NICOTINE MODULATION OF ANTI-VIRAL IMMUNITY IN PERIADOLESCENT MALE AND FEMALE C57BL/6J MICE

A Dissertation in
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by
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Abstract

The link between tobacco use and poor health outcomes was clearly outlined in the 1964 Surgeon General’s report (United States Department of Health, Education, and Welfare, 1964). However, the causal relationship between nicotine, the addictive component in tobacco, and its effects on immune function was not examined until the 1990’s. Immune cells have been exposed to nicotine via *in vitro* and *in vivo* designs. However, the suppression of T- and B-lymphocytes, dendritic cell, macrophage, and neutrophil functioning by nicotine has only been observed using *in vitro* immune stimulation (e.g., concanavalin A or lipopolysaccharide exposure). Although valuable, these *in vitro* immune stimulation designs provide only a partial picture of the overall *in vivo* immune cell functioning.

This dissertation sought to understand how nicotine modulates the anti-viral immune response of male and female periadolescent C57BL/6J mice to herpes simplex virus (HSV)-1 infection. For seven days, male (n=59) and female (n=58) C57BL/6J mice were exposed to nicotine-spiked water at one of three concentrations (0 μg/mL, 50 μg/mL, and 200 μg/mL). On day 8, all mice were infected with HSV-1 in both rear foot pads. Nicotine exposure continued until day 12, when mice were sacrificed and popliteal lymph nodes were removed to observe the effects of nicotine on the HSV-1 anti-viral response (i.e., number of lymphocytes isolated, lymphocyte production of interferon (IFN)-γ, HSV-1 specific T-lymphocyte lysis).

Females exhibited a greater anti-viral response (e.g., number of lymphocytes isolated and lymphocyte production of IFN-γ) to HSV-1 compared to their male counterparts. In addition, the 50 μg/mL nicotine exposure group had a reduction in IFN-γ production compared to the control group, which suggests a reduction in T-lymphocyte activation. However, there were no sex or nicotine treatment group differences in HSV-1 specific immunity. Therefore, the *in vivo* viral
challenge resulted in a robust HSV-1 anti-viral response that was difficult to modulate with nicotine exposure in the present study. A discussion of the results and suggestions for future studies are presented.
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Abbreviations

\(^{51}\text{Cr} = \) Chromium 51 radioisotope
\text{ACTH} = \text{adrenocorticotropin hormone}
\text{APC} = \text{antigen presenting cell}
\text{ConA} = \text{concanavalin A}
\text{COPD} = \text{chronic obstructive pulmonary disease}
\text{CORT} = \text{corticosterone}
\text{CRH} = \text{corticotrophin releasing hormone}
\text{CSE} = \text{cigarette smoke extract}
\text{CTL} = \text{cytotoxic T lymphocytes}
\text{DC} = \text{dendritic cells}
\text{EIA} = \text{enzyme immunoassay}
\text{EPI} = \text{epinephrine}
\text{Ig} = \text{immunoglobulin}
\text{IL} = \text{interleukin}
\text{IFN-}\gamma = \text{interferon-gamma}
\text{HPA} = \text{hypothalamic-pituitary-adrenal}
\text{HSV} = \text{herpes simplex virus}
\text{LPS} = \text{lipopolysaccharide}
\text{mCTL} = \text{memory cytotoxic T lymphocytes}
\text{MHC} = \text{major histocompatibility complex}
\text{nAChR} = \text{nicotinic acetylcholine receptor}
\text{NE} = \text{norepinephrine}
\text{NK} = \text{natural killer}
\text{PN} = \text{post natal day}
\text{T_c} = \text{T cytotoxic cells}
\text{T_H} = \text{T helper cells}
\text{TNF-}\alpha = \text{tumor necrosis factor-alpha}
\text{TNF-}\beta = \text{tumor necrosis factor-beta}
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Chapter I: Introduction

Adolescence and Risky Behaviors

Adolescence is a critical period for brain development (e.g., Dahl, 2004), hormonal changes (e.g., Cameron, 2004) and physical and emotional development (e.g., Susman & Rogol, 2004). In addition, during adolescence, stress, social influences, and risk-taking behaviors become more salient (e.g., Steinberg, 2004). These risk-taking behaviors are typically labeled as sensation- or reward-seeking (e.g., Dahl, 2004), and include experimenting with illicit substances like tobacco and alcohol, fighting and/or violence, and early initiation of sexual behaviors (e.g., Bartlett, Holditch-Davis, & Belyea, 2007). There are several recent studies that report a link between substance use (such as nicotine via tobacco smoking or alcohol consumption) and early sexual intercourse (e.g., Porter, Oakley, Ronis, & Neal, 1996; for a review, see Irwin & Millstein, 1992), sexually risky behaviors [i.e., no condom or sex when drunk; (e.g., Kalina et al., 2009)], and earlier reproductive onset (e.g., Waldron et al., 2009). Therefore, adolescence is a critical time to study how these novel experiences, including exposure to drugs (i.e., nicotine) and sexually transmitted diseases [i.e., herpes simplex virus (HSV; See Figure 1)], affect health.

Research focusing on how nicotine use affects immune function during adolescence is minimal. However, nicotine exposure and cigarette smoke increase susceptibility to infection in rodent models and humans (Friedman, Pross, & Klein, 2006) and decreases positive serological protective responses to vaccines in humans (e.g., Baynam et al., 2007; Hagedorn, Rettmann, Dieperink, Durfee, & Aqel, 2010; Lin, Liao, Lin, & Wang, 2008). Due to the unfortunate relationship between tobacco use and sexually risky behaviors during adolescence, investigating the combined effects of tobacco (i.e., nicotine) and exposure to a
sexually transmitted disease (i.e., HSV) on the functioning of the immune system is an important and logical next step.

**Figure 1. Theoretical model of age, sex¹, and nicotine effects on HSV exposure and pathogenesis.** HPA = hypothalamic-pituitary-adrenal; HSV = herpes simplex virus

**Herpes Simplex Virus**

Herpes simplex virus (HSV), a sexually transmitted virus, belongs to a family of viruses that is relatively common, such as the chicken pox virus and the Epstein-Barr virus [Center for Disease Control and Prevention (CDC), 2008]. There are two types of HSV: HSV-1 and HSV-2. HSV-1 typically is connected to orofacial infections (i.e., cold sores) and HSV-2 is associated with genital lesions. However, once infected, both forms of the virus and virus-specific antibodies can be found throughout the body (CDC, 2008). It is estimated that 16.2% of teens and adults in the U.S. have HSV-2 infection (CDC, 2008). However, according to the CDC (2008), HSV-2 infection is more common in women (1 in 5)

¹ The term *sex* refers to the classification of human and nonhuman animals according to reproductive anatomy as male or female. In this dissertation, the term *sex* was chosen due the use of a murine model because the term *gender* implies a self-representation as a male or female that mice are not known exhibit (Institute of Medicine, 2001; Klein, Corwin, & Ceballos, 2006).
compared to men (1 in 9). With regard to HSV-1, about 25% of teens and 50%-90% of adults have antibodies to HSV-1 [American Social Health Association (ASHA), 2009]. The large prevalence range in adult HSV-1 exposure may have to do with knowledge of the individuals being sampled (e.g., HSV-1 can be asymptomatic). Prevalence also increases with age because the likelihood of HSV-1 contact increases across the lifespan. HSV transmission occurs when bodily fluids are exchanged between mucosal tissues; therefore, adolescence, a time when risky sexual behaviors are more prevalent, is a common period for unintended viral exposure. Either type of the virus is rarely fatal except to newborns; however, once infected, there is a lifetime chance of painful recurrent ulcer-like lesions that also can spread the virus to others (ASHA, 2009; CDC, 2008).

A HSV murine model has been used for several decades to help develop an understanding of the intricate biological pathways associated with primary viral infection and the effects of stress on HSV pathogenesis observed in humans (for a review, see Bonneau & Hunzeker, 2006; 2007). This murine model has provided an ethical method in which it is possible to study how human experiences (e.g., acute and chronic stress) can affect HSV pathogenesis. The effects of stress have been investigated because in epidemiological data an increase in psychological stress in humans is associated with an increase in latent viral reactivation of HSV. For example, it has been observed in humans that stress can cause oral and genital herpes reactivation of the latent virus and the development of ulcer-like lesions (e.g., Friedmann, Katcher, & Brightman, 1977). In addition, academic stress in medical students increases HSV antibody levels, a proxy for latent viral reactivation (Glaser et al., 1987). The immune system response to HSV involves both the innate and adaptive branches of the immune system (discussed in greater detail below). Therefore, there are many
physiological levels on which stress can affect the disease progression of HSV. Although the HSV immune response and effects of stress on its pathogenesis has been carefully studied, the investigation of stress-induced appetitive behaviors (e.g., smokers report an increase in tobacco use during stressful periods) on HSV pathogenesis has yet to be understood.

**Health and Tobacco Use**

The 20th century was filled with landmark findings associating tobacco use with disease. Smoking has been linked to respiratory disease, cardiovascular disease, many forms of cancer, loss of bone density, eye diseases, infertility and reproductive effects, dental diseases, erectile dysfunction, and peptic ulcers [United States Department of Health, Education, and Welfare (USDHEW), 1964], and tobacco use continues to be the most preventable cause of illness and death in the United States [United States Department of Health and Human Services (USDHHS), 2001]. Between 1997 and 2001, more than 450,000 deaths each year were the result of cigarette smoking (CDC, 2005). Unfortunately, approximately 1.5 million adolescents begin to smoke every year, with more than 25% becoming daily cigarette smokers [Substance Abuse and Mental Health Services Administration (SAMHSA), 2008].

Data suggest that the earlier adolescents begin to smoke, the harder it is for them to quit (USDHHS, 1988). Individuals who begin smoking during adolescence are likely to smoke cigarettes for an additional 20 to 30 years (Pierce & Gilpin, 1996) and suffer the health consequences of long-term tobacco use. On average, adults who smoke cigarettes die 14 years earlier than do nonsmokers (CDC, 2002; Doll, Peto, Boreham, & Sutherland, 2004). Lung cancer, heart disease, and chronic lung diseases such as emphysema, bronchitis, and chronic airway obstruction are responsible for the largest number of smoking-related deaths
(CDC, 2005). The immune system plays a key role in the development of these smoking-related diseases. However, the mechanisms through which nicotine, the primary addictive ingredient in tobacco, or other components of tobacco smoke affect the immune system have not been thoroughly investigated. Further, these diseases develop over the course of 10-20+ years in the life of an adult smoker. It is known that nicotine and cigarette smoking increase susceptibility to viral infection (Friedman, Pross, & Klein, 2006). However, the extent to which the immune system may play a role in rendering adolescent tobacco-users vulnerable to health risks is unknown.

**Pharmacology of Nicotine**

Smoking cigarettes is the most common form of tobacco use to self-administer nicotine, the primary addictive ingredient in tobacco. Nicotine can interact with the body, including the immune system, via cholinergic receptors (e.g., Collins, Marks, & Pauly, 1989; Friedman, Pross, & Klein, 2006). Thus, research has focused on studying the effect of nicotine on the immune system in an attempt to understand how smoking tobacco results in negative health outcomes.

Although the effect of nicotine on the immune system has not been thoroughly investigated, the pharmacological effects of nicotine in general have been thoroughly documented. Smoking cigarettes leads to nicotine absorption through the lungs and then into the bloodstream (USDHHS, 1988). Nicotine quickly is absorbed through cells on or in the body (USDHHS, 1988). Once in the bloodstream, nicotine is widely distributed throughout the body. Prior to excretion via the kidneys, 85 to 90% of nicotine is metabolized (USDHHS, 1988). Nicotine is oxidized in the liver by CYP-2A6 into its primary metabolites, cotinine and nicotine-N’-oxide (USDHHS, 1988). The elimination half-life for
nicotine is approximately 2 hours in humans (USDHHS, 1988). Therefore, many cigarette users smoke regularly throughout the day to keep blood nicotine levels elevated.

Once absorbed into the bloodstream, nicotine binds to cholinergic receptors. In the brain, nicotine increases psychomotor activity, cognitive functioning, sensorimotor performance, attention, memory consolidation (USDHHS, 1988), and activates the hypothalamic-pituitary-adrenal (HPA) axis (discussed in more detail below) by binding to cholinergic receptors throughout the brain with different affinities for nicotine (Collins, Marks, & Pauly, 1989; Pidoplichko et al., 2004). Similar to other drugs of abuse, nicotine addiction is believed to be the result of increased dopamine stimulation of the mesocortico-limbic system, particularly the ventral tegmental area (Clarke & Pert, 1985; Clarke, Schwartz, Paul, Pert, & Pert, 1985; Koob & Bloom, 1988; Pontieri, Tanda, Orzi, & Chiara, 1996). The mesocortico-limbic system is responsible for the stimulant, antidepressant, and behavioral reinforcement effects of nicotine (Clarke & Pert, 1985; Corrigall, Coen, & Adamson, 1994; Koob & Bloom, 1988; Pidoplichko et al., 2004; Pontieri et al., 1996).

Outside the brain, nicotine increases blood pressure, heart rate, and the release of epinephrine (EPI) and norepinephrine (NE) via stimulation of the cholinergic receptors in the peripheral nervous system and target organs.

Nicotine also has immunosuppressive effects on both the innate and adaptive responses of the immune system by activation of the HPA-axis (e.g., Matta, Fu, Valentine, & Sharp, 1998; Sopori & Kozak, 1998), stimulation of the central cholinergic system (e.g., Borovikova et al., 2000; Watkins, Maier, & Goehler, 1995), and through direct interaction with nicotinic acetylcholine receptors on immune cells (e.g., Saeed et al., 2005; Skok, Graihe, & Changeux, 2005). Nicotine administration has suppressed the ability of immune
cells to properly function when stimulated in vitro (e.g., Kalra, Singh, Savage, Finch, & Sopori, 1999; Navarro, Basta, Seidler, & Slotkin, 2001; Petro, Schwartzbach, & Zhang, 1999). In addition, smoking tobacco and exposure to second-hand smoke reduces the preventative nature of vaccinations due to a lower antibody serological response (e.g., Baynam et al., 2007; Hagedorn et al., 2010; Lin et al., 2008).

**Overview of the Immune System**

It is important to understand the immunomodulatory role of nicotine because the primary objective of the immune system is to rid the body of foreign or non-self cellular material, such as bacteria, viruses, fungi, and parasites. There are two branches that make up the immune system: the innate and adaptive branches. Although each branch has distinctly different responsibilities in preserving the integrity of the body, they work in concert to most efficiently remove harmful antigens (non-self cellular matter) from the body. The innate immune system is a rudimentary first line of defense and is responsible for initiating the inflammatory response. The adaptive immune system is more highly evolved and designed to “learn” and create “memory” as the organism is exposed to antigen throughout its life. Both branches are responsible for monitoring the entire body for antigen presences; however, activation of each branch requires different mechanisms, which are discussed below (Janeway, Travers, Walport, & Shlomchik, 2005; Kindt, Goldsby, & Osborne, 2007).

The anatomy of the immune system includes primary and secondary organs. Primary organs, where lymphocytes are generated and differentiated, include the bone marrow and the thymus. All immune cells originate from the bone marrow via the hematopoietic stem cell, which gives rise to the lymphoid progenitor and myeloid progenitor (Janeway et al., 2005). The lymphoid progenitor generates T cells, B cells, natural killer (NK) cells, and
dendritic cells (DC). T cells differentiate in the thymus and undergo positive and negative selection, which allows T cells into the bloodstream that will be activated only by recognizing a cell with major histocompatibility complex (MHC) class I markers and presenting an antigen (Kindt et al., 2007). B cells on the other hand mature in the bone marrow and are only subjected to negative selection, such that self-reactive B cells are eliminated prior to release into the bloodstream. B cells can be activated with or without T helper cells, but must have antigen present (Kindt et al., 2007). The myeloid progenitor gives rise to leukocytes, erythrocytes, and megakaryocytes, which are platelet generators (Janeway et al., 2005). Additional types of leukocytes, or white blood cells, are generated in the bone marrow and include neutrophils, eosinophils, basophils, monocytes, immature DCs, and the unknown precursor to mast cells (Janeway et al., 2005). The leukocytes travel throughout the body in an inactive state (i.e., not producing cytokines) monitoring the blood and tissue for foreign antigens until they find foreign material or are recruited by cytokines, (immune messengers) to a site of inflammation. Once recruited, they help in clearing the area of foreign antigen, infected, injured or dead cells, and aid in repair (Kindt et al., 2007).

