et al., 2005).

Two hours after severe exercise (e.g. bicycle exercise, 1 hour, 75% of VO_2max) the neutrophil count is still elevated, whereas the lymphocyte concentration declines below pre exercise values (Bury and Pirnary, 1995). The number of lymphocyte mostly increases during acute moderate and strenuous exercise but rapidly falls to baseline levels or below in post exercise period (Mackinnon, 1999; Pedersen et al., 1997; Tanimura et al., 2008; Wang and Huang, 2005; Wang and Lin, 2009). Although there is an increase in the numbers of lymphocytes during exercise, the ability of lymphocytes to proliferate decrease. If the exercise lasts at least one hour, lymphocyte number may fall below resting levels even during the exercise session (Levada et al., 2008; Levada et al., 2009). Lymphocyte subsets respond differently to exercise, resulting in changes in relative proportions of T, B and NK cells (Gleenson, 2007).

T cells show a greater response to exercise than B cells and memory T ($CD45RO^+$) cells circulate at a greater proportion than naive T ($CD45RA^+$) cells during and after acute exercise (Malm et al., 1999; Pedersen and Hoffman-Goetz, 2000). Although both helper and cytolytic T cell counts increase, the

CD4/CD8 ratio decreases because of relatively higher increase of cytolytic T cells than helper T cells (Gleenson, 2007; Gleeson et al., 1995; McFarlin et al., 2005). Not only the number, but also activity of T cells increased during strenuous exercise (Ibfelt et al., 2002) whereas eccentric exercise caused a decrease in cytolytic T cell activity, T cell proliferation rate and helper T cells number (Malm et al., 1999). Malm indicated that moderate exercise enhances a TH1-type cytokine response, while strenuous exercise augments TH2 cytokines and, thus, promotes protection against bacterial infection. Exercise induces only a little change in B cell numbers persisting for a short time after exercise (Mackinnon, 1999; Pedersen et al., 1997; Plowman and Smith, 2007). B cells increase after anaerobic exercise or exercise having an eccentric component (Malm et al., 1999; Malm et al., 2000). All lymphocytes decline below pre-exercising counts between 1-3 hours after intense exercise.

NK cells are thought to play an important role in the first line of defence and play role in resistance to cancer. It is well known that NK cells are the most influenced cells by exercise (Pedersen, 2005; Sharon and Plowman, 2007; Wang et al., 2009). Both absolute concentration and the relative percentage of NK cell and their activity are increased during and after acute exercise. Endurance type of exercise may cause an increase during and decrease after exercise in a natural killer cell function on a per cell basis (McFarlin et al., 2005).

Circulating monocyte numbers and activity increased after moderate acute exercise and remained for up to 6 hours post exercise, however, decreased during endurance training periods. However, the decreased activity of monocytes could be enhanced by decreased training programmes in endurance athletes (Guereschi et al., 2008; Hack et al., 1994; Mackinnon, 1999; Plowman and Smith, 2007). Monocyte cell surface receptor expression was also affected by exercise depending on the mode, duration and intensity (Guereschi et al., 2008; Malm et al., 2000; Prestes et al., 2008; Starkie et al., 2000).

Of all immune measures, mostly NK cell activity has emerged as a consistent indicator differentiating the immune systems of athletes and

nonathletes. In a study comparing elite female rowers and controls, NK cell activity measured 1.6-fold higher in the rowers (Nieman, 2000b). This increase in NK cell activity during most hours of the day should help the athlete clear viruses from the body, countering the stressful changes that occur in the immune system during the several hours after each bout of intensive exercise. However, recent literature argued these outcomes so that chronic resistant exercise training increased both NK cell number and natural killer cekk activity (NKCA) after a bout of acute exercise but had no effect in resting status in both parameters (McFarlin et al., 2005).

Neutrophil function has been reported to be both suppressed and enhanced in athletes, depending on the intensity of exercise (Mackinnon, 1999). Although there was no difference in resting levels of neutrophil numbers between athletes and sedentary controls, neutrophil activity was found to be higher in athletes. Lower leukocyte and NK cell counts were identified in athletes after prolonged periods of endurance exercise (Gleeson, 2007). Other studies on the contrary stated higher baseline levels of natural killer cell activity in endurance athletes (Nieman et al., 2000; Shore et al., 1999). Nieman (2000) stated significantly elevated natural killer cell activity in rowers compared to non-athletes. Other similar studies involving moderate activity for eight to fifteen weeks showed no significant increase. Thus, endurance exercise may have to be intense and prolonged to obtain this effect. It remains unclear whether the increased natural killer cell activity described is due to an increased number of cells, enhanced activity of individual cells or both (Nieman et al., 2000).

Studies comparing athletes with sedentary controls found no changes in the resting lymphocyte numbers, even in overtrained athletes (Mackinnon, 2000). Although it is accepted that both helper and cytolytic T cells are resistant to chronic exercise (Mackinnon, 2000; Pedersen and Hoffman-Goetz, 2000), a study showed an increase in these T cell subsets in the pre-season of soccer training (Malm et al., 2004). B cells, however, has been found to decrease in the same study. A recent study, showed that resistance training increased NK cells,

helper and cytolytic T cells immediately after an acute exercise session, but had no effect on the basal resting levels of these cells (McFarlin et al., 2005).Resting salivary IgA levels measured in swimmers showed significant correlations with infection rates and number of infections observed could be predicted by the pre-season salivary IgA levels (Gleeson, 2007). Long term training had also an effect on serum cytokines and altered them as in the state of fever (Rowsey et al., 2009).

2.1.2 Effect of exercise intensity

The intensity of exercise more than the duration seems to determine to which degree the immune system is enhanced during exercise. However, a combination of intensity and duration determines whether the immune system is enhanced and/or suppressed following exercise. The exercise induced immune response is a function of exercise intensity and duration and is thought to be enhanced during moderate and severe exercise, but suppressed only after exercise of high intensity and long duration (Kumae et al., 2009; McFarlin et al., 2004; Murakami et al., 2009; Pyne et al., 2000). At rest the immune response is slightly elevated in trained compared to untrained. Research showed that during moderate exercise, several positive changes occur in the immune system (Cara and Chaiken , 2006; Cooper, 2006; Nieman et al., 2000; Oguma and Shinoda-Tagawa, 2004; Pedersen, 2006; Prestes et al., 2008; Shea et al., 2004; Strasser et al., 2006). Stress hormones, which can suppress immunity, and pro- and anti-inflammatory cytokines are not elevated during moderate exercise, but increases with prolonged exercise (Steinberg et al., 2007; Stewart et al., 2007). Although the immune system returns to pre-exercise levels very quickly after a single bout of exercise, each bout represents an augmentation in immune surveillance that appears to reduce the risk of infection over the long term (Nieman et al., 2005; Nieman et al., 2006).

Numerous surveys in the fields of exercise immunology and sports science indicate that physically active individuals experience fewer colds than

their sedentary peers, indicating that moderate type of exercise reduces the number of days with sickness (Davis et al., 2004; Martin et al., 2009; Nieman, 2000b; Shore et al., 1999).Walking exercise for 45 minutes per day and performed for 3 days weekly for three months during the winter/spring or fall reduced the number of days with cold symptoms by half compared to the control groups which remained inactive (Nieman et al., 1990). Another study including 547 adults demonstrated a reduction in risk of upper respiratory tract infection (URTI) in those engaging in regular versus irregular moderate-to-vigorous physical exercise (Matthews et al., 2002).

It has been theorized that each bout of prolonged and intensive exercise such as endurance exercise leads to transient but clinically significant dramatically changes in immune function (Nieman, 1997). Some believe a time exists immediately following intense exercise, referred to as an “open window”, when infection risk is increased (Pedersen and Bruunsgraad, 1995). Although it is assumed that “open window” can last from 3 to 72 hours depending on the intensity and duration of exercise, it has been shown that lymphocytes and NK cells may also change parallel to this theory after moderate exercise (Ortega et al., 2005).

Although it is believed that infection risk decreases with regular moderate exercise and increases with strenuous long duration exercise (Martin et al., 2009; Wong et al., 2008), a study showed that triathletes had normal antibody production to pneumococcal, tetanus, and diphtheria vaccines following a half-Ironman triathlon competitive event. However, the skin test response to seven recall antigens applied after the race and measured 48 hours later was suppressed when compared to control subjects (Bruunsgaard et al., 1997). These data suggest that the short term but complex immunological reaction to the delayed type hypersensitivity skin test is negatively affected by prolonged and intensive exercise, whereas the longer-term, antibody-titer response to vaccination is not affected. While literature consists of data giving support to the “open window” theory (Chubak 2006; Ekblom et al., 2006; Wong et al., 2008), additional research is needed to establish a link with infection risk.

Epidemiological studies suggest that URTI risk is elevated during periods of heavy training and in the 1-2 week period following participation in competitive endurance races especially when athletes exceed individually identifiable training thresholds (Ekblom et al., 2006; Nieman, 2003; Suzuki et al., 2002; Suzuki et al., 2003). Many elite athletes have reported significant bouts with URTI that have interfered with their ability to compete and train. Studies performed with runners showed that sickness rates following a competitive marathon race have been observed to be highest in those exhibiting the lowest salivary IgA levels. Following high intensity exercise for long duration may cause decreased nasal and salivary IgA concentration, nasal mucociliary clearance, and nasal neutrophil function which indicate an impaired ability of the upper respiratory tract to clear external pathogens and may explain the increased risk of URTI in endurance athletes (Martin et al., 2009).

2.2 Endocannabinoids and the immune system

The presence of endogenous cannabinoid ligands, N-arachidonoyl ethanolamine, called ANA (Devane et al., 1992), and 2-AG (Lee et al., 1995; Mechoulam et al., 1995), with their receptors on immune cells suggests that there is a close relationship between the endocannabinoid and immune system. Both endocannabinoids are arachidonic acid derivatives arising from the membrane fatty acid matrix of various cells including neurons and immune cells (Ahn et al., 2008; Di Marzo et al., 2004; Sugiura et al., 2002).

Both CB1 and CB2 mRNA have been detected in various human immune effector cells with the order of B cells, NK cells, T cells, T cell subtypes (helper and cytolytic T cells), neutrophils and macrophage/monocytes (Bouaboula et al., 1993; Galieque et al., 1995; Munro et al., 1993; Rayman et al., 2004). RT-PCR results showed that the expression level of CB1 and CB2 receptors vary among immune organs and is quite higher in mouse spleen when compared to thymus (Kaminski et al., 1998; Lee et al., 1995; Schatz et al., 1997). CB2 cannabinoid receptor mRNAs were also detected in preimplantaion mouse

embryo (Paria et al., 1995), macrophage/monocytes of the marginal zone of the spleen (Munro et al., 1993), in the cortex of the lymph nodes and the nodular corona of Peyer's patches (Lynn and Herkenham, 1994). The importance of CB2 receptor activation in the immune modulatory effects of endocannabinoids is supported by CB2 receptor activation in autoimmune diseases and many pathological conditions such as inflammatory pain, myocardial infarction, stroke, hepatic I/R injury, gastrointestinal inflammatory disorders, liver inflammatory disorders and atherosclerosis (Marquez et al., 2008; Massa et al., 2004; Montecucco et al., 2009; Pacher and Gao, 2008; Sipe et al., 2005). Moreover, studies performed on mice having CB2 receptor null mutation has clearly shown that B and T lymphocyte subset formation requires CB2 receptor (Ziring et al., 2006). Although expression level of CB2 gene in immune tissues appears to be 10–100 times more than CB1 (Shen and Thayer, 1999), studies showed that CB1 receptor is also involved in the regulation of the immune system (Sugamura et al., 2009). In spite of structural difference between the two types of cannabinoid receptors, most exogenous and endogenous cannabinoids showed similar binding affinity to both receptor subtypes (Pertwee, 1999). Not only the receptors, but also their endogenous ligands show variety in mammalian tissues. Further detailed data for CB receptor genes, distribution and functional properties and their ligands can be reviewed by Pacher (2006).

It has been suggested that endocannabinoids have local and limited effects since they are mainly lipophilic and it would be difficult for them to pass the cell membrane However, it has been shown that they travel as far as 20 μm from their sites of origin (Wilson et al., 2001) and for long-distance-travel, the existence of endocannabinoid binding proteins has been proposed (Glaser et al., 2003; Glaser et al., 2005), such as albumin (Bojensen and Hansen, 2003). They can move sideways within the plasma membrane, where they eventually interact with intramembranous binding-sites within the CB1 receptor (Song and Bonner, 1996).

Literature has shown that exogenous and endogenous cannabinoids have various effects on innate and adaptive immune response (Facchinetti et al., 2003; Waksman et al., 1999), such as impairing macrophage functions (Klein et al., 2003; Sugamura et al., 2009), reducing macrophage chemotaxis (Steffens et al., 2005), downregulating activation of helper T cells (Malfitano et al., 2006; Rockwell et al., 2006), disturbing Tcell CD4/CD8 ratio (Hegde et al., 2008; McKallip et al., 2005), downregulating NK cell activity (Klein et al., 1987; Massi et al., 2000; Specter et al., 1986), decreasing cytolytic T lymphocyte activity (Klein et al., 1991), inhibiting cytolytic T cell migration(Joseph et al., 2004), effecting B cell differentiation (Carayon et al., 1998; Ziring et al., 2006), and giving rise to impaired host defense against viral, bacterial, or protozoan infections (Cabral and Dove Pettit, 1998; Cabral and Marciano-Cabral, 2004; Friedman et al., 1995; Klein et al., 1998). While they have regulatory effects on T and B lymphocyte proliferation and apoptosis, they also modulate macrophage-mediated phagocytosis, inflammatory cytokine production, chemotaxis and inflammatory cell migration (Klein et al., 2003; Klein, 2005). Moreover, studies have shown that exogenous and endogenous cannabinoids are able to inhibit tumor angiogenesis and induce apoptosis (Blazquez et al., 2004; Carracedo et al., 2006; Sarfaraz et al., 2008).

In recent studies, it has been suggested that endogenous and exogenous cannabinoids may control or suppress immune response through their ability to induce apoptosis not only in thymocytes and splenocytes (McKallip et al., 2002), but also in several other cell types (Fernandez-Ruiz et al., 2007; Filippis et al., 2008; Jacobsson et al., 2000). Kaminski (1998) suggested that CB2 mediated inhibition of the cAMP/protein kinase A (PKA) pathway may be responsible for the immunosuppressive action of cannabinoids. In addition, cannabinoids may act also at the nuclear level for apoptosis in the immune effector cells (Do et al., 2004; Malfitano et al., 2006; Rockwell et al., 2006). Moreover, VR1 vanilloid receptor activation by ANA has been reported to trigger apoptosis through a cascade of events, including increase in cytosolic Ca2+ concentration, cyclo and lipoxygenase activation, drop in mitochondrial

membrane potential, cytochrome c release and caspase activation, that finally execute apoptotic cell death (Maccarrone et al., 2000). However, there are contradictory results whether ANA binds to TRPV1 receptors or not (Contassot et al., 2004; Contassot et al., 2004; Pacher et al., 2004; Zygmunt et al., 1999).

Cannabinoids were shown to impair antigen processing in macrophages but not their presentation to T-lymphocytes mediated by the cannabinoid CB2 receptor (Buckley et al., 2000). A study confirmed impaired macrophage/Tcell cooperation through cannabinoid induced decrease in IFN-γ and TNF-α production by stimulated mouse splenocytes and peritoneal macrophages (Banchereau et al., 2000). Disturbed macrophage/Tcell cooperation and functioning has a direct effect on antibody production by B cells. This finding was supported by several in vivo (Titishov et al., 1989; Nahas and Osserman, 1991; Schatz et al., 1993) and in vitro (Kaminski et al., 1994; Klein and Friedman 1990) studies which demonstrated inhibition of antibody formation by natural and synthetic cannabinoids at μmolar concentrations.

In addition to TNF-α, cannabinoids downregulated also the expression of class II, Ia molecules of the major histocompatibility complex (MHC) that has also a major importance for macrophage/Tcell cooperation (Fischer-Stenger et al., 1993; Zheng and Specter, 1996). Moreover, cannabinoids inhibited the secretion of IL-1β in addition to TNF-α (Klegeris et al., 2003), enhanced the transcription of several apoptotic genes regulated by NFkappaB (Doet al., 2004) and blocked the G1/S phase transition in stimulated peripheral blood mononuclear cells (Malfitano et al., 2006).

Although the precise mechanism of cannabinoid induced immune dysfunction is not clear, cannabinoids may cause disturbance in the cytokine production by immune cells (Do et al., 2004; Klein et al., 2000). It is hypothesised that cannabinoids may stimulate the inhibition of adenylyl cyclase in thymocytes and splenocytes (Croxford and Yamamura, 2005), which in turn effects cAMP dependent signalling pathways that regulate cytokine mRNA transcription in macrophages. Cannabinoids caused suppression in IL-2 expression in leukocytes (Rockwell et al., 2006; Rockwell et al., 2008) and

decreased levels of IL-1α, IL-1β and TNF-α mRNA (Cabral and Marciano-Cabral, 2004). A study showed that cannabinoids suppressed IL-12 and enhanced IL-10 production in LPS stimulated mouse macrophages (Correa et al., 2005). The relationship between exogenous and endogenous cannabinoids and cytokines is bidirectional. While cannabinoids may inhibit IFN-γ production and IL-12 secretion, cytokines influences directly the endocannabinoid system by regulating the degrading enzymes IL-4 or IL-10 stimulated FAAH activity and IL-12 and IFN-γ reduced FAAH activity and protein expression of FAAH (Maccarrone et al., 2001; Sacerdote et al., 2005).

However the effect of exogenous and endogenous cannabinoids may be differ in their effect on the immune response since the administration dose of synthetic cannabinoids is mostly above the biological concentrations (Kraft et al., 2004). A study showed inhibition of T cell proliferation in response to cannabinoids after administration of high doses to animals (Klein et al., 1998), whereas low nanomolar concentrations showed the opposite, a stimulatory effect on human B cell proliferation (Derocqet al., 1995) and mouse splenocyte poliferation (Luo et al., 1992). Schwarz (1994) stated that ANA caused dose dependent inhibiton in T and B cell proliferation at concentrations likely to be found in biological systems. Analogues of ANA were shown to downregulate activation of helper T cells and inhibit adenylate cyclase activity in splenocytes and thymocytes (Malfitano et al., 2006). As compared to synthetic cannabinoids, ANA was 3 fold less potent and low concentrations caused significant inhibition of lymphocytes proliferation and DNA fragmentation, whereas high concentrations induced apoptosis. ANA also inhibited macrophage killing of TNF-α sensitive cells, and downregulated IL-4, IL-6 and IL-8 production in the same cells at concentrations parallel to be found in living organisms (Berdyshev et al., 1997; Cabral et al., 1995; Carrier et al., 2006; Kishimoto et al., 2004).

Both ANA and 2-AG have diverse and common effects on the immune effector cells depending on the cell type and concentration of the endogenous cannabinoid. Although LPS led to a high increase in the production of ANA by

lymphocytes, 2-AG concentration remained unaltered. Therefore, it is thought that ANA participate in blood cell to cell communication, since, ANA can cross the blood-brain barrier to inhibit cytokine production (Puffenbarger et al., 2000), NO and TNF α response to endotoxins (Molina-Holgado et al., 1997) and stimulation of NO synthase (Stefano et al., 1998). Moreover, ANA at high concentrations caused cell rounding, loss of motility, blocked chemotaxis and stimulated NO release in macrophages (Klein, 2005; Klein et al., 2003; Stefano et al., 1998). It also decreased TNF-α level in bronchoalveolar lavage and diminished neutrophil recruitment in the lung of mice (Jeon et al., 1996). ANA and N-palmitoylethanolamine stimulated growth of murine hematopoietic cell lines in serum free medium (Derocq et al., 1998). Other studies however, proposed that 2-AG rather than ANA is the true endogenous ligand for both CB1 and CB2 receptors located on immune cells membranes (Gonsiorek et al., 2000; Sugiura et al., 1999, Sugiura et al., 2000). 2-AG but not ANA could inhibit lymphocyte response, T and B cell proliferation (Lee et al., 1995) and downregulation of IL-2 synthesis by mouse splenocytes (Ouyang et al., 1998; Rockwell et al., 2008). Moreover, 2-AG suppressed IL-2 expression in leukocytes (Rockwell et al., 2006), induced the production of IL-8 (Kishimoto et al., 2004) and enhanced the adhesion of peripheral blood monocytes (Gokoh et al., 2005). In addition, 2-AG inhibited IL-6 production, but increased iNOS dependent NO and resulted in NO release, which was blocked by the NOS inhibitor, l-NAME and a CB1 antagonist, but not by a CB2 antagonist production (Chang et al., 2001). There is no doubt that both ANA and 2-AG are able to modulate immune response but the role for cannabinoid receptors in this response is completely unclear.

As a summary, it is likely to conclude that endocannabinoid system with its various receptors, ligands and pathways, has very complex effect on the immune system with an influence on almost every component of immune system in order to maintain or restore the well-controlled and finely tuned balance in immune response. Although the results of the studies focused on the relationship between endocannabinoid and immune systems were conflicting, it

seems possible that the endocannabinoid signalling system plays a negative role in the onset of the immune response, but the exact role for endocannabinoids and cannabinoid receptors in the maintenance of immune system homeostasis and the development of immune response needs to be defined in future.

CHAPTER 3

MATERIALS AND METHODS

3.1 Study design

A longituidinal, prospective, randomized and controlled study was designed. The study consisted of two parts. The first part of the study was conducted to determine whether moderate and/or endurance type of exercise has an effect on the endocannabinoid system. The independent variable was the intensity of exercise and the groups were labeled as Control (C), Moderate type of exercise (M) and Endurance type of exercise (H). (Figure 3.1)

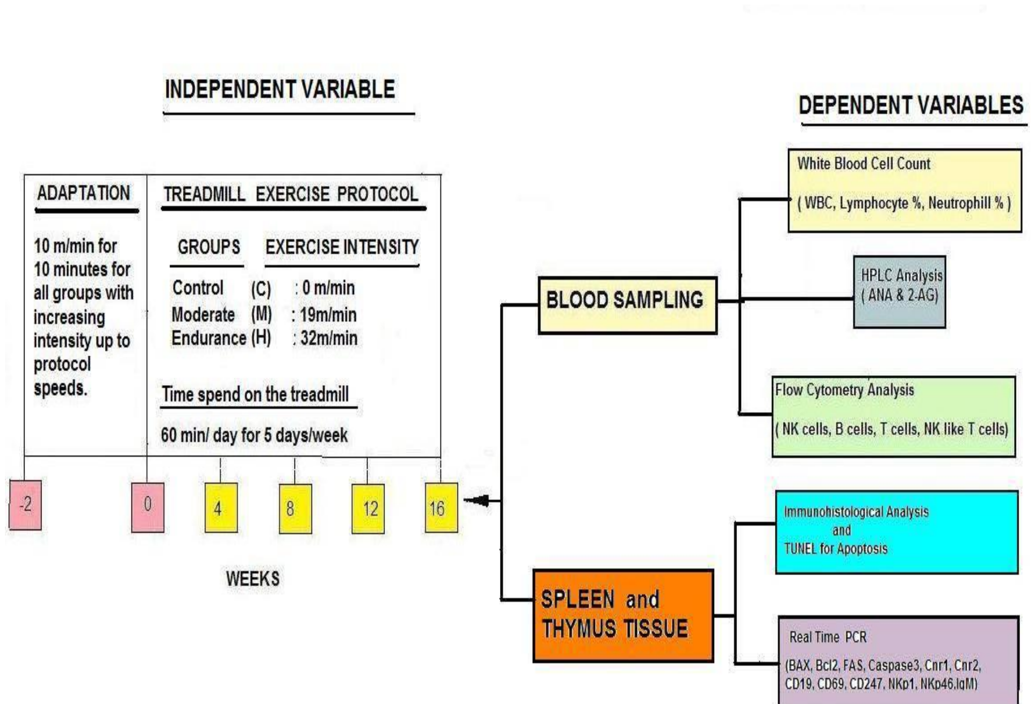


Figure 3.1 The design of the first part of the study

The dependent variables in part 1 of the study were;

(a) Endocannabinoid serum levels: Anandamide, 2-Arachidonylglycerol

(b) Immune effector cells: Natural killer cell ($CD3^{-}CD161a^{+}$), B lymphocyte cell($CD3^{-}CD45RA^{+}$), T lymphocyte cell ($CD3^{+}$), natural killer- like T lymphocyte ($CD3^{+}CD161a^{+}$) cell counts and percentages in addition to white blood cell count, lymphocyte percentage and neutrophil percentage in blood.

(c) Adaptation of the immune system at the lymphoid tissue level: Immunohistological analysis CD3, CD8, CD45, CD45RA, CD56 in spleen and thymus.

(d) Alterations in transcription levels in the endocannabinoid and the immune systems genes in lymphoid organs**:** Up or down regulation of the genes coding for CB receptors (Cnr1, Cnr2), apoptosis pathway (BAX, Bcl2, FAS, Caspase 3), B lymphocytes and their activity (CD19, IgM), T lymphocytes and their activity (CD247,CD69) and natural killer cells and their activity (NKR-P1a, NK-p46) in spleen and thymus tissues.

Results of the first part of the study revealed that endurance type of exercise affected the endocannabinoid system and caused alterations in the immune system. Therefore, the second part of the study was performed to characterize the effect of endurance type of exercise and to determine which receptor(s) take part in this mechanism. Independent variables were endurance type of exercise and two different antagonists for CB1and CB2 receptors blockade (AM281 and AM630), respectively. Groups were labeled as in Figure 3.2.

The dependent variables in part 2 of the study were;

(a) Anandamide plasma level

(b) Immune effector cells: Natural killer cell ($CD3^{-}CD161a^{+}$), B lymphocyte ($CD3^{-}$ $CD45RA^{+}$), T lymphocyte($CD3^{+}$), helper T lymphocyte($CD3^{+}CD4^{+}$), cytolytic

T lymphocyte ($CD3^+CD8a^+$), double positive T lymphocyte ($CD3^+$ $CD4^+CD8a^+$) and natural killer- like T lymphocyte ($CD3^+CD161a^+$) percentages in addition to T cell activity ($CD3^+CD25^+$ and $CD3^+RT1b^+$) in blood, spleen and thymus.

(c) Adaptation at tissue level in lymphoid organs: Immunohistological analysis of CD3, CD8, CD45, CD45RA, CD56 in spleen and thymus.

(d) Adaptation at gene level in lymphoid organs: Up or down regulation of the genes coding for endocannabinoid and immune system parameters in spleen and thymus tissues analyzed by microarray.

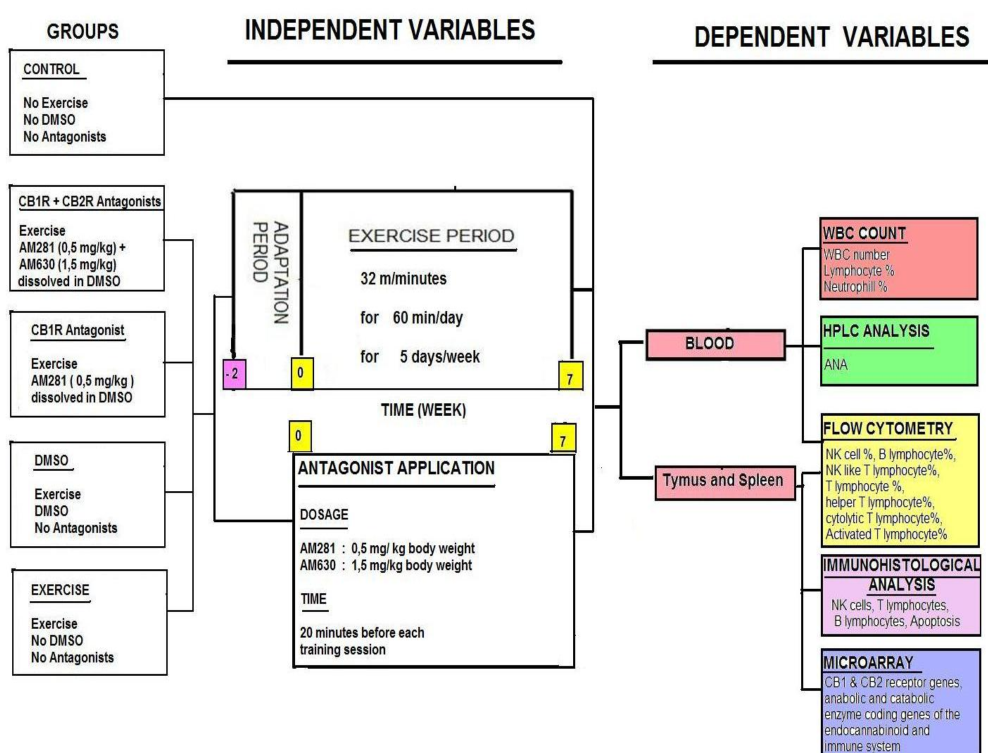


Figure 3.2 Design of the second part of the study

3.2 Chemicals

❖ **Antagonists**

- CB1 receptor antagonist AM 281 (Tocris Bioscience, United States)
- CB2 receptor antagonist AM 630 (Tocris Bioscience, United States)
- Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, United States)

❖ **Blood Sampling and Tissue Extraction**

- PBS (Sigma-Aldrich, United States)
- Squeeze bottle with 70% ethanol (Sigma-Aldrich, United States)
- Scissors and forceps
- Cutting board or paper towels
- 60x 15-mm Petri dishes
- Stainless steel screen (Thomas)
- Glass tissue homogenizer (Fisher)
- Fetal calf serum (FCS) (Sigma-Aldrich, United States)
- 15-ml conical centrifuge tube
- Vacutainer tubes

❖ **Quantitative Assessment of Endocannabinoids**

- Phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich, United States)
- Affi-Prep Polymyxin matrix (Bio-Rad Laboratories, Hercules, CA).
- Synthetic Anandamide (Sigma-Aldrich, United States)
- Synthetic 2-Arachidondylglycerol (Sigma-Aldrich, United States)
- Ethanol and acetonitrile (Sigma-Aldrich, United States)
- Ethylendiaminetetraacetic acid (EDTA) (Beckton Dickenson, France)
- Saline solution (Sigma-Aldrich, United States)

❖ **Flow Cytomerty Analysis**

- Ig Isotype Control Cocktail-A (BD Pharmingen, United States)
- T/B/NK Lypphocyte Cocktail-A (BD Pharmingen, United States)
- T Lympocyte Coctail (BD Pharmingen, United States)
- Activated T Lympocyte Coctail (BD Pharmingen, United States)
- Cell Wash Solution (BD Pharmingen, United States)
- FACS Lysing Solution (BD Pharmingen, United States)
- Phosphate Buffered Saline Solution (Sigma-Aldrich, United States)

❖ **Immunohistological Analysis**

- Anti-mouse HRP-DAB staining kit (R&D, United States)
- (BALB/c) IgG_3 (CD3) (BD Pharmingen, United States)
- Mouse(BALB/c) IgG_1,k (CD8a) (BD Pharmingen, United States)
- Mouse(BALB/c) IgG_1,k (CD8b) (BD Pharmingen, United States)
- Mouse IgA,k (CD11b) (BD Pharmingen, United States)
- Mouse(BALB/c) IgG1,k (CD25) (BD Pharmingen, United States)
- Mouse(BALB/c) IgG1,k (CD45) (BD Pharmingen, United States)
- Mouse IgG_1,k (CD45 RA) (BD Pharmingen, United States)
- RatIgG2a (CD56) (BD Pharmingen, United States)
- Mouse IgG2a (CD56E) (BD Pharmingen, United States)
- TrisHCl containing 0.1% tween 20 (Dako, Denmark).
- Entellan paraformaldehyde (Merck, Germany).
- 3.3’-diaminobenzidine-tetrahydrochloride (DAB)(Sigma-Aldrich, USA)
- 0,01 M phosphate buffered saline (PBS) (Sigma-Aldrich, United States)
- biotinilated goat anti mouse secondary antibody kit (R&D, United States)
- Streptavidine peroxidase (R&D, United States)
- Xylene and alcohol (Merck, United States)
- ApopTag in situ detection kit (Q-biogene, United States).

- **RT-PCR**

- Thin wall, flat cap, DNase-free, RNase-free 0.2µl and 0.5 µl PCR tubes (Greiner Bio One, United States)
- DNase-free, RNase-free 20 µl, 100µl, 1000µl filter tips (Greiner Bio One, United States)
- TRIZOL Reagent (Fluka, Switzerland)
- 3-[N-Morpholino] propanesulfonic acid (MOPS) (Sigma, St Louis, United States)
- ImProm-II™ Reverse Transcription System (Promega Corporation, United States)
- dNTP Mix (Promega Corporation, United States)
- 100bp DNA Ladder(Promega Corporation, United States)
- 1kb DNA Ladder (Promega Corporation, United States)
- 50bp DNA StepLadder (Promega Corporation, United States)
- SYBR Gren JumpStart Taq ReadyMIX without MgCl2 (Sigma, St Louis, United States)
- DEPC (Sigma- Aldrich, United States)
- RNase ZAP (Sigma-Aldrich, United States)
- GoTaq Flexi DNA Polymerase (Promega Corporation, United States)

- **Microarray Analysis**

- Affimetrix Rat Genome RAE_230 2.0 expression probe array (Affymetrix, Inc., San Diego, CA, United States)
- Gene Chip Fluidics Workstation (Affymetrix Inc., San Diego, CA, United States)

3.3 Animals

72 male Sprague Dawley rats were obtained from Hacettepe University Animal Research and Production Laboratory (Ankara, Turkey). Animals were maintained under standard conditions at the same university with access to food and water *ad libidum*. Rats were housed 4 animals per cage under a 12 hours light and 12 hours dark cycle during the whole study. Housing environment was 22°C with a 19 % humidity level. The studies were carried out in accordance with the Guiding Principles in the Use of Animals in Experiments as adopted by the Hacettepe University Ethical Committee with the certification on 05/09/2006 and approval number 2006/53-8.

In the first part of the study, 32 male Sprague Dawley rats were categorized randomly into 3 groups labeled as; (a) Control (n=9), (b) Moderate (n=10) and (c) Endurance (n=12) type of exercise groups. In the second part of the study, 40 male Sprague Dawley rats were categorized randomly into 5 groups labeled as; a) Control (n=8), (b) Exercise (n=8), (c) DMSO (n=8), (d) AM281 (n=8) and (e) AM281+ AM630 (n=8). In order to observe adaptation in both immune and endocannabinoid system, young rats were chosen for this study (Quinn, 2005). All rats were 4 weeks old at the beginning of the adaptation period with an average weight of 197,8±17,0 gram.

3.4 Exercise Protocol

3.4.1 Adaptation period:

For both parts of the study, the adaptation period of rats to exercise was two weeks. During the first week, rats were trained at a velocity of 10 meters per minute for 10 minutes and for 5 days per week. During the second week of the adaptation period, the intensity and the duration of the exercise was gradually increased for the moderate and the endurance type of exercise groups up to their experimental exercise protocol levels. At the end of the adaptation level, animals started with their experimental exercise protocols.

3.4.2 Exercise period:

Figure 3.3 Rats were trained on a motor-driven treadmill according to their exercise protocols for 5 days/week

3.4.2.1 Part 1 of the study

Rats were exercised 5 days per week for 16 weeks on a motor-driven treadmill.

a) Non-exercising control group: In order to eliminate the stress caused by the treadmill itself (Moraska et al., 2000), animals were kept on the treadmill without running for 60 minutes for 5 days per week.

b) Moderate type of exercise group: Animals exercised at a velocity of 19 meters per minute for 60 minutes for 5 days per week according to Baptista (2008) and Carmeli (2005).

c) Endurance type of exercise group: Animals exercised at a velocity of 32 meters per minute for 60 minutes for 5 days per week according to Carmeli (2006; 2005). The statistically significant increase in the heart weight/ body weight ratio ($p= .001$) in the endurance type of exercise group at the end of the study was the confirmation of the high exercise intensity and duration in this group.

