Change in gene expression of toll-like receptor pathways in response to preseason conditioning in elite NCAA Division 1 wrestlers

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Change in gene expression of toll-like receptor pathways in response to preseason conditioning in elite NCAA Division 1 wrestlers

by

Cole Rex Sanderson

A thesis submitted to the graduate faculty in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Kinesiology

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ABSTRACT

This study examined the effects of both a single bout of intense exercise and 1 month of intense preseason conditioning on the expression of Toll-like receptor (TLR) proteins and signaling pathways. Eight elite Division I wrestlers were followed through the preseason training period. Blood samples were collected before preseason training started (at rest), after two weeks of training (within 30-60 minutes after completing a ~1.5 hour exhaustive exercise session) and following the pre-season training period (at rest). After the collection of blood samples from the athletes, peripheral blood mono-nuclear cells (PBMC) were separated from the whole blood samples. Messenger RNA was isolated from the PBMC’s and TLR focused gene expression was measured using DNA microarray. Illness records were collected over the wrestling season. The microarray results demonstrated a significant change (p<.05) in gene expression for seven genes following the single bout of intense exercise (decrease in IFNγ, IRF-1, IL-6 and an increase in PGLYRP2, LY86, TIRAP, TLR5), and a decrease in seven genes following the one month training period (PRKRA, SARM1, IFNα, TLR3, PTGS2, TNFSRF1, REL). The decrease in IFNγ in response to one bout of exhaustive exercise was significantly correlated with the number of events with cold symptoms lasting longer than 1 week (p=0.02). Similarly, the decrease in REL and PTGS2 expression after preseason training were correlated with increased in the total number of days with illness symptoms. The findings from this study have demonstrated that changes in TLR gene expression do occur in response to intense exercise training, and that these changes may be correlated with subsequent infection.
CHAPTER 1: INTRODUCTION

Longstanding conventional wisdom tells us that a person who exercises regularly will be healthier and in effect become ill less often and for shorter periods of time. The effects of exercise on immune response likely depend on the intensity, duration, frequency, and type of exercise one performs. (52) Also contributing to the immune response is the influence of stressors not directly caused by the physical activity (i.e. psychological stress, and nutritional status). The prevailing theory on exercise and immune function is called the “Inverted J Hypothesis”. First termed by Nieman (1997), this theory describes the relationship between exercise and infection (49). The theory postulates that exercise of low to moderate intensity, undertaken on a regular basis, will result in an increase in the efficiency of the body’s immune response and an effective decrease in one’s susceptibility to disease and illness. The potential immune benefits of exercise may be optimal with the performance of regular moderate exercise and may begin to deteriorate as exercise intensity, duration, and frequency increase beyond that point. (50) Some data support the possibility that immune response is suppressed at higher intensities. (35,53-56,64,66,67) This effect may have significant consequences for the elite athlete such as increased risk of infection. As suggested by Nieman (52) it is commonly understood and agreed upon that in many athletic events a person is required to train at very high intensities and with relatively high frequency in order to achieve and maintain those physiological adaptations that make it possible for him or her to reach the highest levels of athletic performance. Theoretically athletic involvement then becomes a balancing act between performing the necessary physical training and avoiding a state of immune suppression which may lead to illness and subsequent impairment of athletic
performance. There is much evidence in the literature to support the idea of an inverted J effect though the working mechanisms of this hypothesis are not completely understood or agreed upon. (35,53-56,64,66,67) The question remains what are the specific effects of differing exercise intensities, durations etc. on the athlete’s immune function, and how does the effective immune response play into possible immune suppression and the subsequent increase in susceptibility to illness that follows. (52)

Further investigation of exercise and immunological phenomenon questions the possible role played by exercise-induced immune suppression on the broader condition termed Overtraining Syndrome (OTS). OTS is a condition in which the athlete experiences an unexplained decrease in performance, which can manifest over varying time periods.(16) The causes are not completely understood but are likely the conglomerate of several stresses (psychological, emotional, etc.) in combination with a physical training load in which the degenerative effects are greater than the body’s ability to recover (catabolism exceeding anabolism). There are several physical and psychological phenomenon considered to be markers of overtraining. These range from decreased VO2 and altered heart rate to a decline in psychological mood state. Among the plethora of possible contributing factors to OTS is the suppressed immune function that may occur with overly intense training. (50,70). The question remains as to what aspects of the immune response are altered by overtraining and do these changes contribute to an increased risk of infection.

One purpose of this paper is to review the literature exploring exercise and immune function outlining what has been found in regard to immune cell function, adaptation, and possible suppression. This information will be evaluated in reference to the commonly accepted “inverted J hypothesis”, and will attempt to identify those factors that may or may
not contribute to overall immune suppression. This study will be conducted from a standpoint supporting the “danger” model of immune function recently suggested by Matzinger (1994). This theory suggests that the body’s immune defenses respond to detect danger and potential destruction (“danger” being defined as anything that causes tissue stress or destruction) as compared to the “classical” immunological theory in which one of the factors most important is the distinction of self and non-self. (39-44) This paper hypothesizes that the “danger theory” of immune response more clearly and effectively explains the body’s immune response to exercise when contrasted with the classical theory emphasizing immune response focused more exclusively on the identification of self and non-self. (39,43)

**Original Research**

The main focus of our research was to elucidate the effects of high intensity training on the selected immunological parameters in the context of the danger model, and to determine the extent to which our findings fit the inverted J hypothesis. This specific athletic team was chosen because they utilize a pre-season conditioning regimen which we believe maximizes physical and psychological stress(63), and which may trigger activation of danger signals, thus increasing immunological stress and in effect the likelihood of immune suppression. This maximal stress response along with high levels of tissue breakdown (i.e., damage) will activate the body’s “danger” response.

In this study we examined toll-like receptor activation and subsequent cell signaling as the primary pathways by which exercise alters immune response. We extracted blood from the subjects at several time points throughout the pre-season training period. From the whole blood leukocytes were isolated (specifically the peripheral blood mononuclear cells) followed by mRNA isolation. The mRNA was analyzed through the use of a microarray kit
which effectively demonstrated changes in gene expression. The up or down regulation of genes involved in toll-like receptor signaling was performed to better understand how the immune system reacts to the type of training our subjects performed. There is little information in the literature (to date) that focuses on toll-like receptor activation in response to exercise as a potential means of assessing the extent to which exhaustive exercise may result in the production of factors which act as danger signals. In addition, two psychological questionnaires were administered in order to assess the possible contribution of anxiety to the athlete’s immune responses.(29,43,45) Illness data questionnaires were also administered to the athletes at the conclusion of the preseason-training period and throughout the regular season training period in order to identify illness patterns in the athletes in an attempt to correlate changes in gene expression with subsequent illness.
CHAPTER 2: LITERATURE REVIEW

Exercise and Infection

There are two general beliefs concerning exercise and illness held by the population at large as well as among athletic populations. The first of these common beliefs is that regular exercise increases a person’s resistance to illness. The second belief, more applicable in athletic populations, is that with overexertion there is a decrease in immune competency and an increase in a person’s risk of infection and illness. (49) These beliefs generally adopted by intuition may be considered “common sense” for many people, and indeed the epidemiological evidence seems to support these beliefs overall. (35,53-56,64,66,67) However, clear recommendations for preventing “overexertion” and “overtraining” are equivocal, presenting difficulty for elite athletes who are often required to exercise at high intensities (which can border and occasionally cross into the realm of overexertion) in order to create and maintain those biological adaptations that allow them to compete at an elevated level. It appears from the research that regular low intensity exercise increases resistance to illness and that that immune competency increases with increased regularity and intensity of exercise as long as exercise intensity and duration remain at the low-moderate level. (53,54)

The question arises; at what level does exercise cease to confer enhanced resistance to infection and instead increase the risk of infection? Even more critical to the elite athlete is the question of how to avoid activity that may increase susceptibility to illness and infection while maintaining the required intensity and duration to remain competitive. The findings from current literature can provide some insight into these questions but answers are far from definitive, and our understanding of the mechanisms involved is limited. One potential
mechanism by which exhaustive exercise may alter immune responsiveness is through the production of danger signals resulting in toll-like receptor activation and subsequent expression of inflammatory factors. (29, 43, 69) Perhaps prolonged activation of inflammatory factors disrupts the normal immunological balance between pro and anti-inflammatory factors leading to an impaired host defense against pathogens. Consideration of the danger signal model in the context of exercise immunology may ultimately lead to a better understanding of the causes of impaired immunity that accompany overtraining as well as the immunological benefits derived from regular low-moderate intensity exercise.

**Epidemiological Evidence of Exercise and Infection**

Data from epidemiological data studies have allowed us to move beyond intuition and the subjective observations of coach and athlete. The data generally support the idea of the “J” curve model proposed by Neiman (49) in which the amount and intensity of exercise influence risk of infection (low-moderate intensities may be beneficial, whereas very high intensity exercise for a prolonged period of time compromises immune competency). In a 1998 review Neiman divided the research data categorically into “heavy exertion” and “moderate exertion”. (51) Heavy exertion has been associated with increased infection. The most common form of infection reported in epidemiological studies in response to exercise is an upper respiratory tract infection (URTI) with symptoms suggestive of viral rather than bacterial infection. URTI is the most common type of infection reported by athletes and the literature has focused typically on URTI symptoms rather than identification of the pathogen.