Secondary organs of the immune system include the lymph nodes and the spleen, which are reservoirs of immune cells. The lymph nodes act as a filter of the lymphatic system. It is here where antigen and activated DCs and neutrophils interact with B and T cells that drain from the tissue (Kindt et al., 2007). The spleen is a reservoir of erythrocytes and leukocytes and removes dead or dying cells. In addition, it filters the blood for antigens and is critical in mounting a response to blood-borne pathogens (Kindt et al., 2007). Finally, mucosal areas of the body that regularly interact with outside particles have lymphoid tissue associated with them. For example, the lungs are exposed to foreign matter every time a
breath is taken; therefore, bronchus-associated lymphoid tissue exists and includes the tonsils and adenoids (Kindt et al., 2007). Peyer’s patches are an example of gut-associated lymphoid tissue, which is available to combat food ingested antigens (Kindt et al., 2007).

Immune cells use cytokines to communicate as autocrine, paracrine, or endocrine messengers between one another and with other biological systems. Cytokines can be synergistic, antagonistic, or have multiple effects. The production of cytokines can modulate the type of adaptive immune response. For example, the production of interleukin (IL)-2 and interferon (IFN)-γ support the activation of cytotoxic T-lymphocytes, which is necessary to fight off bacteria and viruses. However, the production of IL-4 and IL-6 support the activation and proliferation of B lymphocytes, which are necessary to develop antibodies. In addition, an imbalance of cytokines can influence the development of disease. For example, bacterial septic shock is caused by overproduction of IL-1 and tumor necrosis factor (TNF)-α; however, bacterial toxic shock is caused by the overproduction of IL-2 and IFN-γ (Kindt et al., 2007). Recently, anti-cytokine and cytokine therapies are being developed and used to treat autoimmune diseases and neuropathic pain (e.g., Ding & Jones, 2006; Milligan et al., 2005; Morgan et al., 2008; Tayal & Kalra, 2008).

Innate immune system

The innate immune system not only involves cellular defenses, but also physical and chemical barriers (Kindt et al., 2007). Physical barriers include the skin and mucous membranes. For example, a chemical barrier would be the acidic pH of the stomach. Although the innate immune system is simpler, without it a human being would be unable to effectively mount a life-protecting response to an antigen. It is responsible for the immediate, non-specific inflammation such as the warmth, redness, pain, and swelling
associated with a cut on the skin. Using receptors (i.e., pattern recognition) that identify common membrane ligands on bacteria, the innate immune system is able to detect a wide range of microbial antigens and instigate an inflammatory response. In addition, soluble pattern recognition receptors are found in the blood, known as the complement system. Complement is a cascade of proteins that results in holes being “punched” into the membrane of microorganisms and aids in the recruitment of inflammatory cells (Kindt et al., 2007).

The immune cells actively involved in the innate immune response include macrophages (activated monocytes), neutrophils, NK cells, and DCs (Kindt et al., 2007). Macrophages, neutrophils and DCs use phagocytosis to clear antigen or microbes and produce reactive oxygen species to kill microbes. NK cells lyse virally infected cells. Once activated, NK cells continue to recruit new immune cells via cytokines especially chemokines. Activated DCs and macrophages, also known as antigen presenting cells (APCs), migrate to lymph nodes to elicit activation of the adaptive immune system.

Cytokines that are critical to effectiveness of the innate immune system include IL-1, IL-6, IL-9, tumor necrosis factor (TNF)-α, interferon (IFN)-α, and IFN-β. These messengers are able to induce fever, pain, and fatigue, also known as sickness behavior (Maier & Watkins, 1998), and the HPA-axis (Wrona, 2006).

Adaptive immune system

The adaptive immune system is only found in vertebrates (Kindt et al., 2007). Its job is to increase the intensity and specificity of antigen clearance as well as developing memory to allow of a faster removal of the antigen during a secondary exposure. The adaptive response is delayed as compared to the innate response. In humans, it takes 10-14 days to mount a primary antigen specific response. Key immune cells involved in the adaptive
immune system include activated DCs, T cells, B cells, and NK cells (Kindt et al., 2007). DCs migrate from the site of inflammation into the lymphatic system and travel to the closest lymph node(s) to present the antigen to the adaptive immune system. In the lymph node, T cells are activated by the presentation of antigen by the DCs and macrophages (Kindt et al., 2007).

There are two major types of T cells, T helper (T_H) cells (CD4+) and T cytotoxic (T_C) cells (CD8+) that are released in an immature form from the thymus. A typical CD4+ to CD8+ ratio is 2:1 in the bloodstream. Both T cells circulate throughout the blood and lymphatic system and reside in the 2nd lymph organs. Once the T cells are presented with antigen via an APC, they differentiate into memory and effector cells. T_C effector cells are cytotoxic T lymphocyte (CTL) and lyse the antigen-bearing cells. T_H effector cells are responsible for cytokine production in high amounts. The current cytokine (created by the innate immune response) environment influences the development of T_H cells, which in turn impacts the course of the immune response to favor one of two directions. T_H1 cells produce cytokines IL-2, IFN-γ and tumor necrosis factor (TNF)-β, which drives the CTL activation and supports a cell-mediated immune response. This cell-mediated immune response is critical to mount a response to intracellular bacteria and viruses. On the other hand, T_H2 cells produce IL-4, IL-5, IL-6 and IL-10, which support B cell activation and differentiation, as well as a humoral response. This humoral immune response is important in battling parasites and mounting an antibody response during primary and secondary responses to antigen. T_H and T_C memory cells monitor the body via the blood and lymphatic systems for recurrent exposure to antigen. Both cell types are critical in mounting a fast, efficient secondary response to an antigen (Kindt et al., 2007).
Once released into the periphery from the bone marrow, immature B cells travel throughout the body and are found in high concentration in the lymph nodes. Similar to T cells, when B cells are activated, they become an effector cell or, specifically, plasma cells, which manufacture antigen-specific antibodies, or memory cells, which in turn manufacture antigen-specific antibodies during a secondary exposure. However, the antibodies or immunoglobulin (Ig) are structurally modified based on the cytokine environment in which the memory B cells are surrounded. For example, T_{H1} cytokines like IFN-γ and TNF-β result in antibodies such as IgG2 or IgA, while T_{H2} cytokines like IL-4 and IL-2 result in antibodies such as IgE, IgG1, or the maintenance of IgM. Unlike plasma cells, memory B cells do not undergo apoptosis at the end of the immune response. Memory B cells are long-lasting cells partly responsible for the immune system’s faster response to the 2nd exposure to the antigen. Antibodies provide several effector responses for the immune system. Antibodies form immune complexes by binding and neutralizing antigens and communicating with NK cells and macrophages, which leads to the phagocytosis of the antibody-neutralized antigen. In addition, antibodies can activate or perpetuate the complement cascade (Kindt et al., 2007).

Due to the multi-faceted and complex nature of the immune system, it may be apparent that studying the modulation of the immune system could be overwhelming. In fact, research designs can over simplify the immune system. In research on the immune system, many investigators often focus on one lymphocyte or immune cell in vitro to study the effects of environmental factors on the effectiveness of the immune response. This narrow focus eliminates the true harmonious nature of the immune system and possibly the ability of the immune system to adjust or compensate in vivo. Therefore, it is necessary to
investigate the manipulation of the immune system in vivo to determine how environment or behavioral factors impact its overall functioning. The infection of mice with HSV provides the perfect opportunity to observe how behavior (i.e., nicotine exposure) can affect the anti-viral immune response of the whole organism, not just observe if a T-lymphocyte is suppressed by nicotine exposure.

**HSV Infection and Physical Stress in a Murine Model**

The HSV murine model has been used to study the intricate details surrounding the effect of physical stress on suppressing the anti-viral immune response. However, the effects of stress on the two branches of the immune system have not been equally studied. The innate immune response to HSV is much less studied as compared to the adaptive immune response. Recall that the innate immune system is the first responder, such that if it is not activated the activation of the adaptive immune response will most likely not occur as well. The innate immune response includes the mass production of pro-inflammatory cytokines, which aids in the recruitment of other immune cells. Using the murine model, it was determined that restraint stress during the primary infection of HSV resulted in the decrease of type I and type II interferon (Ortiz, Sheridan, & Marucha, 2003) and splenic IL-6 (Bonneau, Zimmerman, Ikeda, & Jones, 1998) production. In contrast, a study by Noisakran, Halford, and Carr (1998) found an increase in IL-6 production in mice exposed to hyperthermic stress. The decrease in cytokine production observed could lead to a reduction in T- and B-lymphocyte activation, which would compromise the immune system’s ability to fight HSV and create memory.

As noted above, leukocyte recruitment is a critical function of the innate response. In a report by Anglen, Truckenmiller, Schell, and Bonneau (2003), restraint stress caused a
delay in CD4+ and CD8+ T-cell recruitment into the brains of mice with HSV encephalitis. Macrophages and natural killer (NK) cells are even less understood. However, previous studies using mice depleted of macrophages prior to HSV infection and found that macrophages are critical to the resistance of HSV infection (Morahan & Morse, 1979; Pinto, Stewart, van Rooijen, & Morahan, 1991). In a study by Koff and Dunegan (1986), EPI and NE, which are elevated during stress, reduce the ability of macrophages to lyse HSV infected target cells in vitro. Similar inconsistent results to stress on macrophage activation in HSV are also found for NK cells. NK cells lyse virally infected cells; therefore, the reduction in lytic activity observed in restraint stressed mice is concerning (Bonneau, Sheridan, Feng, & Glaser, 1991). Exercise stress had no effect on splenic NK activity in mice (Davis et al., 2004). Freeman, Sheridan, Bonneau, and Hendricks (2007) determined that restraint stress reduced the number and functionality of HSV-specific CD8+ cells in the trigeminal ganglion, which resulted in a reduction of IFN-γ production in response to the reactivation of the virus.

Using a vaginal infection HSV model, Ashcraft & Bonneau (2008) found that, in addition to the reduction in NK cell number, there was a decrease in the number of HSV specific-CD8+ cells at the site of infection and its draining lymph nodes. In another report by Ashcraft, Hunzeker, and Bonneau (2008), it was determined that the increase of viral titer in the restraint condition mice was due to the reduction of lymphocytes at the site of infection and their inability to resolve the infection at the site. Restraint stress significantly decreased the number of NK cells available to respond to vaginal herpes infection in female C57BL/6J mice (Ashcraft and Bonneau, 2008). Hence, it is possible to conclude that physical stress has a negative impact on the innate immune response to HSV; however, it is important to consider (1) the type of stressor, (2) the strain of mice, (3) the sex of mice, and (4) where the
initial infection occurred (i.e., footpad, vaginal, intranasal) which are all critical factors that require study to gain a better understanding of how individual and/or environmental factors may or may not affect disease progression.

The adaptive immune system is responsible for helping clear the viral infection as well as in developing memory for future secondary infections or to control latent viral activation. The impact of stress on the adaptive immune response to HSV has been more commonly studied. However, the current literature focuses more heavily on T cell activation and memory T cells than on B cells and antibodies because HSV is an intracellular virus and therefore does not exist for long outside of a cell. However, antibodies provide support during the anti-viral response because antibodies neutralize free viral particles as well as mark infected cells for NK cells and macrophages to recognize and destroy (Kindt et al., 2007).

In a study by Karp, Moynihan, and Ader (1997), group-housing stress resulted in lower cytokine levels compared to control housing conditions (i.e., individual); however, there were no differences in circulating IgM and IgG antibodies between the two groups. In another study, mild electric foot shock exposure during HSV infection resulted in the reduced IgM titers, which exhibits immunosupression, as compared to the controls (Kusnecov et al., 1992). Again, the type of stressor (e.g., acute vs. chronic, social vs. physical) may be the underlying cause for these different results.

Because the transfer of HSV from mom to baby during delivery is possibly fatal, Dr. Robert Bonneau’s laboratory has examined the effects of stress on HSV transplacental and transmammary transfer of HSV-specific immunity from dam to offspring. His lab has demonstrated that previous HSV infection of the dam/mother provided high titers of HSV-
specific antibodies which protected the offspring (Yorty & Bonneau, 2003; Yorty & Bonneau, 2004a). Acute stress (e.g., 45 minute restraint stress exposures for three times a day over 6 days) did not affect the protective transfer of HSV-specific antibodies to newborns or offspring from the dam (Yorty & Bonneau, 2004a; Yorty & Bonneau, 2004b); however, artificially chronic (e.g., 200 μg/ml of corticosterone administered via drinking water for 6 days) elevated corticosterone (CORT) levels in the dams/mothers resulted in a reduction of HSV-specific antibodies in the serum and milk, which results in the offspring being more susceptible to HSV infection (Yorty, Schultz, & Bonneau, 2004; Zahwa, Yorty, & Bonneau, 2008).

Due to the role that T cells play in controlling viral infection, most investigations on the effects of stress on the adaptive immune response have focused on T cell-mediated immunity. Physical restraint in the HSV murine model has suppressed lymphocyte proliferation in draining lymph nodes to site of infection (Bonneau et al., 1991; Dobbs, Vasquez, Glaser, & Sheridan, 1993) and HSV-specific cytotoxic T cell (CTL) and NK cell activity in male mice (Bonneau et al., 1991). Kusnecov and colleagues (1992) found that mild foot shock stress in male mice resulted in suppression of lymphocyte proliferation and HSV-specific CTL activity, which adds to the research supporting involvement of T cells in the stress-mediated immunosuppressive effects on the pathogenesis of HSV. Several studies have examined the underlying mediation of stress on HSV pathogenesis and report that CORT (Bonneau, 1996; Bonneau, Brehm, & Kern, 1997; Bonneau, Sheridan, Feng & Glaser, 1993) and EPI and NE (Leo & Bonneau, 2000) are responsible for immunosuppression. To further understand the mechanism underlying the immunosuppression of lymphocyte proliferation, additional studies have investigated how stress-induced CORT impacts
dendritic cells’ (DC) function as the “professional” antigen presenting cell. DC present antigens to naïve T cells via MHC class I receptor complexes. Truckenmiller, Princiotta, Norbury, and Bonneau (2005) determined that CORT suppressed formation of antigen specific-MHC class I complex. The suppression of antigen specific-MHC class I complex causes a significant decrease in the ability of DCs to activate the naïve T cells (Truckenmiller Princiotta, Norbury, & Bonneau, 2005). Elftman, Norbury, Bonneau, and Truckenmiller (2007) extended the field’s understanding of how CORT effects the maturation of DCs; DCs exposed to CORT and LPS remained immature and production of IL-6, IL-12, and TNF-α was diminished.

In summary, HSV pathogenesis appears to be affected by chronic physical stress in the C57BL/6J mouse model. Suppression of innate immunity has been demonstrated through the decrease in IFN and IL-6 production, as well as through the delay of CTL recruitment and a reduction in NK availability at the site of infection and macrophages’ ability to lyse HSV-infected cells. Adaptive immunity is also suppressed; T cell suppression is mediated by CORT, EPI and NE. Restraint stress reduced the number and functionality of HSV-specific T cells, which research suggests is caused by a decreased in the ability of DCs to activate naïve T cells because CORT suppresses the expression of MHC class I receptor complexes. In addition, passive immunity from mother to neonate is reduced under chronic CORT administration leaving the neonate unprotected against HSV. Although there have been some null and increase in immune function findings following stress, it is critical to keep in mind the type of stressor used (i.e., group housing vs. individual housing or exercise stressor vs. restraint) and that unless studying female-specific-HSV transmission (i.e., vaginal or dam to offspring), studies have typically used male adult mice to study the effects of stress on
HSV pathogenesis. Therefore, it is difficult to predict how sex and physical stress may interact to affect the pathogenesis of HSV.