3.4.2.2 Part 2 of the study

Rats in all groups except the control group were exercised 5 days per week for 7 weeks on a motor-driven treadmill at a velocity of 32 m/min for 60 minutes per day. Antagonists AM281 (0,5 mg/kg body weight) and AM630 (1,5 mg/kg body weight) dissolved in DMSO were applied by intraperitoneal injection 20 minutes before each training session to the AM281 and AM281+AM630 groups (Figure 3.4). The DMSO group received only DMSO which was the vehicle of the CB1 and CB2 receptor antagonists.

Figure 3.4 Intraperitoneal injection of AM281 and AM630 dissolved in DMSO.

Animals in the control group were kept on the treadmill without running for 60 minutes for 5 days per week in order to eliminate the stress caused by the treadmill itself (Moraska et al., 2000).

3.4.3 Termination of the animals

Rats were anesthetized in a chamber containing ether and soaked with ethanol in order to prevent or reduce the possibility of hair becoming airborne. All rats were killed by hypovolemic shock since peripheral blood was collected by intracardiac puncture until death.

3.5 Measurments

3.5.1 Blood sampling and tissue extraction

3.5.1.1 Blood sampling

Rats were anesthetized and needle was inserted just below and slightly to the left of the xyphoid cartilage at the base of the sternum, at a 20° to 30° angle. The needle was advanced slowly, applying very slight negative pressure on the barrel of the syringe. Blood flowed into the hub of the needle when the tip was entered into the left ventricular chambers of the heart. Blood was gently aspirated until blood flow ceased. Death was ensured by cervical dislocation when collection was completed. Blood was handled according to the protocols for endocannabinoid assessment and flow cytometric analysis as described below in measurement section.

3.5.1.2 Lymphoid tissue extraction

Rats were placed on a cutting board (or on paper towel) and soaked it with ethanol to reduce the possibility of hair becoming airborne. A cut through the skin in the inguinal region was made and with fingers on both sides of the cut, it is pulled toward the tail and head until the peritoneal wall was sufficiently exposed. Peritoneal wall was soaked again with ethanol for sterilization and cut with a sterile scissors. The spleen is attached to the greater curvature of the stomach by connective tissue. Spleen was lifted with the

forceps and separated from connecting tissues with scissors. After placing the spleen into a Petri dish containing medium, it was cut into several smaller pieces and separately conserved according to the following protocols of flow cytometry (dipped into saline solution), immunohistological analysis(placed into cryovials and dipped into liquid nitrogen) and RNA isolation protocols (dipped into Trizol containing tube on ice).

The same protocol was performed for thymus. An incision in the chest was made, beginning at the xyphoid and extending to the neck with surgical scissors. The ribs were retracted with a curved forceps. Thymus is a yellowish-white bi-lobed organ found just under the ribs, attached above the heart in the midline. It was grasped with curved forceps and gently placed into a Petri dish containing medium and handled as the spleen described above.

3.5.2 Quantitative assessment of endocannabinoids

A set of parameters were tested to develop a sensitive method for the extraction and quantitative determination of the endocannabinoids, ANA and 2-AG from limited amounts of rat serum. Precise assessment of ANA and 2-AG serum levels is very difficult and important because of their very low serum levels (pmol/ml) and the labile structure. Moreover, pre-analytical blood sampling and handling conditions play important roles in determination of these highly reactive compounds in serum (Woods et al., 2008).

First, the HPLC method was modified by eliminating the time consuming derivatization procedure which contains hazardous chemicals. It was possible to detect ANA and 2-AG in the fractionation step and quantify it with the standard curve area measurement method (Savinainen et al., 2003). Retention time of serum ANA and 2-AG were determined using standard ANA and 2-AG solutions, at 3.2 and 4.2 minutes, respectively (Figure 3.5). By the quantification of ANA and 2-AG without derivatization, it was possible to avoid the usage of hazardous chemicals (Benzene and DBD-COCl) and shorten the time of the HPLC method for at least 1 hour.

While using the original procedure with the derivatization step described by Wang (2001), phase separation was observed in samples after the derivatization step and column atmospheric pressure started to increase over the tolerable levels after the injection of 15 samples. Moreover, sensitivity of the measurement did not increase with the derivatization step. Therefore, modifications were applied to the original procedure by eliminating the derivatization step of ANA and 2-AG with DBD-COCl at 60°C for 40 minutes (Koga et al., 1995; Savinainen et al., 2003; Wang et al., 2001).

Extraction and quantification of ANA and 2-AG in peripheral circulation is challenging. Collecting blood in to the suitable tube and immediate centrifugation is a critical step. Since endocannabinoids are very rapidly released from red blood cells in high quantities in ex vivo conditions (Vogeser et al., 2006), strictly standardized preanalytical protocols such as immediate centrifugation at 0°C was applied. In this study, ANA and 2-AG serum concentrations did not fluctuate due to the standardized pre-analytical protocol (Vogeser et al., 2006; Woods et al., 2008).

The blood volume was decreased from 3 ml to 1 ml whereas percent of serum in a sample solution was also reduced. Reducing the serum volume to 1 ml and diluting it with 3 volumes of ice-cold saline solution improved selective adsorption of the PMB-immobilized beads to ANA and 2-AG and prevented intense interference of other lipids and proteins in the serum. A clear separation between elutes by HPLC was observed as seen in Figure 3.5. Diluted serum naturally prevented non-selective adsorption and improved selective adsorption of ANA and 2-AG to the beads resulting in cleaner samples.

Wang (2001) investigated the effect of serum concentration in saline and serum mix. According to this study, cumulative recovery of ANA decreased with the increasing concentration of ANA in the serum solution. By increasing the dilution of ANA with saline solution, binding efficiency of ANA and 2-AG on PMB-immobilized beads was such improved that no any ANA or 2-AG concentrations could be observed in the washing saline solution before the elution step.

The saline solution used during the protocol was always ice cold. The cold glycerol-saline solution as the promising quenching solution for accurate intracellular metabolite analyses of microbial cells was also investigated recently (Villas Boas and Bruheim, 2007). It has been shown that blood cells could synthesize endocannabinoids in ex vivo conditions (Vogeser et al., 2006), therefore, without any experimentation; it was difficult to decide which storage conditions were appropriate for ANA and 2-AG. After a set of measurements with double injections and measured as triplicates at day 1, 7 and 10, it was determined that ANA and 2-AG are stable in serum and ethanol at -80°C until 10 days (Table 3.1).

Although samples could be stored for at least for 1 week, all conditions on the days of experiment were suitable for the measurements of ANA and 2-AG to be performed freshly on the day of blood sampling.

Table 3.1 ANA and 2-AG stored at -80^{0}C as serum and/or ethanol elute.

		Day 1 (Mean ± SD)	Day 8 (Mean ± SD)	Day12 (Mean ± SD)	Day 18 (Mean ± SD)
In SERUM	ANA (nmol/ 1 ml plasma)	5.14 ± 0.24	5.27 ± 0.20	5.30 ± 0.08	4.19 ± 0.53
	2-AG (nmol/ 1 ml plasma)	5.01 ± 0.29	4.93 ± 060	4.92 ± 0.59	3.60 ± 1.24
In ETHANOL	ANA (nmol/ 1 ml plasma)	-------------	5.81 ± 0.19	4.55 ± 0.22	3.88 ± 0.55
	2-AG (nmol/ 1 ml plasma)	--------------	5.19 ± 0.44	4.98 ± 0.77	5.08 ± 0.17

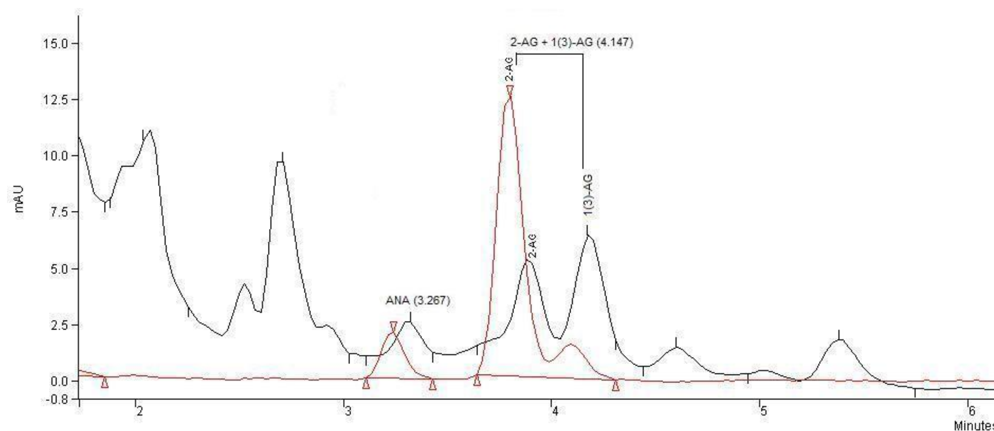


Figure 3.5 Chromatogram of rat plasma sample (black line) and the synthetic standards for ANA and 2-AG (red line).

3.5.2.1 PMB-immobilized beads adsorption assay

Venous blood samples were collected with a syringe by intracardiac puncture immediately after the last bout of exercise into Vacutainer tubes containing 4.5nM EDTA and centrifuged immediately at 3500 rpm for 10 minutes at 0°C in order to prevent the release of endocannabinoids from red blood cells in ex vivo conditions at high quantities (Vogeser et al., 2006). 1 ml serum was taken and 1mM PMSF was added in order to inhibit fatty acid amide hydrolase (FAAH) activity which plays role in the degradation process of ANA and 2-AG. This serum was diluted with 2 ml ice-cold saline solution. Following this dilution, 1 ml of 50 % Affi-Prep Polymyxin B suspension was added and shaken for 1 hour at 40°C. After the adsorption process, beads were transferred to a micro-chromatography column (0,5 x 3 cm) and washed twice with 3 ml saline solution. Remaining saline was removed by centrifugation at 3000 rpm for 3 minutes at 0°C. ANA and 2-AG on the beads were eluted twice with 200 µl of ice-cold absolute ethanol. Elutes were dried under nitrogen gas and re-suspended in 150 µl absolute ethanol for HPLC measurement.

3.5.2.2 High pressure liquid chromatography (HPLC) analysis

The method employed for HPLC analysis was modified from that of Lang (Lang et al., 1996). Briefly, a HPLC System (Varian Prostar, United States) equipped with a 240 pump and a PDA (Varian Prostar, Model 330) detector was used. Separation was carried out on a TSK gel ODS 80 TM (75x4.6-mm i.d. x 5 µm) column (TOSOH, Tokyo, Japan) with an elution solution of acetonitrile and water (8:2, v/v) at a flow rate of 1 ml/minute.

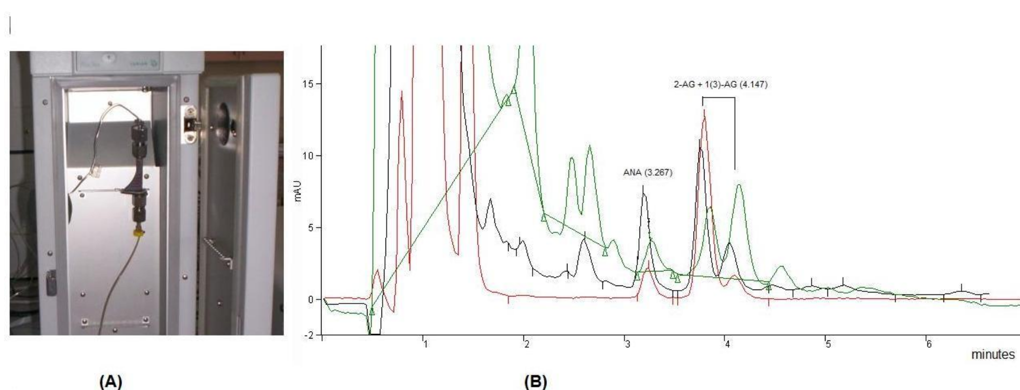


Figure 3.6 (A) TSK gel ODS 80 TM (75x4.6-mm i.d. x 5 micrometer) column (TOSOH, Tokyo, Japan) and the separation graph of ANA and 2-AG with its metabolites 1(3)-AG in a serum sample (green) and synthetic standards (red, black).

Absorbance was monitored at 204 nm wavelength. The dried and re-suspended elute from the PMB-immobilized beads column was fractionated by reverse phase HPLC. The retention time of serum ANA and 2-AG were determined by using synthetic ANA and 2-AG which were found to be 3.2 minutes and 4.2 minutes, respectively. The concentrations of ANA and 2-AG in serum were quantified by comparison with calibration curves constructed using synthetic ANA and 2-AG. Each sample was injected and measured as duplicates and means were calculated (Figure 3.8).

The sample measurement was validated by spiking samples with known concentrations of ANA and 2-AG. The particular sample used for this purpose had previously been shown not to contain any of these analytes. The spiked samples were measured according to the same procedure. These were compared with the results of analyses of standard samples of known composition. The precision was evaluated by analyzing the same sample three consecutive times on three days.

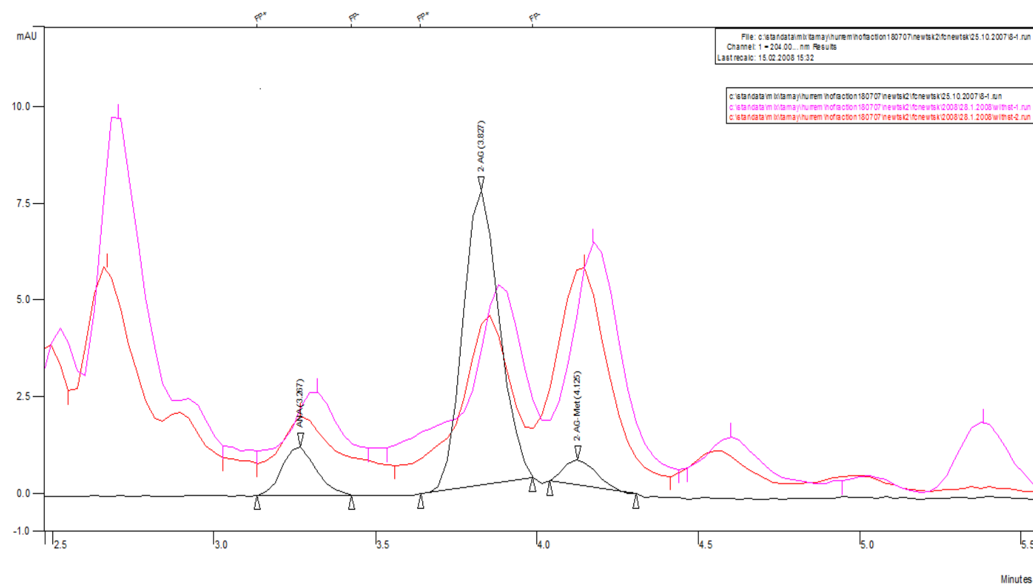


Figure 3.7 The chromatogram of synthetic ANA and 2-AG mixture (black line) and rat serum sample worked as duplicate (pink and red lines) from the same rat.

Calibration and Standard Curve

Varied amounts of synthetic ANA and 2-AG were added to individual rat serum samples. Each sample was adsorbed with PMB-immobilized beads and quantified by RP-HPLC (Figure 3.9 and 3.10).

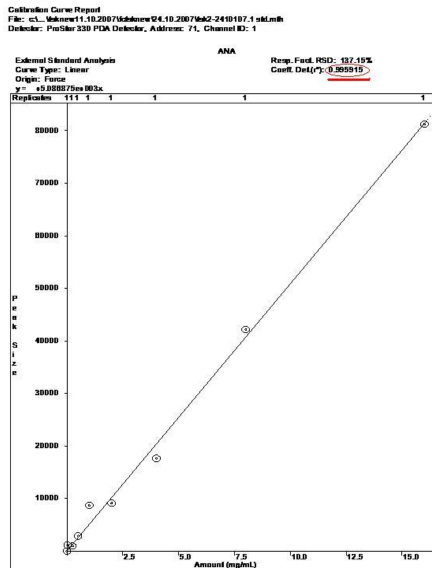


Figure 3.8 Calibration curve of ANA with a coefficient determinant (r^2)of 0.995915. The equation for ANA is: ANA mg/mL = 0.1966 x 10^{-3} x AREA

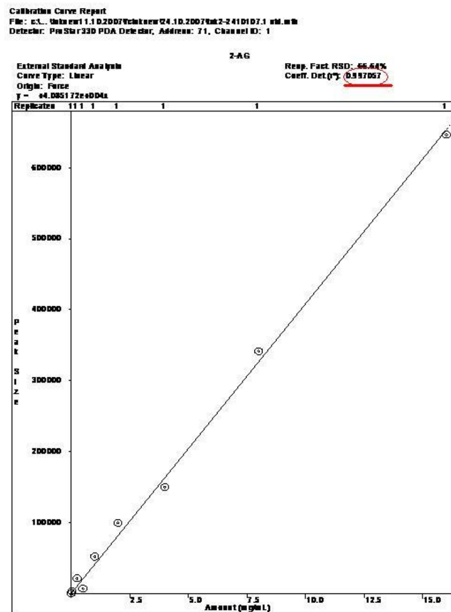


Figure 3.9 Calibration curve of 2-AG with a coefficient determinant (r^2)of 0.997057 . The equation for 2-AG is: 2-AG mg/mL = 0.2448 x 10^{-4} x AREA

3.5.3.Quantitative assessment of immune effector cells

3.5.3.1 White blood cell counts

White blood cell count and differentials were estimated with a Coulter STKS haemocytometer (Beckman Coulter Inc., Fullerton, CA, United States). Because cell numbers were determined in whole blood, corrections for changes in plasma volume were not made.

3.5.3.2 Flow cytometry analysis in blood

100µl blood samples from the same animal were collected into 4.5nM EDTA containing tubes. 20 µl antibody of the kit of interest (Table 3.2) was added separately to each tube and mixed by vortex for 3 minutes. All tubes were incubated in dark at room temperature for 15 minutes. After the addition of 1.5 ml lysing solution, samples were incubated again in dark at room temperature for 8 minutes and centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and cells were re-suspended by adding 1 ml of PBS and centrifuged again at 1200 rpm for 5 minutes. The pellet was re-suspended in 0.5 ml cell wash solution before they are inserted into Flow Cytometry Analyzer (Becton Dickinson C500, United States).

3.5.3.3 Flow cytometry analysis in lymphoid tissues

50 mg of extracted fresh tissue (thymus and/or spleen) was washed with saline solution and placed into the Medimachine (BD, United States) for homogenization. Following the homogenization, cell suspension was transferred into FACS tubes and remaining homogenate was collected by washing the medimachine knife with PBS. The homogenate was centrifuged at 1300 rpm for 5 minutes and supernatant was discarded.

Table 3.2 Antibodies for CD markers and their fluorescent labels used in the commercial available cocktail kits for flow cytometric analysis

		Component	Clone	Isotype	Reacts with
T/B/NK Cocktail	T cells	APC anti-Rat CD3	1F4	mIgM	CD3 cell-surface antigen found on thymocytes and peripheral T lymphocytes
	B cells	FITC anti-Rat CD45RA	OX-33	mIgG1	CD45RA found only on B lymphocytes
	NK cells	PE anti-Rat CD161a	10/78	mIgG1	NKR-P1A (CD161a), 60-kDa homodimer expressed on all natural killer (NK) cells
T cell Cocktail	T cells	APC anti-Rat CD3	1F4	mIgM	CD3 cell-surface antigen found on thymocytes and peripheral T lymphocytes
	Cytolytic T cells	FITC anti-Rat CD8a	OX-8	mIgG1	hinge-like membrane-proximal domain of the 32-kDa alpha chain of the CD8 differentiation antigen
	Helper T cells	PE anti-Rat CD4	OX-35	mIgG2a	CD4 antigen on most thymocytes, a subpopulation of mature T lymphocytes
Activated T cell Cocktail	T cells	APC anti-Rat CD3	1F4	mIgM	CD3 cell-surface antigen found on thymocytes and peripheral T lymphocytes
	Activated T cells	PE anti-Rat CD25	OX-39	mIgG1	the alpha chain of the IL-2 receptor on T lymphoblasts and thymic and splenic dendritic cells
	Activated T cells	FITC anti-Rat RT1B	OX-6	mIgG1	non-polymorphic determinants of the Rat MHC class II antigens, I-A equivalent

The pellet was re-suspended in 1 ml of 2% rat serum containing PBS and incubated at room temperature for 15 minutes. 100 µl of cell suspension was transferred to several tubes and 20 µl antibodies from the kit of interest (Table 3.3) was added separately to each tube and mixed by vortex for 3 minutes.

Table 3.3 Amount and type of tissue and staining kit used in flow cytometric analysis.

Amount of antibody added	100 µl Blood	100 µl spleen cell suspension	100 µl thymus cell suspension
20 µl	Ig isotype Control	Ig isotype control	Ig isotype control
20 µl	T lymphocyte cocktail	T cell cocktail	T cell cocktail
20 µl	T/B/NK cocktail	T/B/NK cocktail	T/B/NK cocktail
20 µl	Activated T lymphocyte cocktail	Activated T cell cocktail	Activated T cell cocktail

All tubes were incubated in dark at room temperature for 15 minutes. After the addition of 1 ml lysing solution, samples were incubated again in dark at room temperature for 8 minutes and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded and cells were re-suspended by adding 1 ml of wash solution and centrifuged again at 1200 rpm for 5 minutes before they are inserted into Flow Cytometry Analyzer.

T/B/NK Cocktail

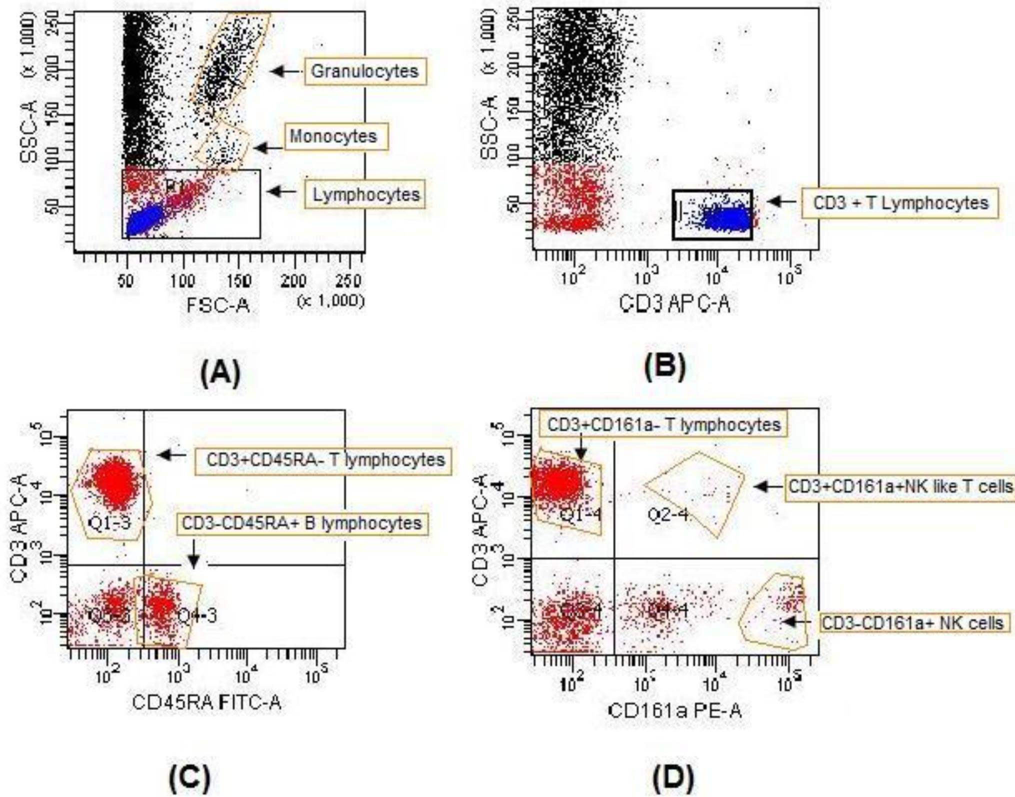


Figure 3.10 The interpretation of flow cytometry results for the T/B/NK cocktail; (A) Discrimination of lymphocyte, monocyte, and granulocyte in the forward scattered and side scattered gate, (B) CD3+ T cell population separated from other leukocytes, (C) separation of T lymphocytes and B lymphocytes, (D) separation of the T lymphocytes, NK cells and NK like T cells.

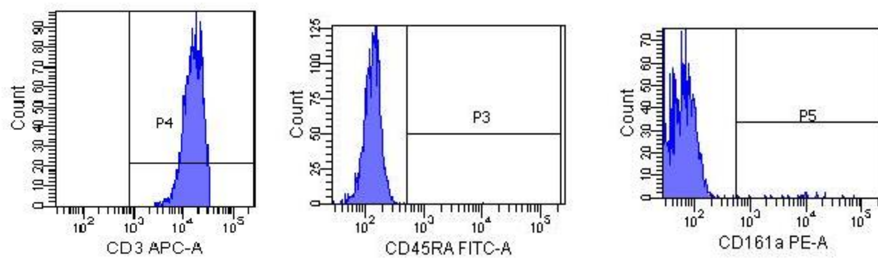


Figure 3.11 The histogram plots show the intensity of staining with the specific antibodies designated on the x-axis

T Cell Cocktail

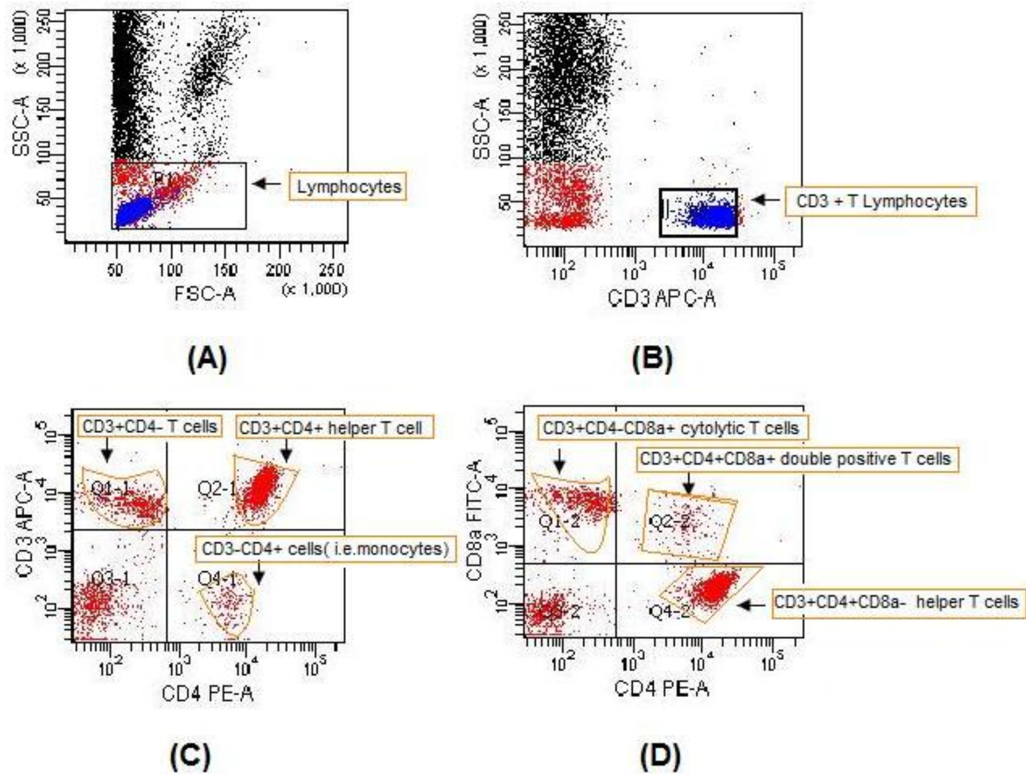


Figure 3.12 The interpretation of flow cytometry results for the T lymphocyte cocktail; (A) Discrimination of lymphocyte, monocyte, and granulocyte in the forward scattered and side scattered gate, (B) CD3+ T cell population separated from other leukocytes, (C) separation of CD3+ T lymphocytes, helper T lymphocytes, and other CD3-CD4+ immune effector cell (i.e. monocytes), (D) separation of T lymphocyte subsets (helper & cytolytic T cells) and double positive T cells.

Activated T Cell Cocktail

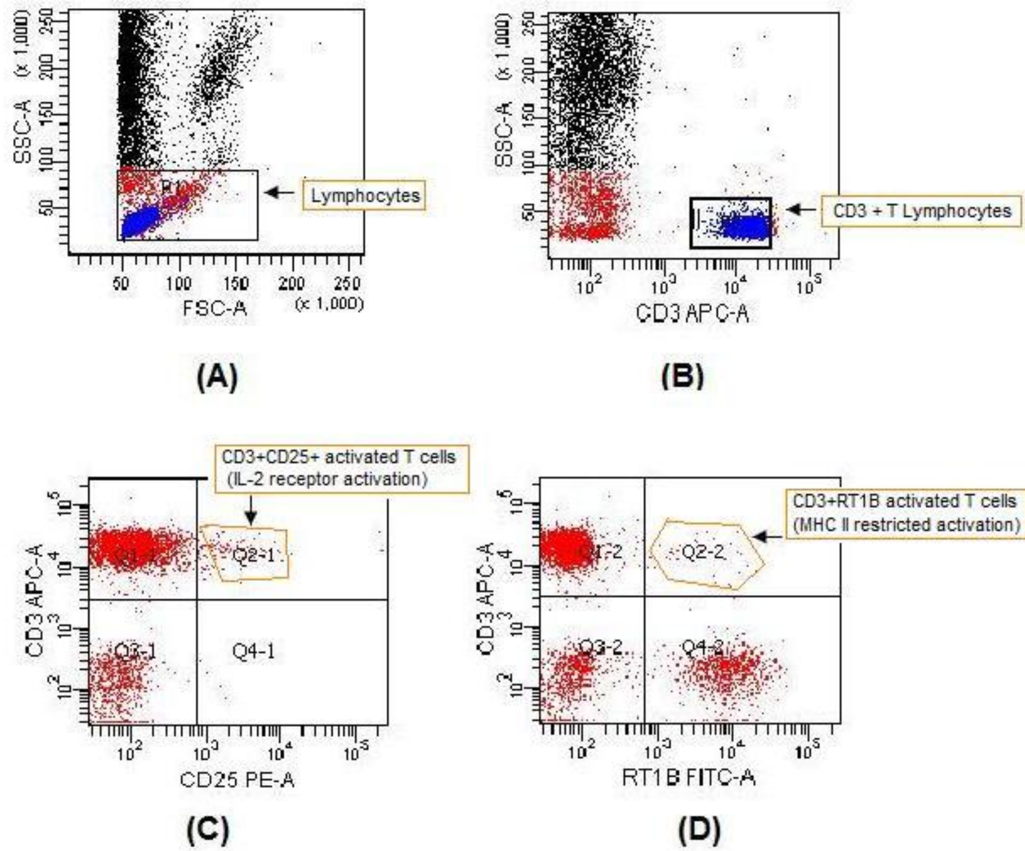


Figure 3.13 The interpretation of flow cytometry results for the activated T lymphocyte cocktail ; (A) Discrimination of lymphocyte, monocyte, and granulocyte in the forward scattered and side scattered gate, (B) CD3+ T cell population separated from other leukocytes, (C) IL-2 receptor activated CD3+CD25+T lymphocytes, (D) MHC II restricted antigen activated T lymphocytes

3.5.3.4 Immunohistological analysis

T, B, NK cells and macrophages were immune-labelled in spleen and thymus by the indirect immunoperoxidase technique. Apoptosis was assessed in situ by the Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) method. All experiments were conducted on fresh frozen tissue samples (Gavrieli et al., 1992; Korkusuz et al., 2002).

3.5.3.4.1 Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) for apoptosis

Apoptosis was determined by enzymatic labeling of DNA strand breaks using terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL). TUNEL labeling was conducted using an ApopTag in situ detection kit. Stainning was then performed according to manufacturer instructions. Briefly, 5 μm frozen sections were mounted on adhesive pretreated glass slides and fixed with 1% paraformaldehyde in PBS, pH 7.4 for 10 minutes at room temperature. After washing with PBS, sections were post-fixed with ethanol: acetic acid (2:1) for 5 minutes at -20°C. Sections were rinsed and incubated with equillibration buffer for 1 minute. Digoxigenin labelled deoxyuridine triphosphate and deoxyadenosin triphosphate in terminal deoxiribonucleotidyl transferase buffer were added to cover each section and the slides were incubated in a humidified chamber at 37°C for 60 minutes. The reaction was stopped by immersion in stop/wash buffer. After washing in PBS (0.01 M, pH 7.4) the sections were incubated in antidigoxigenin rhodamine for 30 minutes at room temperature in the dark. Following washing with PBS, the slides were covered by 1 drop of DAPI. (Sigma--Aldrich, United States). Negative controls were processed at the same time by excluding the deoxiribonucleotidiyl transferase enzyme from the processing steps. All slides were examined and apoptotic cells were photographed with Leica DM6000B (Wetzlar-Germany) with a DC490 digital camera (Leica, Wetzlar-Germany).

3.5.3.4.2 In situ indirect immune peroxidase labeling of effector cells

Six to eight µm thick cryostat sections were fixed in acetone for 10 minutes and air-dried for at least 30 minutes. Anti-mouse HRP-DAB staining kit (CTS002, R&D, United States) was used according to manufacturers' protocol. Unspecific binding was blocked using rat serum at a dilution of 1:10 for 30 minutes at room temperature. Sections were incubated in a humidified chamber for an hour with monoclonal antibodies, which are listed in Table 3.4.

Table 3.4 Monoclonal antibodies used for immunohistology

Antibody	**Clone**	**Isotype**	**Dilution**
CD3	G4.18	Mouse(BALB/c) IgG_3	1:20
CD8a	OX-8	Mouse(BALB/c) IgG_1,k	1:20
CD8b	341	Mouse(BALB/c) IgG1,k	1:20
CD11b	WT.5	Mouse IgA,k	1:20
CD25	OX-39	Mouse(BALB/c) IgG1,k	1:20
CD45	OX-1	Mouse(BALB/c) IgG1,k	1:20
CD45RA	OX-33	Mouse IgG_1,k	1:20
CD56	N12F11	RatIgG2a	1:20
CD56E	N-CAM13	Mouse IgG2a	1:20

After washing in 0,01 M PBS at pH 7.4, the sections were incubated with biotinilated goat anti mouse secondary antibody kit for 10 min at room temperature and washed in PBS. After incubation with streptavidine peroxidase for 30 min, slides were washed with PBS and covered with HSS-HRP for 30 minutes at room temperature. The sections were washed with PBS and incubated with 3.3'-diaminobenzidine-tetrahydrochloride (DAB) for 2 minutes following suppliers' written steps. After washing with tap water, sections were dehydrated through graded alcohols and cleared in xylene prior to mounting with entellan. All antibodies were diluted in a background reducing buffer solution in 0.05 M TrisHCl containing 0.1% Tween 20 (Dako, Denmark). Negative control staining was performed by omitting the initial primary antibody staining step and using a control mouse Ig G. Positive control staining

was performed by using appropriate control tissue samples and sections were photographed by using Leica DM6000B (Wetzlar-Germany) with a DC490 digital camera (Leica, Wetzlar-Germany).

3.5.3.4.3 Quantitative evaluation of immune labeling

The digital images were captured using the Leica DC500 digital camera (Germany). Quantitative image analysis was carried out by using Leica Application Suit and Qwin Plus computer image analysis system (Germany) by modifying the literature (Goyal et al., 2004;Inan et al., 2006; Uğur et al., 2003).