**Heavy exertion and risk of infection**

Several factors associated with heavy training might be related to infection. Some of the factors contributing to URTI risk are: the stress of participation in competition events,
event duration/distance, training duration/distances, and nutritional status and supplementation. In this section, the influence of exercise duration, distance, and training status on the risk of infection will be described. In a 1987 study by Linde et al., 44 Danish orienteers (matched with 44 non-athletes by age, sex and occupational distribution) showed that orienteers suffered 2.5 cases of URTI each year compared with a significantly lower incidence 1.7 in non-athlete counterparts. (35) This study showed a contrast between non-athletic types and those performing regular exhaustive/intensive exercise. This study suggests that non-athletes generally have greater immune competency than the athlete who regularly experiences heavy exertion.

Average training intensities have also been shown to influence URTI risk. Nieman showed in a 1989 study surveying 294 recreational California runners (during preparation for a race) that those running ~ 42 km/wk had lower URTI incidence than those running only ~12 km/wk.(55) The findings from this study support the “J curve” model. Nieman’s 1990 study of 2311 Los Angeles marathon runners showed that an increase in immune response seen in the before mentioned study does not continue indefinitely.(56) Those marathon runners training for greater than or equal to 97 km/wk were at higher risk for URTI than those running only 32 km/wk. In fact it was determined that those running greater than 96 km/wk were at double the risk for infection and illness as those running less than 36 km/wk. Heath et al. (23) in a study utilizing older men (an average age of 40 years) those running less than or equal to 16 km/wk had fewer URTI symptoms than those running more than 27 km/wk. When comparing these data with the data from Nyman’s 1989 study with the recreational runners it is possible that the total number of miles run per week correlates with infection, but that this effect varies by age. (55)
The stress of competition during an actual event (race) has been shown to play a role in the relationship of infection and exercise. Peters and Bateman (1983) showed that in the 2 weeks following a 56 km race incidence of URTI was 33.3% compared with 15.3% in a group of controls matched for activity that did not participate the competitive event.(65) Peters repeated the study in 1996 this time finding 28.7% incidence of URTI compared with 12.9% in the control group. (67)

**Exercise and on leukocyte function**

In a review by Nieman (1997) it was reported that Natural Killer Cells, neutrophils, and macrophages appear to be the immune cells most affected by acute exercise. The response of these cell types is dependent on exercise intensity and duration. There are many potential mechanisms by which exercise might influence immune response including cytokine concentrations, stress hormone changes, and the relocation of many cells and humoral factors. (52)

**Natural Killer Cell Activity**

Natural killer cells are large granular lymphocytes that play an important role in innate immune function. These cells help to inhibit infection by viruses, bacteria, fungi and parasites. They are effective in killing a multitude of different target cells. NK are able to attack a wide variety of virally infected and tumor cells. (49,85) NKC usually bind to target cells, disrupt cell membranes through the use of perforin (pore forming protein) and introduce toxic granzymes into the target cell to destroy it. (49,85) NKC are one of the first cellular responses to viral infections, they aid in antibacterial and anti-tumor functioning, and kill cells opsonized with antibody. NKC cells do not require antigen presentation by MHC. In contrast to T cell, the NK cells are able to lyse target cells without any apparent previous
sensitization and do not require the expression of MHC on target cells. (49, 51, 85) They have the ability to lyse target cells that have undergone malignant transformation. (85) NK cells cytolytic activity is enhanced by interferon and interleukin 2. (61) NK cells are characterized by a set of markers (CD3-, CD2+, CD16+, and CD56+). Cytokines (INF-α, IFNγ, IL-2, and IL-12 have been identified as efficient activators of NKCA.) (85) These cells are important in innate immune function and they play an important role in preventing and combating infectious agents that can lead to illness for the athlete especially during times of heavy training or possible immune suppression.

The effects of exercise on NK cell function have been shown to follow a few general patterns. During exercise NK cells are recruited to the blood and NK cell activity increases as the concentration of NK cells in an area is increased. (61) The NKCA has been shown to be enhanced in endurance athletes vs. non-athletes (49). A study by Nieman et al (1995) showed NKC activity to be 57% higher in experienced marathon runners vs. sedentary controls. It has also been suggested in some studies that NKCA may actually be enhanced during periods of heavy training. Tvede 1991 compared cyclists during heavy training vs. light training and showed an increase in NK cell activity during the heavy training period. (78) Though this trend is seen consistently among those performing heavy exertion exercise there have been a number of studies using moderate exercise that showed no increase in NKCA. These data suggest that the increase in NKC # and NKC activity are dependent to some extent upon intensity and duration of exercise requiring that certain levels be achieved in order to facilitate the chronic increase in NKCA activity. (50, 52, 61, 85)
Neutrophil Function

Another important part of the body’s innate immune response is neutrophil activity. The neutrophils are granulocytes with multi-lobed nuclei whose main function is to phagocytize bacteria and viral pathogens. They are considered to be very effective phagocytes. (52) Instead of showing an increase in number and function in response to exercise, neutrophil activities tend to be suppressed. No researchers have shown increases in neutrophil function in endurance athletes vs. non-athlete controls. Neutrophils function has been shown to be suppressed during periods of heavy training. They show a decrease in phagocytic activity in response to exercise that decreases further following a bout of heavy exertion. Pyne et al. (1994) showed lower neutrophil activity in elite swimmers during intense training than in sedentary controls. (70) Hack et al. (1994) showed that neutrophil function was similar to controls when training is of low-moderate intensity but that it became suppressed during times of intensive training. (15) The suppression of this important infection barrier is a possible candidate in explaining the increase of infection in those performing regular intensive exercise. (49,50,52,57,61,85)

Macrophages

Macrophages are 1st line defenders against multiple pathogens and malignant cells due to their phagocytic, cytotoxic, and intracellular killing abilities. Macrophages have potent phagocytic capabilities. Monocytes are derived from precursor cells in the bone marrow. They mature and differentiate as they leave the blood and travel to target locations often in response to cytokine messengers (IL-3, IL-6, IL-1, TNF, and others.) where they mature into macrophages (85). Each tissue determines the functional phenotype of its macrophage population. Therefore work performed on macrophages from one anatomic location does not
necessarily apply to macrophages from another. The diversity of the macrophage populations give the non-specific immune system flexibility and an increased ability to respond to diverse challenges. Macrophages are not easily accessible in human subjects because they reside within target tissues for which they have been adapted. Therefore, much of the research involving macrophages often macrophage research is conducted using animal models.

Peritoneal macrophages have been shown in both moderate and exhaustive exercise to enhance a variety of functions including chemotaxis, adherence, phagocytic, and anti tumor activity. However, macrophages obtained from the respiratory tract (alveolar macrophages) have been shown to be suppressed by exercise. It has been shown that the stress hormones associated with prolonged exercise may suppress alveolar macrophages antiviral functions. It has also been suggested that the mechanisms for increased phagocytic and chemotactic activity in response to exercise may be mediated by corticosterone or prolactin (chemo) and thyroxine for phagocytic. (85)

Salivary IgA

Mackinnon et al. 1993 demonstrated low salivary IgA preceded URTI. The secretory immune system of the upper respiratory tract is the first barrier to infection (URTI) it stands as a barrier to pathogens entering the body.(37) IgA is the major defensive player of the mucosal barrier, preventing attachment and replication of pathogens preventing entry into the body. IgA concentration has been shown to decrease after a single bout of intense endurance exercise (37,49) Both intense endurance and interval exercise have been shown to decrease concentration of secretory IgA. (Mackinnon 1993) in this 1993 study showed 11/12 recorded URTI’s (diagnosed by medical practitioner) were preceded within 2 days by decreased salivary IgA concentration.(37) Thus IgA may be a contributor to increased incidence of
URTI among athletes. (Gleeson 1999) showed an inverse relationship between URTI and salivary IgA level, with an increase in illness as at decreased levels of salivary IgA. (21) They showed IgA to be 4.1% lower than in controls for each additional month of training over a 7 month training period, also showing a 5.8% decrease in salivary IgA with each additional infection.