Taken together, this literature suggests that physical stress has an impact on multiple levels of HSV pathogenesis, which is typically negative for immunity (i.e., compromised immunity or immunosuppression). For a more detailed review of stress-mediated effects on viral pathogenesis, please see review articles by Bonneau, Padgett, and Sheridan (2007) and Truckenmiller, Bonneau, and Norbury (2006), as well as book chapters by Bonneau and Hunzeker (2006, 2007). However, the exact mechanisms need to be elucidated for adrenal-independent effects (Bonneau & Hunzeker, 2007), as well as how the anti-viral response to HSV might be affected by pharmacological stressors, such as nicotine exposure (i.e., tobacco use, cigarette smoking) and how sex may affect the anti-viral response when using the same route of HSV infection.

**Nicotine Stimulation of the Hypothalamic-Pituitary-Adrenal (HPA) Axis**

The effect of tobacco use on the stress response systems, sympathetic adrenomedullary- and HPA- axes, has been studied since the 1960’s (i.e., Cryer, Haymond, Santiago, & Shah, 1974; Hokfelt, 1961). As suggested by Rosecrans and Karin (1998), nicotine, the psychoactive and addictive ingredient in tobacco, activates the HPA-axis via stimulation central nervous system nicotinic acetylcholine receptors (nAChRs), which are found throughout the brain in a variety of forms and densities (Pidoplichko et al., 2004). Specifically, nAChRs are located in the parvocellular paraventricular nucleus of the hypothalamus and the brainstem catecholaminergic regions, including the locus coeruleus (Matta et al., 1998). It was determined that a nicotine-induced increase in adrenocorticotropic hormone (ACTH) occurs only when nicotine crosses the blood-brain
barrier (Matta et al., 1998). Therefore, nicotine is a potent stimulator of the HPA-axis via rapid secretion of ACTH (Seyler, Pomerleau, Fertig, Hunt & Parker, 1984; Balfour, 1989). In addition, it has been demonstrated that nicotine also increases endogenous opioid peptides (Pomerleau, 1998) and plasma EPI and NE (Cryer et al., 1976; Seyler et al., 1984) via central nervous system stimulation. Therefore, nicotine is a sympathomimetic and can be classified as a pharmacological stressor. Recent reviews have shown that HPA-axis basal function among human smokers is elevated compared to non-smokers (Steptoe & Ussher, 2006; Rohleder & Kirschbaum, 2006; Badrick, Kirschbaum, & Kumari, 2007).

As a pharmacological stressor, nicotine triggers the HPA-axis cascade by stimulating the release of corticotropin releasing hormone (CRH) from the hypothalamus. CRH then stimulates the release of ACTH from the anterior pituitary, which in turn stimulates the adrenal cortex to produce glucocorticoids (GCs), cortisol (humans) or CORT (rodents), which are responsible for a sustained stress response. Glucocorticoids are chemical messengers that support the physiological changes necessary to sustain the fight-or-flight response. In addition, GCs are responsible for completing the HPA-axis in that they provide negative feedback to the hypothalamus which inhibits production of CRH and ACTH, and consequently, ends stimulation of the HPA-axis (e.g., Stratakis & Chrousos, 1995; Klein & Corwin, 2002; Webster & Sternberg, 2004). Specifically, GCs bind to GC receptors in the hippocampus which mediates the negative feedback to the hypothalamus to end production of CRH (Sapolsky, McEwen, & Rainbow, 1983; Sapolsky, Krey, & McEwen, 1984a).

Additional research has shown HPA-axis regulation is affected by the sex of the subject due to the presence of androgens and estrogens [gonadal hormones; (Torpy & Chrousos, 1996; Da Silva, 1999; Kajantie & Phillips, 2006)], arginine vasopressin (Gibbs, 1986; Kajantie &
Phillips, 2006) and oxytocin [posterior pituitary hormones; (Gibbs, 1986; Taylor et al., 2000)], and the increased presence of circulating cytokines that occurs when a person is fighting an infection [e.g., IL-1; (Maier & Watkins, 1998; Wrona, 2006)].

Chronic GC elevation can impair immune function. For example, cortisol inhibits inflammation, blocks B-cell and T-cell functioning (e.g., Chrousos, 1995; Glaser & Kiecolt-Glaser, 2005; Glaser, 2005), and blocks the production of interleukins, which allow for communication among white blood cells (Watkins, Nguyen, Lee, & Maier, 1999; Steptoe, Hamer, & Chida, 2007). Cortisol stimulates gastric acid secretion, which may lead to a breakdown of the gastric mucosa (Klein & Corwin, 2007). Damage (e.g., lesions) to the hippocampus has resulted in a reduction of the feedback regulation of the HPA-axis (Sapolsky, Krey, & McEwen, 1984b; Saplosky, 2000; Kellendonk et al., 2002), which leads to problems in learning, memory, and attention, as well as the development of psychiatric disorders such as episodes of repeated and severe depression (Klein & Corwin, 2007).

Impaired CRH function leads to lethargy and fatigue, which could be a contributing factor in diseases like chronic fatigue syndrome, fibromyalgia, and seasonal affective disorder (Sternberg & Gold, 2002).

The biological stress response involves multiple physiological systems. Critical factors in the stress response are related to the length of the stressor, the severity of the stressor, and the organisms’ genetics and life experiences. Although stress is not the focus of this dissertation, a working knowledge of how the HPA-axis functions is critical because of the known immunosuppressive effects of elevated levels of CORT, EPI, and NE on the HSV-1 anti-viral response (Bonneau, 1996; Bonneau & Hunzeker, 2007; Bonneau et al., 1991; Bonneau et al., 1993; Yorty & Bonneau, 2004a; Yorty & Bonneau, 2004b; Yorty et al., 2004;
Zahwa et al., 2008). In addition, the HPA-axis plays a role in the physiological effects of nicotine on the body (e.g., Matta et al., 1998). The use of animal models allows for controlled examination of the extremely complex immune-brain interactions and individual differences in HPA-axis stimulation.

**Nicotine Stimulation of the Cholinergic Anti-Inflammatory System**

There are two types of nAChRs in the brain and the periphery. Heteropentameric nAChRs consisting of 5 subunits including α2 – α10 and β2 – β4 with the vast majority being one of the following combinations: α4β2, α3β2, α3β4, and α6β2 subunits. The homopentameric α7 nAChR is the most common receptor throughout the brain, and other central and peripheral nervous tissue and can be found on non-neuronal tissues (Morely & Rodriguez-Sierra, 2004). Once thought to only exist as a homopentameric nAChR, evidence is beginning to suggest that the α7 subunit could exist as a heteropentameric nAChR (Wang, Orr-Urtreger, & Korczyn, 2002; Pidoplichko et al., 2004). This α7 heteropentameric nAChRs discovery could result in the reason behind individual differences in response to effect of nicotine on the immune system because nicotine may bind with varying affinities to the different α7 nAChRs. Future studies will need to investigate this possibility further. The different subtypes of nAChRs vary in specificity for nicotine and are present throughout the brain in differing concentrations which results in varying effects (Collins et al., 1989; Pidoplichko et al., 2004).

The vagus nerve traditionally is known for its regulation in the parasympathetic part of the autonomic nervous system (e.g., heart rate and bronchoconstriction; Czura, Rosas-Ballina, & Tracey, 2007). The vagus nerve has afferent and efferent nerve fibers for bidirectional communication between the brain and the periphery (Czura et al., 2007).
Researchers discovered a brain-immune connection that is ultimately affected by nicotine when they observed that a subdiaphragmatic vagotomy disrupts hyperthermia induced by IL-1β (Watkins et al., 1995). It was hypothesized that the vagus nerve was responsible for immune-brain communication (Maier & Watkins, 1998; 1999), and was later labeled the cholinergic anti-inflammatory pathway (Borovikova et al., 2000; Pavlov & Tracey, 2005) because the major neurotransmitter of the vagus nerve is acetylcholine and its stimulation has an anti-inflammatory role.

Electrical stimulation of the vagus nerve resulted in reduced serum cytokines TNF-α, IL-1β, and IL-6 in wild-type mice, but not in α7 nAChR deficient mice (Wang et al., 2003). In a follow-up study, it was shown that α7 nAChR deficient mice do not experience the decrease in cytokine production from nicotine treatment during endotoxin challenge (Wang et al., 2004). Wild type mice exposed to nicotine via intraperitoneal injections had reduced serum TNF-α, IL-1β, and IL-6 levels and significantly increased survival from sepsis induced by lipopolysaccharide injection compared to α7 nAChR deficient mice (Wang et al., 2004). It was these landmark studies that found the α7 nAChR to be absolutely critical in the communication between the brain and the periphery for this pathway (Wang et al., 2004; Wang et al., 2003); therefore a more specific name was coined, the *nicotinic* cholinergic anti-inflammatory pathway (Ulloa, 2005). Therefore, much of cholinergic anti-inflammatory pathway research has focused on nicotine and stimulants of the cholinergic system.

Other rodent experimental models of disease have shown that nicotine exposure and vagus nerve stimulation are associated with anti-inflammatory responses in a variety of diseases, such as sepsis, hemorrhagic shock, inflammatory bowel disease (Ghia, Blennerhassett, Kumar-Ondiveeran, Verdu & Collins, 2006; Guarini et al., 2003; Guarini et
al., 2004; Huston et al., 2006; Luyer et al., 2005; van Westerloo et al., 2005; Wang et al., 2004). In addition to IL-1, several other pro-inflammatory cytokine levels are suppressed by the cholinergic anti-inflammatory pathway including TNF-α (Wang et al., 2004), nuclear factor-kappa B (Guarini et al. 2003), IL-6, and IL-18 (Borovikova et al., 2000), but not anti-inflammatory cytokines, like IL-10 (Borovikova et al., 2000). The stimulation of the nicotinic cholinergic anti-inflammatory pathway results in the inhibition of pro-inflammatory cytokines (Gallowitsch-Puerta & Pavlov, 2007), which aids the body when the immune system has a deadly hyper response (e.g., sepsis); however, this reduction of pro-inflammatory cytokines in a healthy organism could diminish the effectiveness of the immune system in response to a foreign antigen.

**Nicotine and the Immune System**

Nicotine can be administered to animals as cigarette smoke, cigarette smoke extract, or nicotine alone. Cigarette smoke is compromised of thousands of chemicals (USDHHS, 1988); therefore, when using cigarette smoke and its extract, it is difficult to determine which tobacco smoke constituent (or some combination) contributes to the observed effects. Administration of nicotine alone allows for examination of the effects of the primary addictive ingredient in tobacco on the immune system without confounding effects of other key constituents in tobacco that are known carcinogens. Thus, the effects observed by nicotine administration result from the neuro-active and peripheral properties of nicotine. In addition, research has shown that nicotine is responsible for the immunomodulatory effects observed in the body (for a review, see Friedman, Pross, & Klein, 2006).

Sopori and colleagues (1998) proposed that nicotine regulates the immune system via central and peripheral mechanisms. In the periphery, they observed that nicotine treatment
impaired T-cell receptor-mediated signaling, which led to a reduction in mature, activated T cells and the antibody plaque-forming cell response to sheep red blood cells in young adult (60-90 days old) Lewis rats and Swiss-Webster mice. In addition, the chronic nicotine administration in the intracerebroventricular region of the brain resulted in decreased antibodies to the sheep red blood cells; however, when a similar concentration was administered peripherally, nicotine had no effect (Sopori et al., 1998). These data suggest that when keeping the nicotine dose constant, nicotine has an immunosuppressive effect when delivered directly into the brain compared to delivery in the periphery.

In a report by Singh and colleagues (2000), it was determined that length of nicotine administration is critical to the impact that nicotine has on the immune system in adult Lewis rats. Acute nicotine exposure results in the activation of the HPA-axis and reduction of peripheral blood T-cell proliferation, which can be reversed through the administration of mecamylamine, a competitive nicotine antagonist that crosses the blood-brain barrier. The effect of chronic nicotine administration could not be reversed by administration of mecamylamine. Chronic nicotine administration led to the reduction in T-cell proliferation in the peripheral blood and the spleen, which resulted in T cell anergy or inability for the T cell to be stimulated (Singh et al., 2000).

Effect of Nicotine on Immune Cells

Alpha-7 nAChRs also are expressed on non-neuronal cells including macrophages, endothelial cells, dendritic cells, keratinocytes, B and T lymphocytes (e.g., Grando, Kawahima, & Wessler, 2003; Kawashima, Yoshikawa, Fujii, & Moriwaki, 2007; Saeed et al., 2005). In a study by Saeed and colleagues (2005), it was determined that nicotine treatment suppresses the in vitro (i.e., cell cultures) and in vivo (i.e., Shwartzman reaction)
activation of endothelial cell adhesion molecules, which is essential for the recruitment of leukocytes into the tissue. Nicotine also suppressed the production of TNF-α by human neutrophils and monocytes in vitro (Saeed et al., 2005). In a study by Takahashi and colleagues (2006), it was determined that in vitro nicotine treatment inhibited the IL-18 effects on human monocytes. IL-18 enhances monocytes production of cytokines, TNF-α and IL-1β, and expression of adhesion molecules (Dai, Matsuno, Nakamura, Nishioka, & Yudoh, 2004). Nicotine blocked the effect of IL-18 on monocytes’ expression of adhesion molecules such as ICAM-1, B7.2 and CD 40. Nicotine also decreased the production of IL-12, TNF-α, and IFN-γ in the presence of IL-18 (Takahashi et al., 2006). However this effect was blocked by nAChR antagonists, mecamylamine and α-bungarotoxin. Therefore, nicotine suppresses the adhesion molecules on monocytes and endothelial cells. This reduction demonstrates that there are two pathways in which nicotine decreases the effectiveness of the immune system in recruiting inflammatory cells from the blood to the site of infection.

Hallquist, Hakki, Wecker, Friedman, & Pross (2000) used BALB/c murine splenocytes to determine the effects of nicotine on the response to concanavalin A (ConA) at two time points in the lifespan. Age was an important determinant in immune responses to nicotine: young adult splenocytes (8-9 wks) produced significantly higher IL-10 and lower IFN-γ than did the aged splenocytes (18-24 mos). IL-10 production was inhibited by 48 hr in vitro nicotine exposure for both the young adult and the aged splenocytes in an unchallenged state. IFN-γ cytokine production was not altered by nicotine exposure. However, in the presence of the ConA challenge, a 3-hr nicotine pretreatment resulted in a significant increase in IFN-γ in the young adult, but not the aged splenocytes. It was concluded that
nicotine shifts the cytokine balance to support the T_h1 environment, which is supportive of cytotoxic T cell activation, opposed to a T_h2 because of the increase in IFN-γ and the decrease in IL-10, especially in the young adult (Hallquist, Hakki, Wecker, Friedman, & Pross, 2000). These findings imply that nicotine could influence the development or environment of the immune system early in life differently than later in life.

A series of published studies from different laboratories have examined nicotine’s effects on dendritic cells (DCs). Recall that DCs are critical mediators between the innate and the adaptive immune systems by presenting antigen to naïve T cells. In 2003, Nouri-Shirazi and Guinet reported that _in vitro_ nicotine is immunosuppressive to human DCs. Specifically, they found that DCs uptake of antigen was suppressed by nicotine; therefore, DCs maturation and cytokine (IL-12, IL-1β, IL-10, and TNF-α) production were decreased. In addition, T cell responses to DCs were diminished and suppressed IFN-γ production during a re-stimulation without nicotine present. These findings demonstrate that nicotine compromised the antigen presenting process, which directly diminishes/compromises a robust immune response.