Maximum staining intensity was determined from the positive control tissue sections by using the image analysis system. Accordingly each spleen and thymus section was graded for cellular immune reaction on a scale of 0 to +++ with DAB. 0 was given to no immune reactivity, + to weak but continuous reactivity, ++ for moderate but continuous reactivity and +++ to intense but continuous immunostaining. The number of immune positive cells was expressed as a percentage of positive cells over total cells at 200x magnification. In every specimen the average of screened/analysed 3 non over-lapping fields was reported. Staining intensity was combined with the percentage of stained cells by using the HSCORE that was calculated with the following equation: H score = $\Sigma\rho$ (i+1), where i = intensity of staining with a value of (1 for +), (2 for ++) or (3 for +++) and ρ is the percentage of positive cells stained with each intensity, varying between 0-100. Σ:: The sum of stained cells at different intensities. For cell apoptosis determination, cells exhibiting red labeling with rhodamine were considered apoptotic. All counts were carried out on a light microscope using 200x magnification. Ten visual fields were selected randomly and the average ratio of the number of apoptotic cells to the number of DAPI labeled only cells (blue) was considered apoptosis rate; that is to say apoptosis rate=n the number of apoptotic cells / number of all cells (Ferrini Magee et al., 2001; Zhang and Yang, 2006).

3.5.4 Quantitative assessment at gene level

3.5.4.1 RNA isolation from lymphoid tissue

100 mg of spleen and thymus were harvested on ice and placed in screw-cap tubes containing 1 ml of acid guanidinium thiocyanate-phenol-chloroform extraction (TRIZOL, Invitrogen Life Technologies, United States). Tissues were homogenized using The FastPrep® bead-beater system and incubated on ice for 5 minutes to permit the complete dissociation of nucleoprotein complexes. 300 μl chloroform was added to ensure phase separation and samples were agitated vigorously and incubated on ice for approximately 10 minutes. Tubes were centrifuged at 12,000 x g for 15 minutes at 8°C. RNA found in the aqueous phase was precipitated by adding 0.5 ml of ice-cold isopropyl alcohol per 1 ml of reagent used for the initial homogenization. The samples were incubated at -20°C for 10 minutes to facilitate precipitation and centrifuged at 12,000 g for 10 minutes at 4°C. The supernatant was discarded and the pellet was washed with 75% ethanol, with at least 1ml of 75% ethanol per 1 ml of initial volume used for homogenization. After vortex for 2 minutes, the sample was centrifuged at 7,500 x g for 5 minutes at 4°C to achieve complete precipitation of the RNA. The supernatant was discarded and the RNA pellet was briefly dried for approximately 5 to 10 minutes.RNA was dissolved in RNase-free water by incubating in a water bath for 10 minutes at 55 to 60 °C.

3.5.4.2 RNA quality control

The quality of the extracted total RNA was assessed via measurement of optical densities (OD) at 230, 260 and 280nm using NanoDrop (ND-1000) spectrphotometer. RNA concentrations were calculated using absorbance at A260 nm with the following equation:

[C] = A260 x RNA constant (40)

A230 and A280 measurements were used to assess the RNA purity and to assure that the samples were devoid of contaminants such as phenol, protein or salts.The integrity and the quality of the total RNA were assessed via denaturing gel electrophoresis on 1% agarose gel containing 37 % formaldehyde and 3-[N-Morpholino] propanesulfonic acid (MOPS). The visualization of 18S and 28S ribosomal RNA provides information on the integrity of the isolated total RNA. The gel electrophoresis has been documented using BioRad Chemidoc XRS system. After the quality control of mRNAs extracted from spleen and thymus tissues, it has been decided to use only one sample from control and moderate groups whereas endurance type of exercise group data is the average of three independent samples (Yuzbasioglu et al., 2009).

3.5.4.3 Reverse transcriptase and cDNA synthesis

One to two µg of isolated total RNA was used for the synthesis of cDNA via incubating at 37°C using 0,5 µg random hexamer oligonucleotide primers and RNAse H(+) reverse transcriptase enzyme Improm II (Promega Corporation, United States). The volume of cDNA's were completed to 80µl with distilled water and reserved in -20°C. The reaction was carried out on a Perkin-Elmer 9700 thermocycler via an initial denaturation of 10 minutes at 65°C followed by incubation at 37°C for 10 minutes to ensure the adequate hybridization of the oligo dT oligonucleotides. Following the primer binding, reverse transcriptase enzyme was added to the mixture and the reaction was carried out at 42°C for 60 minutes followed by 15 minutes at 75°C to achieve the complete denaturation of the reverse transcriptase.

3.5.4.4 Quantitative RT-PCR

The cDNAs was subjected to semi-quantitative PCR analysis for the genes Cnr1, Cnr2, BAX, Bcl2, FAS, Caspase 3, CD19, IgM, CD247, CD69, NKR-P1a, NK-p46. The gene of interest was amplified from 2 μl of cDNA using gene specific primers via SYBR-green technique. Four serial dilutions of a control sample were utilized to establish the regression curve to interpolate relative expressions of the samples using ΔCt method. A special hot-start DNA polymerase enzyme mix was utilized to avoid non-specific miss-priming at lower temperatures and to ensure the specificity of the product. The mix for PCR reaction was composed of 25 μl of 2x SYBR green mix, 6 μl of 25 mM MgCl2, 2,4 μl of both 10 μM reverse and forward primers, 1,5 μl of cDNA completed to 50 μl with double distilled, deionized water. The PCR conditions were programmed as 2 minutes at 94°C for initial denaturation, followed by 2 seconds at 94°C for denaturation, and 15 seconds at 60°C for annealing and extension. The denaturation and annealing-extension cycles were repeated 40 times and the SYBRgreen incorporation at each cycle was detected on fluorometric quantitative thermal cycler Rotorgene 6500 (Corbett scientific, Brisbane, Australia).

The expressions of the gene of interest were normalized to the expression of the house-keeping gene beta-actin which was used as the normalizator. The primer pairs used for the amplification of the target genes were provided in Table 3.5.

Table 3.5 Primer pairs used for amplification of target genes

Amplicon		Sequence
CD247 (CD3-zeta)	Sense	5'-GCAGAAAGACAAGATGGCAGAG-3'
	Anti-sense	5'-AGCAGGGTGAAGCCTTTGAG-3'
IgM	Sense	5'-TGTTCCAACCTTACCTTC-3'
	Anti-sense	5'-ACAACCATACCAGAAGTG-3'
Bax	Sense	5'-TGGGCTGGACACTGGACTTC-3'
	Anti-sense	5'-GAGCGAGGCGGTGAGGAC-3'
Bcl2	Sense	5'-GGGAGCGTCAACAGGGAGATG-3'
	Anti-sense	5'-CAGCCAGGAGAAATCAAACAGAGG-3'
Casp3	Sense	5'-GCCAGAAGATACCAGTGGAG-3'
	Anti-sense	5'-ACGAGTGAGGATGTGCATG-3'
CD19	Sense	5'-GGCAGGACGATGGACTTC-3'
	Anti-sense	5'-TGGGACCAGACCGAATTG-3'
CD69	Sense	5'-AACACTGGATTGGGCTGAGAAACG-3'
	Anti-sense	5'-GCCTCGCAGTCTACAGAAGCAAC-3'
CnR1	Sense	5'-ACCTACCTGATGTTCTGGATTGG-3'
	Anti-sense	5'-CCATGCGGGCTTGGTCAG-3'
CnR2	Sense	5'-CTGCTCCATGCTGTGCCTTG-3'
	Anti-sense	5'-ATCCTTCAGGACCAGAGTCTCAG-3'
Fas	Sense	5'-CCGTGTCAGCCTGGTGAAC-3'
	Anti-sense	5'-TCCACTTCTAAACCATGCCCTTC-3'
NKp46	Sense	5'-GTTGAGAACACCAGCCTTGC-3'
	Anti-sense	5'-AGCCATACTAGAGCCATCACG-3'
NKR-P1A	Sense	5'-TGTGTATCACCTCCATCTCTTCC-3'
	Anti-sense	5'-CGGCATGGCTCCACTGATG-3'
B-Actin	Sense	5'-GTGCTATGTTGCCCTAGACTTCG-3'
	Anti-sense	5'-GATGCCACAGGATTCCATACCC-3'

3.5.4.5 Microarray analysis

All GeneChip experiments were conducted in the Hacettepe University Transcriptomics Laboratory, as recommended by the manufacturer of the GeneChip system (Affymetrix, Santa Clara, CA). The GeneChip array used was the Rat Genome RAE_230 2.0 expression probe array (Affymetrix, Inc., San Diego, CA, United States), which allows for analysis of more than 31,000 transcripts.

Double stranded cDNA was synthesized with a starting amount of approximately 100 μg full-length total RNA using the 3'IVT Express System in the presence of a T7-(dT)24 DNA oligonucleotide primer. Following synthesis, the cDNA was purified by sample cleanup module. The purified cDNA was then transcribed in vitro using the High Yield RNA Transcript Labeling Kit in the presence of biotinylated ribonucleotides form biotin labeled cRNA. The labeled cRNA was then purified on an affinity resin, quantified and fragmented. An amount of approximately 10 μg labeled cRNA was hybridized for approximately 16 hours at 45ºC to an expression probe array. The array was then washed and stained twice with streptavidin-phycoerythrin using the GeneChip Fluidics Workstation (Affymetrix). The array was then scanned twice using a Confocal laser scanner (GeneArray Scanner) resulting in one scanned image. This resulting ".dat-file" was processed using the GCOS program (Affymetrix) into a ".cel-file" which was captured and loaded into the Affymetrix GeneChip Laboratory Information Management System (LIMS). Raw data was converted to expression levels using TMEV software. After checking for quality, data were exported to the Microarray analysis suite software for analysis.

Data analysis was performed by the transcriptomics & expression profiling researchers using the gene array tools package. Principle component analysis (PCA) was used to identify outliers (defined as samples with potential technical flaws such as tissue contamination by other surrounding organs) based

upon overall gene expression values prior to continuing with analysis.

Individual experiment groups were analyzed separately, with the gene expression levels for all samples from each time point normalized to the median of their respective control samples prior to statistical analysis. Filtering was performed to remove low or non-expressing genes using the following criteria: raw expression value of >50 and a present call in at least two-thirds for any treatment group within a time point. One-way analysis of variance (ANOVA) by treatment was performed on this filtered list of genes with a p-value of 0.05, which would result in 5% of the genes identified as being expected by chance. To further refine the resulting gene list, only those genes that demonstrated at least a 2-fold difference in expression levels between the controls and the individual treated group were included for subsequent investigation. Other genes and probe sets which do not fulfill the ANOVA mentioned criteria are also taken into account according to the scientific relevance.

The information content of these data sets is a conjunction of numerical changes and biological information. The decision to consider a specific gene relevant was based on a conjunction of numerical changes identified by comparative and statistical algorithms and the relationship to other modulated genes that point to a common biological theme. The value of that relationship was assessed by the analyst through a review of the relevant scientific literature. Any hypothesis generated from this analysis would need further validation with other analytical and experimental techniques.

3.6 Statistics

Several statistical methods were used for different sets of data in order to evaluate the hypothesis that endurance type of exercise would increase plasma ANA and 2-AG levels, and thereby giving rise to alterations in immune effector cell counts and percentages in blood and lymphoid tissues. One way ANOVA in addition to Kruskal-Wallis and Mann-Whitney U tests were used for the data obtained from the assessment of endocannabinoids and immune

effector cells measured by flow cytometry.. İmmunohistologic data were analysed by the same nonparametric variance analysis tests. Since overall tests were significant, follow-up tests were conducted to evaluate pair wise comparisons among the groups, controlling for Type I error across tests using the Holm's sequential Bonferroni approach. The relationship between endocannabinoids and immune effector cells was assessed with the Pearsons Product Correlation Coefficient. The SPSS 11.0 Software for Windows was used.

CHAPTER 4

RESULTS

4.1 Part 1 of the study

4.1.1 Measurement of the endocannabinoid serum levels

ANA and 2-AG serum levels were significantly higher in the endurance type of exercise group when compared to the moderate type of exercise and the control groups (p< .001) (Figure 4.1) (Table 4.1).

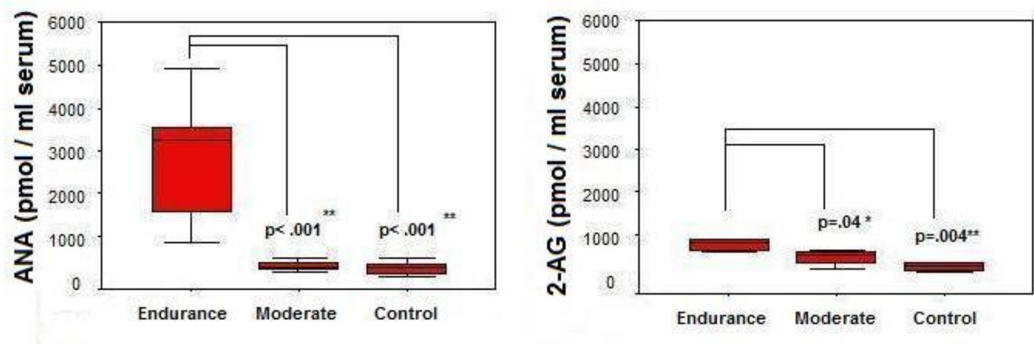


Figure 4.1 Endocannabinoid serum levels in the endurance type of exercise, the moderate type of exercise and the control groups.

Table 4.1 Z scores and p values of Mann-Whitney U tests for endocannabinoids between three groups; the endurance type of exercise, the moderate type of exercise and the control groups.

	Moderate Type of Exercise	**Control**	
Endurance Type of Exercise	-3.42* (p= .001)	-3.67 *(p< .001)	**ANA**
	-2.70* (p= .007)	-3.46* (p= .001)	**2-AG**

*Correlation is significant at the α = 0.05 level (2- tailed).

4.1.2 White blood cell counts

White blood cell counts and lymphocyte percentage were significantly lower in the endurance type of exercise group when compared to that of the moderate type of exercise and the control groups (p< .001) (Figures 4.2 and 4.3.A). On the other hand neutrophil percentage was significantly higher in the endurance type of exercise group when compared to the other two groups (p< .001) (Figure 4.3.B) (Table 4.2)

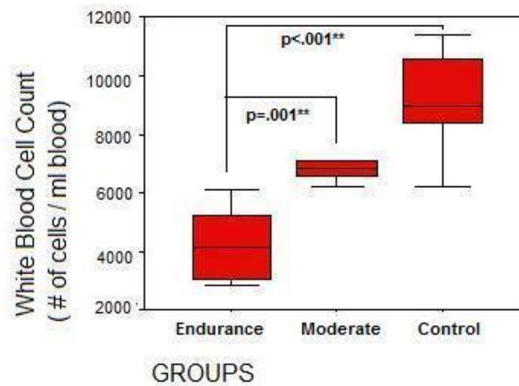


Figure 4.2 WBC counts in the endurance type of exercise, the moderate type of exercise and the control groups.

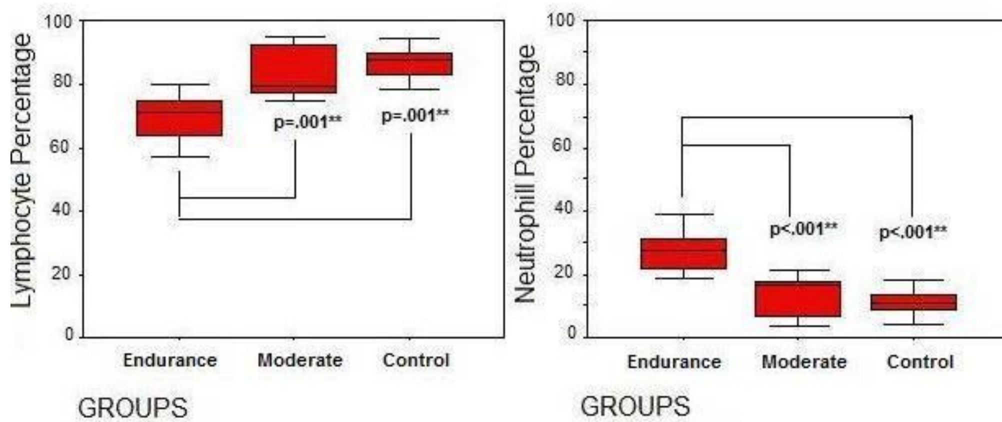


Figure 4.3 Lymphocyte and neutrophil percentages in the endurance type of exercise, the moderate type of exercise and the control groups.

Table 4.2 Z scores and p values of Mann-Whitney U tests for WBC count, lymphocyte % and neutrophil % between the three groups.

		Moderate Type of Exercise	Control Group
Endurance Type of Exercise	WBC count	-3.46 *(p= .001)	-3.77 *(p<.001)
	Lymphocyte %	-2.96 * (p= .003)	-3.69 *(p< .001)
	Neutrophil %	-3.53 *(p= .001)	-3.76 *(p< .001)

*Correlation is significant at the $\alpha = 0.05$ level (2- tailed).

4.1.3 Flow cytometry (Immune effector cell counts)

($CD3^-CD45RA^+$) B lymphocyte (p= .001), ($CD3^+$)T lymphocyte (p= .003) and ($CD3^-CD161a^+$)natural killer like T lymphocyte (p< .001) counts were significantly lower in the endurance type of exercise group when compared to the moderate type of exercise group (Figure 4.4).

When the endurance type of exercise group is compared to the control group, test results showed that there were statistically significant differences between all immune effector cell counts and percentages, except ($CD3^+CD161a^+$) the natural killer like T lymphocyte percentage. ($CD3^-CD161a^+$) natural killer cell (p=.017), ($CD3^-CD45RA^+$) B lymphocyte (p< .001), ($CD3^+$) T lymphocyte (p= .001) and ($CD3^+CD161a^+$) natural killer like T lymphocyte (p< .001) counts were lower in the endurance type of exercise group when compared to the control group. Moreover, ($CD3^-CD45RA^+$) B lymphocyte percentage (p< .001) was significantly lower, whereas ($CD3^-CD161a^+$) natural killer cell (p= .023) and ($CD3^+$) T lymphocyte percentages (p= .002) were higher in the endurance type of exercise group when compared to the control group (Figure 4.5). When the moderate type of exercise group was compared to the control group, ($CD3^-CD45RA^+$) B lymphocyte (p= .007) and ($CD3^+CD161a^+$) natural killer like T lymphocyte (p= .012) counts were significantly lower in the moderate type of exercise group (Figure 4.5).

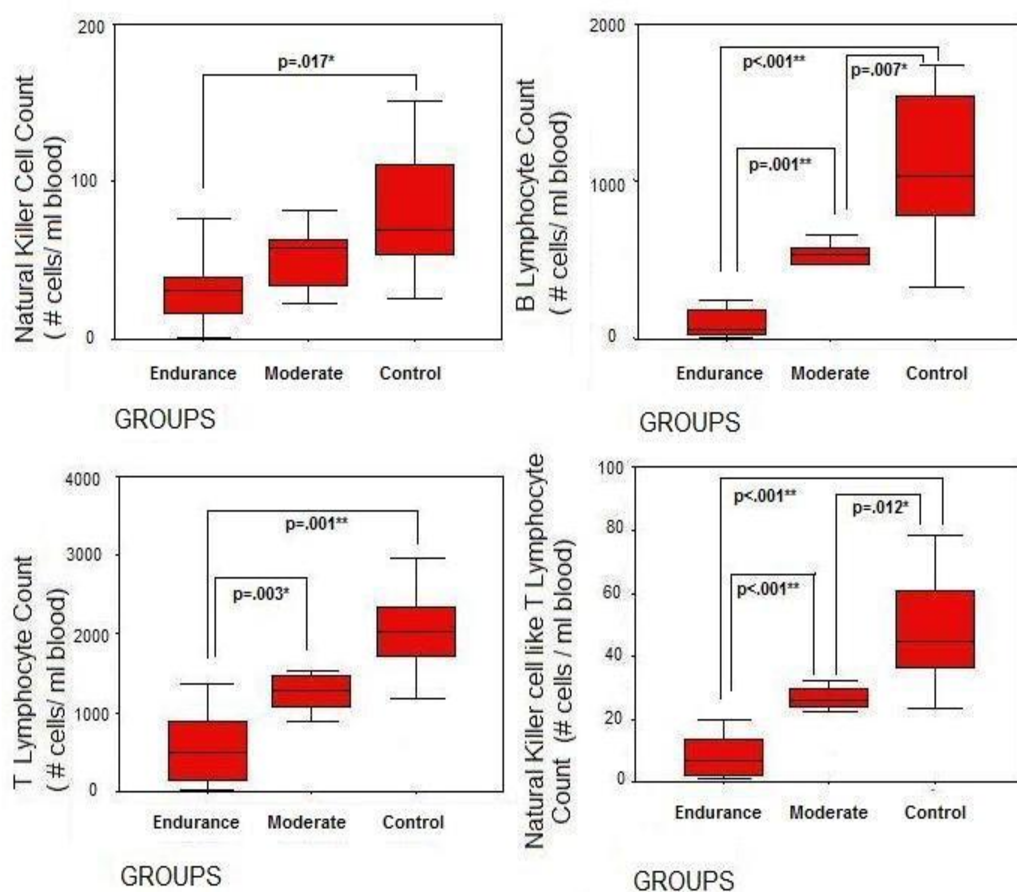


Figure 4.4 Immune effector cell counts in the endurance type of exercise, the moderate type of exercise and the control groups.

Table 4.3 Z scores and p values of Mann-Whitney U tests for immune effector cell counts

	Moderate Type of Exercise	**Control Group**	**Immune Effector Cell Counts**
Endurance Type of Exercise	Not Significant	-2.39 (p< .017)	**NK Cell**
	-3.38 (p=.001)	-3.67 (p< .001)	**B Lymphocyte**
	-2.93 (p=.003)	-3.23 (p= .001)	**T Lymphocyte**
	-3.53 (p< .001),	-3.76 (p< .001)	**NK like T Lymphocytes**
Moderate Type of Exercise		Not Significant	**NK Cell**
		-2.69 (p= .007)	**B Lymphocyte**
		Not Significant	**T Lymphocyte**
		-2.52 (p= .012)	**NK like T Lymphocytes**

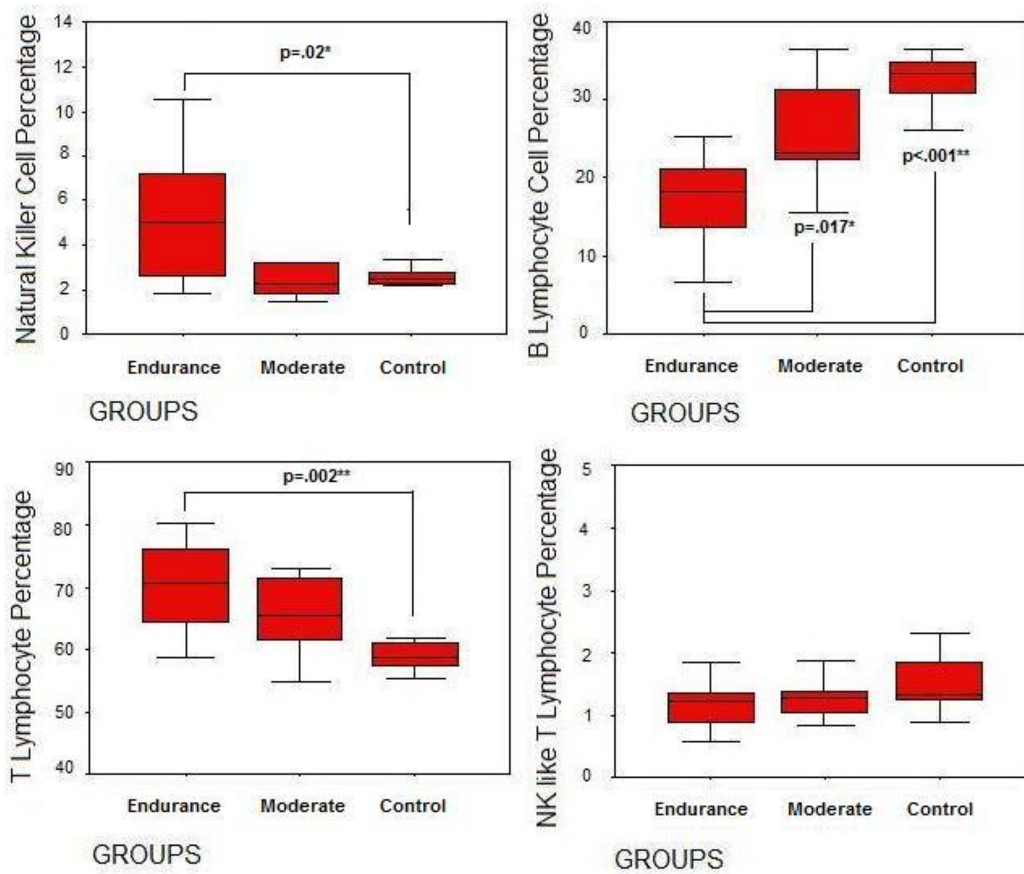


Figure 4.5 Immune effector cell percentages in the endurance type of exercise, the moderate type of exercise and the control groups.

Table 4.4 Z scores and p values of Mann-Whitney U tests for immune effector cell percentages

	Moderate Type of Exercise	**Control Group**	**Immune Effector Cell Percentages**
Endurance Type of Exercise	-2.01* (p< .044)	-2.28* (p< .023)	NK Cell
	-2.39* (p< .017)	-3.76* (p< .001)	B Lymphocyte
	Not Significant	-3.15* (p= .002)	T Lymphocyte
	Not Significant	Not Significant	NK like T Lymphocytes

*Correlation is significant at the α = 0.05 level (2- tailed).

4.1.4 Immunohistology

4.1.4.1 Assessment of apoptosis with TUNEL labeling

Both endurance ($p < .001$) and moderate ($p < .001$) type of exercises caused a statistically significant increase in apoptotic index when compared to the control group by using the TUNEL method in the spleen specimens (Figure 4.6). The thymic apoptotic index, however, did not show any statistically significant difference between groups.

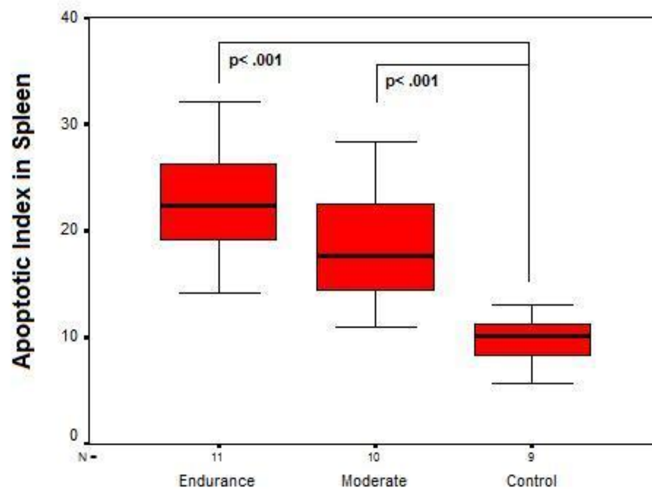


Figure 4.6 Apoptotic index in the endurance type of exercise, the moderate type of exercise and the control groups in the spleen.

In spleen, the apoptotic cells were located at the periphery of the periarteriolar lymphatic diffuse and nodular tissue of the white pulp. They were also noted within the marginal zone and venous sinusoid walls adjacent to the white pulp. These were the places where blood born antigen presentation occured. The apoptotic cell population consisted of lymphocytes and phagocytotic cell (Figure 4.7).The thymic apoptotic cell groups consisted of the

T lymphocytes and the epithelial reticular cells. These cells groups were sparse, exhibited similar number and location patterns (scattered at the corticomedullary border or within the medulla of the lobule) in the exercise groups when when compared to the control group (Figure 4.8).

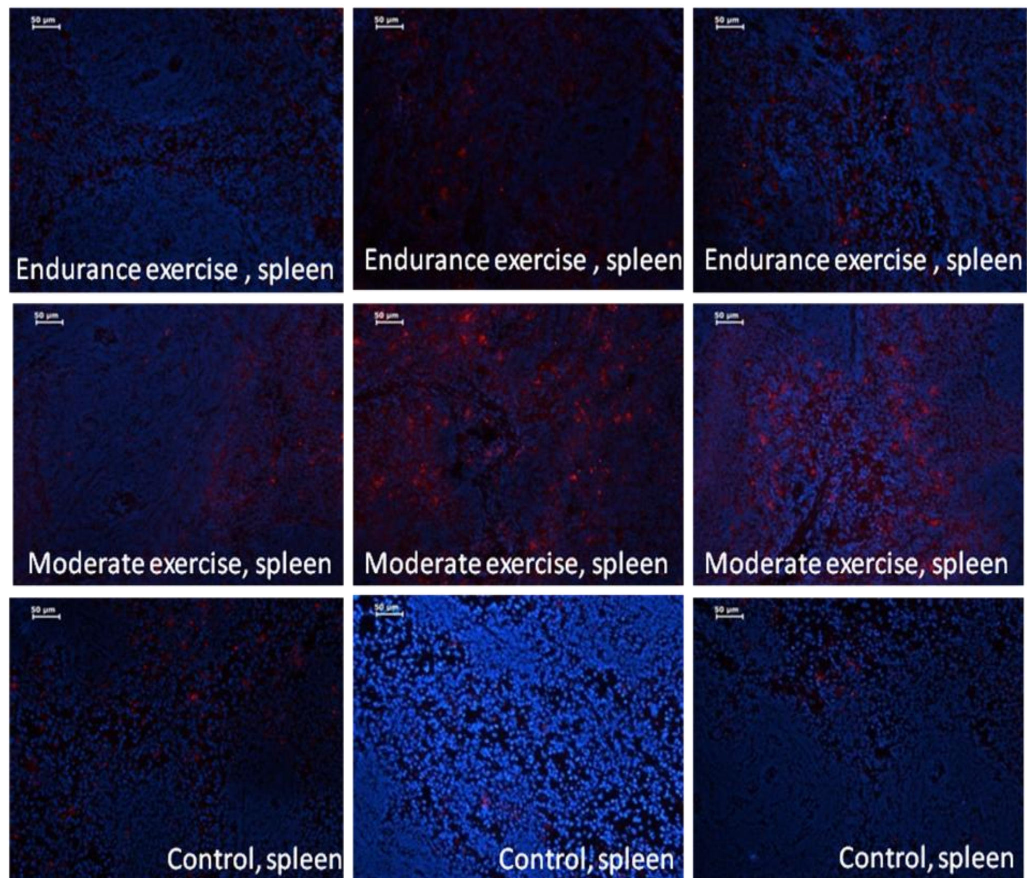


Figure 4.7 Micrographs of the spleen specimens belonging to the endurance type of exercise, the moderate type of exercise and the control groups labeled by the TUNEL method for apoptosis (200x). Lymphoid follicles (left column), periarteriolar diffuse lymphoid tissue (central column) and the venous sinusoids (right column) contain red rhodamine labeled apoptotic cell groups. All cell nuclei appeared blue with DAPI. Note that the apoptotic cells are numerous in the exercise groups compared to that of the control group.

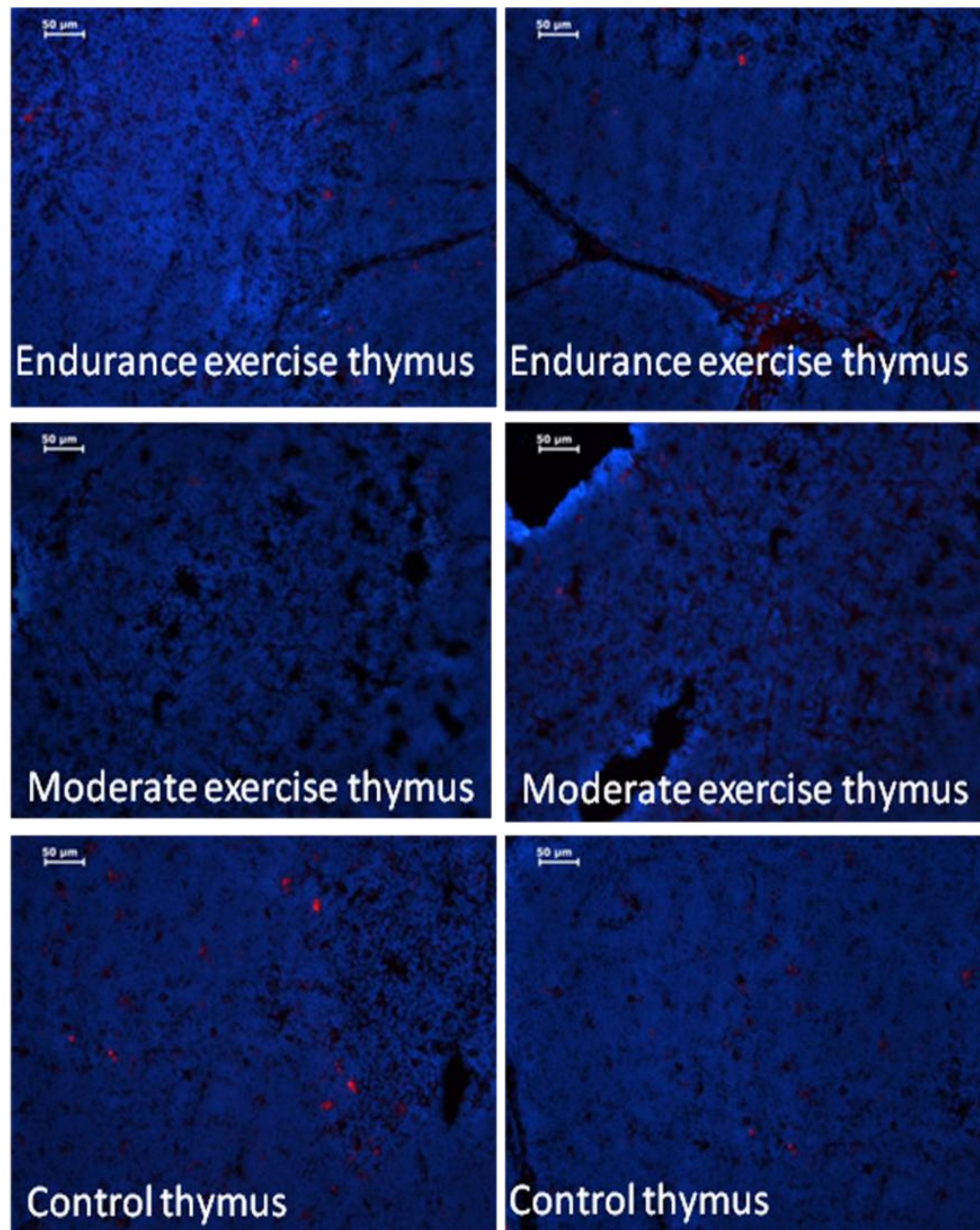


Figure 4.8 Micrographs of the thymus specimens belonging to the endurance type of exercise, the moderate type of exercise and the control groups labeled by the TUNEL method for apoptosis (200x). Thymic lobules exhibited similarly few numbers of apoptotic cells in their medulla (left column) and their cortical compartments (right column) in all groups. All cell nuclei appeared blue with DAPI.

4.1.4.2 Quantitative evaluation of immune labeling

CD 3 exhibited a diffuse immune staining pattern in all groups. CD3 was expressed in different stages of developing thymocytes in both cortex and medulla. CD3 was mainly expressed in the T zones of the spleen. The majority of the thymocytes and the T cells that are specific antigens presented by the MHC class 1 molecule that exhibited CD8a and 8b immunoreactivity (Figures 4.9, 4.10,). CD11b was expressed by a relatively low number of cells probably containing granulocytes, NK cells and subsets of T and B cells in the spleen (mainly red pulp) and thymus (Figure 4.9, 4.10).