**Summary of changes in leukocyte function and exercise**

Though a few general patterns seem to be established in the literature with respect to NK cells, neutrophils, macrophages and salivary IgA, the effect of exercise on immune cell function is often unclear and contradictory. More research is needed in order to determine definite patterns. (17,18,21,37,49)

**The Danger Model**

An underlying tenet of this literature review and the original research being presented in this paper is the idea that the body’s immune system is activated in response to the detection of danger or alarm signals that comes from injured cells. These injured cells could be damaged by pathogens, toxins, mechanical stress, etc. Regardless of the cause of injury the body launches a defensive reaction initiated by the release of danger/alarm signals from the cell. This idea of a danger based reaction process is a relatively new theory, but has gained in acceptance and in experimental support over the past 10 years. The basic ideology of the Danger Model theory separates it from the self-non-self model that has dominated immunological theory and research from the time of their introduction in the early 1950’s-1960’s. The basic tenet of self/non-self theory is that the immune system functions by making a distinction between self and non-self. In other words the immune system has the ability to recognize foreign entities, such as foreign bacteria and viruses, and that upon
detection of foreign entities it subsequently launches an immune response attacking and killing the invading entity. Some difficulties which have risen, with the use of this base ideology, relate to the fact that in some circumstances “self” can be dangerous (tumors) and non-self can be harmless and necessary (healthy fetus). (39-44)

History

Australian scientist Frank Macfarlane Burnet introduced the original self-non-self model (self/non-self) which was built on three basic principles: 1) Each lymphocyte expresses multiple copies of a single surface receptor specific for a foreign entity 2) Signaling through this surface antibody initiates the immune response 3) The self reactive lymphocytes are deleted early in life. (11,43) Burnett based this third principle on a study done by Owen (1945) who found while working with cattle that non-identical twin cows were tolerant of each other’s blood cells. (59) Medeware et al. helped Burnett’s theory to gain acceptance with their research, finding that adult mice would accept foreign skin grafts if they had been injected as babies with cells from the donor. (8) This finding supports Burnett’s theory that self reactive lymphocytes are deleted early in life. If the baby mice were injected with the donors cells their immune system would consider the cells to be self. Identifying the donor cells as self lead to the deletion of the lymphocytes which would recognize these cells as foreign. This recognition allows the body to accept the grafts as non-foreign entities in the adult mice. Frank Macfarlane Burnet and Peter Brian Medewar received the 1960 Nobel Prize for their efforts. (43) Since that time the theory of self-non-self has governed both research and theory surrounding immune function.

During the past 40+ years that followed, several questions and inconsistencies rose in relation to this immune system base, which forced modification of the original theory. In
1969 Bretscher and Cohn presented the concept of the helper cell (T-cell) being necessary for the activation of immune response. (10) Later, Lafferty and Cunningham (1975) proposed that T-cells also needed a second signal (Co-stimulation), which they received from the Antigen Presenting Cells (APC’s). (32,43). The need for co-stimulation presented problems for the self/non-self model because the self/non-self model assumes that the decision to react is made by surface receptors that are specific for a foreign entity. However, APC’s are not antigen specific. They capture many self and non-self substances. Janeway (1989) came up with a solution to the problem of non-specific action by APC’s. (27) He suggested that APC’s have the ability to discriminate between self and non-self by their own mechanisms, and that they are at rest until said mechanisms are activated. Janeway proposed that the APC’s have receptors (pattern recognition receptors (PRR)) that can recognize pathogen associated molecular patterns (PAMP’s) on bacteria. These receptors allow the APC’s to discriminate between infectious self and non-infectious non-self. (27) Even with Janeway’s insight there are still many questions that the infectious non-self can’t answer satisfactorily. (i.e. why transplants are rejected?, and what causes autoimmunity?)

Thinking that the explanations provided by the self/non-self and infectious non-self were not sufficient in explaining immune function, Matzinger abandoned convention and presented an entirely new perspective on immune function. Matzinger suggested that the body launches immune defenses in reaction to danger/alarm signals from injured/stressed cells. These signals may be produced in reaction to pathogens, viral damage, mechanical damage, etc. Cells that die by normal apoptotic processes are scavenged before disintegration preventing molecules that could be identified as dangerous from stimulating APC’s. Cells that die by necrosis, however, spill their contents into the surrounding environment.
Theoretically, any intracellular product could be a danger signal. For these to be danger signals they should not be the same as a signal sent by those cells that undergo normal cell death. This new theory recognizes the fact that self tissues can be harmful and non-self tissues can be harmless. In the past few years some of the key entities functioning as danger signals have been identified, and will be addressed in a later section of this review. (39-44)

**Commonalities between the Danger and Self/Non-self – Infectious Non-self models.**

Both theories assume that APC’s are at rest until activated and that they can be activated by signals in the environment surrounding them. The Infectious Non-self theory receives support from the recognized action of Toll-Like Receptors (TLR), which act as PRR’s recognizing patterns in bacteria and fungi and thus are able to initiate immune responses. (45) There are currently 10 known mammalian TLR’s. These receptors are able to bind many different types of biological entities and in effect stimulate the resting APC’s. The danger model relies on the detection of danger signals to activate APC’s. The discovery of endogenous non-foreign danger signals lends support to the danger theory showing that a substance need not be foreign to induce APC activity. A few of the many candidates for alarm signals are: mammalian DNA, RNA, Heat shock proteins, interferon α, interferon β, CD40-L, and others. (43,45)

APC’s can be activated by both endogenous and exogenous signals. Often one receptor can serve multiple signal stimulators. Matzinger suggests that pathogens may have evolved to bind to receptors instead of receptors evolving to existing pathogens, and that they may have adapted in order to use receptors to enhance their own survival. There is evidence to support multiple activation pathways for immune system. The immune system may act to recognize danger signals, but is also capable of differentiating self and non-self. Ultimately
both the recognition of danger signals and antigen specific recognition may both be required to effectively clear pathogens. In the context of strenuous exercise it is more likely that the endogenous factors produced by exercise may lead to the activity of danger signal pathways rather than acting to activate antigen specific pathways. (43,45)

**Mechanisms of the Danger Model**

The Danger Model works via APC (i.e. dendritic cells and macrophages) recognition of pathogens. These cells detect “dangerous” entities by recognizing pathogen associated molecular patterns (PAMP) on bacteria and other pathogens. They are also able to recognize certain endogenous products released by stressed, damaged, or dying cells. The APC’s detect these molecular danger signals by using pattern recognition receptors (PRR) that reside intracellularly and as transmembrane receptors. In APC’s there are several classes of PRR’s which include nucleotide oligomerization domain (NOD), Dectins, retinoic acid inducible gene I (RIG-I), and the receptor class which has been most commonly studied and most completely defined, toll-like receptors (TLR). These different receptors vary in cellular location. TLR’s, for example, are generally trans-membrane receptors while NOD’s are intracellular cytoplasmic receptors. All PRR’s act through the identification of PAMPs. PRR’s are able to detect conserved microbial structures/patterns that are not found in human cells but which may constitute vital components of microbial organisms like bacteria. (43-46) APC’s have also been shown to be stimulated by the detection of endogenous non-microbial entities such as Uric Acid. Uric Acid is released when cells are killed or damaged, but is not released during normal cell death processes (apoptosis). When cells die by necrotic process; cells spill their intracellular products (any of which, theoretically, could work as a danger signal alerting the immune system) into the surrounding environment. (73)
One of the most important APC types in this danger model process (and the one which has been studied most) is the dendritic cell (DC). Dendritic cells are very efficient APC’s. They are even referred to as “professional” APC’s. DC’s are found throughout the body, but are found in greatest concentration at places where pathogen or infectious encounters are most likely to occur such as mucosal surfaces and the skin. DC cells remain in an immature form until “danger” has been detected. Once the DC’s have been activated by PRR’s they often produce inflammatory cytokine via NFκB, that may influence immune response. DC’s may also phagocytize and process antigens so they can be loaded onto MHC molecules to present as antigen to antigen–specific T and B lymphocytes. As part of the DC maturation process, DC’s upregulate CCR7 and travel to the lymphatic system toward draining lymph nodes. On the way DC’s mature and become able to present their antigenic information to T-cells. This process links innate and adaptive immunity as the presentation of antigen not only helps to activate innate immune responses, but with the information provided the immune system develops immune memory to aid in future battles. This memory keeps track of the antigenic encounter, and can then be recalled for a long time period, sometimes for the entire life of the host. (34,43,69)

Danger Signals

The immune system can be triggered by a multitude of varying molecules/molecular structures that the body recognizes as damage causing or “dangerous”. These molecules or danger signals are detected by pattern recognition receptors (i.e. Toll-like receptors) located both on the surface and intracellularly in antigen presenting cells like dendritic cells. These danger signals can be exogenous or pathogen-linked patterns displayed by microbial intruders. They may also be endogenous molecules made by the host organism and released
by cells that are stressed, damaged, or in the process or necrotic cell death. Both types of signals activate antigen presenting cells via pattern recognition receptor detection. (18,39-44) Pathogens which display exogenous danger signals are phagocytized by APC’s which carry them to the lymph nodes to be presented to naïve T-cells. This directs T-cell development and creates a specific immune response to the antigen as well as the development of memory cells that can act quickly and directly in the event of future infection by the same pathogen.