In two reports, (Vassallo, Tamada, Lau, Kroening, & Chen, 2005; Vassallo, Kroening, Parambil, & Kita, 2008), human monocyte-derived DCs from non-smokers were used to investigate cigarette smoke extract (CSE), which contains nicotine, on the functionality of the DCs. First, they determined that CSE inhibited DC-mediated T cell activation by inhibiting IFN-γ secretion (Vassallo et al., 2005). In addition, the IL-12p70 production from matured DCs stimulated by lipopolysaccharide (LPS) was significantly inhibited by the CSE; however, the IL-10 production was significantly increased (Vassallo et al., 2005). Vassallo and colleagues (2005) concluded that the effects of CSE on the immune
system leads to Th2 cytokine environment or supportive of B lymphocyte activation, which opposes Hallquist and colleagues’ (2000) conclusion. However, the studies examined two different pathways and two different species in which nicotine may be affecting the overall effectiveness of innate immunity. In 2008, Vassallo and colleagues investigated the effects of CSE on DCs in the context of chronic obstructive pulmonary disease (COPD). It was determined that DCs in the presence of CSE resulted in significant production of neutrophilic chemokines, which could be the basis for the increased inflammation associated with COPD, a common smoking related disease (Vassallo et al., 2008).

In the only other available published study on nicotine and DCs, Aicher and colleagues (2003) report that nicotine strongly activates DCs and increases their capacity to stimulate T cell proliferation and cytokine secretion, which contradicts Vassollo and colleagues’ (2005) previously described report. Using human monocyte-derived DCs, the investigators found that nicotine exposure increased cytokine (IL-12 and IL-10) production and upregulation of proinflammatory surface molecules (e.g., CD86), adhesion molecules (e.g., CD11a/CD18) and CD83, a DC maturation marker (Aicher et al., 2003). They also found similar results in BALB/c murine DCs, which included upregulation of CD54, MHC class II, and CD40, costimulatory molecule (Aicher et al., 2003). In addition, they injected dye labeled DCs to determine the effect of nicotine on DCs adhesion to atherosclerotic lesions in 50-week old male apolipoprotein E- knockout mice, known for their atherosclerotic lesion near the aortic valve. The nicotine-treated mice had a significantly higher number of DCs in the plaque near the aortic valve. The authors were studying the effects of nicotine on DCs in the context that smokers are at risk for developing atherosclerosis (Aicher et al., 2003). Being the first in vivo investigation of atherosclerotic
plaque development, it is clearly important that the effects of nicotine on DCs must be studied in context of specific disease pathogenesis and the rest of the immune system.

Not only does nicotine affect antigen presenting cells, but it also has a direct impact on T cells in rats and mice (e.g., Thomas, Holt, & Keast, 1973; Holt, et al., 1976; Chang, Distler, & Kaplan, 1990), monkeys (Sopori, Gairola, DeLuncia, Bryant, & Cherian, 1985) and humans (e.g., Silverman, Potvin, Alexander, & Chretien, 1975; Peterson, Steinmel, & Callaghan, 1983). The molecular mechanisms through which nicotine affects T cells have been investigated. In 1999, Kalra and colleagues demonstrated that chronic nicotine exposure in vivo resulted in splenocytes that have a decreased ability to raise intracellular Ca2+ and depleted inositol-1,4,5-triphosphate-sensitive Ca2+. Cigarette smoke causes T cell anergy (unresponsive to stimuli; unable to proliferate and reduces number of T cells available during anti-viral response) and may contribute to the immunosuppressive effects of tobacco smoking (Kalra et al., 1999), which could contribute to disease progression seen in long-term smokers. Petro and colleagues (1999) investigated the effects of nicotine and smokeless tobacco on memory T cells production of cytokines after stimulation without the presence of nicotine or smokeless tobacco. Using BALB/c splenic mononuclear cells, in vitro nicotine exposure lead to a greater production of IFN-γ, IL-10, and IL-4 at 48h during re-stimulation as compared to the non-nicotine exposed splenic mononuclear cells. In vitro exposure to smokeless tobacco, which contains nicotine and multiple carcinogens, resulted in significant increases in production of IL-2, INF-γ, IL-10 and IL-4 during a re-stimulation at 24h and 48h after tobacco exposure. However, mRNA expression for cytokine production results suggest that nicotine alone will cause greater cytokine production for more than 4 days, while the smokeless tobacco reaches maximum cytokine production 2 days post re-stimulation. These
data suggest that nicotine can cause excessive and prolonged memory T-cell cytokine expression, which could be the underlying factor in several inflammatory diseases, such as periodontitis (Petro et al., 1999). Therefore, these data suggest that nicotine can cause an exaggerated response after the primary anti-viral response which could lead to excessive inflammation throughout the body.

Skok and colleagues (2005) found that B lymphocytes in male C57BL/6J mice have both \( \alpha_4\beta_2 \) and \( \alpha_7 \) nicotinic receptors. Using \( \alpha_4, \beta_2, \) and \( \alpha_7 \) knockout mice with the genetic background of C57BL/6J mice, the investigators found that knockout mice had similar levels of serum IgM, but significantly less serum IgG and splenic IgG-producing cells compared to the wild-type, which suggests that the B lymphocyte variability was decreased. In addition, they found that B lymphocytes from \( \beta_2 \) knockout mice had a greater response to anti-CD40 in the presence of nicotine than did wild-type mice; therefore, nicotine suppresses B lymphocyte activation via the T cell-dependent pathway. Previous research (Rinner, Kawashima, & Schauenstein, 1998) demonstrated that T lymphocytes are capable of producing acetylcholine, which, until this report, seemed fruitless. Not only does endogenous acetylcholine affect B lymphocytes, but exogenous nicotine could interfere with B to T lymphocytes communication, thus causing a decrease in both T and B lymphocytes and their activation.

As reviewed here, the nicotine appears to have a negative impact on the proper functioning of the immune system. However, the data are not always consistent. When trying to decipher the effect of nicotine on the immune system, one must consider (1) the administration of nicotine (e.g., injected or infused), (2) the study design (e.g., \textit{in vitro} vs. \textit{in
(3) the source of immune cells (e.g., species, non-smoker, or smoker), and (4) whether or not it is being studied within a contextual disease model (e.g., COPD or atherosclerosis).

There are several areas that still need to be thoroughly investigated, such as the effect of nicotine on the pathogenesis of disease in vivo. A robust immune system response to viruses relies on activation and communication between the innate and adaptive immune systems. To date, nicotine has been shown to affect the immune system indirectly via stimulation of the nicotinic cholinergic anti-inflammatory pathway and the HPA-axis (see Ulloa, 2005; Gallowitsch-Puerta & Tracey, 2005; Gallowitsch-Puerta & Pavlov, 2007, for reviews) and by direct communication with immune cells (e.g., Grando et al., 2003; Saeed et al., 2005; Kawashima et al., 2007). Since the mid-1960’s, a link between tobacco use and poor health outcomes has been observed (USDHEW, 1964); however, research has neither investigated the impact of early nicotine exposure (i.e., adolescence) on the trajectory of the immune system nor how nicotine affects the immune system’s response to an initial viral exposure.

**Effect of Nicotine on the Immune System in Adolescent Rodents**

Published data suggest that nicotine interacts with the body to suppress the immune system on varying levels, both indirectly and directly. Most of the reports have used adult animals; therefore, what has not been elucidated is how the age of exposure to nicotine may have differential effects on the responsiveness of the immune system. As an organism ages, the immune system becomes less adaptive to new antigen because the thymus atrophies and there are less naïve T- and B-cells to be trained (e.g., Graham, Christian, & Kiecolt-Glaser, 2006; Weiskopf, Weinberger, & Grubeck-Loebenstein, 2009). Yet, an aged immune system has been exposed to more antigens and able to produce secondary immune responses to more
antigens than a young immune system (e.g., Weiskopf, Weinberger, & Grubeck-Loebenstein, 2009). Because the adolescent immune system is relatively untrained, the effect of the exposure to second-hand smoke or use of tobacco products by adolescents on the functionality of the immune system must be investigated.

The majority of reports on the direct molecular effects of nicotine on the immune system have been *in vitro* study designs. The focus on *in vitro* study designs has allowed for better mechanistic understanding of the effects of nicotine. However, using an *in vivo* study design is absolutely necessary to understand how all the biological systems interact. Nicotine can be administered *in vivo* through a variety of mechanisms including, osmotic mini-pump (e.g., Grunberg, Bowen, & Morse, 1984; Navarro et al., 2001), injection (e.g., Aicher et al., 2003; Navarro, Basta, Seidler, & Slotkin, 2003), nicotine-spiked water (e.g., Klein, Stine, Pfaff, & Vandenergh, 2003; Klein, Stine, Vandenergh, Whetzel, & Kamens, 2004), and the nicotine patch (e.g., Myles et al., 2003; Kalra et al., 2004). As opposed to other animal models that use chronic nicotine exposure, the oral self-administration model of nicotine intake more closely mimics the “peak and trough” blood nicotine concentrations that a regular smoker experiences throughout the day and night (Klein et al., 2004). Therefore, self-administration of nicotine-spiked water appears to be more generalizable than the other nicotine administration models. In addition, this model of self-administration can be used with adolescent mice because there is (1) no need to train the animals how to administer the nicotine to themselves, (2) no stress of surgery to insert pumps or repeated nicotine injections, and (3) the animals have control over how often they are exposed to or “use” the nicotine (Klein et al., 2004).
There is a lack of data on how nicotine affects the function of the immune system during adolescence; therefore, it is especially important to focus resources in this dissertation to determine whether or not nicotine exposure has detrimental effects on the immune system during this sensitive period. In a report by Navarro, Basta, Seidler, and Slotkin (2001), it was noted that the effects of nicotine on the developing immune system of adolescents are completely understudied. Adolescent exposure to nicotine in rats via an osmotic mini-pump did not affect splenic T cell response to a mitogenic challenge during adolescence [post natal (PN) day 45], but did significantly reduce the splenic T cell response to the mitogenic challenge during adulthood (PN 80). This inhibited T cell response in adulthood was found only in rats treated with nicotine during adolescence even though nicotine was no longer detectable in the rats (Navarro et al., 2001). The authors concluded that cholinergic stimulation caused by nicotine modified the T cells in a manner that had lasting effects despite removal of the cholinergic stimulation on PN 47. Navarro and colleagues (2001) believe these findings should be included in the negative ramifications that nicotine has on the health of adolescent and adult human tobacco users.

In a follow up study by Navarro, Basta, Seidler, and Slotkin (2003), adolescent (PN 30) male and female rats were exposed to nicotine via (1) mini-pump infusion or (2) 2 daily subcutaneous injections for 7 days at 2 different dosages (2 mg/kg/day or 6 mg/kg/day). The nicotine-infused rats displayed reduced T lymphocyte responses to ConA mitogenic challenge for both nicotine doses on PN 37; however, only rats exposed to 6 mg nicotine/kg/day (equivalent to about half a pack of cigarettes/day) displayed a significant decrease compared to control rats (Navarro et al., 2003). The ConA mitogenic challenge was also done on PN 45 and 65. There were no differences between treatment groups at PN 45;
however, at PN 65 (young adulthood), T lymphocytes from nicotine-infused rats were significantly suppressed in response to ConA challenge in both nicotine dosage groups. The nicotine-injected rats did not display any significant suppression of their T lymphocyte response to ConA at any time point. However, rats injected with 6 mg nicotine/kg/day had an exaggerated B lymphocyte response compared to control rats on PN 65 (Navarro et al., 2003). The authors concluded that just one week of nicotine exposure can compromise the immune system. Even though nicotine was removed on PN 37, in young adulthood (PN 65) the nicotine-exposed rats showed dysregulated immune response compared the non-nicotine exposed rats which varied dependent on nicotine administration (Navarro et al., 2003).

These two studies (Navarro et al., 2001; Navarro et al., 2003) were the first steps to understanding how nicotine exposure during adolescence could have a negative impact on the functionality of the immune system. In the first study, it was determined that nicotine exposure during adolescence did not affect splenic T cells during adolescence but did suppress them in adulthood (Navarro et al., 2001). In the follow-up study, the mini-pump infusion administration was effective at suppressing the T lymphocyte response during adolescence (PN 37) and young adulthood (PN 65), even with nicotine treatment ending in young adulthood (Navarro et al., 2003). However, the use of the mitogenic challenge to activate the immune system is not necessarily relevant when investigating how nicotine exposure affects the overall functionality of the immune system. Being that there are only 2 published studies, it also is clear that the immune system has been ignored in prior adolescent nicotine exposure studies. The need to study the effects of nicotine on the immune system during adolescence is necessary to understand what underlying mechanisms are causing the immunosuppression that could continue into adulthood and be responsible for the increase in
diseases found in adult human smokers. The use of the HSV mouse model in conjunction with an adolescent mouse model of nicotine consumption will allow for the landmark investigation of how nicotine may affect the antiviral response to an in vivo immunologic challenge during adolescence.

**Effect of Nicotine on HSV Pathogenesis**

Nicotine has suppressive effects on the immune system; however, the effects of nicotine on the pathogenesis of HSV are not clearly defined. During the 1980’s HSV was thought to have oncogenic properties (Hirsch, Svennerholm, & Vahine, 1984; Oh, Paik, & Park, 1989). Therefore, early studies investigated the effect of smokeless tobacco on HSV replication and protein synthesis due to the link between the increase rates in oral cancer observed in tobacco users. For HSV to become oncogenic, it must be inactivated and lose its cytolytic activity (Oh et al., 1989). For example, Hirsch, Svennerholm and Vahine (1984) found that tobacco snuff extracts, including nicotine, inhibited the DNA replication of HSV in vitro. The inhibition of replication occurred during the early stages of virus infection; therefore, it was suggested that the combination of HSV oral shedding and snuff use might be responsible for the increase in oncogenicity of HSV and result in cancerous oral lesions. The inhibition of DNA replication and cytolytic activity of HSV has been found using snuff extract (Stich, Li, Chun, Weiss, & Park, 1987; Hirsch, Svennerholm, & Vahine, 1984; Oh, Cherrick, & Park, 1990; Dokko, Min, Cherrick, & Park, 1991; Larsson, Hirsch, Gronowitz, & Vahlne, 1992) and smoked tobacco tar (Oh et al., 1989).

Much of the findings discussed thus far have been the result of in vitro study designs; such as, exposing HSV infected cells via submersion in snuff extract or smoked tobacco tar. There only have been a few in vivo studies using mice and rabbits. In a report by Park,
Herbosa, and Sapp (1987), using BALB/c male mice, they found that oral exposure to tar condensate at the site of inoculation (HSV or mock) for 2 or 3 months led to reactivation of latent HSV in 10 – 20% of the mice and a significant number of dysplastic lesions in the mouth of HSV inoculated mice as compared to non-HSV inoculated mice. In a study using ICR/CD-1 mice, it was determined that nicotine suppressed the interferon production that was induced by HSV-2 if nicotine was administered 2 hours after a lower jaw inoculation of the virus (Fukuma et al., 1986).

The use of the HSV rabbit eye model helped gain an understanding of how nicotine results in HSV reactivation via ocular shedding (Myles et al., 2003; Myles et al., 2004). In the first study, Myles and colleagues (2003) reported an increase in reactivation and ocular shedding of HSV in rabbits treated with a nicotine transdermal patch for 21 days as compared to placebo animals. It was proposed that HPA-axis and cholinergic system stimulation by nicotine were the mechanisms underlying the immunosuppressive effects observed. In the 2nd study, Myles and colleagues (2004) used bupropion, which inhibits neuronal uptake of NE, EPI, and dopamine, to investigate the increased link between nicotine and HSV reactivation. It was observed that nicotine/bupropion treated rabbits had lower reactivation and ocular shedding of HSV compared nicotine/placebo treated rabbits (Myles et al., 2003). These findings suggest that bupropion, which can be used as a smoking cessation tool, could be reducing the immunosuppressive effect of nicotine on the immune system by interfering with the neurotransmitter reward of nicotine in the brain.