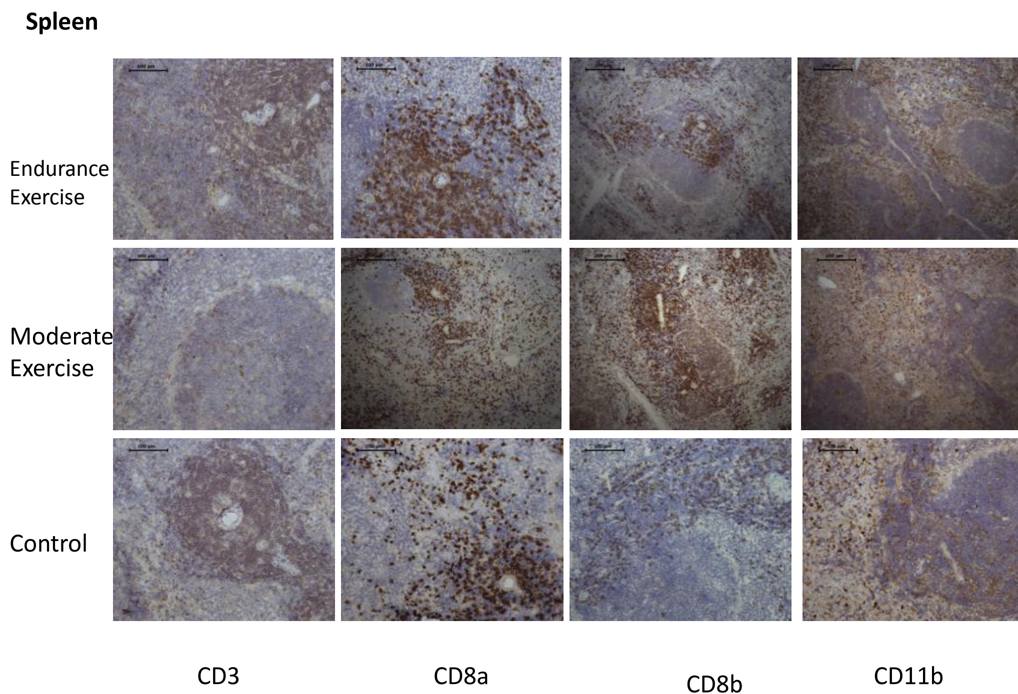


Figure 4.9 Immunohistological labeling of several CD markers in the spleen (rows present the groups and columns present the surface antigens)

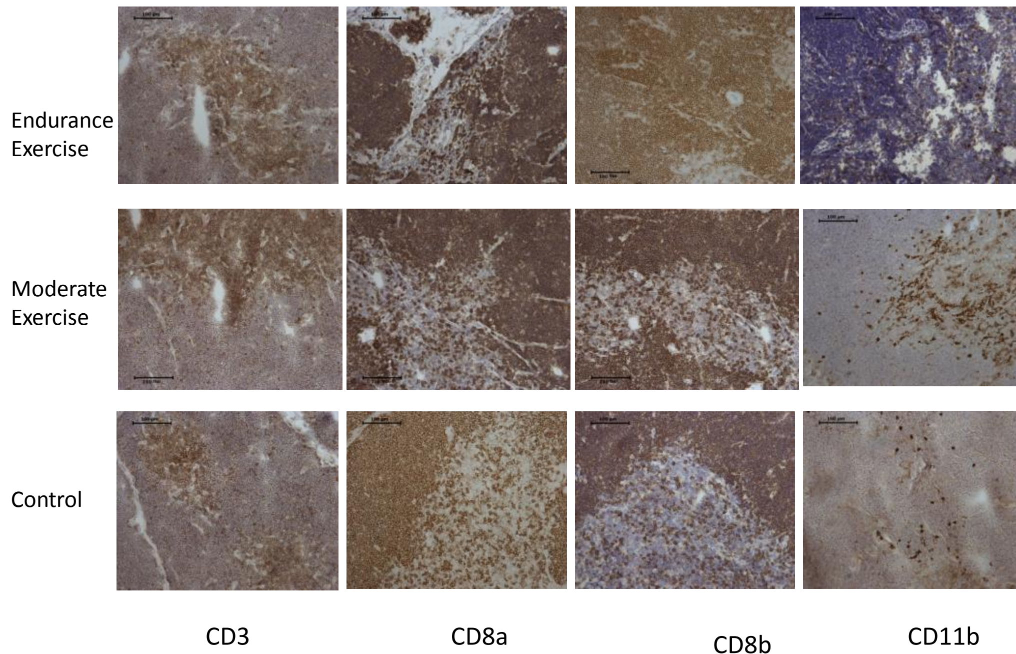
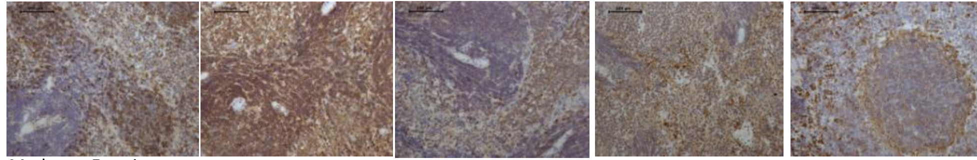


Figure 4.10 Immunohistological labeling of several CD markers in the thymus (rows present the groups and columns present the surface antigens)

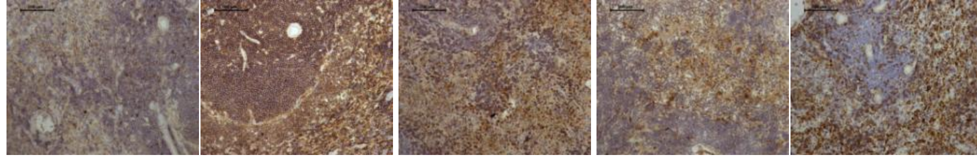
CD25 which is known as a B cell marker stimulated by anti IgM antibody, was typically expressed within the lymphatic nodules of the white pulp of the spleen in all groups. It was also expressed in a low number of the medullary lymphocytes in thymic lobules (Figures 4.11, 4.12). CD45 exhibited a broad and strong expression pattern in both the thymus and the spleen parenchymes. CD45 RA was expressed on resting T cells (Figures 4.11, 4.12). CD56 which is known as a NK cell marker was also expressed on a subset of T and B cells. In this study, CD56 exhibited a limited expression pattern within the red pulp of the spleen and the medulla of thymic lobules. Anti phagoytic antibody was strongly expressed in all of the parenchymal and stromal macrophages of the spleen and the thymus (Figures 4.11, 4.12).

Spleen

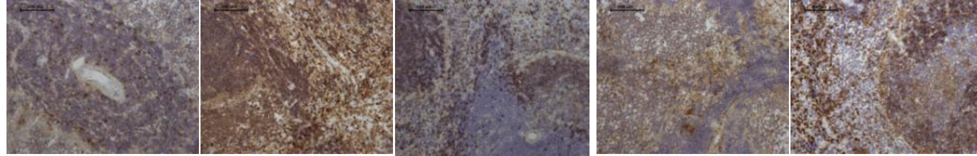
Endurance Exercise



Moderate Exercise



Control

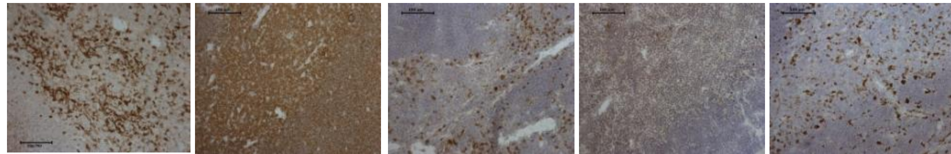


CD25 CD45 CD45RA CD56 pha

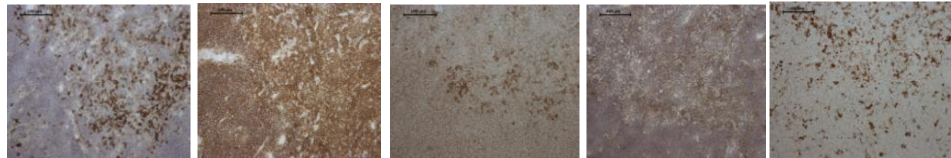
Figure 4.11 Immunohistological labeling of several CD markers in the spleen (rows present the groups and columns present the surface antigens)

Thymus

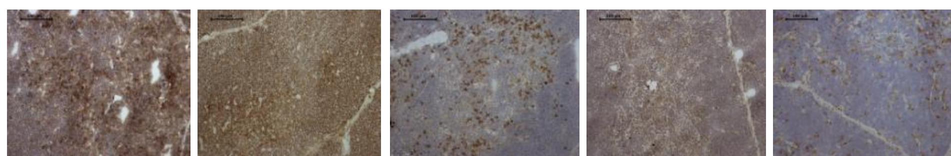
Endurance Exercise



Moderate Exercise



Control



CD25 CD45 CD45RA CD56 pha

Figure 4.12 Immunohistological labeling of several CD markers in the thymus.

CD8a$^+$ and CD8b$^+$ cell numbers increased in both the moderate and the endurance type of exercise groups when compared to the control group in the spleen (Figure 4.13). Moderate and endurance type of exercise groups did not differ in terms of CD8a$^+$ and CD8b$^+$ cell numbers. On the contrary, CD11b$^+$ cells numbers decreased only in the endurance type of exercise group compared to the moderate type of exercise ($p= .024$) and the control ($p= .007$) groups in the spleen (Figure 4.14). Although CD25$^+$ cells also decreased with exercise, only the difference between the control and the moderate type of exercise group was statistically significant ($p= .022$) (Figure 4.15). On the other hand, PHA$^+$ cell number increased in the endurance type of exercise group when compared to the control group ($p= .006$) (Figure 4.16).

Unlike the spleen, apoptotic cell percentage did not differ between groups in the thymus. Only the CD8a$^+$ cell number increased significantly in the endurance type of exercise group when compared to the control group in the thymus ($p= .038$) (Figure 4.17).

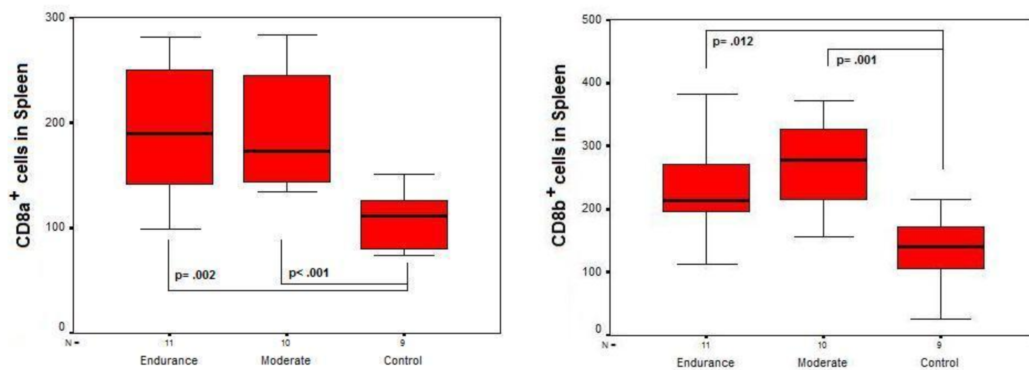


Figure 4.13 CD8a$^+$ and CD8b$^+$ cell numbers in the endurance type of exercise, the moderate type of exercise and the control groups in the spleen.

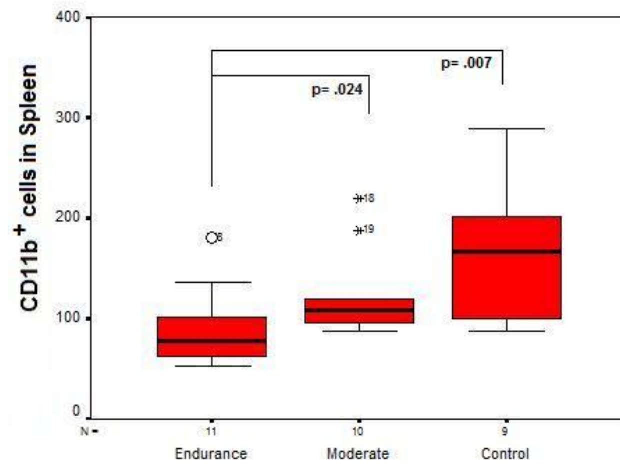


Figure 4.14 CD11b$^+$ cell numbers in the endurance type of exercise, the moderate type of exercise and the control groups in the spleen.

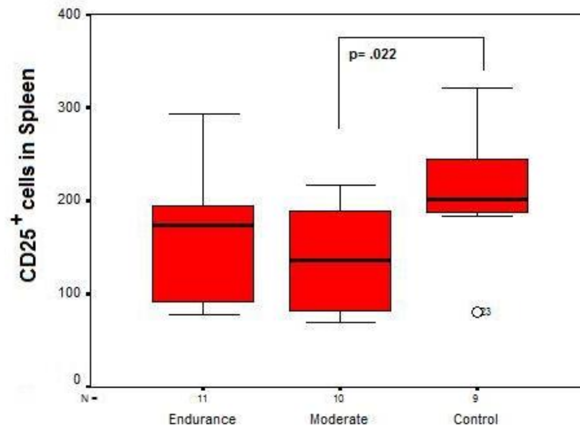


Figure 4.15 CD25$^+$ cell numbers in the endurance type of exercise, the moderate type of exercise and the control groups in the spleen.

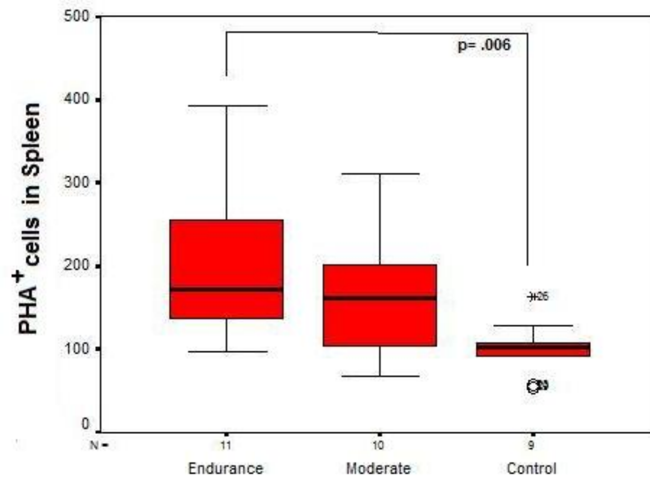


Figure 4.16 PHA$^+$ cell numbers in the endurance type of exercise, the moderate type of exercise and the control groups in the spleen.

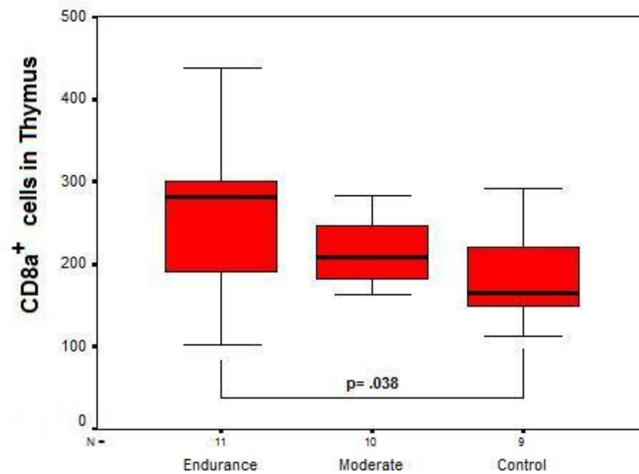


Figure 4.17 CD8a$^+$ cell numbers in the endurance type of exercise, the moderate type of exercise and the control groups in the thymus.

4.1.5 RT-PCR results

There was a statistically significant increase in the splenic CB1 receptor mRNA expression in the endurance type of exercise group compared to that of the moderate type of exercise and the control groups (Figure 4.18). The increase of plasma ANA concentration assisted the increase of CB1 receptor mRNA expression in these groups.This was meaningful since ANA has been shown to be the primary ligand of the CB1 receptor and the spleen tissue reflects the peripheral blood. It can be concluded that exercise causes augmentation of ANA in the serum and up regulates the CB1 receptor in the spleen. Thus ANA may act via its CB1 splenic receptor in exercise mediated immune modulation.

CD 247 mRNA expression which exhibits the presence of T cells did not reveal any significant difference between the control and the exercise groups. CD 69 was used as a marker of T cell activity. A significant increase in the CD69 mRNA expression was noted in the moderate type of exercise group when compared to that of the endurance type of exercise and the control groups. CD69 mRNA expression level was higher in the endurance type of exercise group when compared to the control group; but this difference was not statistically significant (Figure 4.19).

The apoptotic pathway components BAX, caspase 3, Fas and the anti-apoptotic component Bcl2 mRNA expression remained unaltered in both the spleen and the thymus which leaded to the conclusion that either apoptosis is not favored at the cellular level or the apoptotic cascade depends on phosphorylation rather than nuclear changes at the gene level (Figure 4.20).

No significant difference was noted in NK cell gene expressions (NKRp46 and NKRp1A) among the endurance type of exercise, the moderate type of exercise or the control groups in both the spleen (Figure 4.22).and the thymus (Figure 4.26). B cell gene expression pattern (CD 19 and Ig M) did not show any significant difference between the groups in the spleen and thymus by RT-PCR. (Figure 4.21, 4.26).

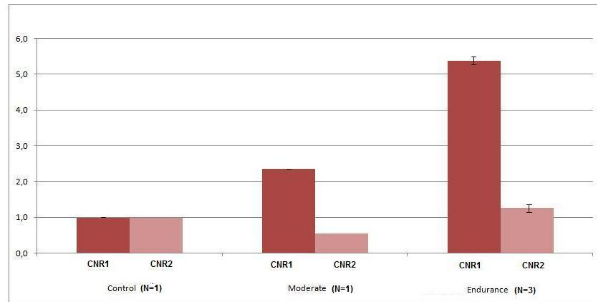


Figure 4.18 Splenic CB1 (CNR1) and CB2 (CNR2) receptors mRNA/gene expression level of the endurance type of exercise, the moderate type of exercise and the control groups.

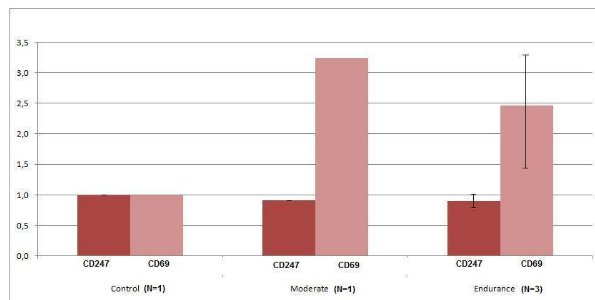


Figure 4.19 Splenic CD247 and CD69 mRNA expression levels of the endurance type of exercise, the moderate type of exercise and the control groups.

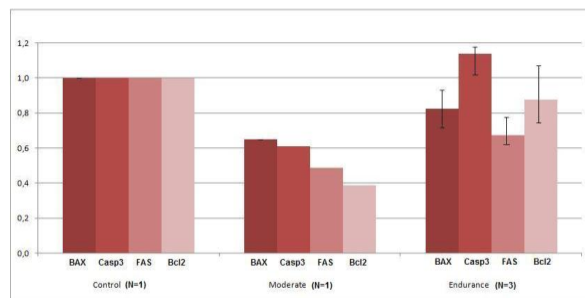


Figure 4.20 Expression of the apoptotic pathway genes BAX in the spleen

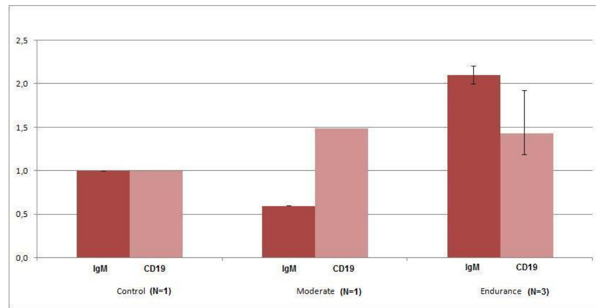


Figure 4.21 CD19 gene expression reflecting the presence of B lymphocytes and IgM gene expression specific for B cell activity revealed no significant difference between the groups in the spleen.

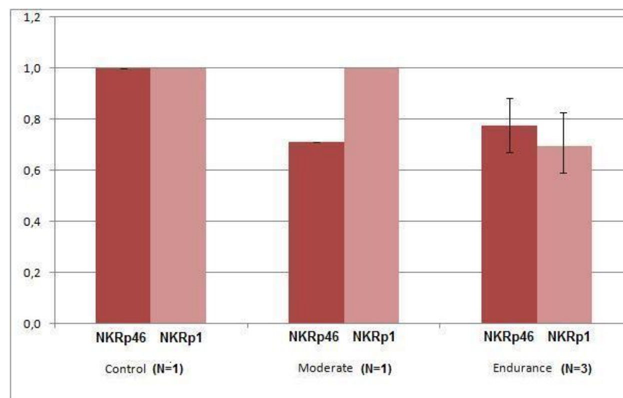


Figure 4.22 NKRp46 gene expression reflecting the presence of NK cells and NKRp1A gene expression specific for NK cell activity showed no significant difference between the groups in the spleen.

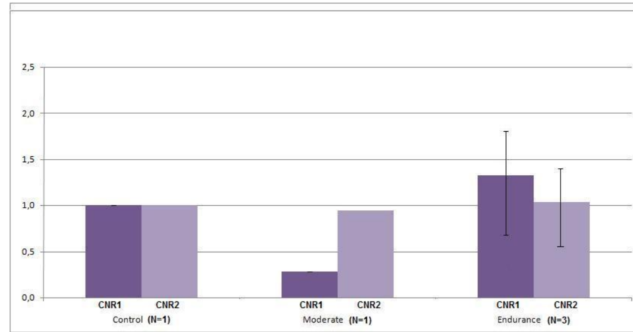


Figure 4.23 CB1 (CNR1) and CB2 (CNR2) receptor mRNA expression levels of the endurance type of exercise, the moderate type of exercise and the control groups in the thymus.

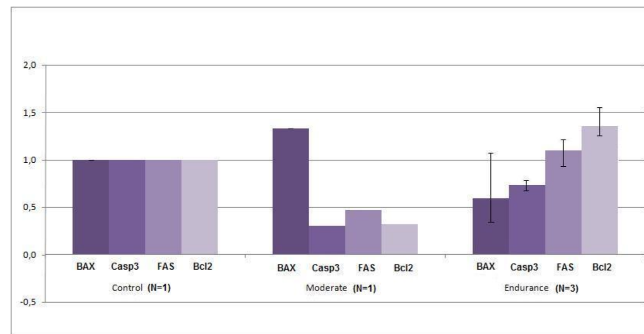


Figure 4.24 Expression of apoptotic pathway genes BAX, caspase 3, FAS and Bcl2 in the thymus in the exercise and the control groups.

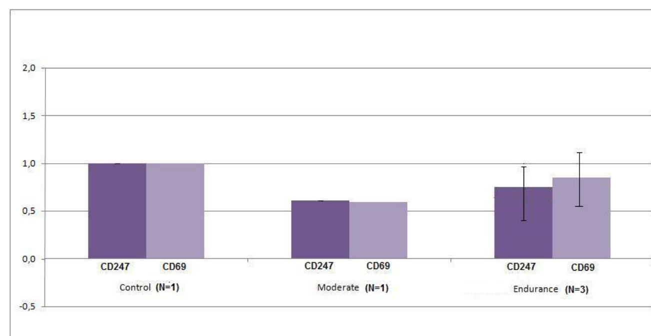


Figure 4.25 CD247 gene expression which shows the presence for T cells and CD69 gene expression which is specific in for T cell activity in the thymus in the control and the exercise groups.

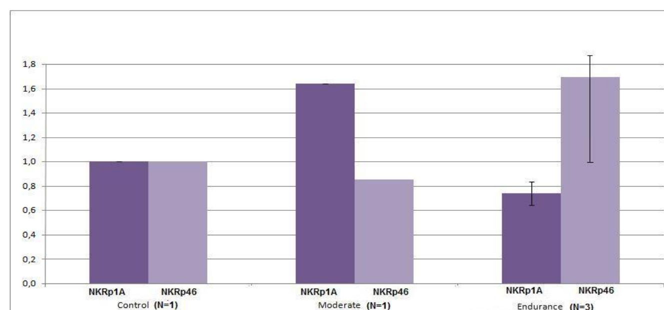


Figure 4.26 NKRp46 and NKRp1A gene expressions in the thymus.

4.1.6 Relationship between endocannabinoid serum levels and immune effector cell counts

There were statistically significant negative correlations between white blood cell counts, number of lymphocytes, neutrophils and endocannabinoid levels (Table 4.5). Results showed statistically significant negative correlations between 2-AG levels and the number of natural killer cells (p=.001) and natural killer like T lymphocytes (p= .004). On the other hand, there was a statistically significant negative correlation between the ANA levels and the number of B lymphocytes (p< .001), whereas the same endocannabinoid was significantly positively correlated to the T lymphocyte counts (p< .001) (Table 4.5).

Table 4.5 Correlation coefficients between white blood cell counts, immune effector cell counts and endocannabinoids.

	ANA (P; r values)	2-AG(P; r values)
White Blood Cell Count	-.80** (p<.001)	-.52** (p=.004)
Lymphocyte Percentage	-.66** (p<.001)	-.52** (p=.004)
Neutrophil Percentage	. 72** (p<.001)	.56** (p=.002)
Natural Kill Cells	Not significant	.60**(p= .001)
B Lymphocyte Cells	-.74** (p<.001)	Not significant
T Lymphocyte Cells	.67**(p<.001)	Not significant
NK-like T Lymphocyte Cells	Not significant	-. 52** (p= .004)

** Correlation is significant at the α = 0.01 level (2- tailed).

4.2 Part 2 of the study

4.2.1 Measurement of ANA serum levels

ANA serum levels were significantly higher in all exercising groups with or without antagonist application when compared to the control group (Figure 4.27). On the other hand, there were no statistically significant differences among the endurance exercise, the AM281 and the AM281+AM630 groups in terms of ANA serum levels.

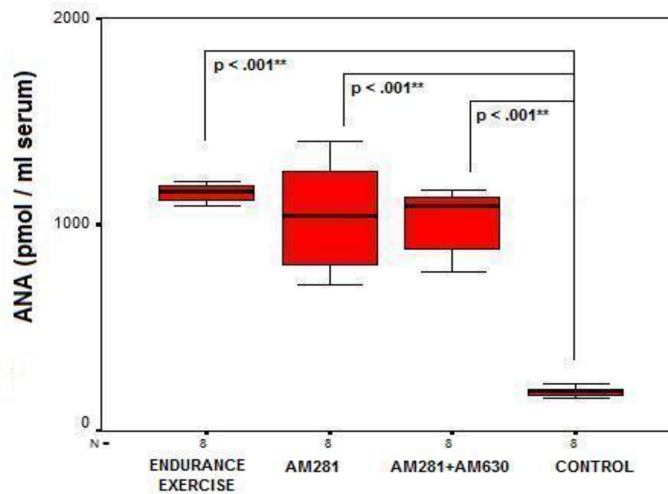


Figure 4.27 ANA serum levels in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups

Table 4.6 Z scores and p values of Mann-Whitney U tests for ANA between the control group and the other three (endurance exercise, AM281 and AM281+AM630) groups.

	Endurance Exercise Group	AM281	AM281+AM630
Control Group	-3.34 **(p= .001)	-3.47** (p= .001)	-3.34 **(p= .001)

** Correlation is significant at the $\alpha = 0.01$ level (2- tailed).

4.2.2 White blood cell counts

Lymphocyte percentage in blood decreased significantly in the endurance exercise group when compared to the control group (F= -40,26; p<.001). CB receptor antagonist applied groups, AM281 (F= 22,91; p=.005) and AM281+AM630 (F= 18,90; p=.023), had higher blood lymphocyte percentages compared to the endurance exercise group. However, these groups showed a statistically significant decrease in blood lymphocyte percentage when compared to the control group; the AM281 (F= -17,34; p=.027) and the AM281+AM630 (F= -21,36; p=.005) groups, respectively (Figure 4.28). On the other hand, lymphocyte percentages of these antagonist applied groups were not statistically different from each other.

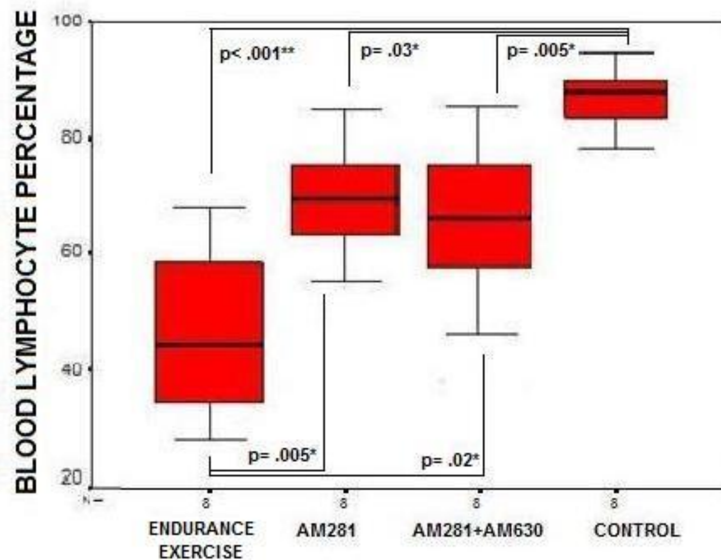


Figure 4.28 Lymphocyte percentage in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in blood

4.2.3 Flow cytometry (Immune effector cell counts)

Blood T lymphocyte ($CD3^+$) percentage increased significantly in the endurance exercise group when compared to the control (Z= 2,42; p=.016) and the AM281+AM630 (Z= 2,05; p=.04) groups. On the other hand, there was no statistically significant difference among the control, the AM281, and the AM281+AM630 groups which may indicate that these antagonists were able to eliminate the exercise dependent increase in blood T lymphocyte percentage (Figure 4.29.A). Moreover, blood helper T ($CD3^+CD4^+$) lymphocyte percentage did not differ between these groups. Blood cytolytic T ($CD3^+CD8a^+$) lymphocyte percentage however was significantly lower in the endurance exercise group when compared to control (Z= -2,21; p= .027) and AM281 (Z= -2,73; p= .006) groups. There was also a statistically significant difference between the AM281 and the control groups (Z= 2,47; p= .013)(Figure 4.29.B).

Difference was not observed between the groups in terms of NK ($CD3^+CD161a^+$) and B ($CD3^-CD45RA^+$) cells of blood and tissue samples (spleen and thymus). T lymphocyte and T lymphocyte subset percentages did not differe between the control, the endurance exercise and the antagonist groups in thymus samples. However, interesting results were noted for some immune effector cell parameters in the spleen. Splenic T lymphocyte ($CD3^+$) percentage increased significantly in all exercising groups with or without antagonist application when compared to the control group (Table 4.7) (Figure 4.30). Moreover, the same increase in these groups was observed also for the double positive T lymphocyte ($CD3^+CD4^+CD8a^+$) percentage (Table 4.7) (Figure 4.31).

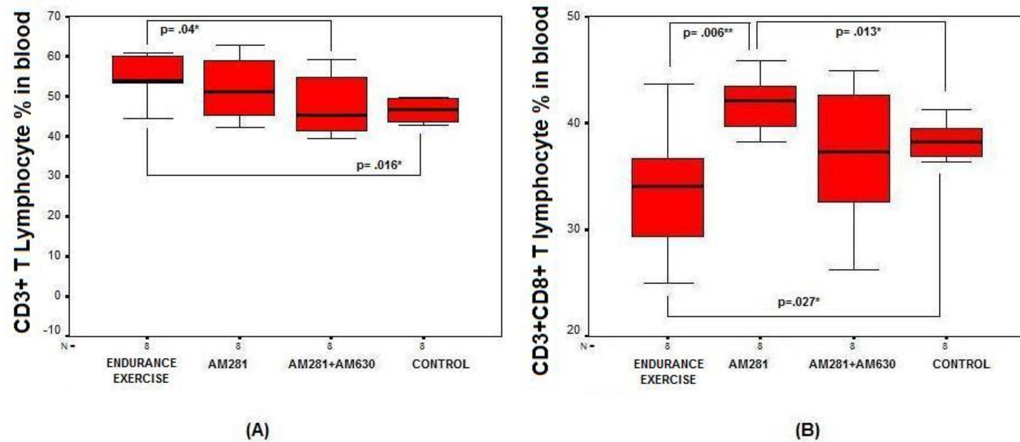


Figure 4.29 (A) T ($CD3^+$) lymphocyte percentage and (B) cytolytic ($CD3^+CD8a^+$) T lymphocyte percentages in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in blood

On the other hand, there was a statistically significant decrease in terms of NK like T lymphocyte ($CD3^+CD161a^+$) percentage in the endurance exercise, the AM281, the AM281+AM630 groups when compared to the control group (Table 4.7) (Figure 4.32). NK like T lymphocyte ($CD3^+CD161a^+$) percentage did not differ between the endurance exercise, the AM281, and the AM281+AM630 groups.

Not only the percentages of T lymphocytes, but also their activity was assessed by flow cytometry in blood, the thymus and the spleen. $CD3^+RT1b^+$ T lymphocyte percentage decreased significantly in the endurance exercise, the AM281 and the AM281+AM630 groups when compared to the control group (Table 4.7) (Figure 4.33). IL-2 receptor activated T ($CD3^+CD25^+$) lymphocyte percentage however did not show any difference among groups. Although AM281 alone could not avoid the exercise dependent decrease in the activated T ($CD3^+RT1b^+$) lymphocyte percentage, AM281 + AM630 caused a significant increase in the T ($CD3^+RT1b^+$) lymphocyte percentage when compared to the endurance exercise group (Z= 2,47; p= .013) (Figure 4.33).

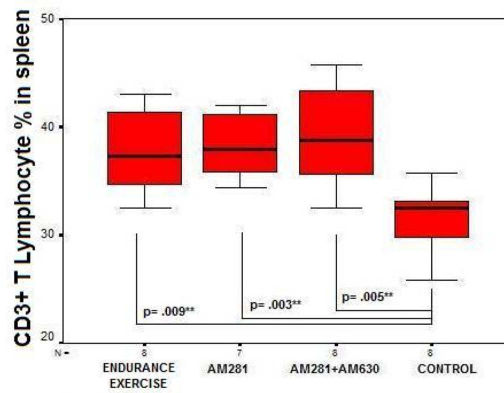


Figure 4.30 ($CD3^+$) T lymphocyte percentages in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in the spleen.

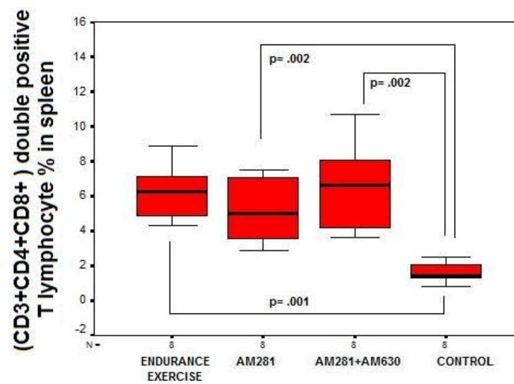


Figure 4.31 ($CD3^+$ $CD4^+CD8a^+$) double positive T lymphocyte percentages in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in the spleen.

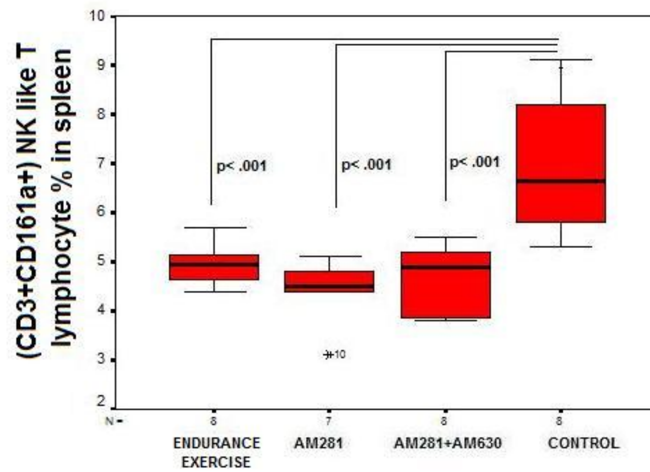


Figure 4.32 ($CD3^{+}CD161a^{+}$) NK like T lymphocyte percentages in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in the spleen.