Endogenous signals are created by the host, and alert the immune system to cellular stress, damage, and necrotic death. In general these signals are released in response to stress or damage and are sensed by PRR’s on APC’s. (19) These signals can be primal (not requiring prior APC activation) or feedback signals (released by activated APC’s to continue or enhance the immune response already in action). Some endogenous signals can act as both primal and feedback signals, initiating the immune response and then helping to continue or enhance the already moving immune activity. Three such danger signals are CD40L, TNF α, and IL-1β. (Caux 1994). CD40L is normally a ligand requiring the activation of dendritic cells in order to be expressed. However when tissue is injured and bleeding, platelets expressing CD40L come into contact with tissue-bound dendritic cells, and may act as a primary dendritic cell activator. TNF-α and IL-1β act in a similar manner. These cytokines are typically released by active APC’s, and they are able to act as primal signals under some circumstances (i.e. TNF-α following myocardial injury). (12,18) Many molecules/molecular patterns that exist normally in the cell become danger signals when released by stress or damage or necrotic death. (3) For example, the nucleotides ATP, and UTP are normally found only intracellularly, however when a cell is damaged the release of these nucleotides into the surrounding extra cellular space may act as an APC stimulator. (18,39-44,69)
**Heat Shock Proteins (HSP)**

Heat shock proteins are another set of proteins that upon cellular stress, or damage can become danger signals. HSP are an ancient and conserved set of proteins present in both prokaryotes and eukaryotes. Regularly HSP’s of different specificities are contained in certain areas of the cell. Though regularly residing in a specific area HSP’s can be translocated through cellular stress and released into the extracellular space when cells undergo necrotic death. Basu et al. 2000 were able to show that HSP released by necrotic death could act as danger signals activating dendritic cells. (6) Cells dying by regular apoptotic process did not act as stimulators of dendritic cell activity. Additionally Aseu et al. conducted a series of experiments using HSP showing that extracellular HSP results in the stimulation of immune response of proinflammatory cytokines 2-4 hours post exposure (TNF α, IL-1β, IL-6, and IL-12) HSP 72 induces dendritic cell maturation by augmenting surface expression of CD40,CD83,CD86 and MHC II on DC cells , and influences migration of DC and NK cells. TLR2 and TLR4 are two PRR’s which have been shown to respond to HSP 70. (6,47,68,80)

**Uric Acid Crystals**

In 2003 Shi et al. identified uric acid as another possible danger signal. Uric acid may serve as a principle endogenous danger signal released from injured cells. Uric Acid stimulates dendritic cell maturation when co-injected with antigen in vivo enhancing the generation of responses from CD8+ Tcells. Uric Acid is present regularly in the blood of mammals. Mammals have relatively high constitutive concentrations of uric acid in the blood. High concentrations of uric acid are present in cellular cytosol . When cells are damaged they release these high concentrations of uric acid and the area around the dying
cells is supersaturated with uric acid favoring the formation of uric acid crystals. It is the crystallized form of uric acid that is thought to act as a danger signal. Uric acid has been identified to be a good indicator of cellular stress and damage, and a good stimulator of innate immune response. (69,73)

**Purpose and Rationale**

It is clear from the literature reviewed that exercise has the ability to influence immune function. It has been shown that intense exercise and heavy training can lead to immune suppression and increased risk of infection. It is the purpose of this study to identify changes in gene expression in the peripheral blood mononuclear cells following bouts of intense exercise performed during a period of heavy training. Identifying altered gene expression will help to increase our understanding of how the immune system responds to heavy exertion and will help us to identify the mechanisms of this response. It is possible that the knowledge gained from the proposed experiments may ultimately help athletes to prevent infection by identifying biomarkers of the toll-like receptor signaling pathways that are altered by exercise and are predictive of infection.

**Hypothesis.**

We hypothesized that TLR signaling pathways would be activated in response to one month of intense exercise training and illness would be correlated with a greater degree of TLR activation. Also, we hypothesized that TLR signaling pathways would be activated after one session of intense acute conditioning exercise.
CHAPTER 3: MATERIALS AND METHODS

Subjects

Eight college age (18-23 yrs.) male athletes from the Iowa State University men’s wrestling team volunteered to participate in the study. Iowa State’s wrestling team is a nationally competitive program among the top Division One wrestling programs in the NCAA. The particular set of athletes from which the subjects were recruited were successful in achieving a 2007 Big Twelve conference championship and were NCAA tournament runners up for the 06-07 season. These evidences of athletic success are indicative of the intense level of training undertaken by this group of athletes. This intensity was of primary import in the selection of this population for study and in the design and results of the study. Inclusion/exclusion criteria were as follows: 1) The athlete had to be uninjured at the time of sign-up and thus able to participate in all preseason training activities and, 2) The athlete had to be able to participate in all blood draws and pre-draw training sessions, having no known schedule conflicts which would prevent their participation on those days.

The athletes who volunteered for the study represented a variety of weight categories. The distribution was as follows: One subject at the 133, 141, 149, 157, 165, and 197 pound weight categories, and two subjects at the 184 pound weight category. The athletes were instructed by their coaching staff to continue jogging, weight-lifting, and wrestling during the off-season period. They were also instructed to be “in shape” before the start of preseason training period. Thus we assume that they were exercising regularly before the start of the preseason training period. However, the level of intensity and the degree of physical and psychological stress involved in the preseason training period is usually (if not always) much greater than that undertaken during off-season activity.
**Overall Exercise Protocol**

The preseason training period is designed to prepare the athletes for the rigors of regular season training and competition. The athletes are expected to continue regular exercise throughout the off-season period. In-season training, however, requires more frequent and more stressful exercise to develop and maintain the physiological and psychological adaptations required for top performance during regular season competition. During each week of preseason training the coaching staff scheduled 3 wrestling practices, 3 weight-lifting practices and one practice designed primarily for stress and conditioning.

**Weight training**

The weight lifting schedule was designed so that each practice focused on a general type of action being performed by the athletes (“push day”, “pull day”, leg exercise – Refer to Appendix B). In preseason, weightlifting emphasis was placed on the building of “explosive power” with high weight/low repetition lifting. This schedule shifts to low weight/high repetition lifting during the last week of preseason training to help facilitate muscle recovery.

**Wrestling practices**

The ISU wrestling coaching staff includes three training sessions each week which consist primarily of wrestling drills, exercises, and “live,” or full speed wrestling. One unique aspect of wrestling is that it requires a wide variety of conditioning methods to reach peak performance. In addition to cardiovascular excellence and muscular power, there are a multitude of strength, balance, and speed building requirements that can only be developed through wrestling specific drills and practices (refer to Appendix B for complete description) Briefly, the average pre-season wrestling practice schedule was run as follows: 1) 10-15
Conditioning Practices

Conditioning practices are designed to increase the athlete’s ability to endure physical and psychological stress. Appendix B describes this type of practice in detail along with heart rates measured during this practice. Briefly, the athletes warm up for 10-15 minutes in the wrestling room performing gymnastic-type exercises. The athletes then jog a 1-1.5 mile track to a coliseum-type location (the ISU football stadium). This track is run at a fast pace and in race-like manner. Once all team members reach their stadium destination, the athletes select a partner of similar weight and size. Each athlete then crawls in wheelbarrow fashion (with a partner holding their legs) uphill a distance of ~ 50 yards. This exercise may be performed multiple times. The athletes then line up at the bottom of the stadium stairs and sprint to the top of the stair case. The exercise is performed 4-6 times with only a small break between sprints (3-5 seconds at the top, and then the time required for the athlete to walk at a brisk pace back to the bottom of the stair case). Following the regular sprint exercises the athletes are given a one minute break. The athletes then carry a partner of similar size and weight on their backs to the top of the staircase. The athletes alternate carriers and usually perform this...
exercise 2-3 times each. After the “buddy-carries” the athletes are given a five minute break in which they get a drink and walk to a hill behind the stadium. They line up at the bottom of the hill and then perform 4-6 sprints from the bottom to the top a distance of 40-yards. After the sprints they again work with a partner and are required to carry their partner on their back as they crawl “bear style” up the hill. This is usually done two times by each athlete, alternating between crawling and riding. Following the bear crawls the athletes walk a distance of two-three hundred yards to a near-by parking lot where they line up and perform 4-30 second sprints from one side of the parking lot to the other. After a short team meeting (1-2 minutes) they jog back to the recreation center a distance of .7 miles. This conditioning bout of exercise is characterized by nearly constant effort with little rest time between the different exercise drills.

Blood Collection and PBMC Isolation

Following the cessation of exercise the athletes were allowed time to rest and the blood extraction was 30-120 minutes post exercise. Approximately 30 ml of blood was collected from each subject. The draws were taken via the anticubital veins of either arm. Blood was collected using a syringe and vacutainer tube (containing sodium-heparin to prevent blood coagulation). Following the blood collection the subjects were asked to sit for 10-15 minute observation period for safety purposes.

PBMC isolation

Whole blood samples were diluted using a 1:1 mixture with 1xPBS. PBMC’s were separated using Ficoll Paque Plus (Amersham Pharmacca Biotech, Pisataway, NJ) The lymphocytes were collected and washed with HBSS. After the last wash the leukocytes were
suspended in AIM-V media and placed in an incubator at 37º C to proliferate overnight (at 5x10^6 cells/ml.)

Isolation of RNA and use of Micro array technology for gene expression

RNA was isolated from the cells using the “Array Grade Total RNA Isolation Kit” (Super Array Bioscience Corporation) according to the manufacturer’s instructions. This kit was used to purify and amplify the RNA. The RNA was then copied and hybridized to complementary DNA sequences arranged in specific order on a microarray strip. The specific set of genes being studied (in this case those involved in toll-like receptor signaling) were adhered to the microarray strip surface. Fluorescently labeled nucleic acids were used for identification. The complementary RNA segment was bound to the appropriate DNA segment on the array membrane. The bound RNA interfereed with waves of light passing through the strip. This interference appeared as color. Thus, the greater the expression of the gene, the darker the color appeared on the image of the array membrane.(61) Both the “True Labeling-AMP 2.0 kit and the 2.5 hr cRNA Target Labeling for Oligo GEArray Hybridization Kit” were used to copy and bind the RNA to the membrane.