Three out of the four studies studying in vivo nicotine effects on HSV were investigating reactivation of HSV (Park et al., 1987; Myles et al., 2003; Myles et al., 2004), not the effect of nicotine on the primary HSV infection. The report by Fukuma and
colleagues (1986) was the only report studying the effects of nicotine during the primary infection and found suppression of interferon, a pro-inflammatory cytokine. It appears that nicotine increases the recurrence of latent HSV, but it is still necessary to determine how adolescent and adult smokers are affecting their immune system’s response to initial exposures to a virus. Therefore, the field needs to more thoroughly investigate how nicotine might affect the primary response to a viral infection, such as HSV, in the organism.

**Purpose of the Dissertation**

In light of the understudied effects of nicotine on the adolescent immune system and on the primary exposure to HSV in males and females, the purpose of this dissertation was to investigate the immunologic effects of nicotine on the pathogenesis of HSV during adolescence in male and female mice. As one might think, there are ethical constraints surrounding examination of the causal effects of tobacco smoking and HSV infection in human adolescents. Fortunately, animal models are available to examine the casual effects of nicotine on immune function that otherwise would be impossible to examine in humans. This dissertation bridged a murine model of adolescent nicotine exposure (Klein et al., 2004) with a HSV exposure murine model (Bonneau, 1996; Bonneau et al., 1991; Bonneau et al., 1998; Leo & Bonneau, 2000) to investigate the effects of nicotine on HSV-1 pathogenesis. In addition, sex differences in the anti-viral response to HSV-1 during adolescence were studied to do the lack of sex comparisons in the current literature. This is the first experiment in a series that will decipher the importance of nicotine use during adolescence and how it affects the primary exposure to HSV-1 during adolescence and later in adulthood (i.e., nicotine exposure may alter anti-viral immunity trajectory).
Chapter II: Experiment

Overview

The purpose of this dissertation was to examine the effects of nicotine exposure on the pathogenesis of HSV among 120 infected adolescent male and female C57BL/6J mice. Adolescent (PN 30) male (n=59) and female (n=58) mice were exposed to one of three concentrations of nicotine via their drinking water: 0 μg/mL, 50 μg/mL, or 200 μg/mL (Klein et al., 2003). After 7 days of nicotine exposure, all mice were infected with HSV-1 (Bonneau, 1996; Bonneau et al., 1991; Bonneau et al., 1998). Nicotine exposure continued for 5 more days post-infection. Following this initial anti-viral response, mice were sacrificed via cervical dislocation followed by blood and tissue collection. Serum was tested for cotinine and corticosterone. Lymphocytes from popliteal lymph nodes were isolated and incubated for 3 days. Functionality of HSV-specific T lymphocytes lysis was analyzed using a 51 Chromium (51Cr) release assay. Additionally, IFN-γ levels were analyzed from the supernatant of the 3-day incubation period.

Hypotheses

A between-subjects design was used to assess the dose-dependent effects of nicotine on the primary HSV-1 exposure among C57BL/6J adolescent male and female mice. The following hypotheses were tested:

Hypothesis 1: Nicotine exposure would result in a dose-dependent elevation in cotinine levels such that 200 μg/mL group > 50 μg/mL group > 0μg/mL group.

Rationale: Cotinine is the primary metabolite of nicotine (USDHHS, 1988); therefore, the greater the nicotine exposure, the more nicotine available to be metabolized into cotinine.
Hypothesis 2: Nicotine treatment would result in dose-dependent stimulation of the HPA-axis, as indexed by serum corticosterone levels, such that 200 \( \mu \text{g/mL} \) group > 50 \( \mu \text{g/mL} \) group > 0\( \mu \text{g/mL} \) group.

Rationale: Nicotine has been shown to stimulate the HPA-axis (e.g., Matta et al., 1998); therefore, greater exposure to nicotine should result in greater stimulation of the HPA-axis and production of corticosterone. Previous research has shown a dose-dependent increase in corticosterone of adult rats that were treated with nicotine via osmotic mini-pump as adolescents (Klein, 2001). Additionally, a recent article by Lamota and colleagues (2008) showed that daily nicotine subcutaneous injections increased corticosterone levels in both male and female adolescent rats.

Hypothesis 3: Regardless of sex, nicotine exposure would result in a dose-dependent reduction in anti-viral immunity such that 0 \( \mu \text{g/mL} \) group > 50 \( \mu \text{g/mL} \) group > 200 \( \mu \text{g/mL} \) group, as indexed by the number of lymphocytes that were isolated from the popliteal lymph nodes. With regard to sex, among nicotine-exposed mice, females would demonstrate a greater suppression of their anti-viral immunity such that the females’ number of isolated lymphocytes from the popliteal lymph node should be less than their male counterparts’ number of isolated lymphocytes.

Rationale: Previous research has shown that chronic exposure to nicotine in rats leads to T cell anergy, which is a state of unresponsiveness (Kalra et al., 2004; Singh et al., 2000; Sopori & Kozak, 1998); therefore, the T cells will be unable to proliferate. Because nicotine stimulates the HPA-axis (e.g., Matta et al., 1998), previous HSV-1 and physical restraint models in C57BL/6J mice have shown that the mice exposed to restraint stress (activation of the HPA-axis) have a reduction in the number of T lymphocytes compared to
control animals (Bonneau et al., 1991). Additionally, previous research has shown that female mice self-administer a greater nicotine dosage (mg/kg) than their male counterparts in a nicotine choice paradigm (Bennett, Leslie, McClearn, & Klein, 2007; Klein et al., 2004). Given the increased exposure to nicotine for female mice as compared to their male counterparts, a greater suppression of the anti-viral T cell proliferation would occur in the female mice. Although there are two studies on the effect of nicotine administration on T cell function in adolescent rat models (Navarro et al., 2001; Navarro et al., 2003), neither study investigated an in vivo immunological challenge and how nicotine modulates the T cell proliferation in vivo nor did the studies investigate whether or not sex of the rodent had any effect on T cell proliferation within the same paradigm.

**Hypothesis 4**: Regardless of sex, the production of IFN\(\gamma\) by T cells during the cell culture incubation would be reduced by nicotine treatment such that 200 \(\mu\)g/mL group < 50 \(\mu\)g/mL group < 0 \(\mu\)g/mL. With regard to sex, among the nicotine-treated mice, the production of IFN\(\gamma\) by T cells during cell culture incubation will be reduced by nicotine exposure such that female’s T cells should secrete less IFN\(\gamma\) compared to their male counterparts’ T cells.

**Rationale**: Previous research has shown that IFN\(\gamma\) production is correlated with the ability of T cells to proliferate (Bonneau, 1996; Bonneau, et al. 1991; Bonneau et al., 1998). When activated T cells proliferate, the T cells produce IFN\(\gamma\) to communicate with other immune cells and continue to support an anti-viral immune environment (Kindt et al., 2007). Therefore, the dose-dependent reduction in the ability of T cells to proliferate caused by nicotine exposure should result in a dose-dependent reduction in IFN\(\gamma\) levels in the supernatant from the cell culture. Additionally, the T cells from the female mice which are
exposed to a greater level of nicotine should be less able to proliferate than their male counterparts’ and produce less IFNγ during the cell culture incubation period.

**Hypothesis 5**: Regardless of sex, nicotine exposure would result in a dose-dependent suppression of HSV-specific T cell functionality, as indexed by the ⁵¹Cr release assay, such that 200 μg/mL group > 50 μg/mL group > 0 μg/mL group. Among nicotine-exposed mice, there will be a sex difference such that females T cells should display greater suppression compared to males.

**Rationale**: To date, no studies have investigated the effects of nicotine exposure on HSV-specific T cells’ ability to lyse infected cells or whether sex of the animal has an impact. However, previous research using an adolescent rat model (Navarro et al., 2003) found that nicotine administered via osmotic mini-pump resulted in a significantly reduced T lymphocyte response to ConA mitogenic challenge compared to control rats on PN 37. Additionally, activation of the HPA-axis by physical restraint has been shown to suppress the ability of T cells to lyse HSV-infected cells (Bonneau et al., 1991). Therefore, nicotine’s stimulation of the HPA-axis (e.g., Matta et al., 1998) could increase the suppression of the ability of T cells to lyse HSV-infected cells in addition to directly suppressing the functionality of the T cells. Because previous research has shown that female mice self-administer a greater nicotine dosage (mg/kg) than their male counterparts in a nicotine choice paradigm (Bennett et al., 2007; Klein et al., 2004), the effect of increased nicotine exposure in female mice should result in a greater suppression of the T cell’s ability to lyse infected cells. Therefore, it is necessary to determine whether or not the effect of sex is significant when investigating nicotine’s effect on the T cells’ ability to lyse HSV-infected cells.
Experimental Design

Table 1 presents an overview of the experimental design. One hundred and seventeen periadolescent C57BL/6J mice (59 male and 58 female) were treated with one of three nicotine-spiked water bottles [CONTROL (N=19 males and 20 females): 0 μg/mL, LOW (N=20 males and 19 females): 50 μg/mL, HIGH (N=20 males and 19 females): 200 μg/mL] starting on PN 30. After 7 days of nicotine treatment, mice were infected with HSV-1 via rear foot pad injections (PN 37). On PN 42, mice were euthanized to collect blood and tissue samples. All procedures were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (IACUC #31606) and the Pennsylvania State University Institutional Biosafety Committee (IBC #31626).

Table 1: Overview of experimental design

<table>
<thead>
<tr>
<th>Nicotine Treatment Groups</th>
<th>CONTROL (0 μg/mL)</th>
<th>LOW (50 μg/mL)</th>
<th>HIGH (200 μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males:</td>
<td>N= 19</td>
<td>N= 20</td>
<td>N= 20</td>
</tr>
<tr>
<td>Females:</td>
<td>N= 20</td>
<td>N= 19</td>
<td>N= 19</td>
</tr>
<tr>
<td>Total:</td>
<td>N= 39</td>
<td>N= 39</td>
<td>N= 39</td>
</tr>
</tbody>
</table>

Methods

Animals

C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were chosen because previous research in our laboratory has demonstrated that these mice will voluntarily consume nicotine in drinking water (Klein et al., 2003; Klein et al., 2004). Additionally, the immune response to HSV-1 in C57BL/6J mice is well characterized (e.g., Bonneau & Hunzeker, 2006). Mice arrived in the Central Biological Laboratory on post natal (PN) day 25.
**Nicotine**

(-) Nicotine freebase (Sigma Chemicals, St. Louis, MO) was provided to mice in a concentration of 50 μg/mL (LOW) or 200 μg/mL (HIGH) dissolved in tap water (Klein, et al., 2003; Klein, et al., 2004); tap water was used as the control solution. These nicotine concentrations were selected based on a forced nicotine paradigm with pregnant dams, which yielded measurable levels of cotinine, the primary metabolite of nicotine (Klein, et al., 2003). See Appendix A for the nicotine solution preparation procedures.

**Herpes Simplex Virus-1**

Herpes simplex virus (HSV)-1 strain Patton virus stocks were propagated in Vero cells by infection at a multiplicity of infection of 0.01 and titers were determined by plaque assays on Vero cells. Virus stocks were stored at -70°C. The HSV-1 strain Patton virus was provided by Dr. Robert H. Bonneau (Penn State Hershey, College of Medicine).

**Procedure**

Mice were tested in six cohorts (at least 19 animals per cohort; 3-4 animals/experimental group) to allow time for data collection with minimal disruption of animals in the housing room and at necropsy where it was critical to maintain biological integrity of the lymphocytes for the HSV-1 specific immune outcome measure. Cohorts were run in pairs (cohort 1 & 2, cohort 3 & 4, cohort 5 & 6) with start dates offset by one week. Specifically, mice in odd cohorts (1, 3, & 5) arrived in a housing room, and on day 3 of nicotine treatment the matching even cohort (2, 4, & 6) arrived in the same housing room. Due to this cohort overlap, the animal room environment was reviewed with extreme caution and attention to detail with regard to the protocol. To reduce a possible difference in cohort effect due to the animal room experience, care was taken to minimize disruptions in the
housing room across both the odd and even cohorts. For example, the researcher entered the room each morning during acclimation of the odd cohorts to mirror the experience of the even cohorts. Another example, the researcher brought the isoflurane machine into the animal room and ran it for about 1 hour on day 1 of nicotine treatment for cohort 1 to mirror the disruption that cohort 2 would experience on their first day of nicotine treatment due to the HSV-1 injection of cohort 1. Due the importance of the fluid consumption (mL), bottle flips and fluid changes were handled consistently across baseline and treatment periods across all cohorts.

Upon arrival, mice were individually housed in standard shoebox style plexi-glass cages, with filter tops, filled with ¼ inch bedding (Bed-o’Cobs, The Andersons Agriservices, Inc., Maumee, OH). Mice were kept in climate-controlled rooms, with a temperature of 21 ± 2 ºC and a relative humidity of 51.2%, and maintained on a 12-hour light-dark cycle (lights on at 0800 hrs). Throughout the study, mice had continuous access to standard rodent chow (LabDiet 5001 Rodent Diet, PMI Nutrition International, Brentwood, MO). For three days (PN 25 – PN 27), mice were allowed to acclimate to their new environment undisturbed (e.g., cages were not moved and animals were not handled).

**Baseline (3 days).** Figure 2 presents a timeline of the experiment. During baseline (PN 28 – PN2 9), mice had 24-hour access to tap water in a single water drinking bottle and were handled to obtain their body weight and fluid and food consumption, which continued throughout the remainder of the study. Based on baseline body weight, food and fluid consumption, mice were assigned to one of three nicotine treatment groups to ensure that the groups did not significantly differ on these measures at the start of the experiment.
Figure 2. Experimental timeline

Nicotine Treatment (7 days). On PN 30, mice had access to one bottle for drinking either no nicotine (CONTROL), LOW nicotine (50 μg/mL) or HIGH nicotine (200 μg/mL) for 7 days (Navarro et al. 2001; Navarro et al., 2003). Body weight, food consumption and fluid intake were measured daily.

HSV-1 Infection and Nicotine Treatment (5 days). On PN 37, mice were anesthetized with 5% isoflurane inhalation for 2 minutes. Plaque-forming units (1 x 10⁶) HSV-1 Patton was injected subcutaneously into each footpad in a volume of 30 μL. See Appendix B for the HSV-1 injection preparation and injection protocol. Mice were returned to their home cage and monitored until the mice were able to access food and water (about 3 to 8 minutes). Daily body weight, food consumption and fluid intake measurements continued for the next 5 days.

Blood and Tissue Collection. On PN 42 (5 days post-infection), mice were euthanized via cervical dislocation beginning at the onset of the light cycle. Blood was collected by cardiac puncture or as trunk blood in the thoracic cavity and was allowed to sit at room temperature for 15 to 25 minutes. Samples then were centrifuged for 15 minutes at 1500 x g.
Serum was aliquoted and stored at -70°C for later assessment of cotinine and corticosterone. Cotinine and corticosterone were measured by commercially-available enzyme-linked immunosorbent assays (EIA; Immulanalysis; Pomona, CA and Arbor Assays; Ann Arbor, MI, respectively). See Appendices C and D, respectively, for assay kit protocols.