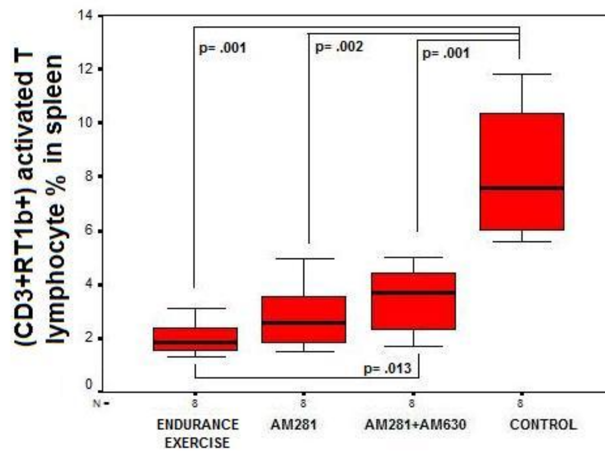


Figure 4.33 ($CD3^{+}$ RT1b) activated T lymphocyte percentages in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in spleen.

Table 4.7 Z scores and p values of Mann-Whitney U tests for splenic immune effector cell percentages between the groups.

	Exercise Group	AM281	AM281+AM630	Cell type
Control Group	-2.63 **(p= .009)	-3.01** (p= .003)	-2.84 **(p= .005)	**T lymphocytes**
	-3.20 **(p= .001)	-3.24** (p= .001)	-3.26 **(p= .001)	**NK like T lymphocytes**
	-3.36 **(p= .001)	-3.16** (p= .002)	-3.34 **(p= .002)	**Double positive T lymphocytes**
	-3.36 **(p= .001)	-3.36** (p= .001)	-3.15 **(p= .002)	**Activated T lymphocytes**

** Correlation is significant at the $\alpha = 0.01$ level (2- tailed).

4.2.4 Immunohistology

4.2.4.1 Assessment of apoptosis with Tunel labeling

Apoptotic cells increased in the endurance exercise group compared to the AM281 (p= .007), the AM281+AM630 (p= .002) and the control (p< .001) groups in the spleen (Figure 4.34), but the difference was not statistically significant in the thymus. The endurance exercise and the antagonist groups revealed similar locations for apoptosis in both the spleen and the thymus compared to the first part of the study (Figure 4.35, 4.36).

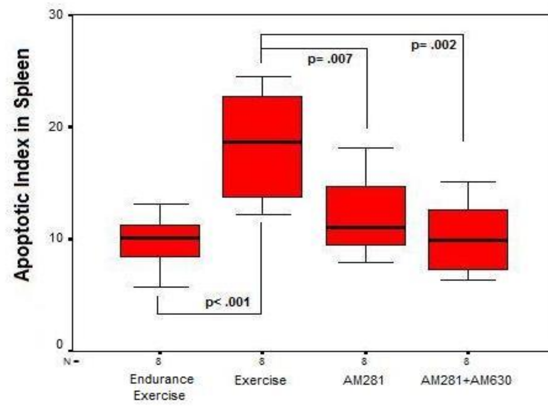


Figure 4.34 Apoptotic index in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in the spleen.

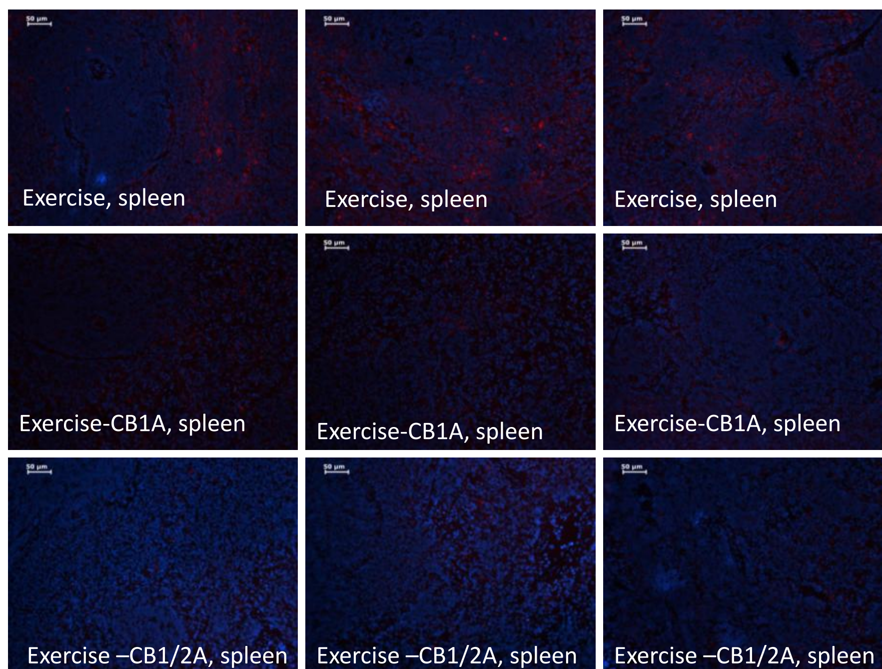


Figure 4.35 Labeling of apoptotic cells by the TUNEL method in the endurance exercise, the AM281 (CB1A) and the AM281+AM630 (CB1/2A) groups in the spleen

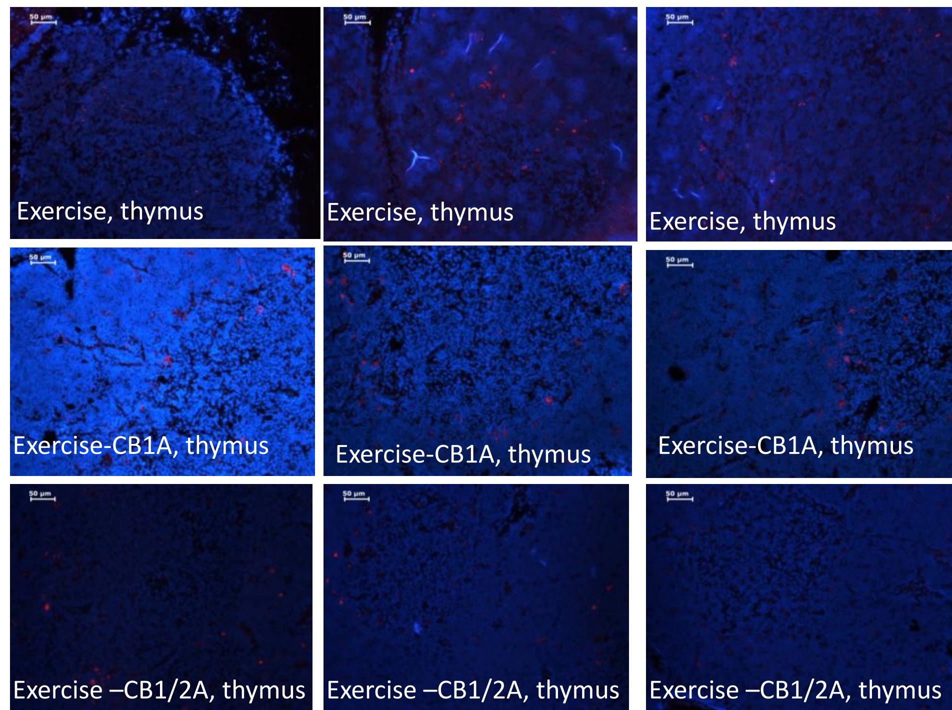


Figure 4.36 Apoptotic cells in the endurance exercise, the AM281 (CB1A) and the AM281+AM630 (CB1/2A) groups in the thymus

4.2.4.2 Quantitative evaluation of immune labeling

Immune efffector cell subsets presented similar immune morphology and tissue distribution pattern in the control, the endurance exercise and the antagonist groups in the white pulp of the spleen and the thymic lobules (Figure 4.37, 4.38, 4.39, 4.40).

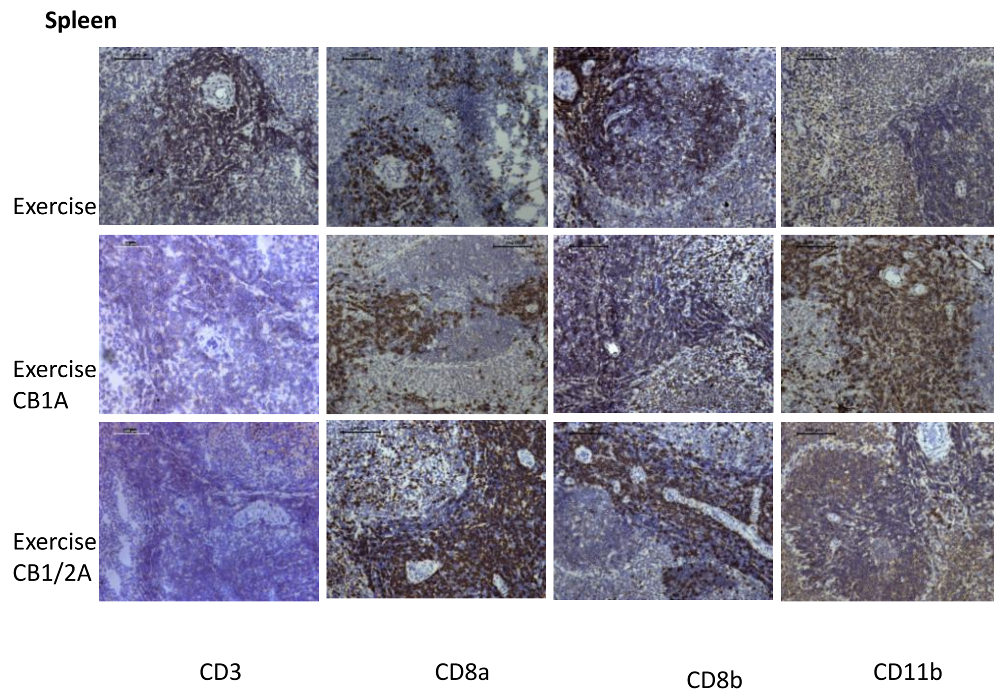


Figure 4.37 Immunohistological labeling of several CD markers in the spleen (rows present the groups and columns present the surface antigens)

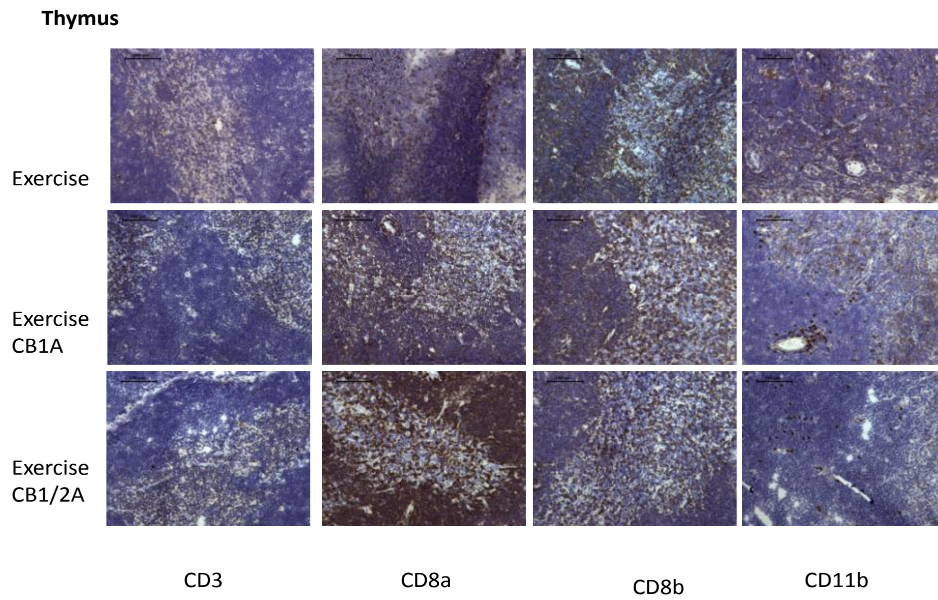


Figure 4.38 Immunohistological labeling of several CD markers in the thymus (rows present the groups and columns present the surface antigens)

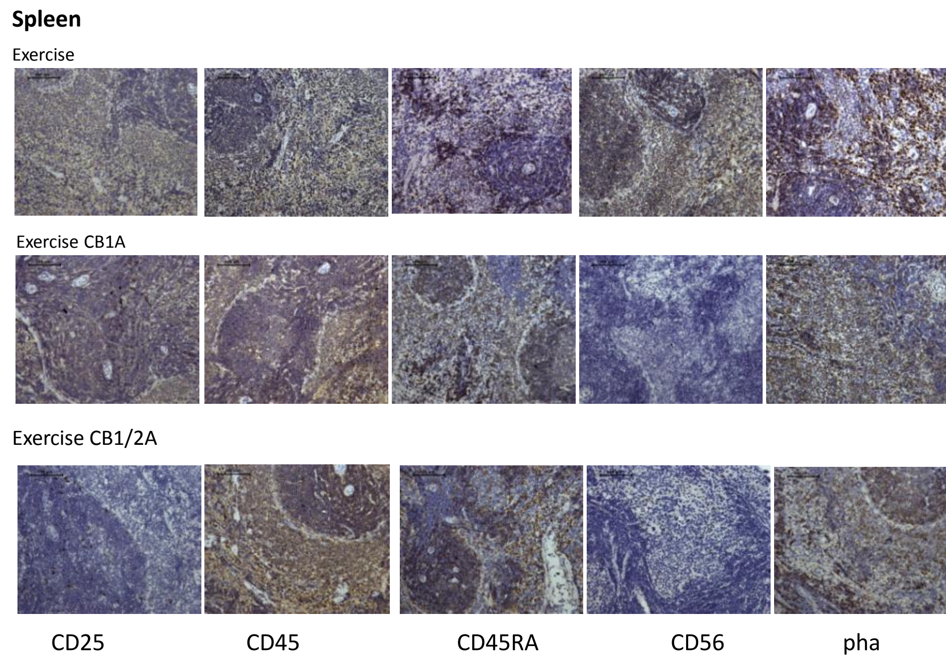
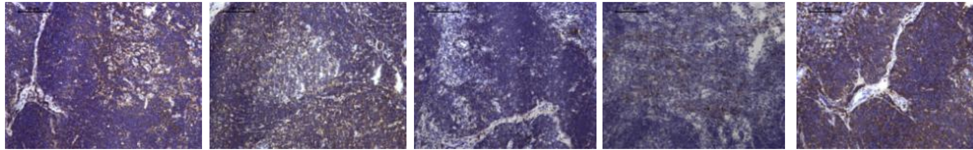


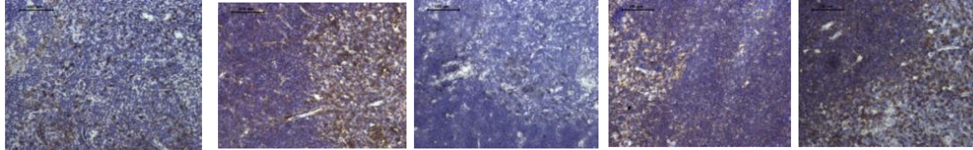
Figure 4.39 Immunohistological labeling of several CD markers in the spleen (rows present the groups and columns present the surface antigens)

Thymus

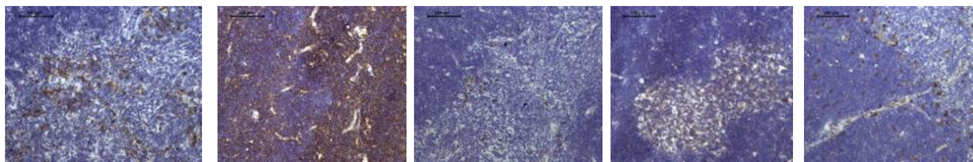
Exercise



Exercise CB1A



Exercise CB1/2A



CD25 CD45 CD45RA CD56 pha

Figure 4.40 Immunohistological labeling of several CD markers in the thymus (rows present the groups and columns present the surface antigens)

CD8a^{+}cells increased significantly in the endurance exercise group (p= .004) and the AM281+AM630 group (p= .015) when compared to the control group (Figure 4.41). CD8b^{+} cells, on the other hand, increased slightly in all exercising groups, however the only significant difference was observed between the endurance exercise and the control groups (p= .011) (Figure 4.41). Moreover, there was a significant increase in the number of PHA^{+} cells in the endurance exercise group when compared to the AM281 (p= .001), the AM281+AM630 (p= .01) and the control (p= .001) groups in the spleen (Figure 4.42). Unlike the spleen, the thymus resisted to any kind of alteration..

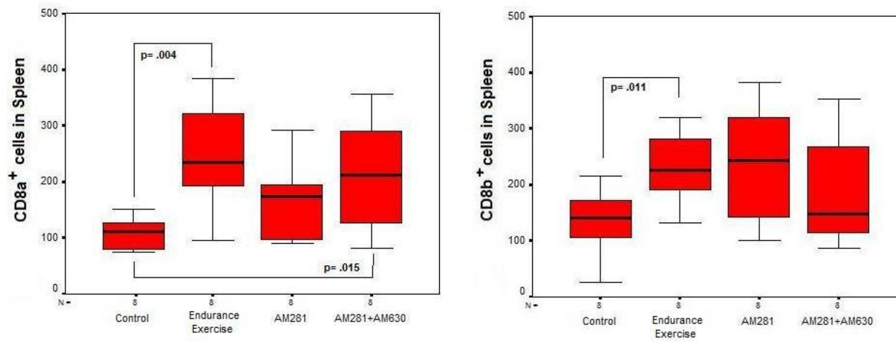


Figure 4.41 ($CD8a^+$ and $CD8b^+$) cytolytic T lymphocyte cell number in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in the spleen.

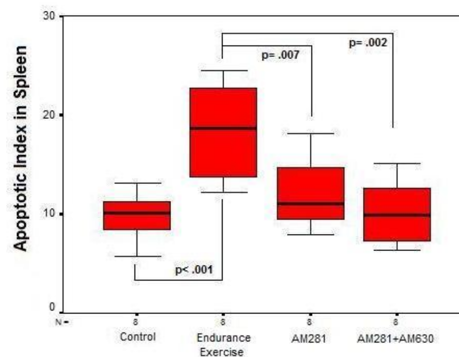


Figure 4.42 PHA^+cell number in the endurance exercise, the AM281 (CB1R antagonist), the AM281+AM630 (CB1+CB2 R antagonists) and the control groups in the spleen.

4.2.5 Microarray analysis results

The quality of the extracted and purified mRNA from the thymus and the spleen tissues fitted the criteria for the microarray analysis except the mRNA of the thymus in C5 (control 5th animal) (Table 4.8)(Figures 4.42 and 4.43).

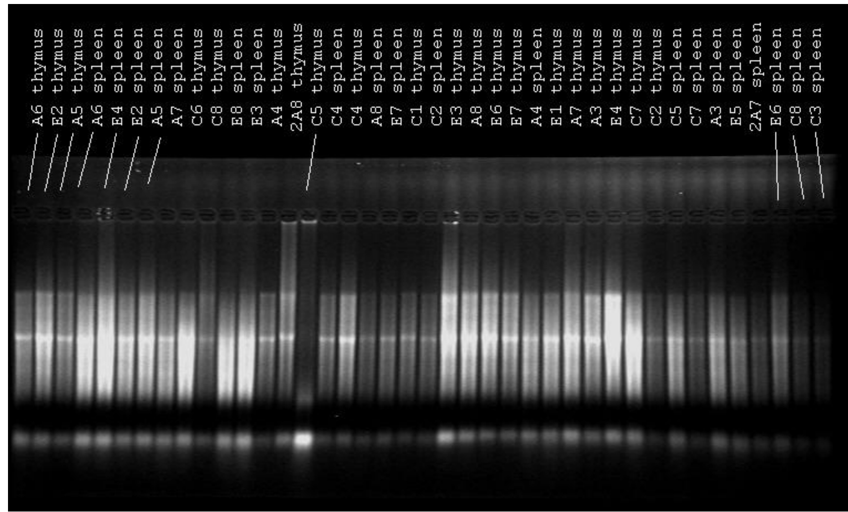


Figure 4.43 Western blotting of the isolated mRNA from the spleen and the thymus of the endurance exercise (E), the control (C), the AM281 (A) and the AM281+AM630 (2A) groups.

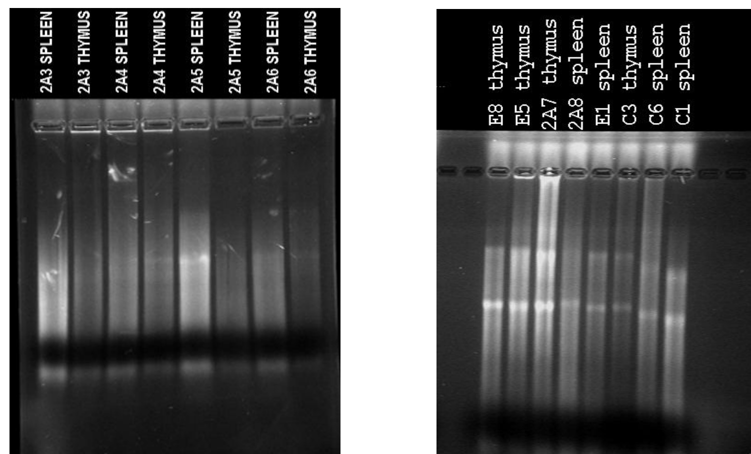


Figure 4.44 Western blotting of the isolated RNA from the spleen and the thymus tissues of the endurance exercise (E), the control (C), the AM281 (A) and the AM281+AM630 (2A) groups.

Table 4.8 Quality control results of the mRNA isolated from the spleen and the thymus tissues in the endurance exercise (E), the control (C), the AM281 (A) and the AM281+AM630 (2A) groups.

tissue	sample ID	sample no.	ID	ng/µl	260/280	260/230
SPLEEN	C1	22	C4SPLEEN	2123	1,98	2,07
	C2	46	C3SPLEEN	3140	1,94	1,76
	C3	35	C6SPLEEN	3911	1,82	1,91
	C4	42	C2SPLEEN	3153	1,96	2,07
	E1	26	E1SPLEEN	3695	1,86	1,92
	E2	37	E7 SPLEEN	3651	1,87	1,86
	E3	33	E5 SPLEEN	4195	1,62	1,55
	E4	39	E6 SPLEEN	4423	1,42	1,43
	A1	36	A8 SPLEEN	3844	1,83	1,84
	A2	30	A3 SPLEEN	4377	1,43	1,47
	A3	20	A6 SPLEEN	4455	1,17	1,31
	A4	11	A4 SPLEEN	4374	1,31	1,46
	2A1	38	2A7 SPLEEN	3979	1,78	1,77
	2A2	18	2A8 SPLEEN	2762	1,93	2,1
	2A3	53	2A5 SPLEEN	2399	2	1,85
	2A4	55	2A6 SPLEEN	2247	1,99	2,11
THYMUS	C1	34	C4THYMUS	2612	1,97	2,13
	C2	31	C3THYMUS	3330	1,92	1,99
	C3	41	C1THYMUS	3229	1,94	2,11
	C4	23	C2THYMUS	4432	1,45	1,64
	E1	2	E8 THYMUS	3603	1,88	2,03
	E2	6	E6THYMUS	4228	1,64	1,67
	E3	9	E5THYMUS	4151	1,73	1,95
	E4	10	E7THYMUS	4282	1,6	1,68
	A1	8	A4 THYMUS	3488	1,9	2,11
	A2	15	A3 THYMUS	4176	1,7	1,68
	A3	13	A7 THYMUS	4342	1,54	1,61
	A4	5	A8 THYMUS	4413	1,43	1,55
	2A1	16	2A8 THYMUS	2786	1,96	1,98
	2A2	14	2A7 THYMUS	3917	1,8	1,98
	2A3	50	2A3 THYMUS	3412	1,9	1,83
	2A4	52	2A4 THYMUS	3088	1,96	1,73

302 genes out of 31099 genes were significantly altered in their transcription level in the exercising group at the nominal 0.001 level of the univariate test. 190 of these genes were named whereas 112 of them were annotated in the array and could therefore not be investigated.

There was a significant alteration in the transcription of the CB2 receptor gene Cnr2 ($p= .003$) and FAAH enzyme protein coding gene ($p= .04$) between the control and the AM281+AM630 antagonist groups in spleen. The gene responsible for IgD was also downregulated significantly in the endurance exercise ($p= .016$) and the antagonist groups, AM281 ($p< .001$) and the AM281+AM630 ($p= .002$) when compared to the control group (Figure 4.44). Moreover, there was a significant fold change in CD8 marker protein coding gene between exercise, control and antagonist groups (Figure 4.45). Also perforin coding gene showed alteration in the transcription level between the endurans exercise and the AM281+AM630) groups (Figure 4.46).Moreover, alterations in the transcription of the CD38 marker protein coding gene ($p= .017$) and IL-15 protein coding gene ($p= .005$) were observed between the control and the AM281 antagonist groups in spleen.

Interestingly, there were also significant alterations in the transcription of the CD4 ($p= .018$), CD8 ($p= .022$), CD48 ($p= .009$) marker protein coding genes, IL 4 protein coding gene ($p= .03$), IL15 protein coding gene ($p= .002$), IL-1a protein coding gene ($p= .015$), INFγ protein coding gene ($p= .03$), Caspase 8 protein coding gene ($p= .017$), NFκB protein coding gene ($p= .026$) in addition to endocannabinoid system related genes coding for CB2 receptor ($p= .015$), FAAH enzyme ($p= .004$), MGL enzyme ($p= .046$) and DGL enzyme ($p= .004$) between the control and the AM281+AM630 groups in the thymus. CD48 marker protein coding gene between the endurance exercise and the AM281+AM630 groups ($p= .03$). Apoptotic pathway related genes coding for caspase8 ($p= .006$) and NFκB ($p= .037$) were upregulated and showed altered expression levels between the endurance exercise and the AM281+AM630 groups.Caspase8 coding gene showed also altered expression between the control and the endurance exercise groups ($p= .05$).

Genes coding for CD69, CD4, CD 244, CD14, CD48, IgM, did not show any changes in transcription in the spleen. The expressions of the apoptotic pathway related genes Bax, Fas, casp3, Bcl2, Fasl, Casp8, and NFkB were not altered between experiment groups. Moreover, genes coding for IL-4, IL-10, IL-6, IL-2, IL-1a, IFNγ did not show any significant difference in their expression levels between groups in the spleen.

CD3 zeta, CD25, CD45RA, CD45RO, CD19, CD10, CD21, CD27, CD11c, CD11b, CD16, CD56, CD57, CD161a, NKp46, rNKp30 and RT1b coding genes were not available or yet annotated in the array and could therefore not be investigated.

However, several genes were disregulated during exercise. Genes with maximum impact in spleen were tabulated in Table 4.10. Moreover, there were also significant alterations in the transcription of several genes related to the immune function (Table 4.9), however these data needs to be further elucidated and confirmed at protein level with upcoming experiments.

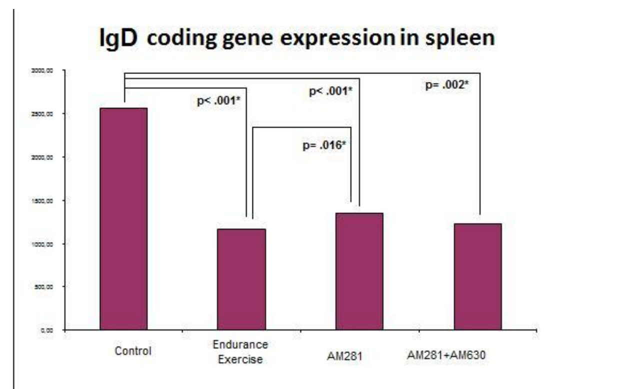


Figure 4.45 Alterations in the expression rate of IgD protein coding gene in the control, the endurance exercise, the AM281 and the AM281+AM630 groups.

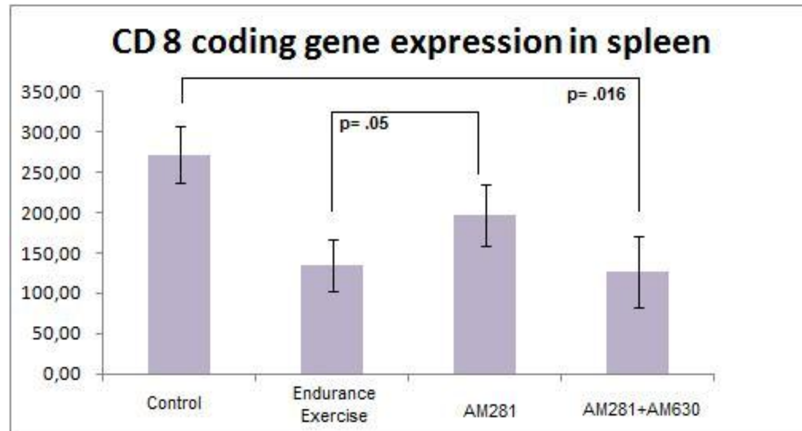


Figure 4.46 Alteration in the expression level of CD8 marker protein coding gene in the control, the endurance exercise, the AM281 and the AM281+AM630 groups.

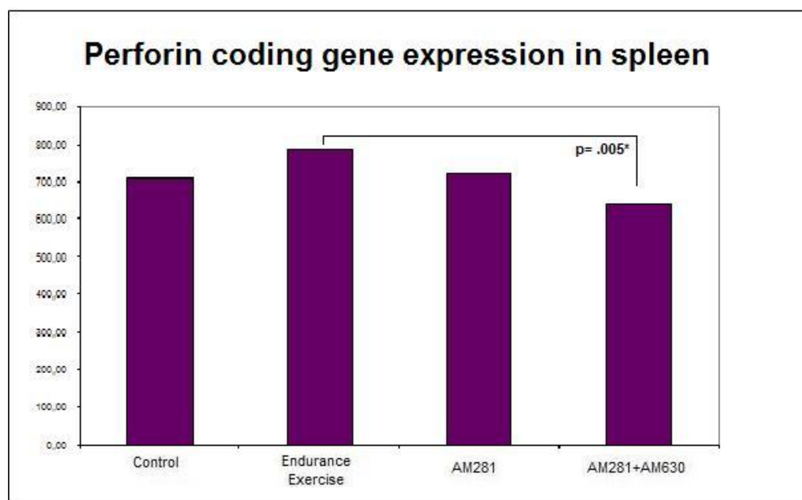


Figure 4.47 Alteration in the expression level of perforin protein coding gene Prf1in the endurance exercise and the AM281+AM630 groups.

Table 4.9 Fold change of genes related with the immune system

Description	Fold change
similar to killer activatory receptor-like protein p91D	7.26
killer cell lectin-like receptor subfamily B, member 1A	2.16
leukocyte immunoglobulin-like receptor, subfamily B, member 4	1.99
Fc receptor, IgE, low affinity II, alpha polypeptide	1.87
killer cell lectin-like receptor, family E, member 1	1.77
leukocyte receptor cluster (LRC) member 9	1.68
CD180 molecule	1.67
thymus cell antigen 1, theta	1.65
granzyme K	1.62
Ret proto oncogene	1.62
major histocompatibility complex, class II, DM beta	1.58
Sialophorin	1.55
presenilin associated, rhomboid-like	1.50
chemokine (C-C motif) ligand 3	1.49
lymphotoxin B	1.37
fos-like antigen 2	0.67
chemokine (C-X-C motif) receptor 4	0.50

Table 4.10 Genes with maximum impact in spleen

Description	Fold change
Heat shock 70kD protein 1B (mapped)	49.8
Heat shock 70kD protein 1A	44.4
FBJ osteosarcoma oncogene	8.1
Heat shock 105kDa/110kDa protein 1	8.0
Heat shock protein 1	7.4
DnaJ (Hsp 40) homolog, subfamily A, member4	5.0
Heat shock protein 90, alpha (Cytosolic), class A, member1	4.8
Similar to death effector domain containing DNA binding protein 2	3.8
Secretin receptor	3.8
Cysteine and histidine rich domain (CHORD)-containing zinc binding protein...........	3.5
Proprotein convertase subtilisin/kexin type 1	3.4
Calcium/calmodulin dependent protein kinase II, alpha	3.3
DnaJ (Hsp40) homolog, subfamily B, member	3.3
Fos-like antigen 2	2.7
Similar to AHA1, activator of heat shock 90kDa	2.7
NUAK family, SNF1 like kinase, 2	2.7
ADP ribosylation factor like 11	2.5
Heat shock protein 90kDa alpha (cytosolic)	2.2
Lipin2	2.4
TSC22 domain family, member 4	2.2

4.2.6 Relationship between ANA serum levels and immune effector cells percentages.

Correlation coefficients were computed between ANA and the immune system parameters (total lymphocyte, NK cell, B lymphocyte, T lymphocyte, NK like T lymphocyte, helper T lymphocyte, cytolytic T lymphocyte, and activated T lymphocyte percentages) in blood, the spleen and the thymus. NK like T lymphocyte ($CD3^+CD161a^+$) and activated T lymphocyte ($CD3^+RT1b^+$) percentages were significantly negatively correlated to ANA in the spleen (Table x). Moreover, T lymphocyte ($CD3^+$) and double positive T lymphocyte ($CD3^+CD4^+CD8a^+$) percentages were significantly positively correlated to ANA in the spleen (Table 4.9). On the other hand, there was no statistically significant correlation between ANA and immune system parameters in blood and the thymus.

Table 4.11 Correlation coefficients between ANA and immune effector cell percentages in the spleen

	ANA (r value ;p value)
NK like T lymphocyte % ($CD3^+CD161a^+$)	-.45* (p=.014)
Activated T lymphocyte % ($CD3^+RT1b^+$)	-.62** (p<.001)
T lymphocyte % ($CD3^+$)	. 40* (p=.03)
Double positive T lymphocyte % ($CD3^+CD4^+CD8a^+$)	.67** (p<.001)

** Correlation is significant at the $\alpha = 0.01$ level (2- tailed).

* Correlation is significant at the $\alpha = 0.05$ level (2- tailed).

CHAPTER 5

DISCUSSION

At the beginning of the study, it was assumed that exercise intensity may have an effect on both (1) the endocannabinoid and (2) the immune systems. Therefore, in the first part of the study, moderate and high intensity protocols were utilized to observe the effect of intensity on the endocannabinoid system by monitoring the changes in ANA and 2-AG serum levels. In the second part of the study, the relationship between exercise induced increase in the ANA serum levels and immune effector cell alterations (B cell, NK cell, NK like T cell, T cell, helper T cell, cytolytic T cell, double positive T cell and activated T cell) in three different tissues (blood, the spleen and the thymus) were researched at the cellular and the molecular levels using flow cytometry, immunohistological analysis, RT-PCR and microarray methods. Moreover, with the use of CB1 and CB2 receptor antagonists, AM281 and AM630 respectively, it was aimed to determine the involvment of these receptors in the exercise dependent alteration in the endocannabinoid and the immune systems.

Findings of this study provided the first evidence that exercise performed at moderate and high intensities activated the endocannabinoid system differently and are suggestive of a new neurohumoral mechanism for the endurance type of exercise dependent immune system alterations. As endurance type of exercise dramatically elevated ANA and to some extend 2-AG serum levels, we hypothesized that endocannabinoids might be produced in the peripheral system as a response to the endurance type of exercise and act on the immune effector cells to regulate the immune system. This hypothesis is supported by two findings: First, ANA and 2-AG are synthesized in and released from a variety of peripheral cells, including the immune effector cells (Klein et al., 2003). Second, ANA and 2-AG caused profound immune suppressive and stimulating effects (Berdyshev et al., 2006; Klein et al., 2003), which are mediated by the CB1 and/or CB2 cannabinoid receptors located on

the B, T and NK cells (Bouaboula et al., 2003).