There are several qualities that make microarray technology a superior choice for the proposed experiment. The first is that the NB method, which utilizes gel electrophoresis and radioactively labeled nucleotides, is limited in the number of genes that can be assayed allowing only a handful of gene sequences to be studied at a time. Microarray methods allow for the assay of hundreds or even thousands of genes in a single experiment. The microarray’s ability to survey a large number of genes in a single experiment is very helpful in exploratory research and trying to identify physiological markers of cellular response to the changing needs of the cell. With the northern blotting (NB) method a scientist is most
effective when he or she has a small set of specific genes to assay. In our experiment we studied genes related to toll-like receptor-mediated signal transduction. The chosen microarray allowed us to assay 113 genes at a time in contrast to the very limited number that would have been allowed by a NB protocol.
CHAPTER 4: RESULTS

Response to one month of intense pre-season exercise training

Gene expression was compared in PBMC’s isolated from subjects at rest prior to one month of intense pre-season training and again at rest (>24 hrs since last exercise session) after one month of intense pre-season training. The results show a significant decrease in the expression of seven genes, TNFSRF1, REL, PKRKA, SARM 1, IFNα1, PTGS2, TLR3 (Figures 1 & 2). There were no genes that were up-regulated in response to one month of intense conditioning.

Response to one acute session of intense conditioning exercise (~ 1.5 hours).

Gene expression was measured in PBMC’s isolated from subjects at rest prior to exercise and again after one session of acute intense conditioning exercise. The post-exercise blood sample was collected within 30-60 minutes following exercise. The results of the microarray demonstrated that 3 genes were significantly decreased after exercise, IL-6, IRF1, and IFNγ (Fig 3 and 4). In contrast, 4 genes were significantly increased following exercise, PGLYRP2, LY86, TIRAP, and TLR5 (Fig 3 & 4).

Relationship between change in gene expression and illness

Although there was only one incident of a more severe infection with fever during the competitive wrestling season, the athletes reported multiple occasions of cold-like symptoms, such as runny nose, coughs, scratchy or sore throat. A correlation was run between the change in gene expression either in response to one session of acute exercise or 1 month of intense pre-season training and illness symptoms. Illness symptoms were categorized by 1) number of events with cold-symptoms lasting for longer than 1 week, 2) total number of days during the wrestling season with cold-like symptoms, 3) percentage of weeks during
wrestling season in which cold-like symptoms were reported, 4) number of events of more severe illness with fever (Table 1). Pearson correlations results showed that the decrease in IFN\(\gamma\) in response to acute exercise was correlated with the number of events with cold symptoms lasting longer than 1 week (\(p=0.022\), Pearson correlation = .782). The total number of reported days with cold-like symptoms was correlated with downregulation of two genes that occurred in response to the one month of intense training (REL, \(p=0.06\), Pearson correlation=.687; PTGS2, \(p=0.004\), Pearson correlation=.9=882).
CHAPTER 5: DISCUSSION AND CONCLUSIONS

Overview

Although multiple investigators have attempted to identify changes in one or more immune parameters that predicts increased susceptibility to infection during periods of intense training, to date there are no clearly identified biomarkers. In the past, exercise immunologists have focused primarily on traditional measures such as NK cell function, mitogen-activated T and B cell response, immunoglobulin levels in serum or saliva, neutrophil or macrophage monocytosis, however, none of these have yielded consistent results. In recent years, the discovery of toll-like receptors (TLR’s) with the ability to recognize conserved pathogen repeats provides immunologists with the tools to examine early innate defenses against multiple pathogens. Perhaps more relevant to exhaustive exercise are the data suggesting that TLR’s sense innate danger signals such as those that might be produced during intense prolonged exercise (ie., HSP’s). Given that there is very little data on the effects of exercise on TLR function, initially a broad approach using a microarray was chosen to identify pathways of interest that might be affected by prolonged intense exercise and/or prolonged intense exercise training.

The data collected in this experiment is a slightly different approach to gain a better understanding of in vivo effects of exercise. In many studies, peripheral blood mononuclear cells are isolated from the blood and then activated by addition of a mitogen to stimulate T or B cell function and cytokine production. Although this method allows for an easily detectable change in cell function, the relevance of this model with respect to how the body might defend against a pathogen is questionable. For example, it has been shown that the effect of stress on immune response to mitogen differs from the immune response to antigen
In the model that was used in this experiment, RNA is isolated from PBMC shortly after blood is collected. Changes in gene expression reflect changes that occur in vivo in response to exercise stress and/or any danger signals that might be induced or present in vivo, rather than an external stimuli such as mitogen. Of course, there are also limitations in characterizing gene expression such as determining whether the change in gene expression translates to actual changes in protein levels. However, the approach used in this study may be useful in identifying key pathways influenced by acute exercise or periods of intense exercise training. In subsequent studies, after identifying important pathways, it will be essential to determine actual changes in protein levels and or functional measure related to the protein expressed.

The results of this study show that modification of PBMC gene expression occurred in response to ~1.5 hours of intense exercise, as well as in response to 1 month of intense training. Seven genes showed modified expression in response to acute exercise, and seven genes showed modified expression in response to 1 month of training. Interestingly, none of the genes shown to modify expression in acute exercise were shown to be modified in 1 month of intense training, suggesting that the change in gene expression following acute exercise was likely shorter-term (perhaps inducible expression that lasts for hours rather than days). In contrast, the change in gene expression following the training period was measured at rest (at least 24 hours post-exercise) and therefore, these changes may reflect an overall change of immune responsiveness rather than a short term response to stress.

**Characterization of Genes showing modified expression**

Following one month of intensive pre-season training, the results of the microarray showed that gene expression for the following genes was significantly decreased (PRKRA,
SARM1, IFNα, TLR3, PTGS2, TNFSRF1, REL) with a trend toward a decrease in CCL2. Also, although only eight subjects were tested, these genes decreased in either all 8 subjects or in 7 of the 8 subjects. That point is important to note because the same pattern of change in the majority of subjects strengthens the results (as opposed to a large change of gene expression in only one or two individuals which could skew the mean). Another important observation is that all of these genes may play a role in defense against viral infection (rather than other types of pathogens such as bacteria or parasite). The reason that this finding is interesting is because the type of infections that are typically reported in athletes who are over-trained are viral infections (based on symptoms), rather than bacterial, fungal or parasitic infections. Therefore, it is possible that the genes most affected during periods of intense training are those responsible for defense against viral infections. A summary of each gene follows along with a description of potential relevance to our study.

**IFNα**

In response to viral infection, one of the most prominent classes of cytokines produced are the Type I interferons (IFN-α and IFN-β). These cytokines were originally named for their ability to inhibit viral replication. IFNα not only has potent antiviral effects, but also serves as an immunomodulator of the adaptive immune response. IFNα antiviral activity is based on the expression of IFN-inducible protective genes that confer cellular resistance to viral infection, inhibit viral replication, and impede viral dissemination. Antiviral effects involve at least 3 pathways, the PKR (dsRNA-dependent protein kinase), the 2,5A system involving 2-5A synthetases, and the Mx pathway (74). IFNα also modulates other immune responses critical in clearance of viral infection. These immunoregulatory properties include maturation of dendritic cells for antigen presentation via increased co-
stimulatory molecule and MHC expression, increased surface expression of peptide-MHC complexes, enhancement of NK cytotoxicity, bias towards Th1 differentiation and expansion of antigen-specific CD8+ cells (9, 20, 36, 38, 71). Taken together, the results from many studies highlight the importance of IFNα as a key cytokine in defense against viral infection. The results from our study showed a significant decline in IFNα gene expression after one month of intense exercise training. To our knowledge, there are no other studies that have examined intense periods of training and IFNα level in plasma or the ability of PBMC’s to produce IFNα. One study demonstrated a significant increase in IFNα plasma level at 24 and 48 hours after completing a half-ironman triatholon (5). However, given that we observed a decrease in IFNα gene expression, it is possible that the short-term increase post-intense exercise reflects an inflammatory response, whereas a reduced level of IFNα at rest may reflect impaired response to viral infection.

**PRKRA**

PRKRA (protein kinase interferon-inducible ds RNA dependent activator) is also known as PACT (PKR activating protein). PKR (IFNα-induced, ds RNA activated, serine-threonine protein kinase) is an important cellular mediator of the antiviral, and anti-proliferative abilities of the IFNs. PKR is constitutively present in cells, but requires the help of an “activator” to become activated and to perform its mediating functions. An important activator of PKR is ds RNA. Once a virus infects a cell which has been exposed to IFNα, PKR is activated by ds RNA from the virus. Once activated, PKR affects a block in protein synthesis inhibiting viral replication in the cell (60). PKR is also involved in the regulation of cellular apoptosis, cell proliferation, signal transduction and cell differentiation. Over-
expression of PKR in various cell types has been shown to lead to apoptosis, whereas the use of PKR inhibitors have been shown to protect cells from apoptosis in response to TNF-, dsRNA, and other cell stressors (60). In cells which are free from viral infection PRKRA has shown itself to be an effective activator of PKR. Patel et al. have shown PRKRA to be “stress modulated” reacting to various stressors by PRKRA phosphorylation, which increases its affinity for PKR. Over-expression of PRKRA leads to apoptosis. As PRKRA has been shown to become phosphorylated in response to cellular stress, one possibility it that PRKRA expression may be down regulated after a month of intense training (as was seen in our study) in order to protect the PBMC’s from stress-induced apoptosis. Another possibility is that upon viral infection, if PRKRA is required to activate the PKR pathway, downregulation of PRKRA could impair defense against viral infection.