Popliteal lymph nodes also were removed from the mice. Based on previous protocols by Pfizenmaier and colleagues (1977) and Bonneau and colleagues (1991), single cell suspensions were prepared and cell viability was determined by trypan blue dye exclusion. Total cell count was determined using a Coulter counter. Then, in 12-well tissue culture plates, 4 x 10^6 lymphocytes/mL were cultured at 37°C in 5% CO₂ for 3 days in 0.8 mL of supplemented IMDM. See Appendix E for popliteal lymph node removal and lymphocyte isolation procedure.

**[^1]Cr Release Assay.** Cell-mediated cytotoxicity was determined by ^[^1]Cr release assay. This assay tests the ability of HSV-1 specific T<sub>C</sub> cells (effector cells) from the *in vivo* HSV-1 infected mice to lyse HSV-1 specific-pulsed and MOCK-pulsed target cells. The target cells were cultured B6/WT-3 cells, fibroblasts, *in vitro*.

On the day of the assay, half of the target cells were pulsed with synthetic gB<sub>498-505</sub>, an immunodominant H-2K<sup>b</sup> restricted HSV-encoded epitope specific to C57BL/6 mice exposed to HSV-1, while simultaneously being ^[^1]Cr labeled for 2 hours at 37°C (Bonneau, et al., 1991; Carter, Schaffer, & Tevethia, 1981; Jennings, 1985). In a report by Bonneau (1996), the effectiveness of T lymphocyte lysis of HSV-1 labeled target cells was compared across different HSV-1 label types. Two synthetic epitopes, gB<sub>498-505</sub> and RRI<sub>822-829</sub>, were compared to HSV-1 infected target cells. The gB<sub>498-505</sub> synthetic epitope was the best label at activating the T lymphocytes, which was determined by the greatest % ^[^1]Cr released and the
most linear line of % $^{51}$Cr release across the effector:target ratios (Bonneau, 1996). Therefore, in this dissertation the gB$_{498-505}$ synthetic epitope was used to label the target cells because it has provided better $^{51}$Cr release assay data and it is safer for the researcher conducting the assay as compared to infecting the target cells with HSV-1 or labeling with RRI$_{822-829}$ (Bonneau, 1996).

MOCK-pulsed or non-HSV-1 specific labeled B6/WT-3 cells (control) also were incubated with the equivalent volume of media without the gB$_{498-505}$ synthetic peptide at $37^\circ$C in the presence of $^{51}$Cr for 2 hours. A volume of 0.1 mL 1 x $10^4$ B6/WT-3 cells was added to a 96-well V-bottom microtiter plate and an equal volume of viable effector cells at a concentration necessary to give the maximally attainable effector-to-target ratio. The effector-to-target cell ratios were serially diluted for each sample and sample were run in triplicate. The serial dilutions for each sample included 100:1 down to 3.12:1. The plates were centrifuged at 60g for 5 minutes and were incubated at $37^\circ$C in 5% CO$_2$ for 4-5 hours. Following the incubation, plates were centrifuged and 100 $\mu$L of supernatant were removed from each well. The amount of $^{51}$Cr for each well was quantified via gamma counter (2470 Automatic, PerkinElmer, Waltham, MA). See Appendix F for the $^{51}$Cr release assay protocol.

Spontaneous release included 100 $\mu$L of target cells with 100 $\mu$L of media. The $^{51}$Cr release from the spontaneous wells indicates the amount of $^{51}$Cr release that occurs due to the function of the assay, such as the cell membrane of target cells bursting due pipetting. Maximum release included 100 $\mu$L of target cells with 100 $\mu$L of SDS, a detergent. The $^{51}$Cr release from the maximum wells indicates the amount of $^{51}$Cr release that occurs when all
target cells are lysed. The percentage of specific $^{51}$Cr release was determined by the following formula:

$$\% \text{ Specific } ^{51}\text{Cr release} = \frac{\% \text{ release (effector cells)} - \% \text{ release (spontaneous)}}{\% \text{ release (maximum)} - \% \text{ release (spontaneous)}}</nolatex>

Area under the curve (AUC) for the $^{51}$Cr release assay was calculated using Excel 2007 (Microsoft Office; Redmond, Washington) to determine the best fit polynomial equation. Due to the results of the assay, the % specific $^{51}$Cr release of the following effector:target ratios were used to create the line from which AUC was calculated: 12.5:1, 6.25:1, 3.12:1. AUC for the $^{51}$Cr lysis allows for the investigation of the ability of the T lymphocytes to lyse across the multiple E:T ratios and provides an “overall” lysis measurement that analyzing one E:T ratio is unable to provide. Using these 3 data points, a line of % specific $^{51}$Cr release was created for each mouse using the AUC graphing function in Excel. The “trendline” option allowed for the calculation of the best fit polynomial equation for each mouse. Best fit is defined by the $R^2 = 1.0$. The polynomial equation of each mouse was then integrated to calculate the area under the curve using “Definite Integral Calculator” from the Solve My Math website (www.solvemymath.com/online_math_calculator/calculus/definite_integral/index.php, 2009) with an upper limit for “x” equal to 1.25 and a lower limit equal to 0.5. Additionally, the supernatant from the 3-day lymphocyte culture was analyzed for IFN$\gamma$ levels by commercially available EIA kits (R&D Systems; Minneapolis, MN). See Appendix G for assay kit protocol.
**Treatment of Data**

Popliteal lymph nodes were not collected at necropsy for one male mouse in the LOW nicotine-treatment group; therefore, immune data from this mouse are not included in the following analyses. Due to expected sex differences in liver weight (reported below), serum cotinine levels (ng/mL) were adjusted for liver weight (g) and nicotine consumption was adjusted for body weight (mg nicotine/kg body weight) for each animal during nicotine treatment and serum corticosterone (ng/mL/g) was adjusted for body weight to allow for comparisons between males and females (Klein et al., 2004). Natural logarithmic transformations were applied to the following dependent variables: cotinine adjusted for liver weight, corticosterone adjusted for body weight, and 3-day tissue culture supernatant IFN-γ levels, because they were not normally distributed; this transformation resulted in a normal distribution of the data (Klein et al., 2004). All reported analyses are based on natural log-transformed values for the listed dependent variables; raw adjusted mean values (+ S.E.M.) were used in tables and to graph data for clarity (Klein et al., 2004) unless otherwise noted.

**Statistical Analyses**

Data were entered into Statistical Program for the Social Sciences [SPSS (SPSS; Chicago, IL)] for statistical analyses. A 2 (sex; male and female) x 3 (nicotine treatment; CONTROL, LOW, and HIGH) between-subjects design was used to examine the effects of sex and nicotine treatment on (a) baseline weight, food and water intake, (b) body weight, food consumption, fluid intake, and nicotine consumption by pre- and post- HSV-1 injection phases, (c) serum cotinine levels, (d) serum corticosterone levels, (e) number of lymphocytes isolated from the popliteal lymph nodes, (f) IFN-γ levels from the 3-day incubation supernatant, and (g) the area under the curve (AUC) of $^{51}$Cr release assay % specific lysis.
All statistical analyses included cohort as a covariate to control for potential cohort differences in body weight, food consumption, fluid intake, and other items beyond control of experimenter (e.g., dam, transportation, birth order, litter size). Specifically, cohort was dummy coded into five new variables that were entered into separate analysis of covariance (ANCOVA) models.

Separate, repeated-measures, 2-way ANCOVAs, with sex (two levels) and nicotine treatment (3 levels) as the independent variables, time (e.g., baseline, pre-injection nicotine treatment, post-injection nicotine treatment) as the within-subjects variable, and cohort as the covariate, were conducted to examine group differences in body weight (g), food consumption (g), fluid intake (water/nicotine; mL) and nicotine consumption (mg/kg) among nicotine treated animals. Bottle weights were measured daily from which volume of intake was calculated. In addition, dosage of nicotine consumed each day was calculated to control for animal body weight when comparing consumption. Separate, 2-way ANCOVAs, with sex (two levels) and nicotine treatment (3 levels) as the independent variables, were conducted to examine group differences in serum cotinine (ng/mL) and serum cotinine adjusted for liver weight (ng/mL/g) in nicotine treated animals and serum corticosterone (ng/mL), corticosterone adjusted for body weight (ng/mL/g), number of lymphocytes, IFN-γ from the 3-day incubation supernatant (ng/mL), % $^{51}$Cr release at each E:T ratio and the area under the curve (AUC) of $^{51}$Cr release assay % specific lysis from all animals.

All statistical tests were two-tailed and significance level was $\alpha=0.05$ level. Tukey’s B and Bonferroni were used as appropriate for post-hoc analyses to explore group differences. ANCOVA estimated adjusted marginal means for the cohort covariate ($\pm$ SEM) are reported in the results section, tables, and figures, unless otherwise noted.
Chapter III: Results

Baseline

Table 2 presents the averaged two-day baseline body weight (g), food consumption (g), and water intake (mL) for all animals by sex and nicotine treatment group.

Body Weight (g). All mice gained weight across the 2-day baseline period \( F(2,212) = 207.98, p<0.05 \). Males weighed more \( F(1,106) = 24.01, p<0.05 \) and gained weight at a faster rate [Time X Sex interaction: \( F(2,212) = 85.04, p<0.05 \)] than did the females across the 2-day time period. Importantly, body weight did not differ among mice assigned to the three nicotine treatment groups. Time did not interact with nicotine treatment group nor was there a statistically significant time X sex X nicotine treatment group interaction.

Table 2: Averaged two-day baseline body weight (g), food consumption (g), and water intake (mL) for all animals by sex and nicotine treatment group (means ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Body Weight (g) ( ^a )</th>
<th>Food Consumption (g) ( ^a )</th>
<th>Water Intake (mL) ( ^a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=19)</td>
<td>14.29 ± 0.42</td>
<td>4.61 ± 0.08</td>
<td>6.30 ± 0.15</td>
</tr>
<tr>
<td>Low (n=20)</td>
<td>14.41 ± 0.43</td>
<td>4.65 ± 0.11</td>
<td>6.52 ± 0.21</td>
</tr>
<tr>
<td>High (n=20)</td>
<td>14.49 ± 0.39</td>
<td>4.61 ± 0.09</td>
<td>6.26 ± 0.17</td>
</tr>
<tr>
<td>Total (n=59)</td>
<td>14.40 ± 0.24</td>
<td>4.63 ± 0.05</td>
<td>6.36 ± 0.10</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>12.86 ± 0.36</td>
<td>4.36 ± 0.12</td>
<td>5.99 ± 0.15</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>13.14 ± 0.39</td>
<td>4.25 ± 0.11</td>
<td>6.21 ± 0.22</td>
</tr>
<tr>
<td>High (n=19)</td>
<td>13.05 ± 0.30</td>
<td>4.32 ± 0.10</td>
<td>5.95 ± 0.16</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>13.01 ± 0.20</td>
<td>4.31 ± 0.06</td>
<td>6.05 ± 0.10</td>
</tr>
</tbody>
</table>

\( ^a \) p<0.05, Males>Females

Food Consumption (g). Food consumption paralleled baseline body weight such that male mice ate more food \( F(1,106) = 19.41, p<0.05 \) and increased their food consumption more quickly across the 2-day baseline period as compared to female mice [Time X Sex interaction: \( F(1,106) = 12.75, p<0.05 \)]. Food consumption did not differ among mice
assigned to the three nicotine treatment groups. Consistent with baseline body weight, time did not interact with nicotine treatment group nor was there a time X sex X nicotine treatment group interaction.

**Water Intake (mL).** Water intake (mL) also increased across the 2-day baseline period \(F(1,106) = 11.81, \ p<0.05\), and male mice consumed more water than did female mice \(F(1,106) = 5.33, \ p<0.05\). Water consumption did not differ among mice assigned to the three nicotine treatment groups. Time did not interact with nicotine treatment group or sex nor was there a time X sex X nicotine treatment group interaction.

**Nicotine Treatment**

Table 3 presents the averaged body weight (g), food consumption (g), and fluid intake (mL) for all animals by sex and nicotine treatment group before (i.e., pre-injection phase) and after (i.e., post-injection phase) the HSV-1 rear footpad injection. Figure 3 presents the body weight (g) for all animals by sex and nicotine treatment group across the 12-day nicotine treatment period.

**Body Weight (g).** Prior to the HSV-1 injection, all animals gained weight across the 7-day forced nicotine treatment period \(time\ effect: F(7,742) = 92.34, \ p<0.05\). Males had more accelerated weight gain than did the females \(time \times sex \ interaction: F(7,742) = 70.66, \ p<0.05\). There also was a significant time X nicotine treatment interaction \(F(14,742) = 5.83, \ p<0.05\) such that body weight gains were different depending on nicotine exposure. The time effect on body weight prior to the injection for the CONTROL and LOW groups followed a quadratic function \(F's(1,33) > 17.74, \ p's<0.05\) and for the HIGH group followed a linear function \(F(1,33) = 21.99, \ p<0.05\). The difference in the pre-injection body weight growth trajectory between the both the CONTROL and LOW groups and the HIGH group
suggests that the growth pattern of the HIGH group was modified by the high nicotine exposure as compared to the growth pattern observed in the CONTROL and LOW groups. There was no time X sex X nicotine treatment group interaction on body weight prior to the injection.

**Table 3: Averaged body weight (g) and food consumption (g) for all animals during the nicotine treatment period before (7 days) and after (5 days) HSV-1 rear footpad injections by sex and nicotine treatment group (adjusted means ± SEM).**

<table>
<thead>
<tr>
<th></th>
<th>Pre-Injection Phase</th>
<th>Post-Injection Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body Weight (g)</td>
<td>Food Consumption (g)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=19)</td>
<td>17.90 ± 0.29</td>
<td>4.78 ± 0.06</td>
</tr>
<tr>
<td>Low (n=20)</td>
<td>18.24 ± 0.28</td>
<td>4.79 ± 0.06</td>
</tr>
<tr>
<td>High (n=20)</td>
<td>17.56 ± 0.28</td>
<td>4.59 ± 0.06</td>
</tr>
<tr>
<td>Total (n=59)</td>
<td>17.90 ± 0.16</td>
<td>4.72 ± 0.03</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>14.81 ± 0.28</td>
<td>4.21 ± 0.06</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>15.34 ± 0.29</td>
<td>4.29 ± 0.06</td>
</tr>
<tr>
<td>High (n=19)</td>
<td>14.74 ± 0.29</td>
<td>4.14 ± 0.06</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>14.96 ± 0.16</td>
<td>4.21 ± 0.03</td>
</tr>
</tbody>
</table>

During the 5-day post-injection phase, all animals gained weight [time effect: $F(4,424) = 46.74, p<0.05$]; males continued to have accelerated weight gain compared to the females after infection [time X sex interaction: $F(4,424) = 3.69, p<0.05$]. There was neither a time X nicotine treatment group nor a time X sex X nicotine treatment group interaction on body weight post-injection.

Male mice weighed more than female mice on each day of nicotine treatment and on the day of sacrifice [$F$’s(1,106) > 50.48, $p$’s<0.05]. In addition, HIGH mice weighed less than did the LOW mice during days 2-8 of the nicotine testing period [$F$’s (2,106) > 3.15, $p$’s<0.05], which occurred prior to the HSV-1 injection. The body weight differences among
the nicotine treatment groups were no longer observed following the injection [F’s (2,106) < 2.82, p’s > 0.05]. The body weight of the control group did not differ from either nicotine treatment group throughout the 12-day nicotine treatment period.

Figure 3. Body weight (g) for all animals by sex (59 males; 58 females) and nicotine treatment groups (39 control; 39 low nicotine; 39 high nicotine) at baseline (day 0) and across the 12-day nicotine treatment period (adjusted means ± SEM).
Due to loss of the nicotine treatment effect on body weight after the injection, it is reasonable to conclude that the infection protocol resulted in an acute stressor. Therefore, the effect of the injection on body weight (g) and 24-hr pre- and post-injection behavioral measures, including food consumption (g), fluid intake (mL), and nicotine consumption (mg/kg), were analyzed. Using a RM-ANCOVA on day 8 and 9 body weights of the nicotine treatment period, all animals gained weight [time effect: $F(1,106) = 5.34, p<0.05$] regardless of sex or nicotine treatment group. Time did not interact with either sex or nicotine treatment group and there was no time X sex X nicotine treatment group interaction.