Exercise intensities activated the endocannabinoid system at different degrees. ANA and 2-AG responded differently to exercise intensity. Only endurance type of exercise performed at high intensity dramatically elevated ANA and to some degree 2-AG levels. Endurance type of exercise caused a steep increase in ANA plasma levels from pmol to nmol/ml concentrations in the serum, whereas moderate type of exercise did not cause any significant alteration compared to the control group. This steep increase of ANA serum level in the endurance type of exercise group might be a response to stress caused by high intensity of exercise. These results are supported by a recent study that clearly showed that exercise-induced analgesia is intensity dependent (Hoffman et al., 2004) suggesting that increases in ANA serum levels and CB1 receptor signaling were exercise intensity dependent.

ANA serum levels were 2 times higher after the 16 weeks of endurance training when compared to 7 weeks training at the same exercise intensity and frequency; approximately 3 nmol and 1,5 nmol, respectively. This outcome indicated that the length of the training period might have an additional effect on the increase of ANA in the peripheral system.

Not only ANA, but also 2-AG serum levels differed in the endurance type of exercise group. However unlike ANA, 2-AG serum level increased gradually with increasing exercise intensity and to a lower extend compared to ANA. Despite the fact that both ANA and 2-AG are endogenous ligands of the CB1 receptor, it is not surprising that the two ligands responded differentially to endurance and moderate types of exercise. ANA and 2-AG do not share common pathways of synthesis and metabolism, with 2-AG being largely produced by phospholipase C-mediated generation of diacylglycerol, which is subsequently converted to 2-AG by the actions of diacylglycerol lipase (Bisogno et al., 2005). Additionally, 2-AG is primarily metabolized by monacylglycerol lipase, while ANA is primarily metabolized by FAAH (Bisogno et al., 2005). As the pathways of synthesis and metabolism of these ligands are dissociable, it is not unexpected that exercise would evoke changes in one of these ligands and not the other at endurance and/or moderate intensity,

indicating that ANA might play a primary role in exercise induced endocannabinoid system alteration. The higher increase in ANA compared to 2-AG was supported by Sparling and his co-workers (Sparling et al., 2003) who stated that running and/or cycling at moderate intensity causes a significant increase in ANA but not 2-AG serum levels in trained athletes. Although Sparling and his co-workers reveal that ANA increased from 1 pmol to 3 pmol after a bout of moderate type of exercise, the increase in ANA and 2-AG serum levels at moderate intensity were very low and not significant in this study. On the other hand, for the first time in the literature, this study showed that endurance type of exercise caused a significant increase in both ANA and 2-AG serum levels.

This is the first study in literature that assessed and also compared the levels of ANA and 2-AG in the peripheral system at two different exercise intensities including moderate and endurance type of exercises. Another study in the literature supported exercise induced endocannabinoid system activation by measuring ANA and 2-AG levels in the central nervous system (hippocampus and prefrontal cortex) after voluntary wheel running with no specific exercise intensity (Hill et al., 2009). Although Hill and his co-workers supported the results of this thesis by stating that the endocannabinoid system is regulated by voluntary exercise; some aspects of that study should be clarified and discussed in details. In the first part of Hill's study, mice were terminated with decapitation but in the second experiment, animals were overdosed with sodium pentobarbital and perfused transcardially. Both termination methods are known to release endocannabinoids and results are not comparable to that of the current study (Schelling et al., 2006; Vogeser et al., 2006). However, together with the results of the studies performed by Hill and his co-workers (Hill et al., 2009), it is possible to state that exercise induced stress might have a stimulatory effect on ANA synthesis and release in both the central and the peripheral systems. In addition to the critical pre analytical conditions affecting the ex vivo endocannabinoid release in blood, employing activity on a treadmill produced physiological adaptations indicative of chronic stress in Sprague Dawley rats (Moraska et al., 2000) and these changes could potentially increase

the ANA plasma levels. Therefore, the control group in this study was placed on the treadmill for an hour without any exercise in order to equalize the stress caused by the treadmill itself (Hill et al., 2009).

It has been determined that although both ANA and 2-AG are components of the endocannabinoid system, they respond differently to endurance type of exercise. One explanation for the difference might be the functional divergences between ANA and 2-AG (Joseph et al., 2004, Sugiura et al., 2006; Sugiura et al., 2002). These endocannabinoids are synthesized through different biochemical pathways and may be produced under different conditions. Although ANA is stable in the presence of PMSF which inhibits FAAH (Cravatt et al., 2001), some of the 2-AG may rapidly converted to 1-AG and 3-AG via monoacyl glycerol lipase and/or diacylglycerollipase (Sugiura et al., 2000). Both pathways showed significant alterations between the control and the exercising groups in the transcription of related enzyme (FAAH, MGL and DGL) and CB2 receptor coding genes indicating that both ANA and 2-AG are degraded at higher rates as a response to endurance type of exercise. Metabolites of ANA and 2-AG showed a great variability according to the pathway used (Sugiura et at., 2002; Wei et.al., 2006). Diverse structures of ANA and 2-AG are strictly recognized by CB1 and CB2 receptors while the metabolites, 1-AG and 3-AG, are not (Sugiura et al., 2002; Sugiura et al., 2000; Valk et al., 1997). In addition, 2-AG may also be oxygenated and thereby converted to PGE2-G which induces a rapid mobilization of Ca+2 during exercise (Nirodi et al., 2004). Therefore, it can be concluded that it is difficult to totally prevent the hydrolysis of endocannabinoids and especially 2-AG. Not only 2-AG but also ANA can bind to CB2 receptors and initiates Ca2+ signaling via phospholipase C and inositol 1,4,5- trisphosphate (IP3) (Zoratti et al., 2003). It has been clearly shown that endurance training may decrease free Ca+2 concentrations through altered intracellular signaling, possibly to maintain lymphocyte function during stress (Broadbent and Gass, 2006). The endurance training induced altered intracellular Ca+2 signaling can be the result of exercise stimulated endocannabinoid system activation and play role in lymphocyte regulation.

The exercise intensity dependent increase in ANA serum levels was accompanied by the splenic CB1 receptor mRNA expression in both exercising groups after a 16 week training period. This was meaningful since ANA has been shown to be the primary ligand of CB1 receptor and spleen tissue reflects the peripheral blood (Bakovic et al., 2005; Frances et al., 2008). It can be concluded that exercise causes augmentation of ANA in plasma and up regulates the CB1 receptor in the spleen. Thus ANA may act via its CB1 splenic receptor in exercise mediated immune modulation.CB2 receptor mRNA expression on the other hand did not show any significant changes among groups in the spleen, but its transcription rate increased in the thymus as a result of AM281 and AM630 application in addition to endurance type of exercise indicating that receptor dependent endocannabinoid system regulation might be tissue specific. Moreover, it is possible that the changes in receptor binding may be a result of changes in ligand availability. Several studies in literature have indicated that the regulation of the cannabinoid CB1 receptor and its endogenous ligands are not coupled in the typical negative regulation relationship, in which the ligand down regulates its own receptor (Hill et al., 2005). Direct infusion of endogenous cannabinoids both up-regulated CB1 receptor mRNA transcription (Kola et al., 2005) and stimulated membrane expression of CB1 receptors (Maccarrone et al., 2008). These results indicated that increased endocannabinoid signaling can promote both the genetic expression and surface recycling of the CB1 receptor as shown in this study. This was also supported by studies with FAAH null mutated mice. Consistent with their inability to efficiently degrade ANA, FAAH(-/-) mice showed different behavioral responses to ANA, which were blocked by pre-treatment with a CB1 receptor antagonist, indicating that ANA acts as a selective CB1 agonist (Wise et al., 2007). Moreover, Keeney (2008) supported the CB1 receptor involvement in the relationship between exercise and endocannabinoids. He and his co-workers stated that injection of CB1 receptor antagonist decreased running speed and distance in endurance exercising mice, indicating that endocannabinoids may also play role in endurance exercise induced neurobiological rewarding through CB1 receptor activation (Keeney et

al., 2008).

Both CB1 and CB2 receptors showed increased mRNA expression levels at different rates in two lymphoid organs; the spleen and the thymus, indicating the involvement of these receptors in the exercise induced immune system alteration. A number of pharmacological studies with high affinity ligands strongly supported the existence of both CB1 and CB2 receptors in immune tissues (Galieque et al., 1995). Experimental results obtained from studies in vitro strongly suggest that both ANA and 2-AG induces the stimulation of several types of inflammatory cells and immune-competent cells, via CB1 and CB2 receptor dependent mechanisms, thereby stimulating inflammatory reactions and immune responses (Klein et al., 2003; Newton et al., 2009) as seen in the results of this study. Both white blood cell count and lymphocyte percentage were lower in the endurance type of exercise group, whereas neutrophil percentage seems to be increased. ($CD3^+$) T lymphocyte and ($CD3^-$ $CD161a^+$) NK cell percentages were higher, whereas ($CD3^-CD45RA^+$) B lymphocyte percentage was reduced after 16 weeks of endurance training. On the other hand, the stress caused by exercise at moderate intensity for 60 minutes had no effect on WBC count, neutrophil and lymphocyte subset percentages. This finding was supported by other studies which clearly showed that immune system alterations after strenuous exercise are neutrophilia and lymphopenia (Nieman, 2000a; Mackinnon, 1999; Krüger et al., 2008; Murakami et al., 2009), whereas moderate exercise did not cause any alteration in lymphocyte subsets (cytolytic T cells, helper T cells, T cells, NK cells and B cells) (Markovitch et al., 2008). Although exercise causes an increase in neutrophil number, neutrophil function has been reported to be suppressed in athletes, and may depend on the severity of training (Kumae et al., 2009). Several studies showed that following severe exercise, the blood lymphocyte levels fall below normal levels and the duration of suppression depends on the intensity and duration of the exercise (Pyne et al., 2000). Tvede and his co-workers showed that the immune system was suppressed following the 60 minutes lasting intense exercise with the intensity above 75% of VO2max, but not at the intensity of 50 % of VO2max (Tvede et al., 1989). Similarly, another

study performed with 22 healthy young males trained for 60 minutes on a treadmill ergometer at moderate speed (60% of the maximum aerobic speed) supported the outcomes of our study that moderate intensity does not cause any significant change in the number and function of T lymphocyte subsets and cytokine production (Romeo et al., 2008).

Because of an overall reduction in the number of immune effector cells in the endurance type of exercise group after 16 week training, it was not surprising to observe lower NK cell, B cell, T cell and NK like T cell counts compared to the control group. Moderate type of exercise also caused a decrease in the number of immune effector cells compared to the control group, but this was to a lesser extent compared to the endurance type of exercise group. This finding indicated that exercise intensity plays an important role in the apoptotic rate in blood immune effector cells (Wang and Huang, 2005). This was mimicked by the splenic immune effector cell population where apoptotic cells increased highly in the endurance type of exercise group and to a lesser extend in the moderate type of exercise group compared to the control group by using the Tunel method. The immune effector cells in the thymus, on the other hand, resisted to the exercise induced alterations and the apoptotic index did not differ significantly among groups. These results were supported by several studies stating that immune effector cell alterations return quickly to baseline levels after 60 minutes of moderate type of exercise but remain altered for 6 hours after endurance exercise (Lagranha et al., 2004, Wang and Huang, 2005, Wang and Lin, 2009). Moreover, it has been shown that ANA can stimulate apoptosis in lymphocytes, especially in the spleen (McKallip et al., 2002; Schwarz et al., 1994). Therefore, it has been suggested that this overall reduction in immune effector cells is endocannabinoid-mediated and may result from the ability of endocannabinoids to induce apoptosis in immune cells, particularly in peripheral NK, T and B cells (Do et al., 2004; Rockwell et al., 2006; Malfitano et al., 2006).

The decline in the lymphocytes was also observed by 7 weeks of endurance training in the second part of the study. Lymphocytes decreased in both the blood and the spleen in the endurance type of exercise group whereas a

change in the thymus was not observed. The CB1 receptor antagonist AM281 and the CB2 receptor antagonist AM630 could partially reverse the apoptotic effect of endurance type exercise in blood and the spleen since antagonist applied groups showed statistically lower lymphocyte numbers than the control group, but higher lymphocyte numbers than the endurance type of exercise group. These results indicated that both CB1 and CB2 are involved in the pathway of endurance exercise induced lymphocyte apoptosis in blood and the spleen, but there might be other receptors and/or receptor independent components taking part in this complex pathway.

Do and his co-workers demonstrated that exogenous and endogenous cannabinoids can induce apoptosis through activation of both CB1 and CB2 receptors and thereby giving rise to cannabinoid-mediated immune suppression and anti-inflammatory response where NF-κB played a critical role in this process (Do et al., 2004). With the use of CB1 and CB2 receptor antagonists in the same study, outcomes suggested the involvement of both CB1 and CB2 receptors in apoptosis and indicated that simultaneous activation through both receptors may be necessary to induce apoptosis. In the first part of the study, RT-PCR results showed that the apoptotic pathway components BAX, caspase 3, Fas, and the anti- apoptotic component Bcl2 mRNA expression remained unaltered in both spleen and thymus which leads to the conclusion that either apoptosis is not favored at cellular level or the apoptotic cascade depends on phosphorylation rather than nuclear changes at the gene level. However, in the second part of the study, microarray technique was used to have an on overall view of the events after endurance exercise. The expression of apoptotic pathway related genes Bax, Fas, FasL, caspase3 and Bcl2 remained unaltered between the exercising groups and the control group, however, NFkB, caspase8, perforin and granzymes were upregulated and showed altered expression levels in the endurance type of exercise group. This may indicate that exercise induced apoptosis is favored by ($CD3^{+}CD8^{+}$) cytolytic T cells which are able to directly induce apoptosis by opening up pores in membranes by secreting perforin and granzymes (Cotran et al., 1998), which were upregulated with fold changes. The increase of perforin as protein levels in

blood was also shown in literature (Staats et al., 2000). This was supported by the incline of ($CD3^+CD8^+$) cytolytic T cell count in the spleen having the possibility to give rise to higher perforin and granzyme secretion which in turn increased the apoptotic index in spleen. Although apoptosis was observed by lower counts of lymphocytes in blood and the spleen by flow cytometric analysis in addition to higher apoptotic index calculated by the TUNEL method, the expression of apoptotic pathway related genes Bax, Fas, FasL, caspase3 and Bcl2 remained unaltered in both parts of the study indicating that apoptosis is a result of phosphorylation rather than using the apoptotic pathway cascade.

Although, immune effector cell subsets exhibited normal distribution patterns within the T and B cell zones at the lymphoid tissue sections, their subsets, in number by blood flow cytometry and immunohistological analysis. ($CD8a^+$) and ($CD8b^+$) cytolytic T cells increased in the spleen in the endurance type of exercise group, but showed a decline in blood when compared to the control group. Blood ($CD3^+$) T cells, on the other hand, increased whereas $CD3^+CD4^+$ T lymphocytes and CD4/CD8 ratio did not change after endurance type of exercise. These outcomes were supported by a study with a similar exercise protocol where rats swam for 60 min/day, 5 days/week for 4 weeks. Results of this study showed that subpopulation of $CD3^+CD4^+$ T lymphocytes and the CD4(+)/CD8(+) ratio did not change significantly whereas $CD3^+CD8^+$ T lymphocytes increased with endurance exercise (Kwon et al., 2008). In this study however, ($CD25^+$) regulatory T cell subtype, declined as a response to exercise, indicating a complex immune response rather than immune effector cell redistribution as a response to exercise. It is accepted that ($CD24^+$) regulatory T cells that co-express CD4 and CD25 (the interleukin-2 receptor alpha-chain) take part in the mechanisms for the active suppression of autoreactivity and are powerful inhibitors of T-cell activation both in vivo and in vitro. Down regulation of these cells as shown in this study as a response to exercise might be beneficial for the enhancement of the immunogenicity of vaccines that are specific for tumor antigens (Shevach, 2002). A study with 12 week period of regular moderate exercise, on the other hand, caused an increase in the absolute value and percentage of ($CD4^+CD25^+$) regulatory T

lymphocytes and a decrease in ($CD3^+CD8^+$) cytolytic T lymphocyte percentages whereas absolute value of ($CD3^+CD8^+$) cytolytic T lymphocytes remained unaltered (Yeh et al., 2007). Another study compared absolute values and fractions of $CD3^+$ T lymphocyte subsets after endurance training and exercise in Alaskan sled dogs bred for endurance racing. Results of this study showed that 1100 mile race did not have any significant effects on absolute numbers or fractions of helper and cytolytic T lymphocytes whereas a 7-month training period caused a significant increase in the fraction of cytolytic T (Davis et al., 2008). The alteration in cytolytic T lymphocytes after training, but not after a single bout of endurance exercise, indicate that cytolytic T lymphocyte alteration is associated with the chronic effect of endurance exercise rather than acute effect as supported by this study (Davis et al., 2008). Endurance type of exercise had no effect on thymic T lymphocytes subsets and other thymic cell populations in this study and these outcomes were supported by Krüger (2008) who showed that exercise cause a release of T lymphocytes from spleen rather than thymus (Krüger, 2008). Another study showed that even voluntary wheel running exercise could induce alterations in splenic lymphocytes (Suzuki and Tagami, 2005) indicating that the spleen is the primary lymphoid organ effected by exercise rather than the thymus as shown in this study.

$CD11b^+$ cells showed also a decrease with the increasing exercise intensity and were lowest in the endurance type of exercise group. CD11b antigen is expressed on microglia, monocytes/macrophages, and to a lower extent on granulocytes, NK cells, $CD5^+$ B cells, and subsets of dendritic cells. Monocytes/macrophages are antigen presenting cells that play a key role in the T cell mediated immune response and decrease with endurance type of exercise (Guereschi et al., 2008; Timmerman et al., 2008). Moreover, it has been shown that endocannabinoids can regulate their activity through the CB1 and CB2 receptors located on their membrane surfaces (Galieque et al., 1995; Rajesh et al., 2007). When taken all these together with the results of this study, it can be suggested that endurance exercise induced increases in the endocannabinoids may play role in the regulation of ($CD3^+$) T cell by altering the antigen presentation process carried out by ($CD11b^+$) monocytes in blood and

macrophages in tissue (Klein and Cabral, 2006). This could not be confirmed with microarray analysis, since monocyte/macrophage markers such as CD11b, CD11c and related enzyme protein coding genes were not available or yet annotated in the array.

When immune effector cell percentages were considered instead of absolute values after a 16 week training period, it was observed that B lymphocyte percentage was significantly reduced, whereas T lymphocyte and NK cell percentages increased in the endurance type of exercise group compared to the moderate type of exercise and the control groups. The increase in T lymphocyte percentage was supported by a study in literature showing increased T lymphocyte proportions in peripheral blood as a result of high intensity exercise in human subjects (Simpson et al., 2007). These results showed that different types of immune effector cells show different responses to ANA and/or 2-AG at the same exercise intensity. Literature stated that exogenously applied endocannabinoids may have different effects on different immune effector cells such as T and B cells which participate in all elements of cell-mediated and humoral immunity (Derocq et al., 1995; Joseph et al., 2004). Although the inhibitory effect was seen after acute or chronic administration of high doses of endocannabinoids to animals, recent in vitro studies with low nanomolar concentrations of cannabinoids showed a stimulatory effect of cannabinoid receptor ligands on human B-cells (Derocq et al., 1995). Moreover, studies with null mutations in B cell antigen receptors lead to deficiencies of B cell subsets which were reduced in CB2−/− mice (Donahue et al., 2004; Khan et al.,1995), indicating that B lymphocyte regulation is partly CB2 receptor dependent. In the present study, ANA was negatively correlated to ($CD3^-$ $CD45RA^+$) B cells, whereas the same endocannabinoid stimulated the ($CD3^+$) T cell population and caused an increase in their proportion in both blood and the spleen. Moreover, 2-AG was negatively correlated to ($CD3^-CD161a^+$) NK cells and ($CD3^+CD161a^+$) NK like T cells indicating an immune-suppressive effect after 16 weeks of exercise.

The increase in the blood ($CD3^+$) T lymphocyte percentage was rehearsed by the endurance training protocol for 7 weeks and could be prevented by using

both CB1 and CB2 receptor antagonists together, AM281 and AM630 respectively. Using only CB1 receptor antagonist had no effect on the increased blood ($CD3^+$) T lymphocyte percentage, indicating that at least the CB2 receptor is taking part in the endurance exercise induced ($CD3^+$) T lymphocyte shift mediated by ANA. On the other hand, unlike blood ($CD3^+$) T lymphocyte percentage, AM281and AM630 could not inhibit the endurance type of exercise induced increase in the splenic ($CD3^+$) T lymphocyte percentage at the end of the 7 week training period. Splenic ($CD3^+CD4^+CD8^+$) double positive T cell percentage showed an increase where as ($CD3^+CD161a^+$) NK like T lymphocyte percentage decreased as a result of endurance exercise. These alterations, however, could not be reversed with CB1 and/or CB2 receptor antagonists, indicating that exercise induced lymphocyte regulation in spleen may use another pathway than blood. It is either CB receptor independent or there might be a specific receptor for ANA in mammalian tissues (Maione et al., 2009; Tuboly et al., 2009; Zhang and Oppenheim, 2006). There are contradictory results in the literature that discuss the CB receptor dependent immune system regulation (Springs et al., 2008; Newton et al., 2009). Massi (2000) and his co-workers showed that both CB1 and CB2 receptor antagonists completely reversed the immune suppressive effect of the endocannabinoids (Massi et al., 2000). These results suggested that both cannabinoid receptors were involved in the network mediating immune effector cell number and activity. On the other hand, many studies carried out with either CB1/CB2 receptor antagonists or knockout mice showed that cannabinoid effect persists in the functional absence of both CB1 and CB2 receptors. A study focused on the role of CB1 and CB2 receptors in immune competence and modulation showed that cannabinoid injection to wild type and CB1/CB2 receptor knockout (CB1(-/-)/CB2(-/-)) mice does not cause any change in the percentage of splenic T cell subsets, B cells, or macrophages. Conversely, humoral immune responses showed a markedly different profile of activity where synthetic cannabinoid suppressed the in vivo T cell-dependent IgM antibody-forming cell response in wild-type but not in CB1(-/-)/CB2(-/-) mice, and the in vitro IgM response in CB1(-/-)/CB2(-/-) splenocytes was too low to rigorously assess

CB1/CB2 involvement in modulation by cannabinoid. These unexpected findings led to the hypothesis that certain areas of the brain and peripheral tissues contain unknown cannabinoid receptor subtypes (Breivogel et al., 2001). It seems likely therefore that ANA may act through receptors other than the CB1 and CB2, such as the vanilloid receptors and/or undefined ANA specific ones. Another explanation for the effect of ANA could be depend on vanilloid receptors since endocannabinoids also stimulates vanilloid receptors and the stimulation of these receptors cause an increase in the synthesis of opioid peptides mainly endorphins and enkephalins (Giuffrida et al., 1999; Maione et al., 2009; Tuboly et al., 2009; Zhang and Oppenheim, 2006). May be opioid peptides contribute to effect of ANA as stated in literature. This subject needs further investigation. Further in vitro studies performed with splenocytes will define whether this complex relationship between splenic lymphocytes and endocannabinoids is regulated through CB1 and/or CB2 receptors or not.

The endurance type exercise was found to be associated with significant immunological changes, particularly in the T lymphocyte compartment of the splenic cells in rats. There was a significant rise in the percentages of ($CD3^+$) T cells and ($CD3^+CD4^+CD8^+$) double positive T cells. On the other hand, ($CD3^+CDRT1b^+$) activated T cells and ($CD3^+CD161a^+$) NK like T cells showed a significant decline as a response to endurance exercise. It is not surprising to observe immune system alterations primarily in the splenic ($CD3^+$) T cells since it has been shown that exercise is associated with constriction of visceral organs and the spleen represents a major one with high perfusion that constricts during the rather severe stresses of maximal exercise (Frances et al., 2008). Besides providing extra supply of red cells to exercising muscles, significant immune-regulatory changes have also been described in the spleen during exercise (Bakovic et al., 2005; Nielsen, 2003). The spleen is well known for its role in humoral immunity. However, it has been increasingly recognized as an organ of importance for T cell-mediated immunity as well (Krüger et al., 2008).

Exercise and environmental stress have been shown to induce a substantial re-distribution of T-cells within lymphoid and non-lymphoid organs. A uniform response pattern with a decrease in lymphocyte numbers in the

spleen accompanied by an increase in lymphocytes in lung, bone marrow and Peyer's patches has been described and suggested to play a role in immune regulation (Krüger and Mooren, 2007). In the present study, the percentage of lymphocytes in the peripheral blood of rats was found to decrease after exercise. Exercise induced lymphocyte apoptosis has been described as a transient process with tissue type specific apoptosis inducing mechanisms. Apoptotic lymphocytes have been described in the spleen that may contribute to the transient drop in peripheral lymphocyte counts. The apoptotic index was increased in the spleen in the present study whereas the percentage of ($CD3^+$) T cells was increased with an increased ($CD3^+CD4^+CD8^+$) double positive T cell population in the spleen of the exercising groups.

Because of their unconventional phenotype and rarity in human and mice, the extra-thymic ($CD3^+CD4^+CD8^+$) double positive lymphocytes have been ignored by most researchers. Extra-thymic ($CD3^+CD4^+CD8^+$) double positive T cells have been described rarely in peripheral blood of healthy individuals and in persons suffering from certain disease conditions. In humans, and rats, ($CD3^+CD4^+CD8^+$) double positive T cells were shown to appear transiently following activation of their progenitors. On the other hand, extrathymic ($CD3^+$) T lymphocytes exhibiting a $CD4^+CD8^{dim}$ phenotype has been reported to be abundant in the peripheral blood of swine, monkeys and chickens. It is suggested that ($CD3^+CD4^+CD8^+$) double positive T cells in swine are comprised predominantly of MHC class II restricted memory $CD3^+CD4^+$ helper T cells that after activation have acquired the ability to express the CD8α chain and then to maintain this double positive phenotype. Moreover, porcine ($CD3^+CD4^+CD8^+$) double positive T cells appear to be comprised of memory cells due to their ability to respond to recall antigen, resilience to thymectomy, increase in proportion with age, expression of memory T cell markers, production of interferon γ and localization to inflammatory sites. Some of these characteristics are also descriptive of human and monkey ($CD3^+CD4^+CD8^+$) double positive T cells. Thus, in swine, humans and monkeys, these phenotypically distinct lymphocytes appear to represent a primed T cell subset. Extrathymic ($CD3^+CD4^+CD8^+$) double positive T cells have been described in

the intraepithelial lymphocytes of rodents and are strongly suggested to represent mature lymphocytes, likely with an important effector function (Zuckermann, 1999).

Age related changes in ($CD3^+CD4^+CD8^+$) double positive splenic T cells have been defined in chickens. It has been shown that the proportion of αβ TCR expressing ($CD3^+CD4^+CD8^+$) double positive cells in the spleen of chickens had increased three times within 5 weeks. A large population of ($CD3^+CD4^+CD8^+$) double positive cells has been found in the peripheral blood, the spleen and the intestinal epithelium (Luhtala et al., 1997). These $CD4^+$ lymphocytes were shown to express the αβ TCR and also the CD8αα dimer but not the CD8αβ heterodimer (Luhtala et al., 1997). The phenotype of these cells, as for pigs and monkeys, were also shown to be $CD4^+CD8^{low}$ (Luhtala et al., 1997). Studies examining the functional capabilities of the ($CD3^+CD4^+CD8^+$) double positive T lymphocyte subset have not been reported yet.

In two previous studies, it has been stated that endurance type of exercise ameliorates splenic $CD3^+$ T cell immunity by inducing an increase in splenic interferon γ secretion (Kohut et al., 2005; Kwak, 2006). Exercise induced changes in the ($CD3^+CD4^+CD8^+$) double positive T cells subset in the spleen has not been reported in previous studies. In the present study, the increase in splenic ($CD3^+CD4^+CD8^+$) double positive cells in the exercise group may suggest that endurance type exercise may play a role in effector functions.

Another finding in this study was the significant decrease in the ($CD3^+CD161a^+$) NK like T cell population in the spleen of exercising rats. Similarly, a decrease in the NK like T cell population associated with a Th1/Th2 imbalance has been described in rats 7 days after exercise and the authors have suggested the immunoregulatory role of NK like T cells (Ru et al., 2009). It is believed that NK like T cell decrease causes onset of disease (Gombert et al., 1996). Moreover, NK like T cells have been shown to be derived from double positive thymocytes and proportions of doublr negative and CD4+ NK like T cells in the spleen (Hammond et al., 2002). There are several types of NK like T cells with a broad range of function (Kronnenberg and Gapin, 2002). In the present study, it was not possible to categorize the

decreasing NK like T cell subtype and therefore further detailed studies are nedded to determine the subtype of NK like T cells that responded to endurance type of exercise.

The results of the microarray analysis showed an increase in the expression of several heat shock proteins (Hsp) especially, Hsp 70. This was supported by studies that found an increase in serum levels of HSPs and an upregulation in polymorphonuclear cells as a result to increased body temperature induced by exercise (Connelly et al., 2006; Zieker et al., 2005). It has been widely accepted that HSPs elicit a potent anti-cancer immune response through antigen presenting cells, T cells and natural killer (NK) cells (Wells and Malkovsky, 2000). They induce a MHC class I mediated CD8-specific T cell response and thereby provide activatory signals for the innate immune system (Srivastava et al., 1998; Suto and Srivastava, 1995.). HSP70s may stimulate the secretion of several cytokines including interleukin (IL)-1β, IL-6 and tumor necrosis factor (TNF)-α by antigen presenting cells in the absence of tumor (Asea et al., 2000) results in a non-specific stimulation of the innate immune system (Asea et al., 2000). An additional effect of peptide-free Hsp70 is its immunostimulatory activity upon NK cells (Botzler et al., 1996; Multhoff et al., 1999). It can be suggested that HSPs may exert immune activation to protect cells from lethal damage induced by exercise induced stress stimuli.

CONCLUSION

Taken all these results together, we can conclude that endurance type of exercise activates the endocannabinoid system which in turn gives rise to immune system alterations. Exercise intensity and duration play critical roles in the activation of the endocannabinoid system. Serum ANA and 2-AG levels increase with the increasing exercise intensity in endurance type of exercise. The two endocannabinoids, ANA and 2-AG respond differently to endurance exercise. ANA serum levels show a steep increase at high intensity exercise, whereas 2-AG increases gradually with increasing exercise intensity. The increase of endocannabinoid serum levels are assisted by increased splenic CB1 receptor mRNA expression, indicating an adaptation in spleen but not thymus following 16 weeks of endurance training. Moreover, ANA play an important role in peripheral and splenic T lymphocyte regulation as a response to endurance exercise. This modulation might be cannabinoid receptor dependent in the periphery, but CB1 and/or CB2 receptor independent at tissue level such as in spleen. Moreover, exercise induced increase in ANA serum levels might play a role in the exercise dependent increase in splenic $CD3^{+}CD4^{+}CD8^{+}$ double positive T lymphocytes and decrease in splenic $CD3^{+}CD161a^{+}$ NK like T lymphocytes. Moreover, endurance type of exercise for 7 weeks might cause an decrease in splenic $CD3^{+}CdRT1b^{+}$ activated T lymphocytes, but had no effect on splenic $CD3^{+}CD25^{+}$ activated T lymphocyte percentage. Thymus, on the other hand, was affected by neither moderate, nor endurance type of exercise in terms of endocannabinoid and/or immune system parameters. Therefore, it can be concluded that endurance type of exercise cause an increase in endocannabinoids, especially ANA, which in turn cause immune system alterations in blood and splenic T cell subtypes.

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APPENDIX A

CURRICULUM VITAE

PERSONAL INFORMATION

Surname, Name: Özdurak, Rabia Hürrem
Nationality: Turkish (TC)
Date and Place of Birth: 26 April 1974 , Germany
email: hurremo@yahoo.com

EDUCATION

Degree	Institution	Year of Graduation
MS	METU Physical Education and Sports	2004
BS	METU Biology Education	2001
High School	Kadıköy Kız High School, Ankara	1993

WORK EXPERIENCE

Year	Place	Enrollment
2001-Present	MEB	English Teacher

FOREIGN LANGUAGES

Advanced English, Advanced and Fluent German

PUBLICATIONS

1. Özdurak, R.H., Sezgin, Ö.C., Akın, S., and Korkusuz, F. (2006). Vibration Analysis in the in-vivo Strength Determination of Radius in Human. Clin Orthop Rel Res. 443: 94-100.

2. Özdurak, R.H., Düz, S., Arsal, G., Akıncı, Y., Kablan, N., Işıklı, S., Korkusuz, F. (2003). Quantitative forearm muscle strength influences radial bone mineral density in osteoporotic and healthy males. Technology and Health Care. 11: 253-261.

3. Özdurak, R.H., Şeker, T., Korkusuz, P., Korkusuz, F. Anandamide and 2-Arachidonylglycerol Quantification in serum Samples: Optimization of the HPLC Method. Turkish Journal of Biochemistry. Accepted with review

International Congresses and Meetings

Oral presentation

1. Özdurak RH, Korkusuz P, Uçkan D, Kılıç E, Korkusuz F. Endurance exercise induces immune supression through anandamide. 9th Symposium of the International Society of Exercise and Immunology, Tübingen, Germany, September 21-23, 2009. (Abstract Book pp. 39)

Posters

2. Özdurak RH, Korkusuz P, Korkusuz F. High intensity exercise induces immune suppression through endocannabinoid increase. The Integrative Biology of Exercise-V, South Carolina, USA (Abstract book pp 32)(Poster)

3. Korkusuz P, Özdurak-Sıngın RH, Zeybek D, Yağcı M, Korkusuz F. High intensity exercise induces immune suppression through endocannabiniod increase. Microscopy Conference, Graz, Austria, August 30-September 4, 2009. (Abstract Book pp. 373-374)(Poster).

4. Korkusuz F, Özdurak RH, Korkusuz P, Kılıç E, Uçkan D. Endurance exercise increased plasma anandamide levels. 6th European Sports medicine Congress, Antalya, Turkey, October 14-18, 2009 (Published in J Sports Sci Med 2009;8(Supp 11):132-133)(Poster)

National Congresses and Meetings

Posterler

1. Özdurak-Sıngın RH, Zeybek D, Korkusuz P, Şeker T, Yağcı M, Korkusuz F. Egzersizin endokannabinoid aracılı immune supresif etkilerinin immünohistokimya ve akışkan hücre tekniği ile gösterilmesi. 19. Ulusal Elektronmikroskopi Kongresi 22-25 Haziran 2009, Trabzon (Kongre kitabı sf.175)(Poster)

2. Özdurak RH, Seker T, Korkusuz P, Korkusuz F. The optimization for the qunatification of Anandamide and 2-Arachidonylglycerol in blood samples. International Symposium on Biotechnology: Developments and Trends, 27-30 Haziran 2009, Ankara (Kongre kitabı sf.116) (Poster)

3. Şeker T, Ozdurak H, Korkusuz P, Korkusuz F. Endokannabinoid reseptörleri anandamid ve araşidonilgliserol düzeylerinin kan örneklerinde ölçümünün optimizasyonu. 16. Ulusal Biyoteknoloji Kongresi, 13-16 Aralık 2009 Antalya (Kongre kitabı sf.S-TB14)

APPENDIX B

TÜRKÇE ÖZET

GİRİŞ

Klasik yaklaşıma göre düzenli egzersiz yapmak yararlı kabul edilmekte ve bağışıklık sistemini güçlendirmesinin yanı sıra, koroner arter hastalığı riskini azalttığı, psikolojik streslere karşı direnci arttırdığı, yaşlanmayı geciktirdiği ve yaşam sürecini uzattığı görüşü kabul görmektedir (Cara and Chaiken, 2006; Cooper 2006; Pedersen 2006; Raso et al.,; 2007; Strasser and Killeen, 2006). Kardiyovasküler alanda yapılan birçok araştırma düzenli egzersizin yararlarını ortaya koysa da, bazı çalışmalar yoğun egzersiz yapan bireylerin çeşitli hastalıklara yakalanma riskinin sedenter bireylere göre arttığını ve hastalandıklarını bildirilmiştir.(Asgeirsson and Bellanti, 1987; Baron et al., 1982; Droste et al., 2007; Fediuc et al., 2006; Fleshner, 2005; Griesbach et al., 2007; Sasse et al., 2008). Bu çelişkili sonuçlar ile günümüze kadar gelen kutuplaşmanın nedeni, konunun yeterince araştırılmaması değil, immün sisteminin karmaşıklığı, nöroendokrin sistem ile ilişkisinin tam olarak ortaya konulamaması, çalışmalarda uygulanan egzersizlerin ve kullanılan deneklerin heterojenliğidir. Çelişkili sonuçlara rağmen günümüzde kabul edilen görüş; orta şiddetteki düzenli egzersizin immün sistemi güçlendirdiği, buna karşın elit sporcuların yaptığı yüksek şiddetteki egzersizin immün sistemi baskılayıcı etkisi olduğudur (Pyne et al., 2000; McFarlin et al., 2004; Kumae et al., 2009; Murakami et al., 2009).