**TLR 3 and SARM1**

Toll-like receptor 3 (TLR3) receptors are found in intracellular endosomes. TLR3 are involved in host defense against viruses. TLR3 are able to recognize dsRNA associated with viral infection, and via signaling through the TRIF adaptor protein, are able to induce activation of NFκB and production of Type I IFN. (1,4,79) In addition to the important role of TLR3 in inducing Type I interferon that promotes antiviral activity, TLR3 has also been shown to enhance cross-priming of T cells (essential for induction of virus-specific T cells) (72). It has been shown that TLR3 deficient mice have increased susceptibility to CMV infection (76) Therefore, the downregulation of TLR3 observed after one month of intense exercise might contribute to an impaired ability to defend against viral infection involving dsRNA intermediates and/or the ability to enhance T cell function against viral infection. Related to TLR3, but less well studies is SARM1 (sterile α and armadillo-motif containing
protein). It appears that SARM inhibits TRIF (the adapter protein by which TLR3 exerts its effects), and a reduction of SARM expression resulted in increased poly I:C-induced chemokine and cytokine production. (58) Therefore, SARM appears to have a regulatory role in modulating TLR-3 mediated effects. While it is not clear how SARM1 expression may relate to resistance to viral infection, given that it mediates the effects of TLR3 activation, it likely plays some role. The downregulation of SARM1 observed after 1 month of intense training could be related to the TRL3 downregulation, given that there appears to be some type of feedback between the receptor and adapter protein.

**PTGS2**

PTGS2 (prostaglandin endoperoxide synthase 2), also known as cyclooxygenase 2 (COX2), is a key enzyme in prostaglandin biosynthesis involved in inflammation. Although the inflammatory pathways involving COX2 are well-characterized, less is known to what extent viral infection may directly affect COX2. Endoplasmic Reticulum (ER) stress can occur in response to mutant proteins or viral infections and may cause improper protein folding (26). ER stress is thought to contribute to many types of human disease, and NFκB is activated in response to ER stress. The activation of NFκB by ER stress leads to the induction of some cellular genes with antiapoptotic functions (26) which may play a role in clearance of viral-infected cells. ER stress stimulates PTGS2 expression. Given that we observed a downregulation of PTGS2 after the period of intense training, it is possible that some adaptation in PTGS2 occurred in response to the regular cellular stress that might be associated with intense exercise. It is also possible that a downregulation of PTGS2 might result in a reduced ability to respond to viral infection if it involves ER stress.
REL

REL (also known as cREL) is a member of the NFκB family, and it is known that NFκB transcription factors are activated by viral infection. However, it is not clear whether NFκB activation is a requirement for the induction of Type I interferons in response to viral infection. One recent study demonstrated that cREL activation was not essential for production of IFNβ expression in response to Sendai virus and Newcastle disease virus (82). However, the role of cREL in human virus infection remains unclear at this point, and therefore, it is possible that a downregulation of cREL could contribute to an impaired ability to defend against viral infection, although further research is needed.

PBMC gene expression in response to acute bout of intense exercise

Changes in PBMC gene expression were also demonstrated in response to the acute intensive exercise bout. An important variable to take into account in the discussion of these findings is the fact that with acute exercise there are changes in the number of various cell populations circulating in the plasma. Multiple studies have shown that lymphocyte populations change in response to acute exercise with typically an increase in the number of NK cells, and neutrophils, a decrease in CD8+ cells and tendency for a decline in CD4+ cells, and small changes in B cells and monocytes (50). With the change in the leukocyte subpopulations, it is possible that the changes in gene expression post acute exercise reflect changes in number of cells expressing a certain gene rather than an actual change in gene expression on a per cell basis. Thus, our array data may show increased gene expression, but without characterizing the gene expression per cell type, we are unable to be certain if the increase reflects a change in gene expression or a change in the number of cells expressing that gene. Leukocyte subpopulations were not measured in this study. However, it is
important to consider that even though the expression on a per cell basis may not change, if for example, there are a greater number of cells in the circulation expressing that gene, then it is possible that the immune defenses requiring activation of that gene in the peripheral blood would be increased as well.

The genes that changed in response to acute exercise were: IFN$\gamma$, PGLYRP2, LY86, TIRAP, TLR5, IL6, and IRF1. Three of the genes showed decreased expression (IFN$\gamma$, IL-6, and IRF1). Of these three genes, two of them have a role important in antiviral defense (IFN$\gamma$ as activator of NK and T-cell mediated antiviral function, and IRF1 as inducer of Type I interferon). In contrast, the genes that showed an increased expression could be grouped as genes with defense against bacterial infection; (TIRAP as adaptor protein for TLR2 and TLR4, TLR5 the TLR for bacterial flagellin, PGLYRP2 as recognition for bacterial peptidoglycan, and LY86 involved in LPS response). Although to our knowledge no other studies have evaluated the effect of acute exercise on these genes, one study evaluated expression of TLR1, TLR2, and TLR4 expression on CD14+ monocytes after 1.5 hours of acute exercise, and observed a decrease in the expression of these receptors (33). Therefore although the direction of the response differs from our study, the same TLR’s were not evaluated. Also, in our experiments all peripheral mononuclear cells were studied, rather than just the monocyte population. A description of gene function and how it might relate to exercise follows in the text below.

**PGLYRP2 (Peptidoglycan recognition protein #2)**

PGLYRP’s are conserved recognition proteins which have been observed both in insects and mammals. PGLYRP2 is one of four PGLYRP proteins found in mammals. PGLYRP2 hydrolyzes bacterial peptidoglycan and reduces the pro-inflammatory effects of
this bacterial protein. PGLYRP’s are secreted by the liver and are induced in epithelial cells in response to bacterial pathogens (15). PGLYRP’s are not trans-membrane proteins, and may have direct anti-bacterial effects independent of TLRs. Mammalian PGLYRP has been shown to act through direct bacterial recognition, and displays effector function (15). In our study PGLYRP2 expression was shown to increase in response to the acute exercise bout. This increase in expression could increase the body’s ability to recognize and hydrolyze bacterial pathogens involving peptidoglycan proteins. An increase in antibacterial activity could possibly act to decrease a person’s susceptibility to bacterial infection following intense exercise (15).

**TIRAP (Toll-interleukin 1 receptor (TIR) domain containing adaptor protein)**

TIRAP is a protein in the TIR domain containing family. TIRAP plays an important role in TLR2 and TLR4 signaling. It is a component of the MyD88 signaling pathway (the key TLR pathway by which all TLR’s use except for TLR3), and aids in TLR2 and TLR4 signaling by acting as bridge to MyD88. TLR 4 is able to recognize LPS proteins which are a major cell wall component of gram negative bacteria (86). TLR2 is diverse in function, having the ability to recognize PAMPs from bacteria, viruses, fungi, yeast, and parasites. The absence of TIRAP leads to impairment of TLR2 and TLR4 signaling. In our study the expression of TIRAP increased in response to acute exercise training. It is possible that the increase in TIRAP may lead to a more rapid or improved response to bacteria via TLR2 or TLR4, resulting in a more efficient antibacterial host response. (87)

**LY 86 (MD-1)**

Lymphocyte antigen 86 is required for the cell surface expression of the TLR related protein RP105. RP105 has been identified in B cells. The physiological function of LY86 is
largely unknown. However, one recent study showed that LY86 plays a role in recognition of LPS and associates with RP105 (a B cell specific transmembrane protein) (77). Therefore, it is likely that LY86 has some role in defense against bacterial LPS. Until the function is better determined, it is difficult to speculate as to a role in response to acute exercise.

**TLR 5**

Toll-like receptor 5 is expressed on the cell surface of epithelial cells, monocytes, and immature dendritic cells. TLR5 is responsible for the recognition of flagellin which is the major protein found in bacterial flagella. TLR5 is highly expressed in the lungs playing an important role in defense against respiratory tract pathogens (1,80). To our knowledge, there are not any studies that have examined whether an increase in TLR5 expression could occur in the lungs post-exercise. It is interesting to speculate that if this does occur, an individual might be better protected against pulmonary bacterial infection post-exercise, but still susceptible to viral infection. This possibility would be consistent with the studies showing increased viral infection following acute intense exercise, and no increase in bacterial infections. (1,80)

**IRF1**

Interferon regulatory factor one is important in the induction of type 1 interferons, particularly IFNβ, that is expressed in multiple cells types. IFNβ has actions similar to IFNα with respect to antiviral effects (as described previously). In our study, we showed a decrease in IRF1 expression. This decrease in expression may lead to a decrease in the production of Type 1 IFN, and therefore a greater susceptibility to viral infection. This possibility is consistent with the open-window theory proposed by Nieman (49-57) suggesting that after
one session of prolonged intense exercise, there is increased susceptibility to viral infection.(30).