**Food Consumption (g).** Figure 4 presents food consumption (g) for all animals by sex and nicotine treatment group across the 12-day nicotine treatment period. Prior to the HSV-1 injection, on average food consumption increased across the 7-day nicotine testing period [$F(6,636) = 26.47, p<0.05$]. Food consumption prior to the injection varied by nicotine treatment group [$F(12,636) = 4.41, p<0.05$]; the LOW and HIGH groups followed a 6th order polynomial function [$F's(1,33) > 44.96, p's<0.05$] and the CONTROL group followed a 4th order polynomial function [$F(1,33) = 11.48, p<0.05$]. Therefore, food consumption for the nicotine treatment groups was less consistent across the 7-day forced nicotine treatment period (i.e., food consumption increased and decreased more often day to day) compared to the control group. There was neither a time X sex nor a time X sex X nicotine treatment group interaction on food consumption prior to the HSV-1 injection.

During the 5-day post-injection phase, there was a significant time effect [$F(4,424) = 9.24, p<0.05$]. Although males and females increased food intake over this time period, male mice ate more food over time compared to the female mice [time X sex interaction: $F(4,424)$}
= 3.77, \( p<0.05 \)]. There was neither a time X nicotine treatment group nor a time X sex X nicotine treatment group interaction on food consumption post-injection.

Figure 4. Food consumption (g) for all animals by sex (59 males; 58 females) and nicotine treatment groups (39 control; 39 low nicotine; 39 high nicotine) across the 12-day nicotine treatment period (adjusted means ± SEM).
Male mice ate more food than did female mice on each day of nicotine treatment \([F(1,106) > 15.11, p’s<0.05]\). ANCOVAs revealed that nicotine treatment effected food consumption on days 1, 6, and 12 of the nicotine testing period \([F(2,106) > 4.04, p’s<0.05]\). Specifically, on day 1 of nicotine treatment, the HIGH group consumed less food than both the LOW and CONTROL groups, who ate similar amounts. On day 6 of nicotine treatment, the HIGH group consumed less food than the LOW group; however, the CONTROL group did not differ from either nicotine group. On the final day of nicotine treatment and 5 days post-injection, both nicotine treatment groups consumed less food than the CONTROL group, but did not significantly differ from each other. There was no nicotine treatment group effect on food consumption on the days 2-5 or 7-11 of nicotine treatment group \([F(2,106) < 2.60, p’s>0.05]\).

The effect of the injection on food consumption was analyzed using a RM-ANCOVA on day 7 and 8 of the treatment period. All animals consumed less food during the 24-hours following the injection [time effect: \(F(1,106) = 21.44, p<0.05\)] regardless of sex or nicotine treatment group.

**Fluid Intake (mL).** Figure 5 presents the fluid intake (mL) for all animals by sex and nicotine treatment group across the nicotine treatment period. Table 4 presents the averaged fluid intake (mL) and nicotine dosage (mg/kg) for all animals during the nicotine treatment period before (i.e., pre-injection phase) and after (i.e., post-injection phase) the HSV-1 rear footpad injections by sex and nicotine treatment group. Prior to the HSV-1 injection, there was a statistically significant time effect across the 7-day nicotine testing period nicotine \([F(6,636) = 64.21, p<0.05]\) that followed a cubic function \([F(1,106) = 226.40, p<0.05]\). There also was a statistically significant time X sex interaction \([F(6,636) = 2.15, p<0.05]\).
Although the fluid intake during the pre-injection phase of both sexes followed a cubic function, the fluid intake of females followed a cubic function more closely compared to the males \([F(1,52) = 211.05, p<0.05, \text{ and } F(1,53) = 57.25, p<0.05, \text{ respectively}]\). There also was a statistically significant time X nicotine treatment interaction \([F(12,636) = 13.82, p<0.05]\). Although the fluid intake during the pre-injection phase of all nicotine treatment groups was significant and followed a cubic function, the fluid intake of the HIGH treatment group followed the cubic function more closely compared to the LOW treatment group followed by the CONTROL group \([F(1,33) = 162.94, p<0.05, \text{ and } F(1,33) = 74.75, p<0.05, \text{ and } F(1,33) = 58.34, p<0.05, \text{ respectively}]\). The cubic function such that fluid intake increases, then decreases, then elevates slightly and stabilizes is consistent with previous reports on the introduction of nicotine in periadolescent mice (e.g., Klein et al., 2004). There was no time X sex X nicotine treatment group interaction on fluid intake.

During the 5-day post-injection phase, there was a significant time effect \([F(4,424) = 6.37, p<0.05]\). There also was a significant time X nicotine treatment group interaction \([F(8,424) = 13.55, p<0.05]\). The fluid intake across the infection period for both nicotine treatment groups was significant and followed a cubic function \([F(1,33)’s > 71.81, p’<0.05]\); however, there was no significant time effect for the CONTROL group. Similar to pre-injection phase, after the injection, there was an increase, decrease, and increase to stabilization in the nicotine exposed mice, but not the CONTROL mice. There was neither significant time X sex nor time X sex X nicotine treatment group interaction on fluid intake post-injection.
Figure 5. Fluid intake for all animals by sex (59 males; 58 females) and nicotine treatment groups (39 control; 39 low nicotine; 39 high nicotine) across the 12-day nicotine treatment period (adjusted means ± SEM).
Male mice drank more fluid than did female mice \([F'(s(1,106)) > 10.160, p's<.0001]\) except on day 11. In addition, the ANCOVAs revealed a nicotine treatment difference on each day \([F'(s (2,106)) > 5.02, p's<0.05]\). Specifically, on days 1 and 7 of nicotine treatment, the HIGH group consumed less fluid than did the CONTROL, which consumed less fluid than the LOW group. On days 2, 5, 6, 8, and 10 of nicotine treatment, the HIGH group consumed less fluid than did both the LOW and CONTROL groups, who drank similar amounts. On day 3 of nicotine treatment, the HIGH group consumed less fluid than did the LOW group which consumed less fluid than did the CONTROL group. On days 4 and 9 of nicotine treatment, the HIGH group and CONTROL group consumed similar amounts of fluid, however, both consumed less than the LOW group. On day 11 of nicotine treatment, the LOW group consumed similar amount of fluid compared to the CONTROL and HIGH groups; however, the HIGH nicotine group drank less than the CONTROL. On the final day of nicotine treatment and 5 days post-injection, the CONTROL group drank less fluid than the HIGH group, which consumed less fluid than the LOW group.

The effect of the injection on fluid intake was analyzed using a RM-ANCOVA on day 7 and 8 of the treatment period. All animals consumed less fluid during the 24-hours following the injection \([\text{time effect: } F(1,106) = 15.85, p<0.05]\) regardless of sex. The decrease in fluid intake due to the injection was greater for the LOW and HIGH treatment groups (1.9 mL and 1.5 mL, respectively) compared to the CONTROL group \([0.8 \text{ mL}; \text{ time X nicotine treatment effect: } F(2,106) = 11.68, p<0.05]\).
Table 4: Averaged fluid intake (mL) and nicotine dosage (mg/kg) for all animals during the nicotine treatment period before (7 days) and after (5 days) HSV-1 rear footpad injections by sex and nicotine treatment group (adjusted means ± SEM).

<table>
<thead>
<tr>
<th>Sex</th>
<th>Pre-Injection Phase</th>
<th>Post-Injection Phase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Fluid Intake (mL)</td>
<td>Nicotine Dosage (mg/kg)</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=19)</td>
<td>7.31 ± 0.12</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Low (n=20)</td>
<td>7.79 ± 0.12</td>
<td>21.09 ± 0.92</td>
</tr>
<tr>
<td>High (n=20)</td>
<td>6.12 ± 0.12</td>
<td>69.03 ± 0.92</td>
</tr>
<tr>
<td>Total (n=59)</td>
<td>7.08 ± 0.07</td>
<td>n/a</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>6.62 ± 0.12</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>6.65 ± 0.12</td>
<td>21.46 ± 0.94</td>
</tr>
<tr>
<td>High (n=19)</td>
<td>5.37 ± 0.12</td>
<td>72.57 ± 0.94</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>6.21 ± 0.07</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Nicotine Dosage (mg/kg). The control group was not included in the following analyses due to their lack of nicotine exposure. Figure 6 presents the nicotine dosage (mg/kg) for nicotine-treated animals by sex and nicotine treatment group across the nicotine treatment period. Prior to the HSV-1 injection, there was a significant time effect across the 7-day nicotine testing period nicotine \( [F(4,276) = 17.36, p<0.05] \) that followed a cubic function \( [F(1,69) = 62.36, p<0.05] \). There was a significant time X sex interaction \( [F(4,276) = 57.93, p<0.05] \). Males nicotine dosage during the pre-injection phase followed a cubic function \( [F(1,34) = 26.06, p<0.05] \) while the females nicotine dosage followed a linear function \( [F(1,32) = 36.50, p<0.05] \). There also was a significant time X nicotine treatment interaction \( [F(4,276) = 57.93, p<0.05] \). The nicotine dosage of the HIGH treatment during the pre-injection phase followed a cubic function \( [F(1,33) = 140.54, p<0.05] \) while the
nicotine dosage of the LOW treatment followed a linear function \( F(1,33) = 78.68, p<0.05 \). There was no time X sex X nicotine treatment group interaction on nicotine dosage.

During the post-injection phase, there was a significant time effect across the 5-day forced nicotine treatment period plus the infection \( F(4,276) = 17.36, p<0.05 \). There also was a significant time X nicotine treatment group interaction \( F(4,276) = 57.93, p<0.05 \). Although both nicotine treatment groups followed a cubic function, the cubic function for the LOW treatment group was a better fit compared to the HIGH treatment group \( F(1,33) = 114.42, p<0.05 \), and \( F(1,33) = 42.49, p<0.05 \), respectively. Upon reviewing Figure 6, a possible explanation for the differences in degree of fit for the cubic function is that the changes in nicotine consumption of the LOW nicotine mice was less drastic compared to the HIGH nicotine mice. This cubic function mirrors the fluid intake analyses as well. There was neither a time X sex nor a time X sex X nicotine treatment group interaction on nicotine dosage post-injection.

Separate two-way ANCOVAs revealed a sex difference on days 7, 8, 9, 11, and 12 of nicotine treatment \( F'(s)(1,106) > 5.86, p's<0.05 \). Specifically, on days 7 and 8 female mice in the HIGH group consumed a greater nicotine dosage than their male counterparts \( F'(s)(1,32) > 5.91, p's<0.05 \). On days 9, 11, and 12, all female mice consumed greater nicotine dosage than did their male counterparts \( F'(s)(1,32) > 4.96, p's<0.05 \). In addition, separate ANCOVAs revealed a nicotine treatment difference on each day \( F'(s)(1,69) > 660.50, p's<0.05 \). As expected, the HIGH group consumed more nicotine (mg/kg) compared to the LOW group across the 12-day treatment period.

The effect of the injection on nicotine dosage (mg/kg) was analyzed using a RM-ANCOVA on day 7 and 8 of the treatment period. All animals consumed less nicotine
(mg/kg) during the 24-hours following the injection [time effect: $F(1,69) = 7.70, p<0.05$] regardless of sex. The decrease in nicotine (mg/kg) due to the injection was greater for the HIGH treatment group (17.3 mg/kg) compared to the LOW treatment group [5.2 mg/kg; time X nicotine treatment effect: $F(1,69) = 52.17, p<0.05$].

Figure 6. Nicotine dosage (mg/kg) for nicotine treated animals by (40 males; 38 females) and nicotine treatment groups (39 low nicotine; 39 high nicotine) across the 12-day nicotine treatment period (adjusted means ± SEM).
Cotinine and Corticosterone

Table 5 presents the liver weight (g), serum cotinine (ng/mL), serum cotinine adjusted for liver weight (ng/mL/g), and serum corticosterone adjusted for body weight (ng/mL/g) for all animals by sex and nicotine treatment group. Table 6 presents the body weight at sacrifice (g), serum corticosterone (ng/mL), and serum corticosterone adjusted for body weight (ng/mL/g) for all animals by sex and nicotine treatment group.

**Liver Weight (g).** As expected, the livers from male mice weigh more than did the livers from female mice \[F(1,106) = 190.44, p < 0.05\]. Liver weights were similar across nicotine treatment groups and there was no sex X nicotine treatment group interaction.

**Table 5: Liver weight (g), serum cotinine (ng/mL), and serum cotinine adjusted for liver weight (ng/mL/g) for all animals by sex and nicotine treatment group (adjusted means ± SEM).**

<table>
<thead>
<tr>
<th></th>
<th>Liver Weight (g)</th>
<th>Cotinine (ng/mL)</th>
<th>Cotinine Adjusted for Liver Weight (ng/mL/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=19)</td>
<td>1.26 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Low (n=20)</td>
<td>1.27 ± 0.03</td>
<td>71.84 ± 154.59</td>
<td>64.85 ± 114.98</td>
</tr>
<tr>
<td>High (n=20)</td>
<td>1.23 ± 0.03</td>
<td>1505.47 ± 154.74</td>
<td>1179.91 ± 114.92</td>
</tr>
<tr>
<td>Total (n=59)</td>
<td>1.26 ± 0.02</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>0.97 ± 0.03</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>1.00 ± 0.03</td>
<td>202.03 ± 158.46</td>
<td>205.09 ± 117.87</td>
</tr>
<tr>
<td>High (n=19)</td>
<td>0.94 ± 0.03</td>
<td>1722.93 ± 158.62</td>
<td>1775.50 ± 117.92</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>0.97 ± 0.02</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

\[ a \ p < 0.05, \text{Males} > \text{Females} \]
\[ b \ p < 0.05, \text{Females} > \text{Males} \]
\[ c \ p < 0.05, \text{HIGH} > \text{LOW} \]

**Serum Cotinine (ng/mL).** The control group was not included in the following analyses due to their lack of nicotine exposure. Female mice had a significantly higher serum cotinine levels (ng/mL) compared to male mice \[F(1,69) = 14.64, p < 0.05\]; and as
expected cotinine levels (ng/mL) were higher among HIGH nicotine mice compared to LOW nicotine mice \[F(2,69) = 224.99 \, p<0.05\]. It is important to note that the 3-4 fold difference in observed in nicotine consumption (mg/kg) on day 12 was not observed in cotinine levels at sacrifice. One possible reason is the metabolism of nicotine is extremely fast in a mouse; therefore, it is readily converted into cotinine, which has a much longer half-life. Thus, the mouse may continue consume nicotine and metabolize it to cotinine; however, the kidney can only clear the cotinine at a steady rate which results in elevated serum cotinine at a greater concentration than the difference in nicotine consumption (mg/kg) may suggest.

Serum Cotinine Adjusted for Liver Weight (ng/mL/g). The control group was not included in the following analyses due to their lack of nicotine exposure. Female mice had a significantly higher serum cotinine levels (ng/mL/g) compared to male mice \[F (1,69) = 14.64, \, p<0.05\]; and as expected cotinine levels (ng/mL/g) were higher among HIGH nicotine mice compared to LOW nicotine mice\[F(2,69) = 224.99 \, p<0.05\].

Serum Corticosterone (ng/mL). Using a partial correlation, serum corticosterone (ng/mL) was negatively correlated with sacrifice order \[r(110) = -0.46, \, p<0.05\], such that corticosterone levels went down across the sacrifice order. Due to this relationship between the corticosterone and sacrifice order, animals were grouped in 4’s based on sacrifice order for a total of 5 sacrifice order groups. A chi-square analysis was used to confirm that equal representation of nicotine treatment group and sex was present across all sacrifice groups \[\chi^2 (8,117) = 1.79, \, n.s.; \, \chi^2 (4,117) = 0.04, \, n.s.; \, \text{respectively}\] which ensured that there was no dependence among the independent variables.