Ancak egzersiz şiddettinin değişmesine bağlı olarak ortaya çıkan farklı immün yanıtı açıklayacak bir mekanizma henüz bulunamamıştır. Son zamanlarda yapılan birçok çalışma immün efektör hücrelerin belirli bir şiddette yapılan egzersize farklı yanıtlar verdiğini göstermekle birlikte, değişik şiddetlerde yapılan egzersizin aynı immün efektör hücreleri farklı etkilediğini

ortaya koymuştur. Bu sonuçlar ışığında immün sistemin egzersizin şiddettine ve süresine bağlı olarak farklı yanıtlarını açıklayabilecek ve günümüze kadar yapılan çalışmalarda incelenen moleküller dışında bazı immünmodülatör moleküller ve mekanizmalar olduğu düşünülmektedir.

Elit atletlerde, özellikle maraton koşucularında, yaptıkları uzun süreli egzersiz nedeniyle yalnızca bağışıklık sisteminde baskılanma değil, aynı zamanda psikolojik değişiklikler de gözlenmektedir. "Koşucu yüksekliği" (Runner's high) olarak nitelendirilen ve kannabinol içeren madde bağımlılarının gösterdiği tepkilere paralel olan bu psikolojik durum sporcuda bilinç kaybına, saf mutluluk hissine, içsel uyuma, sınırsız enerjiye, saf huzura ve ağrının baskılanmasına neden olmaktadır (Bartholomew et al., 1996; Cook and Koltyn, 2000; Kenney et al., 2008). Opioidlerin keşfedilmesinden önce bu değişiklikler yine endorfin hipotezi ile açıklanmaya çalışılmıştır; ancak egzersize dayalı katekolamin değişimleri ile bu görüş tam olarak açıklanamamıştır. Diğer yönden opioid sisteminin aktive olması ile gelişen solunum depresyonu ve gastrointestinal hareketliliğinin azalması gibi etkilerin sporcularda gözlemlenmemesi bu mekanizmanın varolan değişiklikleri açıklamada yetersiz oluğunu göstermiştir (Droste et al., 2007; Fediuc et al., 2006; Kolata, 2002; Sasse et al., 2008).

Kannabinoidlerin; CB1 ve CB2 reseptörleri üzerinden etki gösterdikleri bilinmektedir (Devane et al., 1992; Munro et al., 1993; Sugiura et al., 2002; Howlett et al., 2004). CB1 ve CB2 reseptörlerinin beyin ve sinir sisteminde bulunması ile birlikte sinir sisteminin endokrin ve immün sistem ile ilişkilendirilmesi yeni bir boyut kazanmıştır (Aso et al., 2008; Aguado et al., 2006; Bouaboula et al., 1993; Galieque et al., 1995; Klein et al., 2003, Molina et al., 2007;). Yapılan çalışmaların sonucunda CB1ve Cb2 reseptörlerinin merkez sinir sisteminin yanı sıra imün efektör hücrelerde bulunduğu ve immün sistem regülasyonunda aktif rol oynadığı düşünülmektedir (Di Marzo et al., 2004; Galieque et al.;1995; Klein et al., 2003; Munro et al., 1993; Pacher et al., 2006; Pacher and Gao, 2008; Yuan et al., 2002). Bu konu üzerine yoğunlaşan araştırmalar CB1 ve CB2 reseptörlerine yüksek afinite ile bağlanabilen ve vücut tarafından üretilen anandamid (ANA) (Mechoulam et al., 1995) ve 2-

arachidonylgliserol (2-AG) (Sugiura et al.; 2000) gibi endojen kannabinoidlerin varlığını ortaya koymuştur.

Günümüzde endokannabinoilerin sinaptik aktivitelerde yer aldığı kesinlik kazanmış olsa ve santral ve periferde ağrı azaltıcı etkileri bilinse de, bu maddelerin immün sistem ile ilişkisi incelendiğinde elde edilen sonuçlar çelişkilidir (Di Marzo et al., 2004; Rice et al., 2002; Pacher et al., 2006). Bu alanda yapılan birçok çalışma endokannabinoidlerin B ve T lenfosit poliferasyon ve migrasyonunda tetikleyici ve/veya baskılayıcı (Derocq et al., 1995; Joseph et al., 2004), timus ve dalak hücrelerinde apoptozu tetikleyici (McKallip 2002), ve IL-2 salınımını azaltıcı (Rockwell et al., 2008), etki gösterdiğini bildirmektedir. Diğer çalışmalar; endokannabinoidlerin adipoz dokuda IL-6 salınımını tetikleyici (Hoareau et al., 2006), makrofajlarda LPS bağımlı TNF-α, IL-4, IL-6 ve IL-8 salınımını baskılayıcı (Berdeshev et al., 1997; Cabral et al., 1995; Kishimoto et al., 2004), NK hücre migrasyonunu tetikleyici (Khishimoto et al., 2005) ancak NK hücrelerinin sitotoksik etkisini baskılayıcı (Massi et al., 2000) ve T lenfositlerde CD4+/CD8+ dengesizliği yaratarak immünoglobulin salınımını arttırıcı (McKallip et al., 2005) etkisi olduğunu göstermiştir. Bu konuda yapılan çalışmalardan elde edilen sonuçlar endokannabinoidlerin CB1 ve CB2 reseptörleri üzerinden dendritik hücrelerde apoptozu tetikleyerek immün sistemi baskıladığı (Do et al., 2004; Rockwell et al., 2006, Malfitano et al., 2006).

Tüm bu çelişkili sonuçları mekanizmanın karmaşıklığı, aynı ligand tarafından active edilmesine rağmen farklı etki mekanizmalarına sahip CB1 ve CB2 reseptör tiplerinin her ikisinin de immün efektör hücreler üzerinde eksprese edilmesi (Nong et al., 2001), CB1 ve CB2 reseptör yoğunluğunun immün efektör hücre tipine göre değişiklik göstermesi (Onaivi et al., 1996), aynı anda üretilen endokannabinoidlerin reseptöre bağlanma afinitesinin konsantrasyon bağımlı olup agonistik ve/veya antagonistik etkilerle modüle edilmesi (Do et al., 2004; Lee et al., 1995), ANA ve 2-AG endokannabinoidlerin aktif metabolitlerinin mekanizmada rolünün olması ve endokannabinoilerin çeşitliliği (Joseph et al., 2004; Sugiura et al., 2006; Sugiura et al., 2002; Cravatt 2001) ile açıklamak mümkündür. Yapılan

çalışmalar sonucunda elde edilen bulgular endokannabinoidlerin bağışıklık sistemi üzerinde etkisinin varlığını ortaya koysa da, bu etkinin öncelikli olarak hangi kannabinoidlere ait olduğu; tetikleyici mi yoksa baskılayıcı mı olduğu açık değildir. Literatürde endokannabinoidlerin egzersize dayalı değişimini gösteren yalnızca tek bir çalışma mevcuttur. Kolej öğrencileri üzerinde yapılan çalışmanın sonuçlarına göre orta şiddette 50 dakika süre ile yapılan koşu veya bisiklet egzersizinin endokannabinoid sistemini aktive ettiği saptanmıştır (Sparling et al., 2003). Adı geçen çalışmada sadece anandamid düzeyi ölçülmüş olup, endokannabinoid sisteminin farklı şiddet ve sürelerdeki egzersizlere yanıtı araştırılmamıştır. Bu sonuçlar literatürde Hill (2009) ve Keneey (2008) adlı araştırmacıların önderlik ettiği iki grup tarafından yapılan çalışmalar ile geliştirilerek desteklenmiştir ancak bu gruplarda egzersizi orta ve düşük şiddette egzersiz olarak yaptırmış ve tek egzersiz şiddetti kullanmışlardır. Bu nedenle imün sistem-endokannabinoid ilişkisinin farklı iki egzersiz şiddettinde incelenerek endurans egzersizin kan, dalak ve timus gibi farklı üç dokuda birden araştırılması literatürde bir ilk olmuştur.

Endokannabinoidlerin egzersizin şiddeti, yoğunluğu ve süresine bağlı olarak immün sistemi uyardığı veya inhibe ettiği varsayılmaktadır. Buna göre projenin amacı;

- Egzersiz şiddeti ve süresinin endokannabinoid serum düzeyine etkisini incelemek
- Endokannabinoid serum düzeyinde meydana gelecek değişikliklerin bağışıklık sisteminin efektör hücre sayıları ve oranlarına etkisini araştırmak
- Egzersize dayalı artış gösteren endokannabinoidler üzerinden bağışıklık sistemine ait bazı hücresel yanıtların uyarılmasını veya baskılanmasını kan, dalak ve timusta tespit etmek
- Bu ilişkide görev alan reseptör tipini (CB1 ve CB2) ve endokannabinoid türünü belirlemek
- Doku, hücre ve gen düzeyinde egzersizin şiddeti ve süresine bağlı gelişecek immün adaptasyonu tespit etmek

GEREÇ VE YÖNTEM

1- Çalışma düzeni

Uzunlamasına, betimleyici, rastgele seçilmiş denklem grubuna sahip ve kontrollü olarak yürütülen bu çalışma iki kısımdan oluşmaktadır (Şekil 3.1, şekil 3.2).

Birinci kısımda orta şiddette ve/veya endurans tipi egzersizin endokannabinod sistemine etkisi incelenmiştir. Bağımsız değişkenler egzersiz şiddeti olarak tanımlanmıştır. Bağımlı değişkenler;

(a) Endokannabinoid serum düzeyi: ANA , 2-AG serum düzeyleri

(b) İmmün efektör hücrelerin akışkan hücre tekniğiyle sayısı: Doğal öldürücü hücre (CD3-CD161a+), B hücre (CD3-CD45RA+), T hücre (CD3+), doğal öldürücü benzeri T hücre (CD3+CD161a+) sayıları ve yüzdeleri ile beyaz kan hücre sayısına ek olarak lenfosit ve nötrofil oranları

(c) Lenfoid dokularda efektör hücrelerin immünohistolojik dağılım paterni: Dalak ve timus dokusunda imünohistokimyasal yöntemlerle CD3, CD8a, CD8b, CD11b, CD25, CD45, CD45RA, CD56, makrofaja özgü antijen eksprese eden hücrelerin haritalanarak normal doku ile karşılaştırılması

(d) Lenfoid dokularda endokannabinoid reseptörleri ve efektör hücrelerin gen profili: Dalak ve timus dokusundan elde edilen örneklerde CB reseptörlerini (Cnr1, Cnr2), apoptoz yolaklarında görevli enzimleri (BAX, Bcl2, FAS, Caspase 3), B hücreleri ve aktivasyonu (CD19, IgM), T hücreleri ve aktivasyonu (CD247,CD69) ve doğal öldürücü hücreleri ve aktivasyonu (NKR-P1a, NK-p46) kodlayan genlerin ekspresyon paternlerinin incelenmesi

Çalışmanın birinci bölümünde elde edilen veriler doğrultusunda endurans tipi egzersizin endokannabinoid sistemini aktive etttiği ve immün sistemde değişikliklere neden olduğu sonucuna varılmıştır. Bu nedenle ikinci bölümde egzersiz protokolü olarak endurans tipi egzersiz seçilmiştir. Bağımsız değişkenler egzersiz ve kannabinoid 1 ve 2 reseptör antagonistleri AM281 ve AM630'un intraperitonel yolla uygulanması olarak tanımlanmıştır.Bağımlı değişkenler;

(a) Endokannabinoid Serum düzeyi:ANA serum düzeyi

(b) Akışkan hücre tekniği ile kanda ve dokuda immün efektör hücre sayımı: Kan, dalak ve timus dokusunda NK hücre (CD3-CD161a+), B

hücre (CD3-CD45RA+), T hücre (CD3+), NK benzeri T hücre (CD3+CD161a+) yardımcıT hücre (CD3+CD4+), sitotoksik T hücre (CD3+CD8a+) ve ikili pozitif T hücre (CD3+ CD4+CD8a+) oranları ile aktive T hücre oranları (CD3+CD25+ ve CD3+RT1b+)

(c) Lenfoid dokularda efektör hücre gruplarının immünohistolojik dağılım paterni: Dalak ve timus dokusunda imünohistokimyasal yöntemlerle CD3+, CD8+ (CD8a+ ve CD8b+), CD11b+, CD25+, CD45+, CD45RA+, CD56+ (CD56e+) ve makrofaj belirtçi ile işaretli hücrelerin haritalanarak normal doku ile karşılaştırılması

(d) Lenfoid dokularda kannabinoid reseptörleri ve efektör hücrelere ait gen profilinin incelenmesi: Dalak ve timus dokusundan elde edilen örneklerde endokannabinoid sistemi ve immün sistemde görev alan molekülleri kodlayan tanımlı genlerin ekspresyon profilinin incelenmesi

2- Deney hayvanları

Yetmiş iki erkek Sprague Dawley türü sıçan Hacettepe Üniversitesi Deney Hayvanları Araştırma ve Yetiştirme Ünitesinden (Ankara, Türkiye) elde edilerek kafeste 4 hayvan olacak şekilde gruplandırılmışlardır. Sıçanlar yem ve suya istekleri doğrultusunda ulaşabilirken 12 saat gündüz 12 saat gece döngüsü sağlanmıştır. Ortamın ısısı 22oC ve nem oranı %19 olarak ayarlanmıştır. Çalışmanın tamamı 5/9/2006 da alınan 2006/53-8 numaralı Hacettepe Üniversitesi Etik Kurul izni doğrultusunda ve Helsinki Hayvan Hakları deklarasyonuna uygun olarak yürütülmüştür.

Çalışmanın birinci bölümünde 32 erkek Sprague Dawley sıçan (a) Kontrol (n=9), (b) Orta Şiddet (Moderate (n=10)) ve (c) Endurans (n=12) tipi egzersiz olarak rastgele gruplandırılmıştır. Çalışmanın ikinci bölümünde ise 40 erkek Sprague Dawley sıçan rastgele seçilerek a) Kontrol (n=8), (b) Egzersiz (n=8), (c) DMSO (n=8), (d) AM281 (n=8) ve (e) AM281+ AM630 (n=8) olarak 5 gruba ayrılmıştır. Tüm sıçanlar deneyin başlangıcında 4 haftalık olup ortalama ağırlıkları 197,8±17,0 gram olarak ölçülmüştür.

3-Egzersiz protokolü

3.1-Adaptasyon süreci

Çalışmanın hem birinci , hem de ikinci bölümünde adaptasyon süresi iki hafta olarak uygulanmıştır. Çalışmanın başlangıcında sıçanlar egzersiz protokolüne ilk hafta boyunca dakikada 10 metrelik bir hız ile 10 dakika boyunca koşturularak başlamışlerdır. İkinci hafta boyunca bu hız ve süre kademeli şekilde arttırılarak orta şiddette ve endurans tipi egzersiz gruplarının protokollerine uygun hız ve süreye çıkarılmıştır.Adaptasyon sürecinin sonunda her grup kendi egzersiz protokolüne uygun şekilde egzersize başlamıştır.

3.2- Egzersiz süreci

3.2.1-Çalışmanın 1. bölümü

Sıçanlar haftada 5 gün olacak şekilde 16 hafta süresince koşubandında aşağıda belirtilen şiddette koşmuşlardır.

a) Egzersiz yapmayan kontrol grubu: Koşu bandının kendisinden kaynaklanan stresin etkisini bertaraf edebilmek için (Moraska, 2000) hayvanlar haftada 5 gün ve günde 60 dakika olacak şekilde çalışmayan koşu bandına yerleştirilerek egzersiz yaptırmadan koşubandında bekletilmişlerdir

b) Orta Şiddette (moderate) egzersiz grubu: Sıçanlar haftada 5 gün olacak şekilde dakikada 19 metrelik bir hızda 60 dakika boyunca koşturulmuşlardır (Su 2005 ;Carmeli2005).

c) Endurans tipi egzersiz grubu: Sıçanlar haftada 5 gün olacak şekilde dakikada 19 metrelik bir hızda 60 dakika boyunca koşturulmuşlardır (Carmeli 2006; 2005).

Endurans egzersiz grubundaki kalp ağırlığı/ Vücut ağırlığı oranındaki istatistiksel olarak anlamlı artış (p= .001), yani kardiomegali, egzersiz şiddettinin doğru seçildiğinin sağlaması olmuştur.

3.2.2-Çalışmanın 2. bölümü

Egzersiz grubu hariç tüm sıçanlar haftada 5 gün olacak şekilde 7 hafta boyunca 32 m/dakikalık hızda ve günde 60 dakika boyunca koşubandında egzersize zorlanmışlardır. Hergün sıçanlara bulundukları gruba göre koşu egzersizinden 20 dakika önce DMSO (dimetil sülfoksit) da çözünmüş olarak AM281 (0,5 mg/kg vücut ağırlığı) ve/veya AM630 (1,5 mg/kg vücut ağırlığı) intraperitoneal enjeksiyonla verilmiştir. DMSO grubuna yalnızca DMSO verilmiştir. Koşu bandının yarattığı stresin etkisini egzersiz yapan gruplarda önleyebilmek için kontrol grubunun hayvanları koşu egzersizi olmaksızın haftada 5 gün 60 dakika boyunca koşu bandında tutlmuşlardır (Moraska, 2000).

3.3-Hayvanların sonlandırılması ve kan ile doku örneklerinin elde edilmesi

Tüm sıçanlarda periferik kan intrakardiyak yöntemle alınarak hayvanların hipovolemik şok ile ölmesi sağlanmıştır. Ardından dokuların izolasyonu için hayvan açılmadan servikal translokasyon yapılmıştır. Doku alımına geçmeden önce eter ile uyutulan hayvanlar etanole batırılarak alınacak doku örneklerine kılların kaçması engellenmiştir. Etanole batırılan hayvanlar dokuların alınması için buz üzerine yerleştirilen kesme tahtasına koyulmuşlardır. Burada steril aletler kullanılarak karın boşluğu açılıp, dalak dikkatlice bağ dokudan ayrılarak serum fizyolojik bulunan ayrı bir petri kutusuna alınmış, göğüs boşluğuna girilerek aynı işlem timus için tekrarlanmıştır. Daha sonra süre geçirmeden bu iki doku daha küçük parçalara ayrılarak akışkan hücre tekniği için serum fizyolojik içeren tüplere, mikroarray için buz üstüne yerleştirilen trizollü tüplere ve immünohistolojik analiz için sıvı nitrojene konacak özel tüplere paylaştırılmıştır.

4-Ölçümler

4.1-Endokannabinoidlerin kantitatif ölçümü

Wang'ın yöntemi (Wang et al., 2001) modifiye edilerek uygulanmıştır.

Yapılan bu modifikasyonlardan sonra uygulanan yöntem kısaca söyledir; sıçanlardan elde edilen 1 ml serum cam tübe koyularak üzerine ANA ve 2-AG'in hidrolize olmasını engellemek için 1mM PMSF eklenmiştir. Üç hacim serum fizyolojik (SF) ile dilüsyon gerçekleştirilip, adsorpsiyon işlemi için 1.5 ml %50'lik Affi-Prep polimiksin B eklenerek; 1 saat boyunca 4°C ısıda sallanmıştır. Adsorpsiyonun sonunda PMB-immobilize boncuklar santrifüj edilerek çöktürülerek iki kez 2 ml SF ile yıkandıktan sonra minikromatografi kolonuna (0.5x3 cm) aktarılmıştır. Son bir kez boncuklarda kalan SF'den kurtulmak için tüpler santrifüjlenmiştir. Boncuklarda kalan ANA ve 2-AG'ün alınması için iki kez 200 µl saf etanol geçirilerek elüsyonlar vakumlu kondansatörde (Heto Holten) kurutulmuş ve HPLC de ölçüm için saf etanolde tekrar çözülmüşlerdir.

HPLC ile fraksiyonlara ayırma yöntemi Lang (Lang et al., 1996)'dan alınarak modifiye edilmiştir. Yöntemde 305 pompa ve 118 UV/V dedektörü ile donatılmış Gilson System (Gilson Inc., Middleton, WI) kullanılmıştır. Ayırma işlemi TSK jel ODS 80TM (50x34.6-mm, i.d. 3,5 mm) kolonu (TOSOH, Tokyo, Japan) içinden akış hızı 1 ml/dakika olacak şekilde asetonitril ve su (8:2 hacim/hacim) geçirilerek gerçekleştirilmiştir. Emilim (absorbans) 204 nm'de oluşmaktadır. Kurutulduktan sonra saf etanolde yeniden oluşturulan PMB ile immobilize edilmiş boncuklar, ters-faz HPLC'de (RPHPLC) fraksiyonlarına ayrıştırılmışlardır. ANA ve 2-AG için belirlenen elüsyon zamanı sırası ile 3.2 dakika ve 4.2 dakikadır. Farklı miktarlarda sentetik ANA and 2-AG sıçan serumuna eklenerek her bir örnek PMB-immobilize boncuklarla adsorbe edilerek RP-HPLC ile arındırılıp, standart eğri setinin her birine farklı miktarlarda sentetik ANA ve 2-AG eklenerek bunlar fluorometrik yöntem ile tespit için sırayla HPLC'ye yüklenerek kalibrasyon eğrisi oluşturulmuştur.

4.2-Akışkan hücre tekniği ile immün efektör hücre sayımları

Egzersiz sonrası elde edilen 0,5 ml'lik kan örnekleri; etilen diamin tetraasetik asit (EDTA) içeren özel plastik tüplerde, doku örnekleri ise serum fizyolojik içeren petri kaplarına toplanarak, akışkan hücre tekniği için uygun

plastik deney tüplerine aktarılmışlardır. Eritrositlerin lizisi orth-mune lysing reagent ile gerçekleştirilmiştir. Monoklonal antikor paneli fluoresan izotiyosiyanat (FITC), fikoeritrin ya da peridinin klorofil protein ile konjuge edilmiş olarak planlanmıştır. Her immün işaretleme seansında mutlaka örnekler ışıktan korunarak negatif kontrol işaretlemeleri de yapılmıştır. Kullanılan yöntemin ayrıntıları şöyledir; bistüri ile küçük parçalara ayrılan taze doku madimachine cihazı ile parçalanmıştır. Hücreler toplanıp FACS tüplerine alınıp 1300 rpm de 5 dakika santifüj edilmiş, % 2 lik rat serumu PBS içinde hazırlanarak 1ml olacak şekilde tüplere ilave edilmiştir. 15dakika oda ısısında bekletildikten sonra bu örneklerden 100 ul alınarak FACS tüplerine ilave edilmiştir. Diğer yandan kan örneklerinden 4 tüp içerisine 100 er µl dağıtılarak her tüpe analizi yapılacak T/B/NK kokteyli, T kokteyli ve aktive T kokteyli kitlerinin antikorundan ölçülmek istenen hücreler doğrultusunda 20 µl eklenmiştir. Tüpler düşük devirde vortexlenerek karanlıkta, oda ısısında 15 dak inkübasyona bırakılmıştır. Tüpler düşük devirde vortexlendikten sonra 1 ml Lysing Buffer eklenerek 8 dak oda ısısında karanlıkta inkübe edilmiş, süre bitimine 1500 rpm de 5 dak santrüfüj edilmiştir. Pellet üzerine 1 ml PBS eklenerek santrifüj edildikten sonra pellet vortekslenmiş ve 500 µl cell wash eklenerek analiz yapılmıştır.

4.3-Lenfoid organlarda immünohistolojik inceleme

Uygun cerrahi yöntemle edle edilen dalak ve timus örnekleri doku dondurma ortamı (OCT) içine alındıktan sonra hızla eksi 196 oC sıvı azotta dondurulmuştur. Dondurulmuş doku örnekleri kesit alınıncaya dek -80oC'de derin dondurucuda ya da azotta saklanmıştır. Dondurulmuş kesitler cryomikrotomda 5-7 µm kalınlığında, özel adeziv (poly L lizin) kaplı yada elektrostatik lamlara alınmıştır. Dondurulmuş kesitler en fazla bir gece nemden arındırılmış kutularda oda ısısında bekletildikten sonra ertesi gün immün işaretleme işlemi uygulanmıştır.

4.3.1-İmmünohistolojik inceleme

Doku kesitlerinde; T lenfosit alt grupları, B lenfositler, NK hücreleri ve makrofajlara özgü bir dizi yüzey antijeni için immün işaretleme yapılmıştır. Bu hücrelerin apoptoza uğrayıp uğramadıkları yine immünohistokimyasal olarak incelenmiştir. Çalışmada kullanılan antikorlar; tablo 3.4'de verilmiştir. İmmünohistokimyasal çalışmada efektör hücre işaretlemesi için indirekt immünoperoksidaz; apoptozis için immünfluoresan yöntemi uygulanmıştır (Korkusuz et al., 2002; Gavrieli et al., 1992).

4.3.2-Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) yöntemiyle apoptozisin değerlendirilmesi

Apoptozis, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) yöntemi kullanılarak DNA kırıklarının enzimatik işaretlemesi ile belirlenmiştir. TUNEL işaretlemesi için ApopTag in situ detection kit kullanılmıştır. İşaretleme kitin kullanma talimatnamesinde önerilen şekilde yapılmıştır. Kısaca, 5 µm kalınlığındaki donmuş kesitler poly L liizin kaplı özel lamlara alınıp PBS içinde hazırlanmış %1 paraformaldehit (pH 7.4) ile oda ısısında 10 dakika inkube edilmiştir. Kesitler PBS ile yıkandıktan sonra 2:1 oranda etanol:asetik asit ile -20°C de 5 dakika tesbit edilmiştir. Yıkanan kesitler 1 dakika nötral dengeleyici solüsyon ile inkube edilmiştir. Digoksigenin-dUTP ile işaretli TdT solüsyonu ile 1 saat süreyle 37°C de nemli ortamda inkübe edilmiştir. Reaksiyonu durdurmak için kesitler stop/wash tampon içine alınıp, PBS ile 5 dakika yıkandıktan sonra kesitler antidigoksinin rodamin ile karanlıkta oda ısısında 30 dakika inkube edilmiştir. Kesitler PBS ile yıkandıktan sonra 1 damla DAPI ile çekirdekler işaretlenmiştir. Negatif kontrol için işaretlenen kesitlerde, deoxiribonucleotidiyl transferase enzyme çıkarılarak, diğer basamaklar aynen uygulanmıştır. Tüm kesitler DC490 dijital kamera (Leica, Wetzlar-Germany) bağlı Leica DM6000B (Wetzlar-Germany) ışık mikroskobu ile incelenerek apoptotik hücreler fotoğraflanmıştır.

4.3.3-İndirekt immünoperoksidaz yöntemiyle efektör hücre gruplarının

incelenmesi

Dondurulan kesitler on dakika süreyle 4oC’de soğuk aseton ile tespit edildikten sonra otuz dakika süreyle açık havada kurutularak kesitlerin tümü pap pen ile sınırlanmıştır. İmmunohistokimyasal işaretleme için anti-mouse HRP-DAB staining kit kullanılmıştır. Kesitler rat serumu ile 1:10 dilusyonda oda ısısında 30 dakika inkube edilerek spesifik olmayan bağlanmalar bloklanmış ve ardından Tablo 3.4 de verilen dilüsyonlardaki primer antikorlar ile 1 saat süreyle oda ısısında nemli ortamda inkübe edilmiştir. 0.01M fosfat tamponlu (PBS) pHsı 7.4 olan yıkama solüsyonu ile 3 kez 5 dakika süre ile yıkandıktan sonra kesitler biyotin bağlı keçi anti-fare sekonder antikor ile 30 dakika oda ısısında inkube edilmiştir. PBS yıkama solüsyonu ile 3 kez 5 dakika süre ile yıkandıktan sonra HSS-HRP çözeltisi ile yine oda ısısında 30 dakika inkube edilmiştir ve PBS ile 3 kez 5 dakika yıkanmıştır. Kesitler uygun şekilde hazırlanmış olan 3.3’-diaminobenzidine-tetrahydrochloride (DAB) ile 2 dakika süreyle nem odasında inkübe edilerek PBS yıkama solüsyonu ile 3 kez 5 dakika süre ile yıkanmıştır. Ardından distile suda yıkanarak 45 saniye Mayer’in hematoksileni ile zemin boyama yapılmıştır. Ardından kesitler musluk suyunda yıkanmıştır. Dehidratasyon için, sırasıyla; %80, %96 ve %100'lük derişime sahip alkollerde 5'er dakika bekletilmiştir. Lamların üzerindeki kesitler kuruduktan sonra 20 dakika ksilolde bekletilerek dokular şeffaflaştırılmıştır. Entellan kullanılarak kesitler kapatılmıştır. Tüm antikorlar 0.05 M TrisHCl içinde % 0.1 tween 20 içeren background azaltıcı tampon solusyonu ile dilue edilmiştir. Negatif kontrol işaretleme amacıyla boyanan kesitlerde, primer antikor basamağı atlanarak, diğer basamaklar aynen uygulanmış ve fare IgG kullanılmıştır. Pozitif kontrol için firma tarafından önerilen uygun kontrol doku örnekleri kullanılmıştır. Tüm kesitler DC490 dijital kamera (Leica, Wetzlar-Germany) bağlı Leica DM6000B (Wetzlar-Germany) ışık mikroskobu ile değerlendirilerek fotoğraflanmıştır.

4.3.3-İmmün işaretlenmenin kantitatif olarak değerlendirilmesi

Tüm doku örnekleri Leica DMR model ışık mikroskobunda değerlendirildi. Görüntüler Leica DC500 dijital kamera ile bilgisayar ortamına aktarıldı. Lenfoid hücrelerin immünreaktivitesi bilgisayar destekli Leica Application Suit ve Qwin Plus görüntü analiz sistemi kullanılarak literatürdeki yöntem modifiye edilerek kantitatif olarak analiz edildi.(Uğur et al.,2003; Goyal et al., 2004; İnan et al., 2006).

İmmünperoksidazla işaretli lenfoid hücre alt gruplarının kantitatif olarak incelenmesi amacıyla pozitif kontrol kesitlerinde en koyu siyah boyanan hücre bölümlerinin tonları analiz programında kaydedildi. Buna göre sistemde en koyu işaretlenen hücreler +++, daha açık olanlar sırasıyla ++ ve +, boyanmayanlarsa negatif olarak değerlendirildi. Timus ve dalakta yerleşik hücre gruplarından 100 tanesi x200'lük mikroskop büyütmesinde en az üç alanda görüntü analiz programıyla sayıldı. Yüzde immün işaretli hücre sayısı ve işaretlenme şiddetinin beraber değerlendirildiği H skoru hesaplandı. Bunun için kullanılan formül aşağıda verilmiştir. +,++,+++ olarak işaretlenmiş ve H skoru hesaplanmış toplam hücre sayıları her örnek için hesaplanarak tablolandı.

H Skoru= Toplam $\rho(i+1)$

ρ: İmmün işaretli % hücre sayısı

İ: İşaretlenme şiddeti

Tunel yöntemi ile apoptozise gittiği saptanan hücrelerin kantitatif olarak incelenmesinde literature uygun olarak apoptotik indeks hesaplaması yapıldı. Buna göre x200lük mikroskop büyütmesinde rastlantısal olarak seçilen on alanda rodaminla kırmızı işaretli apoptotik hücreler sayılarak ortalaması alındı; Apoptotik hücreler toplam çekirdekleri mavi ile işaretli (kırmızı boyanmamış) toplam hücre sayısına oranlanarak apoptoz oranı hesaplandı (Ferrini et al., 2001; Zhang and Yang, 2006).

4.4-Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) ile gen profili çalışması

4.4.1-Dokudan RNA izolasyonu

100 mg dalak ve timus dokuları buz üzerinde alındıktan sonra 1 ml guanidinium thiocyanate-phenol-chloroform ekstraksyonu yapılmıştır. The FastPrep® bead-beater sistemi ile homogenize edilerek 5 dakika buzda bekletilmiştir. Her tüpe 300 µl chloroform eklenerek faz ayrışmasının oluşması beklenmiştir. Karışım 15 saniye vortekslendikten sonra 10 dakika buz üzerinde bırakılarak, 15 dakika boyunca 8 °C de 12000xg de santrifüj edilmiştir. Takiben sıvı faz uzaklaştırılarak 500 µl soğuk izopropanol eklenmiştir. 10 dakika -20°C de bırakıldıktan sonra tekrar 4 °C ısıda 12000xg de 10 dakika santrifüj edilmiştir. Sıvı faz uzaklaştırılarak pellet başlangıç miktarı göz önünde tutularak her 1 ml örnek için 1 ml %75 etanol ile yıkanmıştır. 2 dakika vortekslendikten sonra 4 °C ısıda 7500 xg de 5 dakika sentrifüj edilmiştir. Sıvı faz atıldıktan sonra pellet 5 ile 10 dakika boyunca kurutularak tekrar suda çözünmüştür. Bu işlem 10 dakika boyunca 55-60 °C lik su banyosunda bekletilerek gerçekleştirilmiştir.

İzole edilen RNA örneklerinin, miktarlarını ve saflık oranlarını belirlemek amacı ile optik dansiteleri (OD) 230, 260 ve 280 nm seçerek NanoDrop (ND-1000) spektrometrede ölçümler alınmıştır. RNA konsantrasyonları 260 nm'de alınan ölçüm değerinin

[C] = A260 x RNA constant (40)

formülüne koyulması ile heasaplanmıştır.

230 ve 280 nm'lerde elde edilen ölçümler saflık derecesinin tespitinde kullanılmıştır.

Deney gruplarından elde edilen RNA konsantrasyonları ve saflık oranları Tablo-4.8 de gösterilmiştir.

4.4.2-DNA agaroz jel elektroforezi

Agaroz jeli (%1,w/v) hazırlanırken, 1 gr agaroz (Prona Agarose Basica LE) 100 ml 1x TBE tampon çözeltiye % 37 olacak şekilde formaldehit ve 3-[N-Morpholino] propanosülfonik acid (MOPS) ilave edildikten sonra, konsantrasyonu 0.7 µg/ml olacak şekilde ethidium bromide eklenerek, 1x TBE tampon solusyonu ile jel yürütülmüştür. PCR ürünleri 1/10 oranında 10x yükleme boyası ile karıştırılarak (toplam 40 µl) yükleme yuvalarına yüklenmiştir. Elektroforez sabit voltajda (70 volt) 3-4 saatte tamamlandıktan sonra jeldeki bantların jel dokümantasyon sistemi kullanılarak fotoğrafları çekilerek 18S ile 28S ribozomal RNA veya GAPDH amplifikasyonları ile normalizasyonları yine sistemin programı ile yapılmıştır.

4.4.3-cDNA sentezi

2 µg izole RNA kullanılarak cDNA sentezine başlanmıştır. RNA örneklerinin her birinden 2µg RNA alınarak 37°C de 0,5 µg rastgele hekzamer oligonükleotit primerler ve RNAse H (+) reverse transcriptase enzyme Improm II kullanılarak cDNA yapılmıştır. cDNA'ların hacmi distile su kullanılarak 80µl ye tamamlanmış ve -20°C de saklanmıştır. Reaksiyonlar Perkin-Elmer 9700 thermocycler ile başlangıç denaturasyonu 65°C de 10 dakika bekletilerek başlatılmış, 37°C de 10 dakika, 10 µl Reverse transktripase enzimi ekledikten sonra 42°C de 60 dakika, 75°C de 15 dakika, 1 µl RNase H ekledikten sonra 37°C de 20 dakika bekletildikten sonra saklanmak üzere -20°C'ye kaldırılmıştır.