**IFNγ**

Interferon gamma, originally called macrophage activating factor has antiviral, anti-proliferative, and anti-tumor functions. It is an important stimulator of NK function and promotes CD8+ effector function against viral-infected cells. Our findings of reduced IFNγ following acute exercise are consistent with other studies in human showing decreased IFNγ production in PBMCs following prolonged, exhaustive exercise (7) Similarly in mice, following exhaustive exercise, IFNγ in response to viral challenge was suppressed up to 2 days after exercise (30) Therefore, our results regarding gene expression of IFNγ are consistent with other studies that measured IFNγ protein after prolonged, intense exercise.

**TNFRSF1A- TNF receptor superfamily member 1A (TNFαR1)**

As one of the receptors for TNFα, TNFR1 can activate NFκB, mediate apoptosis, and regulate inflammation. Downregulation of TNFαR1 may prevent important TNF-induced antiviral effects. TNFα is a key cytokine for host antiviral defense through its actions as a potent antiviral cytokine. It demonstrates direct cytotoxic action against cells infected with viruses and is effective against both RNA and DNA viruses. The antiviral effects of TNFα are primarily mediated through the TNFR1. TNFRSF1A is the primary mediator of TNFα activity. TNFα is also involved in the induction of many genes involved in various immune functions such as the inflammatory responses, and is an inducer of multiple genes (48). One of TNFα’s major activities is the induction of cellular apoptosis. With the down regulation of TNFαR1, it is possible that there may be a decrease in the ability of IFNα to induce cellular
apoptosis (48). Also, if TNFRSF1A is down regulated, it is possible that the antiviral function of TNFα may be decreased as was shown by Morrison et al. (2003) in their research with EBV. Our study showed TNFRSF1A to be down regulated following intense training-. It is possible with the down regulation of TNFRSF1A that there is a decrease in the antiviral effects of TNF α which may lead to increased susceptibility to viral infection in the athletes.

**IL-6**

Interleukin 6 – is a cytokine that plays an important role in the body’s inflammatory response (15). Many studies have shown an increase in plasma IL-6 levels in response to acute exercise (62,63). During exercise, it has been shown that IL-6 is released into the blood by contracting muscle and this is thought to account for the majority of increase in circulating IL-6 (62,63). In our study IL-6 gene expression was shown to decrease in PBMC’s. We speculate that this acute decrease in IL-6 expression may occur in response to the high levels release into the serum (negative feedback type of response). Whether this change in IL-6 relates to alteration of host defense following acute exercise is a question for future studies.

**Association between illness and changes of gene expression**

Although with a small sample size, it is often difficult to find a correlation between two variables, we did attempt to assess the extent to which change in gene expression either in response to acute exercise or in response to 1 month of intense training might serve as a predictor of illness. We did find that the degree of decrease in IFNγ in response to just one session of exercise was significantly correlated with the number of periods of cold-type symptoms lasting for over 1 week throughout the competitive season. Our findings are supported by earlier research done Weinstock et al. (1997) and Kohut et al. (2001). (30,84)
Weinstock et al tested cytokine levels in serum, urine, and the supernatants of whole blood cell cultures in response to an exhaustive exercise stress test. Their results showed that the LPS and concanavalinA-induced release of IFN$_\gamma$ was suppressed in whole blood cell cultures at 1 hour post-exercise. Kohut et al. demonstrated that a single bout of intense exercise suppressed virus-specific in vitro production of IFN$_\gamma$ in mouse spleen cells. One way in which IFN$_\gamma$ production may be suppressed in the cell is through the elevation of catecholamines in response to intense exercise. The increase in catecholamines results in an increase in intracellular cAMP which in turn may inhibit the production of IFN$_\gamma$. (30) Clearly, IFN$_\gamma$ plays an important role in host defense against viral infection, and therefore it is possible that those with the greatest impairment of IFN$_\gamma$ are the most likely to experience infection.

After one month of intense exercise training, comparable to what some might term as “overload training”, the decrease in expression of two genes was correlated with the total number of reported days of illness throughout the wrestling season. These genes were PTGS2 (reflective of endoplasmic reticulum stress that could occur in response to viral infection or other stressors) and REL (a member of NF$\kappa$B family important in TLR signaling). Perhaps these genes reflect an overall “stress” response that may or may not involve viral infection initially, but may predict susceptibility to viral infection later in the wrestling season. Clearly caution is warranted in interpreting these results given the small number of subjects and the possibility that subjects did not accurately report the number of days with cold symptoms. However, these findings do provide a basis for future investigations to examine REL and PTGS2 as potential biomarkers of overtraining. Although to our knowledge there are no
other studies that have evaluated the effect of exercise on toll-like receptor signaling / gene expression and the subsequent relationship with illness, other findings suggest that salivary IgA may be predictive of infection. For example, Gleeson et al (1999) in their study following elite swimmers over a 7 month training period showed an inverse relationship between IgA level and the number of infections seen in both the elite swimmers and moderately exercising controls. (21) Salivary IgA levels showed an inverse correlation with the number of infections in both elite swimmers and moderately exercising control subjects. IN this study, the linear regression model predicted an additional infection for each 10% drop in percent decrease (slope) of pretraining salivary IgA level over time (per month) ( R^2_{adj} = .15, P = .031)  

**Conclusion**

Though more work is required to clearly elucidate the immunological consequences of intense exercise training periods on the body of an athlete, this study has shown that changes in PBMC toll-like receptor pathway gene expression do occur with periods of intensive training. Many of the changes in gene expression shown to occur in this study suggest negative immune consequences, and a possible decrease in the body’s ability to fight infection with the greatest decrease in regulation occurring in those genes which mediate anti-viral activities. We suggest that these changes may play a role in the increased incidence of illness which has been shown to occur in many athletes during
Table 1. Reported illness by athletes throughout wrestling season

<table>
<thead>
<tr>
<th>Subject i.d.</th>
<th>Number of events of long illness (&gt;1 wk)</th>
<th>Total number of days reported with cold symptoms</th>
<th>Percentage of total time with mild cold symptoms</th>
<th>Number of events of severe illness</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>15</td>
<td>60</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>0</td>
<td>11</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>13</td>
<td>37.5</td>
<td>1</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>33</td>
<td>44</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
<td>14</td>
<td>22</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>0</td>
<td>12</td>
<td>77</td>
<td>0</td>
</tr>
<tr>
<td>7</td>
<td>1</td>
<td>8</td>
<td>62.5</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>13</td>
<td>54</td>
<td>0</td>
</tr>
</tbody>
</table>
Change in gene expression from pre-training (at rest) to post-training (at rest)

Figure 1. Gene expression of TNFα receptor 1 measured at rest before one month of intense pre-season training (PRE) and measured after 1 month of intense training (POST). A significant decrease in expression was found (p=0.04).
Change in gene expression from pre-training (at rest) to post-training (at rest)

Figure 2. Gene expression of multiple genes measured at rest before one month of intense pre-season training (PRE) and measured after 1 month of intense training (POST). A significant decrease in expression was found for REL (p=0.034), PKRKA (p=0.033), SARM1 (p=0.031), IFNA1 (p=0.04), PTGS2 (p=0.014), TLR3 (p=0.024).
Change in gene expression in response to 1 session of intense acute exercise

Figure 3. Gene expression of Interferon-regulatory factor 1 (IRF1) measured at rest, before exercise, and within 30 minutes following ~ 1.5 hours of intense acute exercise. A significant decrease in IRF1 expression was found in response to acute exercise (p=0.048).
Change in gene expression in response to 1 session of intense acute exercise

Figure 4. Gene expression of multiple genes measured at rest, before exercise, and within 30 minutes following ~1.5 hours of intense acute exercise. A significant decrease was found in response to acute exercise for IFN\(\gamma\) (p=0.022), and IL-6 (p=0.03), whereas a significant increase was found with respect to PGLYRP2 (p=0.011), LY86 (p=0.003), TIRAP (p=0.035), and TLR5 (0.004).
APPENDIX A: Questionnaires
Illness Questionnaire

Date ___________________        Name/ID no _________________________

1. Have you experienced fever, chills, or aches (not aches associated with
typical exercise conditioning) within the past week?

YES   NO
IF yes, how many days with these symptoms? ______

2. Have you experienced runny or stuffy nose, cough, sore or scratchy
throat within the past week?

YES   NO
IF yes, how many days with these symptoms? ______

3. Have you experienced any other symptoms that might suggest you have
been ill?

YES   NO
IF yes, how many days with these symptoms? ______
Mental Skills Questionnaire

**Mental Preparation**
1. I always set myself goals in training.  
2. I always have very specific goals.  
3. I always analyze my performance after I complete a competition. 
4. I usually set goals that I achieve.  

**Imagery Ability**
5. I can rehearse my sport in my mind.  
6. I rehearse my skills in my head before I use them.  
7. It is difficult for me to form mental pictures.  
8. I can easily imagine how movements feel.  

**Self-Confidence**
9. I suffer from lack of confidence about my performance.  
10. I approach all competitions with confident thoughts.  
11. My confidence drains away as competitions draw nearer.  
12. Throughout competitions I keep a positive attitude.  

**Anxiety and Worry Management**
13. I often experience fears about losing.  
14. I worry that I will disgrace myself in competitions.  
15. I let mistakes worry me when I perform.  
16. I worry too much about competing.  

**Concentration Ability**
17. My thoughts are often elsewhere during competition.  
18. My concentration lets me down during competition.  
19. Unexpected noises put me off during performance.  
20. Being distracted is a problem for me.  

**Relaxation Ability**
21. I am able to relax before competition.  
22. I become too tense before competition.  
23. Being able to calm myself down is one of my strong points.  
24. I know how to relax in difficult circumstances.  

**Motivation**
25. At competitions I am usually psyched enough to compete well.  
26. I really enjoy a tough competition.  
27. I am good at motivating myself.  
28. I usually feel that I try my hardest.
INSTRUCTIONS: A number of statements that athletes have used to describe their experiences are given below. Please read each statement carefully and then recall as accurately as possible how often you experience the same thing. There are no right or wrong answers. Do not spend too much time on any one statement.

<p>| | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>On a daily or weekly basis, I set very specific goals for myself that guide what I do</td>
<td>Almost Never</td>
<td>Some- Often times</td>
</tr>
<tr>
<td>2</td>
<td>I get the most out of my talent and skills</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>When a coach or manager tells me how to correct a mistake I’ve made, I tend to take it personally and feel upset</td>
<td></td>
<td></td>
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<tr>
<td>4</td>
<td>When I am playing sports, I can focus my attention and block out distractions</td>
<td></td>
<td></td>
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<tr>
<td>5</td>
<td>I remain positive and enthusiastic during competition, no matter how badly things are going</td>
<td></td>
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<tr>
<td>6</td>
<td>I tend to play better under pressure because I think more clearly</td>
<td></td>
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<tr>
<td>7</td>
<td>I worry quite a bit about what others think about my performance</td>
<td></td>
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<tr>
<td>8</td>
<td>I tend to do lots of planning about how to reach my goals</td>
<td></td>
<td></td>
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<tr>
<td>9</td>
<td>I feel confident I will play well</td>
<td></td>
<td></td>
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<tr>
<td>10</td>
<td>When a coach or manager criticizes me, I become upset rather than helped</td>
<td></td>
<td></td>
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<tr>
<td>11</td>
<td>It is easy for me to keep distracting thoughts from interfering with something I am watching or listening to</td>
<td></td>
<td></td>
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<tr>
<td>12</td>
<td>I put a lot of pressure on myself by worrying how I will perform</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>I set my own performance goals for each practice</td>
<td></td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>I don’t have to be pushed to practice or play hard; I give 100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>If a coach criticizes or yells at me, I correct the mistake without getting upset about it</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>I handle unexpected situations in my sport very well</td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>When things are going badly, I tell myself to keep calm, and this works for me</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>The more pressure there is during a game, the more I enjoy it</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>While competing, I worry about making mistakes or failing to come through</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>I have my own game plan worked out in my head long before the game begins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>When I feel myself getting too tense, I can quickly relax my body and calm myself</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22</td>
<td>To me, pressure situations are challenges that I welcome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>23</td>
<td>I think about and imagine what will happen if I fail or screw up</td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>I maintain emotional control no matter how things are going for me</td>
<td></td>
<td></td>
</tr>
<tr>
<td>25</td>
<td>It is easy for me to direct my attention and focus on a single object or person</td>
<td></td>
<td></td>
</tr>
<tr>
<td>26</td>
<td>When I fail to reach my goals, it makes me try even harder</td>
<td></td>
<td></td>
</tr>
<tr>
<td>27</td>
<td>I improve my skills by listening carefully to advice and instruction from coaches and managers</td>
<td></td>
<td></td>
</tr>
<tr>
<td>28</td>
<td>I make fewer mistakes when the pressure’s on because I concentrate better</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
APPENDIX B: EXERCISE TRAINING PROTOCOLS

Table 1. Conditioning Session

<table>
<thead>
<tr>
<th>Activity</th>
<th>Time (minutes)</th>
<th>Intensity</th>
<th>VO2</th>
<th>Heart Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Warm Up</td>
<td>10 to 15</td>
<td>50%</td>
<td>130-160</td>
<td></td>
</tr>
<tr>
<td>1-mile run (1)</td>
<td>6 to 9</td>
<td>80-85%</td>
<td>155-170</td>
<td></td>
</tr>
<tr>
<td>Active recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30 second sprints (3-5)</td>
<td>6 to 10</td>
<td>95-&gt;100%</td>
<td>160-190</td>
<td></td>
</tr>
<tr>
<td>Active recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stadium sprints (4-6)</td>
<td>6 to 10</td>
<td>95-&gt;100%</td>
<td>189-194</td>
<td></td>
</tr>
<tr>
<td>&quot;Buddy Carries&quot; (2)</td>
<td>6 to 10</td>
<td>95-&gt;100%</td>
<td>185-196</td>
<td></td>
</tr>
<tr>
<td>Active recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hill sprints (4-8)</td>
<td>3 to 5</td>
<td>95-&gt;100%</td>
<td>185-196</td>
<td></td>
</tr>
<tr>
<td>Hill &quot;buddy carries&quot; (1-2)</td>
<td>3 to 5</td>
<td>95-&gt;100%</td>
<td>180-196</td>
<td></td>
</tr>
<tr>
<td>Wheel barrow (1-2)</td>
<td>2 to 4</td>
<td>95-&gt;100%</td>
<td>180-196</td>
<td></td>
</tr>
<tr>
<td>Active recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1-mile jog (1)</td>
<td>7 to 11</td>
<td>50-60%</td>
<td>130-160</td>
<td></td>
</tr>
</tbody>
</table>
Table 2. Weight lifting program

<table>
<thead>
<tr>
<th>Lifting program - 4 week cycle</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Push Day - Monday</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bench or DB Bench</td>
<td>5x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 4</td>
<td>4x10</td>
</tr>
<tr>
<td>DB Incline or Straight Bar Incline</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 3</td>
<td>4x10</td>
</tr>
<tr>
<td>Chair Push-Ups or DB Flies</td>
<td>3x10</td>
<td>3x10</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Triceps Pushdowns or Skull Crunchers</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Military Press - (sitting)</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Dips or Close Grip Push Ups</td>
<td>3xMax</td>
<td>3xMax</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Abs (do enough to feel it the next day)</td>
<td>Burn Out</td>
<td>Burn Out</td>
<td>15 reps/ 5 neg. x 3</td>
<td>Burn Out</td>
</tr>
<tr>
<td><strong>Pull Day - Wednesday</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pull Ups with weighted belt</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 4</td>
<td>4x10</td>
</tr>
<tr>
<td>3 Way Pull Down (Front/ Back/ Reverse)</td>
<td>3x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Seated Row/ Bent Over Row</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>DB Curl</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Straight Bar Curl</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 4</td>
<td>4x10</td>
</tr>
<tr>
<td>Lateral Raises ( 3 Way)</td>
<td>3x10</td>
<td>3x10</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Back Extension (35-50 Total)</td>
<td>reps of 10 or more reps of 10 or more reps of 10 or more reps of 10 or more</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Leg Day - Friday</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cleans or Snatches</td>
<td>5x5</td>
<td>5,4,3,2,1</td>
<td>4x8</td>
<td>4x10</td>
</tr>
<tr>
<td>Squats (Back or Front)</td>
<td>5x5</td>
<td>5,4,3,2,1</td>
<td>5 reps/ 5 neg. x 4</td>
<td>4x10</td>
</tr>
<tr>
<td>Lunges or Dead Lifts</td>
<td>4x5</td>
<td>5,4,3,2,1</td>
<td>4x8</td>
<td>3x10</td>
</tr>
<tr>
<td>Hamstring Curls</td>
<td>4x5</td>
<td>3x10</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Leg Extensions</td>
<td>4x5</td>
<td>3x10</td>
<td>5 reps/ 5 neg. x 3</td>
<td>3x10</td>
</tr>
<tr>
<td>Shrugs</td>
<td>4x5</td>
<td>3x10</td>
<td>3x8</td>
<td>3x10</td>
</tr>
<tr>
<td>Abs (do enough to feel it the next day)</td>
<td>Burn Out</td>
<td>Burn Out</td>
<td>Burn Out</td>
<td>Burn Out</td>
</tr>
</tbody>
</table>

* Examples of weight goals for different repetition types: a) 4x5 = (75%, 80%, 85%, 90%)
  b) 5,4,3,2,1 = (75-80%,80-90%, 90-95%, 95-<100%, Max)  c) 4x10 = (60%,65%,70%,75%)
<table>
<thead>
<tr>
<th>Activity</th>
<th>Description</th>
<th>Time (minutes)</th>
<th>Intensity (RPE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>warm-up</td>
<td>jogging, calisthenics, gymnastic exercises</td>
<td>10 to 15</td>
<td>13</td>
</tr>
<tr>
<td>warm-up drill</td>
<td>practicing skills and techniques at sub-maximal effort level</td>
<td>10 to 15</td>
<td>14</td>
</tr>
<tr>
<td>instruction period</td>
<td>discussion and practice of technical skills</td>
<td>20 to 45</td>
<td>14</td>
</tr>
<tr>
<td>“live” wrestling period</td>
<td>full speed wrestling, 100% effort</td>
<td>30 to 45</td>
<td>17-20</td>
</tr>
<tr>
<td>conditioning</td>
<td>sprinting, partner carry running, pull-ups/push-ups/sit-ups, rope climbs</td>
<td>10 to 15</td>
<td>17-20</td>
</tr>
</tbody>
</table>

Table 3. Wrestling Practice Session
REFERENCES CITED