4.4.4-PCR için Primer Hazırlanması

Cnr1, Cnr2, BAX, Bcl2, FAS, Caspase 3, CD19, IgM, CD247,CD69, NKR-P1a, NK-p46 genlerinin primerleri, ayrıca normalizasyon için B-Actin genlerinin mRNA primerlerinin tasarımı için NCBI network ağları kullanılarak sekanslara ulaşılmış ve DNA-star genetik programı kullanılarak tasarlanmıştır (Tablo 3.5).

4.4.5-RT-PCR koşulları

İlgilenilen genlerin cDNA'ları semi-kantitatif PCR analizi için SYBR green yöntemi kullanılarak amplifiye edilmiştir. Tüm tepkimeler standart koşullarda gerçekleştirilmiştir. Bu koşullar: 25 µl 2x SYBR karışımı, 6 µl 25 mM $MgCl_2$, 2,4 µl 10 µM reverse ve forward primerlerin ikiside, 50 µl'ye çift distile su ile tamamlanan 1,5 µl cDNA ve deionize su.4 ünite Taq Polymerase enzimi.

PCR programlarının optimizasyonu için, öncelikle primerlerin kalıp DNA'ya hidrojen bağları ile bağlanma sıcaklığı olan TA derecesi farklı sıcaklıklarda denenerek optimum amplifikasyonları saptanmıştır. Döngü tamamlandığında iki katına çıkmış kalıp DNA'nın tekrar tek zincir haline dönüşmesi ile yeni döngü başlamakta olup, bunun döngü sayısı boyunca tekrarlanması ile başlangıçtaki DNA miktarının tasarlanan primerlerin kapsadığı gen parçası milyonlarca defa çoğaltılmıştır. Her döngü arasında SYBR green inkororasyonu fluorometrik kantitatif termal cycler Rotorgene 6500 (Corbett scientific, Brisbane, Australia) ile ölçülmüştür. İlgili genlerin ekspresyonları house-keeping gen olan beta-actin ile normalize edilmiştir.

4.4.5 Microarraylerin gerçekleştirilmesi:

Tüm hibridizasyonlar ve prob işaretlemesi Hacettepe Universitesi Transkriptomiks Laboratuarında GeneChip sistem kitlerinin protokolüne uygun koşullarda gerçekleştirilmiştir. Kullanılan membran Rat Genome RAE_230 2.0 ekspresyon probe array kiti olup 31,000 genin transkriptinin analizine olanak sağlamıştır.

Çift sarmal cDNA 100 µg full-length total RNA ve 3'IVT Express System kullanılarak T7-(dT)24 DNA oligonükleotid primer varlığında sentezlenmiştir. Sentezin devamında cDNA saflaştırılarak High Yield RNA Transcript Labeling Kit ile biyotinlenmiş ribonükleotidler varlığında transkibe edilmiştir. Ortalama 10 µg işaretlenmiş cRNA hibridizasyon şişelerine eklenerek 16 saat 45ºCde bekletilmiştir. Hibridizasyon gerçekleştirildikten sonra, yıkama solusyonları ile membranlar 2 kez yıkanarak GeneChip Fluidics

Workstation (Affymetrix) kullanarak streptavidin-phycoerythrin ile boyanmıştır.

Array iki kez Konfokal lazer tarayıcı (GeneArray Scanner) kullanılarak tek görüntü halinde sonuç elde edilmiştir. Bu sonuç ".dat-file" şeklinde olup, GCOS programı (Affymetrix) ile ".cel-file" şekline işlenmiş, bu hali formatı ile Affymetrix GeneChip Laboratuar Bilgi Sistemine (LIMS) yüklenerek kaydedilmiştir. Ham data TMEV yazılımı kullanılarak ekspresyon düzeyleri şeklinde ifade edilmiştir. Kalitesi kontrol edildikten sonra data analiz için Mikroarray analiz Suite yazılımına yüklenmiştir. Elde edilen görüntüler, Scanalyze ve GEArray bilgisayar programları kullanılarak analiz edilip normalizasyonları yapılmıştır.

4.5-İstatistik

Farklı veri setleri için farklı istatistiksel yöntemler kullanılmıştır. Gruplar arası endokannabinoidler ve immün efektör sayı farklarını bulmak için Kruskal Wallis ile Mann Whitney U testi uygulanmış olup, Bonferroni düzeltmesi yapılmıştır. Aynı istatistiksel yöntemler kantifiye edilen imünohistolojik sonuçların değerlendirilmesinde de kullanılmıştır. Gene analizi sonuçları için ANOVA kullanılmıştır. Tüm istatistiksel hesaplamalar SPSS 11 programında yapılmıştır.

BULGULAR VE TARTIŞMA

1-Çalışmanın 1. Bölümü

1.1-Endokannabinoidlerin analizi

ANA ve 2-AG serum düzeyleri endurans egzersiz yapan sıçanlarda kontrol ve orta şiddette egzersiz yapan sıçanlarla karşılaştırıldıklarında istatistiksel olarak anlamlı şekilde yükselmiştir ($p < .001$) (Şekil 4.1). Bu

çalışmanın temel bulgularından biri olarak literatürde ilk defa farklı egzersiz şiddetlerinin endokannabinoid sistemini farklı düzeylerde etkilediği ve ANA ile 2-AG'nin aynı egzersiz şiddettinde farklı düzeyde etkilendiği söylemek mümkündür. Sadece yüksek şiddette yapılan endurans egzersizin dolaşım sisteminde ANA'yı dramatik şekilde, 2-AG'yi ise daha az ve kademeli olarak attırdığı görülmektedir.

ANA serum düzeyi pmol seviyesinden nmol seviyesine belli bir eşik seviyesinden sonra ani bir artış göstererek çıkmıştır. Bu nedenle orta şiddette yapılan egzersizin ANA serum düzeyinde fazla bir etkisi olmadığını, ancak endurans egzersizin ANA serum düzeyini oldukça yükselttiğni görmekteyiz. Endurans grubundaki ANA serum düzeyindeki ani artışın sebebi yüksek şiddette yapılan egzersizin vücutta yarattığı stres olarak düşünülmektedir. Bu bulgular literatürde egzersize dayalı analjezinin egzersiz şiddettine bağlı olduğunu ve ANA serum düzeyi ile CB1 reseptör iletişiminin egzersiz şiddetti ile ilişkili olduğunu gösteren bir çalışma ile desteklenmektedir (Hoffman et al., 2004).

Sadece ANA değil, 2-AG serum düzeyi de endurans egzersiz grubunda artış göstermektedir. Ancak bu artış ANA kadar çarpıcı ve dik değil, aksine kademeli olarak gerçekleşmekte ve egzersiz şiddetti arttıkça 2-AG serum düzeyi artmaktadır. ANA ve 2-AG'nin ikiside endokannabinoid sistemin elemanları olmasına rağmen, ikiside endurans egzersize farklı tepkiler vermişlerdir. 2-AG serum düzeyinin ANA kadar dik ve fazla artış göstermemesi, egzersize dayalı endokannabinoid sistem değişikliğinde ANA'nın başrol oynadığı fikrini desteklemektedir. ANA'nın 2-AG'den fazla artış göstermesi Sparling ve arkadaşalrı tarafından gerçekleştirilen bir çalışma ile desteklenmektedir. Bu çalışmada % 75 VO2max şiddettinde koşu ve bisiklet egzersizi yapan sporcuların egzersiz sonunda alınan kan örneklerinde ANA'nın artış gösterdiğ, ancak 2-AG'nin egzersizden etkilenmediği görülmektedir. Ancak orta şiddette yapılan bu egzersiz ANA seviyesini 1 pmol'den 3 pmol'e çıkarabilmiştir. Oysa bu tez literatürde ilk defa yüksek şiddette egzersiz kullanılarak ANA'nın nmol seviyesine çıkarılabilineceğini, biyolojik olarak aktif sayılabilinecek düzeye ulaşılabilineceğini göstermiştir. Bu tez çalışması

yüksek şiddette egzersiz sonucunda hem ANA, hem de 2-AG'nin arttığını gösteren ilk çalışma olma özelliğne sahiptir.

ANA ve 2-AG endurans egzersize farklı tepkiler vermiş ve farklı artışlar göstermişlerdir. Bunun bir açıklamasının ANA ve 2-AG'nin hem farklı fizyolojik etkilerinin oluşu, hem de farklı biyokimyasal yolaklardan farklı koşullar altında sentezlenmesi olduğu düşünülmektedir (Joseph et al., 2004; Sugiura et al., 2006; Sugiura et al., 2002). ANA PMSF'nin varlığında degredasyona uğramayıp stabil kalabildiği halde bir miktar 2-AG çok çabuk 1-AG ve/veya 3-AG'ye dönüşmektedir (Sugiura et al., 2000). ANA ve 2-AG'nin ikiside CB1 ve CB2 reseptörlerine bağlanabilirken 1-AG ve/veya 3-AG bu reseptörlere bağlanma özelliğine sahip değildir ve vücutta herhangi bir fizyolojik etki göstermemektedir (Sugiura 2002; Sugiura 2000; Valk 1997). Buna ek olarak 2-AG oksijen varlığında PGE2-G'ye dönüşerek egzersiz sırasında Ca+2 hızlıca mobilize olmasını sağladığı savunulmaktadır (Nirodi et al., 2004). Bu nedenle 2AG'nin vücut tarafından kullanılmasını ve/veya birçok yolaktan yıkımını engellemek neredeyse imkansızdır Bu etmenler de 2-AG'nin serum düzeyinin ANA kadar yüksek olmamasını kabul edilir kılmaktadır.

Sadece 2-AG değil, ANA'da CB2 reseptörüne bağlanarak fosfolipaz C ve inositol 1,4,5- trisfosfat (IP3) ile Ca2+ salınımını etkilemektedir (Zoratti et al., 2003). Literatürde endurans egzersizin serbest Ca+2 konsantrasyonunu düşürerek hücre içi konsantrasyonu etkilediği ve lenfositlerin stres altında görevlerini yerine getirmelerine olanak sağladığı savunulmaktadır (Broadbent and Gass, 2006). Bu bilgiler birleştirildiğinde endurans egzersize dayalı hücre içi Ca+2 değişikliğinin egzersiz ile tetiklenen endokannabinoid sistem aktivasyonundan ileri geldiği ve lenfositlerin işlevlerini düzenlemede etkili bir rol oynadığını düşünmek mümkündür.

1.2-İmün efektör hücre sayımları

Çalışmanın birinci bölümünden elde edilen diğer bulgulardan biri lökosit sayıları ile lenfosit yüzdelerinin endurans tipi egzersiz yapan grupta diğer kontrol ve orta şiddette egzersiz yapan gruplar ile karşılaştırıldığında düşüş

göstermesidir ($p < .001$)(Şekil 4.2). Öte yandan nötrofil yüzdesi endurans egzersizde diğer iki gruba oranla artış göstermektedir ($p < .001$) (Şekil 4.3). Bu bulgular literatür ile uyumlu olup yorucu egzersizin nötrofili ve lenfopeniye neden olduğunu, ancak orta şiddette yapılan egzersizin lenfosit alt tipleri (sitotoksik T hücreleri, yardımcı T hücreleri, NK hücreleri ve B hücreleri) ile sitokin üretimini etkilemediğini desteklemektedir (Krüger et al., 2008; Murakami et al., 2009; Romeo et al., 2008). Egzersizin sporcularda nötrofil sayısını arttırmasına rağmen, nötrofil aktivitesini düşürdüğü ve bu düşüşün seviyesinin idmanın şiddettine bağlı olduğu kanıtlanmıştır (Kumae et al., 2009).

B hücreleri ($p = .001$), T hücreleri ($p = .003$) ve NK benzeri T hücreleri ($p < .001$) sayı olarak endurans grubunda diğer iki grup ile karşılaştırıldığında azalma göstermiştir (Şekil 4.4). Birçok çalışma yorucu egzersiz sonrasında kan lenfosit düzeylerinin normal seviyenin altına indiğini ve bu baskılanmanın süresinin egzersiz şiddetti ve süresi ile paralel ve ilişkili olduğunu ispatlamıştır (Pyne 2000). Tvede ve arkadaşları 60 dakikalık % 75 VO2mak ile yapılan egzersiz sonrasında immün sistemin baskılandığını, ancak % 50 VO2mak şiddetinde 60 dakika yapılan egzersiz sonunda herhangi bir baskılanma olmadığını ispatlamıştır (Tvede et al., 1989). Bu çalışmayı diğer bir çalışma ise 22 sağlıklı genç erkek üzerinde yapılmış olup, 60 dakikalık orta şiddette koşu egzersizinin T hücre sayı ve aktivitelerinde fark yaratmadığını savunmaktadır (Romeo et al., 2008).

Lökosit sayısındaki düşüş göz önüne alındığında 16 haftalık endurans egzersizinin sonunda lenfosit sayılarında da genel anlamda bir immün baskılanma beklemek ve görmek olasıdır. Hücre sayılarının genel anlamda azalmasının endokannabinoidlerin NK, T ve B hücrelerinde apoptozu tetiklemiş olmasından kaynaklandığı düşünülmektedir (Do et al., 2004; Rockwell et al., 2006; Malfitano et al., 2006). Orta şiddette egzersiz grubunda da apoptotik hücre sayısı kontrol grubuna göre artış göstersede endurans egzersiz grubu kadar yüksek olmadığı tespit edilmiştir. Bu sonuçlar ışığında apoptozun egzersiz şiddetine bağlı olduğu ve egzersiz şiddettine göre değişkenlik gösterdiğini söylemek mümkündür (Wang and Huang, 2005). TUNEL metodu kullanılarak yapılan immünohistolojik analiz sonucunda dalakta da apoptozun

endurans egzersiz grubunda tetiklendiği, orta şiddette egzersiz grubunda da endurans egzersiz grubu kadar olmasa da apoptotik hücrelerin arttığı tespit edilmiştir (Şekil 4.6). Bu sonuçlar literatür tarafından desteklenmektedir (Lagranha et al., 2004, Wang and Huang, 2005, Wang and Lin, 2009). Bazı çalışmalar özellikle dalakta ANA'nın lenfositlerde apoptozu tetiklediğini göstermiştir (McKallip et al., 2002; Schwarz et al., 1994). Bu nedenle imün efektör hücre sayılarında gözlemlenen düşüşün endokannabinoidlere dayalı apoptozdan kaynaklandığı önerilmektedir (Do et al., 2004; Rockwell et al., 2006; Malfitano et al., 2006). RT-PCR sonuçlarına bakıldığında ise apoptotik yolakların genleri olan BAX, caspase 3, Fas ve antiapoptotik bileşen Bcl2 mRNA ekspresyonları artmadığından, apoptozun hücresel düzeyde gerçekleştiği ve/veya fosforilasyon kaskadını kullanarak gerçekleştiği savunulmaktadır. Do ve arkadaşları egzojen ve endojen kannabinoidlerin apoptozu CB1 ve CB2 reseptörleri üzerinden tetiklediğini, bu arada NF-κB'nın rol oynadığı bir mekanizma ile immün supresyona neden olduğunu göstermişlerdir (Do et al., 2004). Aynı çalışmada kullanılan CB1 ve CB2 reseptör anatgonistleri sayesinde bu iki reseptörün de apoptoz mekanizmasında rol oynadığını göstermiştir.

Hücre oranları göz önüne alındığında ise endurans egzersiz yapan grubun NK hücrelerinde ($p= .02$) ve T hücrelerinde ($p= .02$) artma gözlemlenirken, B hücrelerinde($p< .001$) ise azalma olduğu tespit edilmiştir (Şekil 4.5). Buna karşılık NK benzeri T hücrelerinde anlamlı bir fark oluşmadığı tespit edilmiştir. Orta şidddette egzersizin lenfosit ve NK hücre oranlarını etkilemediği literatürle desteklenmektedir (Raso et al., 2007). Bu sonuçlar immün efektör hücrelerin egzersize dayalı olarak artan ANA ve 2-AG'den farklı oranda etkilendiğini, aynı egzersiz şiddettinde farklı değişimler gösterdiğini ve orta şiddette egzersize karşı duyarsız kaldığını göstermektedir. Literatür egzojen olarak uygulanan endokannabinoidlerin hücresel ve hümoral bağışıklığa katılan T ve B hücreleri farklı yönde etkilediğini göstererek bizim bulgularımızı desteklemektedir (Derocq et al., 1995; Joseph et al., 2004).

1.3-Immünohistoloji

1.3.1-Tunel ile apoptozisin değerlendirilmesi

Kontrol ve deney grupları karşılaştırıldığında, dalakta apoptotik indeksin egzersiz yapan gruplarda artmış olduğu, timusta ise belirgin bir farklılık olmadığı saptanmıştır (Şekil 4.6). Endurans ve orta şiddette egzersiz yapan gruba ait dalak örneklerinde hesaplanan apoptotik indeksler kontrol grubuna göre istatistiksel olarak anlamlı biçimde yüksekti (her iki grup için de $p < .001$). İncelenen tüm dalak örneklerinde apoptoptik hücreler beyaz pulpadaki periarteriolar diffüz ve nodüler lenfoid dokunun dış kısmında, marjinal zonda ve hemen kırmızı pulpa komşuluğunda izlendi. Bu bölgelerin, kan kökenli antijenlerin lenfositlere sunularak venöz sinüzoidlerden beyaz pulpadaki lenfoid kompartmanlara giriş yaptğı alanlar olduğu dikkati çekti. Morfolojik olarak apoptotik hücrelerin lenfomonositer hücre gruplarından olştuğu gözlendi (Şekil 4.7). Timustaki apoptotik hücre grupları T lenfosit serileri ile epitelyal hücreleri içermekteydi. Bu hücreler, kontrol ve egzersiz gruplarında benzer lokalizasyon ve sayılarda, çoğunlukla timik lobüllerin korteks medullla sınırında ya da medullanın içinde izlendi (Şekil 4.8).

1.3.2 İmmün efektör hücrerelerin lenfoid dokuda haritalanması

CD3 tüm gruplarda dalağın beyaz pulpasında PALSta yaygın dağılım gösterdi. Timusta farklı gelişme evrelerindeki timositler korteks ve medullada bu molekülü eksprese etti. Dalakta öncelikli olarak T hücre zonlarında eksprese oldu. Dalakta beyaz pulpada MHC class 1 molekülünün sunduğu antijene spesifik olduğu bilinen T hücreleri ve timustaki timositler CD8a ve CD8b'yi eksprese etti (Şekil 4.9, 4.10). CD11b, dalak ve timusta muhtemelen granülositler, NK hücreleri ve T ile B hücrelerinin alt gruplarını içeren daha az sayıda hücre tarafından eksprese edildi. Dalakta asıl olarak kırmızı pulpada dağılım gösterdi (Şekil 4.9, 4.10). Antikorla uyarılan B hücre işaretleyicisi olan CD25, dalağın B hücrelerini bulunduran nodüler lenfoid dokusunda (beyaz pulpa) dağılım gösterdi. İlginç olarak bu antijen timus medullasında az sayıda

lenfoid hücreyi de işaretledi. CD45 dalak ve timus parankiminde oldukça yaygın ve güçlü bir ekspresyon paterni gösterdi. CD45RA dinlenme halindeki T hücrererince eksprese edildi. NK hücre işaretleyicisi olarak bilinen, ancak bazı T ve B hücre alt gruplarının da barındırdığı bilinen CD56, timus medullasında ve dalakta kırmızı pulpada az sayıda hücre tarafından eksprese edildi. Anti fagosit antikoru ile timus ve dalak parankimindeki makrofajlar yaygın biçimde işaretledi (Şekil 4.11, 4.12). İmmün efektör hücrelerin yüzey antijenlerini eksprese etme gruplara göre oranları kantitatif olarak hesaplanmıştır. Buna göre hem orta şiddette egzersiz, hem de endurans egzersiz yapan hayvanların dalak dokusunda CD8a ve CD8b eksprese etme oranı kontrol grubuna göre artmıştır (Şekil 4.13). CD11b eksprese etme oranı dikkate alındığında ise, endurans egzersiz yapan grubun dalaklarında orta şiddette egzersiz ($p= .024$) ve kontrol ($p= .007$) grupları ile karşılaştırıldığında, daha düşük CD11b ekspresyon değerine sahip olduğu saptanmıştır (Şekil 4.14). CD25 ekspresyon oranının egzersizle düştüğü gözlemlense de, istatistiksel olarak anlamlı tek fark kontrol ile orta şiddette egzersiz grupları arasında tespit edilmiştir ($p= .022$) (Şekil 4.15). Endurans egzersiz grubunun dalak dokusunda da PHA eksprese eden hücre sayılarının kontrol grubu ile karşılaştırıldığında artmış olduğu görülmektedir ($p= .006$) (Şekil 4.16).

Timusta CD8a eksprese eden hücreler dışında, gruplar arasında anlamlı bir farka rastlanmamıştır. Sadece endurans egzersiz ile kontrol grubu arasında CD8a eksprese eden hücreler açısından anlamlı bir fark bulunmuştur($p= .038$) (Şekil 4.17).

1.4-RT-PCR analizi

Çalışmanın birinci bölümünden elde edilen RT-PCR sonuçlarına göre endurans egzersiz yapan sıçanların dalak dokusunda CB1 reseptör mRNA ekspresyonu açısından artış olduğu gözlemlenmiştir (Şekil 4.18). CB2 reseptör geninin ekspresyon düzeyinde farklılığa rastlanmamıştır. Timusta da hiçbir gen açısından fark yoktur. Dalakta CB1 reseptör gen ekspresyonundaki bu artış ANA serum düzeyi ile parallelik göstermekte olup, son derece anlamlıdır,

çünkü literatür ANA'nın daha çok CB1 üzerinden etki gösterdiğini desteklemekte, aynı zamanda dalağın periferdeki kanı yansıttığını belirtmektedir (Bakovic et al., 2005; Frances et al., 2008). Bu sonuçlar ile endurans egzersizin sadece protein düzeyinde değil, gen düzeyinde de endokannabinoid sistemi aktive etmiş olabileceğini, ve bu etkinin ANA molekülünün CB1 reseptör yolağını kullanması ile gerçekleştiğinin göstergesi olduğunu savunmak mümkündür. Bu bulgular literatürde Keeney (2008)'nin çalışması ile desteklenmektedir. Keeney (2008) çalışmasında farelere CB1 reseptör antagonisti vererek egzersiz hızı ve koşu mesafesinin azaldığını göstermiş ve bu sonuçlar ile egzersiz ve endokannabinoid sistemi arasındaki ilişkisinde CB1 reseptörünün varlığını ispatlarken endokannabinoidlerin endurans egzersizine dayalı nörobiyolojik ödüllendirmede CB1 reseptör yolağını kullandığını savunmuştur.

2-Çalışmanın 2. Bölümü

2.1-ANA serum düzeyinin nın ölçülmesi

ANA serum düzeyi kontrol grubu ile karşılaştırıldığında tüm egzersiz yapan gruplarda istatistiksel olarak anlamlı şekilde artmıştır. (Şekil 4.27). Aynı zamanda egzersiz yapan gruplar arasında fark olmadığı tespit edilmiştir. Bu sonuçlar son derece tutarlı olup, aynı egzersiz şiddetti ve süresine maruz kalan tüm sıçanlarda ANA serum seviyesinin aynı oranda arttığını göstermektedir.

ANA serum düzeylerinin çalışmanın birinci bölümüne oranla daha düşük olduğu saptanmıştır. Bu sonuç ile ANA serum seviyesindeki artışın sadece egzersiz şiddetine değil, aynı zamanda antreman süresine de bağlı olabileceğini düşünülmektedir.

2.2-İmün efektör hücre sayımları

Endurans egzersiz yapan grubun kontrol grup ile karşılaştırıldığında kandaki lenfosit oranında egzersize dayalı ciddi bir düşüş olduğu tespit

edilirken CB reseptör antagonistleri olan AM281 ve AM630'un bu düşüşü tamamen olmasa da kısmen engelleyebildiği görülmüştür (Şekil 4.28). AM281 (F= 22,91; p=.005) ve AM281+AM630 (F= 18,90; p=.023) uygulanan gruplar ile egzersiz grubu arasında fark olduğu gibi, kontrol grubu arasında da fark görülmektedir. Ancak antagonist uygulanan iki grup arasında anlamlı bir farka rastlanmamıştır.

Yine kan sonuçları incelendiğinde T hücre (CD3+) oranının egzersiz yapan grupta kontrol (Z= 2,42; p=.016) ve antagonist uygulanan gruplara (Z= 2,05; p=.04) kıyasla yükseldiğini söylemek mümkündür. Bunun yanı sıra kontrol grubu ile antagonist uygulanan AM281 ve AM281+AM630 grupları arasında anlamlı bir fark olmadığı görülmüştür. Bu sonuçlar ışığında kullanılan AM281 ve/veya AM630 antagonistlerin endurans egzersize dayalı T hücre oranındaki artışı kanda engellemeyi başardığı düşünülmektedir (Şekil-4.29). Buna ek olarak T hücrelerinin alt tipi olan yardımcı T hücreleri (CD3+CD4+) oranının kanda gruplar arasında farklılık göstermediği, ancak sitotoksik T hücre (CD3+CD8a+) oranının endurans egzersiz yapan grupta kontrol (Z= -2,21; p= .027) ve AM281 (Z= -2,73; p= .006) gruplarına göre düşüş gösterdiği tespit edilmiştir (Şekil 4.29)

NK ve B hücreleri açısından kanda gruplar arasında ne kontrolle, ne de egzersiz ile fark bulunamamıştır. Timus dokusunda da herhangi bir imün parametre açısından farklılığa rastlanmamıştır. Ancak dalak dokusu incelendiğinde, dalakta da T hücre yüzdesi egzersiz ile artış göstermiştir ancak ne tek başına AM281, ne de AM281 ile AM630'un beraber kullanımı endurans egzersize dayalı bu artışı engelleyebilmiştir (Şekil 4.30).

Splenik T hücrelerinde olduğu gibi, her iki antagonist ne tek başına, ne de beraber kullanıldığında endurans egzersize dayalı değişimleri engellemeyi başaramamışlardır. Bu sonuçlara dayanarak ANA'nın imün efektör hücreleri dalakta kandakinden farklı bir yolak üzerinden regüle ettiği düşünülmektedir. Kanda T hücreleri CB1 ve CB2 reseptörleri üzerinden regüle ederken, dalakta T hücreleri, NK benzeri T hücreleri, aktive T hücreleri ve ikili pozitif T hücreleri CB1 ve CB2 reseptörlerinden bağımsız farklı bir yolak üzerinden regüle ettiği sonucuna varılmıştır.

Endurans egzersize dayalı imün efektör hücre değişiklikleri dalakta T hücreler (CD3+) ile sınırlı değildir. Egzersize dayalı CD4+CD8+ T hücre yüzdesinde de artış gözlemlenirken (Şekil 4.31) , NK benzeri T (CD3+CD161a+) hücre yüzdesi ile aktive T (CD3+RT1b+) hücrelerin yüzdesinde azalma olduğu tespit edilmiştir(Şekil 4.32, 4.33).

Egzersize dayalı ANA serum düzeyindeki artışın kan ve dalak T hücre populasyonunu arttırdığı, buna ek olarak dalaktaki CD4+CD8+ T hücreleri de arttırırken NK benzeri T hücreleri ve aktive T hücreleri azalttığı gözlemlenmiştir. Ancak ANA'daki bu artışın dalakta B ve NK hücrelerini etkilemediği, timusta ise herhangi bir imün efektör hücre populasyonuna etki etmediği tespit edilmiştir. Dalakta da T hücre yüzdesi egzersiz ile artış göstermiştir ancak ne tek başına AM281, ne de AM281 ile AM630'un beraber kullanımı endurans egzersize dayalı bu artışı engelleyebilmiştir (Şekil 4.30).

ANA ve imün efektör hücrelerin arasındaki ilişkiye istatistiksel yöntemlerden Pearson Product ile bakıldığı zaman ANA ve bazı imün parametreleri arasında bağıntı olduğu görülmektedir (Tablo 4.7). ANA'nın dalak dokundan ölçülen NK benzeri T hücreler (CD3+CD161a+) ve aktive T hücreler (CD3+RT1b+) ile ters ilişkili, T hücreler (CD3+) ve double pozitif T hücreler (CD3+CD4+CD8a+) ile doğru orantılı olarak ilişkili olduğu tespit edilmiştir. Diğer yönden ANA ile kan ve timustan elde edilen imün efektör hücreler arasında herhangi bir ilişki bulunamamıştır.

2.3-İmmünohistolojik değerlendirme

2.3.1-Tunel yöntemiyle apoptozisin değerlendirilmesi

Egzersiz ve antagonist gruplarında yapılan incelemede, dalak ve timus örneklerinde ilk çalışmadakine benzer yerleşimdeki hücrelerin apoptoza gittiği izlendi. Dalakta egzersiz grubunda artan apoptotik hücre indeksleri saptandı (Şekil 4.34). Anatagonist uygulamasıyla, egzersiz grubundaki apoptozun istatsitiksel olarak anlamlı biçimde baskılandığı izlendi. Timusta tüm gruplarda benzer apoptoz oranları izlendi.

2.3.2 İmmün efektör hücrelerin lenfoid dokularda haritalanması

Egzersiz ve antagonist gruplarında yapılan incelemelerde immünefektör hücreler dalak ve timusta çalışmanın ilk bölümündeki kompartmanlarında tekrar gözlendiler.

Hücrelerin gruplarına göre kantitatif immün işaretlenme oranları hesaplanarak istatistiksel olarak anlamlı farklar olup olmadığı sorgulanmıştır. Dalak kesitlerinden elde edilen sonuçlara göre CD8a eksprese etme oranı hem endurans egzersiz (p= .004), hem AM281+AM639 antagonist (p= .015) grubunda kontrole göre artmıştır (Şekil 4.41). CD8b eksprese etme oranı ise sadece endurans egzersiz grubu ile kontrol grubu arasında anlamlı olacak şekilde değişmiştir(p= .011) (Şekil 4.41). PHA eksprese eden hücre sayısı ise endurans egzersiz grubunda artmıştır olup AM281 (p= .001), AM281+AM630 (p= .01) ve kontrol (p= .001) grupları ile dalakta anlamlı farklılıklar göstermektedir (Şekil 4.42). Diğer yandan timus kesitlerinde tüm bu hücreler için gruplar arası fark tespit edilememiştir.

Mikroarray sonuçları

31099 gen arasından 302 adet genin transkripsyon düzeyinde α= 0.001 olacak şekilde istatistiksel olarak anlamlı farklar olduğu, bunların arasından 190 adet genin isimlerinin bilindiği, geri kalan 112 adet genin henüz tanımlanmadığı ve bundan dolayı değerlendirilemediği, ancak ileride değerlendirileceği tespit edilmiştir.

CB2 reseptörünü kodlayan Cnr2 (p= .003) ve FAAH enzim proteinini kodlayan genin (p= .04) dalakta kontrol ile AM281+AM630 antagonist grupları arasında transkripsyon düzeylerinde farklılık gösterdiği tespit edilmiştir. IgD imünoglobulin proteinini kodlayan genin kontrol grubu ile karşılaştırıldığında endurans egzersiz (p= .016), AM281 (p< .001) ve AM281+AM630 (p= .002) gruplarında transkripsyonunda azalma olduğu tespit edilmiştir. Buna ek olarak CD8 yüzey antijeninini kodlayan gen ile perforin proteinini kodlayan genin

endurans egzersiz ile AM281+AM630 grupları arasında transkripsyon düzeylerinde istatistiksel olarak anlamlı farklar olduğu gözlemlenmiştir. Bunun yanı sıra CD38 antijen proteinini kodlayan gen (p= .017) ile IL-15 proteinini kodlayan genin (p= .005) transkripsyon düzeyinin kontrol ile AM281 grupları arasında dalakta farklılık gösterdiği tespit edilmiştir.

Timusta kontrol ile AM281+AM630 grupları arasında CD4 (p= .018), CD8 (p= .022), CD48 (p= .009) antijenlerini kodlayan genler, IL 4 (p= .03) ve IL15 (p= .002), proteinlerini kodlayan genler, IL-1a (p= .015) proteinini kodlayan gen, INFγ (p= .03) proteinini kodlayan gen, Caspase 8 (p= .017) proteinini kodlayan gen ve NFκB (p= .026) genine ek olarak endokannabinoid sistem ile ilşkili olan CB2 reseptörünü (p= .015) kodlayan gen ile FAAH (p= .004), MGL (p= .046) ve DGL (p= .004) enzimlerini kodlayan genlerin transkripsyon düzeylerinde fark olduğu tespit edilmiştir. CD48 yüzey antijen proteinini kodlayan genin endurans egzersiz ile AM281+AM630 grupları arasında anlamlı bir fark olduğu gözlemlenmiştir (p= .03). Apoptotik yolak ile ilişkili genlerden caspase8 (p= .006) ve NFκB (p= .037)'nın transkripsyon düzeylerinde artış gözlemlenirken endurans egzersiz ile AM281+AM630 grupları arasında fark olduğu belirlenmiştir (p= .05).

Dalakta CD69, CD4, CD 244, CD14, CD48, IgM proteinlerini kodlayan genlerin transkripsyon düzeylerinde fark olmadığı gözlemlenirken, apoptotik yolak ile ilişkili olan Bax, Fas, casp3, Bcl2, Fasl, Casp8, ve NFkB'yı kodlayan genlerin transkripsyonlarında da egzersiz yapan gruplarda fark gözlemlenmemiştir. IL-4, IL-10, IL-6, IL-2, IL-1a, IFNγ proteinlerini kodlayan genlerin ekspresyon düzeylerinde belirgin farklara rastlanmamıştır.

CD3 zeta, CD25, CD45RA, CD45RO, CD19, CD10, CD21, CD27, CD11c, CD11b, CD16, CD56, CD57, CD161a, NKp46, rNKp30 ve RT1b proteinlerini kodlayan genler kullanılan kitte bulunmamaktadır veya henüz tanımlı olmadığından bu genler açısından tespitte bulunulamamıştır.

Ancak, egzersiz sonucunda içerisinde bağışıklık sistemi ile ilişkileri olanlarında bulunduğu çeşitli genler ekspresyonlarında farklılık göstermiştir. En fazla değişikliğe uğrayan genler tablo 4.9 ve tablo 4.10.gösterilmiştir.

Bu çalışma ile ilk kez egzersiz şiddetine bağlı olarak gelişen

endokannabinoid ve imün sistem yanıtı incelenmiştir. Endurans egzersize dayalı immün sistem değişiklikleri endokannabinoid sistemi üzerinden açıklanmaya çalışılmıştır. Egzersiz şiddetine bağlı olarak ANA ve 2-AG'nin farklı düzeylerde arttığı, bu artışın imün efektör hücreleri etkilediği tespit edilmiştir. Endurans egzersizin ANA serum düzeyini arttırarak hem kanda, hem de dalakta T hücre yüzdesini arttırdığı, ancak bu artışın sadece kanda CB1 ve CB2 reseptörleri üzerinden regüle edildiği saptanmıştır. Bunun yanı sıra dalakta ikili pozitif T hücreler artarken, NK benzeri T hücreler ve aktive T hücrelerin azaldığı, ancak bunlarında CB1 ve CB2 reseptörlerinden bağımsız bir yolak ile düzenlendiği gösterilmiştir. Timustaki imün efektör hücrelerinden hiç birinin egzersize dayalı olarak değişmediği saptanmıştır. İmmün sistemde meydana gelen değişikliklerin bazılarının gen bazında olduğu, immün sistemin egzersize gen bazında adaptasyon geliştirdiği gözlemlenirken özellikle heat shock proteinlerini kodlayan genlerin transkripsyonlarında artış olduğu tespit edilmiştir.