Next, a one-way ANCOVA including this sacrifice group (n= 5 groups) as the independent variable revealed that corticosterone levels from the first group (i.e., the first
four animals of each necropsy session) were significantly higher compared to the later groups, which were all similar to one another \( F(4,107) = 12.11, p < 0.05 \). Therefore, sacrifice group was used as a covariate in the following serum corticosterone (ng/mL) analyses. It is important to note that inclusion or exclusion of sacrifice group in the statistical model did not change the following results.

A two-way ANCOVA did not reveal any significant effects for sex or nicotine treatment or sex X nicotine treatment group interaction \( F's < 1.67, p's > 0.05 \). Using partial correlations, serum corticosterone (ng/mL) did not correlate with self-administered nicotine dosage (mg/kg) on day 12 or total nicotine (mg/kg) across the 12 days or with serum cotinine (ng/mL/g) in nicotine exposed animals.

Table 6: Body weight at sacrifice (g), serum corticosterone (ng/mL), and serum corticosterone adjusted for body weight (ng/mL/g) for all animals by sex and nicotine treatment group (adjusted means ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Body Weight at Sacrifice (g) ( \bar{x} \pm SE )</th>
<th>Corticosterone (ng/mL) ( \bar{x} \pm SE )</th>
<th>Corticosterone Adjusted for Body Weight (ng/mL/g) ( \bar{x} \pm SE )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>20.29 ± 0.30</td>
<td>45.59 ± 8.59</td>
<td>22.12 ± 5.39</td>
</tr>
<tr>
<td>Low</td>
<td>20.74 ± 0.29</td>
<td>34.67 ± 8.31</td>
<td>16.79 ± 5.26</td>
</tr>
<tr>
<td>High</td>
<td>20.24 ± 0.29</td>
<td>42.47 ± 8.31</td>
<td>21.35 ± 5.25</td>
</tr>
<tr>
<td>Total</td>
<td>20.49 ± 0.17</td>
<td>40.90 ± 4.83</td>
<td>20.09 ± 3.06</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>16.65 ± 0.29</td>
<td>57.22 ± 8.31</td>
<td>34.41 ± 5.25</td>
</tr>
<tr>
<td>Low</td>
<td>17.18 ± 0.30</td>
<td>41.52 ± 8.53</td>
<td>26.09 ± 5.39</td>
</tr>
<tr>
<td>High</td>
<td>16.56 ± 0.30</td>
<td>40.31 ± 8.53</td>
<td>24.42 ± 5.39</td>
</tr>
<tr>
<td>Total</td>
<td>16.79 ± 0.17</td>
<td>46.35 ± 4.87</td>
<td>28.31 ± 3.08</td>
</tr>
</tbody>
</table>

\( ^a p<0.05, \) Males>Females

Serum Corticosterone Adjusted for Body Weight (ng/mL/g). Due to the significant body weight differences between the sexes, serum corticosterone was adjusted for sacrifice
day body weight. The same analyses were conducted as described in the previous serum corticosterone (ng/mL) section. It is important to note that the results of the following analyses were similar regardless of whether or not serum corticosterone was adjusted for body weight.

Using a partial correlation, serum corticosterone (ng/mL/g) was negatively correlated with sacrifice order \[ r(110) = -0.44, p<0.05 \], such that corticosterone levels went down across the sacrifice order. A chi-square analysis comparing sacrifice group across nicotine treatment group and sex remained non-significant \[ \chi^2 (8, 117) = 1.79, \text{n.s.}; \chi^2 (4,117) =0 .04, \text{n.s.}; \text{respectively}\].

Next, a one-way ANCOVA including sacrifice group (n= 5 groups) as the independent variable revealed that corticosterone levels from the first group (i.e., the first four animals of each necropsy session) were significantly higher compared to the later groups, which were all similar to one another \[ F(4,107) = 10.90, p<0.05 \]. Therefore, sacrifice group was used as a covariate in the following serum corticosterone (ng/mL/g) analyses. It is important to note that inclusion or exclusion of sacrifice group in the statistical model did not change the following results.

A two-way ANCOVA did not reveal any significant effects for sex or nicotine treatment or sex X nicotine treatment group interaction \[ F’s < 3.00, p’s > 0.05 \]. Using partial correlations, serum corticosterone (ng/mL/g) did not correlate with self-administered nicotine dosage (mg/kg) on day 12 or total nicotine (mg/kg) across the 12 days or with serum cotinine (ng/mL/g) in nicotine exposed animals.
Measures of Immune Function

Table 7 presents the number of lymphocytes isolated per lymph node, number of lymphocytes isolated per lymph node adjusted for body weight at sacrifice \( (g^{-1}) \), and IFN-\( \gamma \) levels \( (\text{ng/mL}) \) from the 3-day incubation for all animals by sex and nicotine treatment group. Tables 8 and 9 present the % lysis of the peptide-pulsed and MOCK-pulsed target cells, respectively, from the \( ^{51} \text{Cr} \) release assay at each effector:target \( (E:T) \) ratio and area under the curve \( (\text{AUC}) \) for all animals by sex and nicotine treatment group. Table 10 provides a summary of all significant and non-significant findings with regards to the measures of immune function.

**Lymphocyte Cell Number.** A greater number of lymphocytes were isolated from the male mice than the female mice \( [F(1,105) = 7.22, p<0.05] \). There was no nicotine treatment effect or sex X nicotine treatment group interaction. However, at sacrifice, males weighed significantly more than did females, and, in rodents, organ weight is correlated with body weight (e.g., Bailey, Zidell, & Perry, 2004); therefore, relative organ weights can created by adjusting for body weight (e.g., Deitch, Xu, Qi, Specian, & Berg, 1992). Using all animals, body weight at sacrifice was positively correlated with number of lymphocytes isolated per lymph node \( [r(109) = +0.32, p<0.05] \). Therefore, the number of lymphocytes isolated per lymph node was adjusted by the sacrifice body weight and analyzed.

Figure 7 presents the number of lymphocytes isolated per lymph node adjusted for body weight \( (g^{-1}) \) at sacrifice for all animals by sex and nicotine treatment group. This ANCOVA revealed that females had a greater number of lymphocytes per lymph node per gram of body weight than the males did \( [F(1,105) = 4.75, p<0.05] \). Upon further investigation, although all females had a great number of lymphocytes per lymph node per
gram of body weight despite nicotine treatment, this sex effect was only statistically significant for the LOW nicotine group \[F(1,31) = 4.44, p<0.05\]. There was no nicotine treatment effect or sex X nicotine treatment group interaction.

Table 7: Number of lymphocytes isolated per lymph node, number of lymphocytes isolated per lymph node adjusted for body weight at sacrifice (g⁻¹), and IFN-γ levels (ng/mL) from the 3-day incubation for all animals by sex and nicotine treatment group (adjusted means ± SEM).

<table>
<thead>
<tr>
<th></th>
<th>Lymphocytes per Lymph Node (x10⁶)⁵</th>
<th>Lymphocytes per Lymph Node Adjusted for Body Weight (x10⁵ g⁻¹)⁶</th>
<th>IFN-γ Levels from 3-day incubation (ng/mL)²,³</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=19)</td>
<td>7.98 ± 0.37</td>
<td>3.90 ± 0.20</td>
<td>47.43 ± 10.52</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>7.35 ± 0.38</td>
<td>3.52 ± 0.20</td>
<td>30.78 ± 10.54</td>
</tr>
<tr>
<td>High (n=20)</td>
<td>7.88 ± 0.37</td>
<td>3.87 ± 0.20</td>
<td>22.80 ± 10.26</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>7.74 ± 0.21</td>
<td>3.77 ± 0.11</td>
<td>33.67 ± 6.02</td>
</tr>
<tr>
<td><strong>Female</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>6.87 ± 0.37</td>
<td>4.14 ± 0.20</td>
<td>64.32 ± 10.26</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>7.07 ± 0.37</td>
<td>4.10 ± 0.20</td>
<td>27.59 ± 10.51</td>
</tr>
<tr>
<td>High (n=19)</td>
<td>6.82 ± 0.37</td>
<td>4.12 ± 0.20</td>
<td>51.78 ± 10.52</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>6.92 ± 0.21</td>
<td>4.11 ± 0.11</td>
<td>47.90 ± 6.02</td>
</tr>
</tbody>
</table>

⁵ p<0.05, Males>Females
⁶ p<0.05, Females>Males
² p<0.05, CONTROL>LOW

Using partial correlations, neither lymphocytes isolated per lymph node nor lymphocytes isolated per lymph node adjusted for body weight from all animals were correlated with serum corticosterone (ng/mL) or corticosterone adjusted for body weight (ng/mL/g). Neither lymphocytes isolated per lymph node nor lymphocytes isolated per lymph node adjusted for body weight for nicotine exposed animals was correlated with self-
administered nicotine dosage (mg/kg) on day 12 or total nicotine (mg/kg) across the 12 days or with cotinine (ng/mL) or cotinine adjusted for body weight (ng/mL/g).

Figure 7. Number of lymphocytes per lymph node adjusted for body weight (g⁻¹) for all animals by sex (58 males; 58 females) and nicotine treatment groups (39 control; 38 low nicotine; 39 high nicotine) at sacrifice (adjusted means ± SEM).

¹ p<0.05, Females> Males
IFN-γ (ng/mL). Using a partial correlation, IFN-γ was positively correlated with sacrifice order \([r(109) = +0.22, p<0.05]\), such that IFN-γ production by lymphocytes increased across the sacrifice session. Therefore, sacrifice group was used as a covariate in the following IFN-γ analyses. It is important to note that inclusion or exclusion of sacrifice group in the statistical model did not change the following results.

Figure 8 presents the natural log transformed IFN-γ (ng/mL) level from the lymphocyte 3-day incubation for all animals by sex and nicotine treatment group. Note that natural log-transformed data were used in this graph for clarity as the abnormal distribution of the raw data greatly skewed group means which did not appear to support the following ANCOVA. For raw values, please see Table 7. T lymphocytes from female mice produced more IFN-γ than did their male mice counterparts \([F(1,104) = 7.44, p<0.05]\). Upon further investigation, although overall lymphocytes from females produced more IFN-γ compared to the lymphocytes from males, this sex effect was only significant for the CONTROL group \([F(1,31) = 9.65, p<0.05]\). The LOW group produced significantly less IFN-γ than did the CONTROL, while the HIGH group produced similar amounts of IFN-γ compared to the LOW and CONTROL groups \([F(2,104) = 4.72, p<0.05]\). Upon further investigation, the nicotine effect was found only in lymphocytes from females \([F(2,49) = 5.76, p<0.05]\). There was no sex X nicotine treatment group effect.

Using partial correlations, IFN-γ levels were negatively correlated with the number of lymphocytes isolated at necropsy per lymph node \([r(109) = -0.22, p<0.05]\). Further investigation revealed that this negative correlation only existed among the HIGH male group mice \([r(13) = -0.60, p<0.05]\). IFN-γ levels for all animals were not correlated with serum corticosterone (ng/mL) or corticosterone adjusted for body weight (ng/mL/g) or
number of lymphocytes isolated adjusted for body weight. IFN-γ levels for nicotine exposed animals were not correlated with self-administered nicotine dosage (mg/kg) on day 12 or total nicotine (mg/kg) across the 12 days or with serum cotinine (ng/mL) or cotinine adjusted for body weight (ng/mL/g).

Figure 8. Natural log-transformed IFN-γ levels (ng/mL) for all animals by sex (58 males; 58 females) and nicotine treatment groups (39 control; 38 low nicotine; 39 high nicotine) from the 3-day lymphocyte incubation (adjusted means ± SEM).
**51Cr Release Assay.** The peptide-pulsed target cells provide the T lymphocytes with HSV-1 specific target cells to lyse. The activated T lymphocytes from the mice are primed to recognize HSV-1 infected or the peptide-pulsed target cells and readily lyse them. Therefore, high $^{51}$Cr release from the peptide-pulsed target cells is expected. On the other hand, the MOCK-pulsed target cells expose the T lymphocytes to non-HSV-1 specific target cells or non-infected cells. The activated T lymphocytes with HSV-1 specific priming should not lyse the MOCK-pulsed target cells because they are not labeled for infection and, therefore, are considered a control for the assay. Thus, $^{51}$Cr release is not expected to vary across the E:T ratios. However, the MOCK-pulsed target cells provide an opportunity to observe how nicotine or sex might impact the ability of T lymphocytes to discriminate between infected and non-infected cells.

**Table 8: Percent lysis of peptide-pulsed target cells from the $^{51}$Cr release assay at each E:T ratio and area under the curve (AUC) for all animals by sex and nicotine treatment group (adjusted means ± SEM).**

<table>
<thead>
<tr>
<th></th>
<th>Peptide-Pulsed Target Cells</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3.12:1</td>
<td>6.25:1</td>
<td>12.5:1</td>
<td>AUC</td>
</tr>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=19)</td>
<td>24.81 ± 2.22</td>
<td>38.87 ± 3.19</td>
<td>45.42 ± 3.42</td>
<td>16.78 ± 1.61</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>25.16 ± 2.23</td>
<td>37.67 ± 3.20</td>
<td>47.45 ± 3.42</td>
<td>17.54 ± 1.61</td>
</tr>
<tr>
<td>High (n=20)</td>
<td>24.98 ± 2.17</td>
<td>38.62 ± 3.11</td>
<td>48.49 ± 3.33</td>
<td>17.22 ± 1.57</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>24.98 ± 1.27</td>
<td>38.39 ± 1.82</td>
<td>47.12 ± 1.95</td>
<td>17.18 ± 0.92</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (n=20)</td>
<td>25.93 ± 2.17</td>
<td>38.77 ± 3.11</td>
<td>50.94 ± 3.33</td>
<td>18.22 ± 1.57</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>25.27 ± 2.22</td>
<td>41.41 ± 3.19</td>
<td>49.77 ± 3.42</td>
<td>16.92 ± 1.61</td>
</tr>
<tr>
<td>High (n=19)</td>
<td>22.91 ± 2.22</td>
<td>35.69 ± 3.19</td>
<td>42.28 ± 3.42</td>
<td>15.51 ± 1.61</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>24.70 ± 1.27</td>
<td>38.62 ± 1.82</td>
<td>47.66 ± 1.95</td>
<td>16.88 ± 0.92</td>
</tr>
</tbody>
</table>

Using a partial correlation, peptide-pulsed E:T ratios 3.12:1 and 6.25:1 and the total AUC for % $^{51}$Cr release were negatively correlated with sacrifice order [$r$'s (109) > -0.22, $p$'s < 0.05], such that the % $^{51}$Cr release decreased across the necropsy session. None of the
MOCK-pulsed E:T ratios nor AUC %$^{51}$Cr release were significantly correlated with sacrifice order. Therefore, sacrifice group was used as a covariate in the peptide-pulsed target cells %$^{51}$Cr release analyses. It is important to note that inclusion or exclusion of sacrifice group in the statistical model did not change the following results.

Two-way ANCOVAs on the %$^{51}$Cr release of the peptide-pulsed target cells at three E:T ratios revealed no significant sex [F’s<0.08, p’s>0.05] or nicotine treatment effects [F’s<0.55, p>0.05], nor was there a sex X nicotine treatment group interaction [F’s<1.44, p’s>0.05]. A two-way ANCOVA on the AUC of the %$^{51}$Cr lysis of the peptide-pulsed target cells did not result in any statistically significant findings [F’s<0.45, p’s>0.05]. Similar analyses were performed for the %$^{51}$Cr release of the MOCK-pulsed target cells, which were not statistically significant [F’s<1.93, p’s>0.05].

Table 9: Percent lysis of MOCK-pulsed target cells from the $^{51}$Cr release assay at each E:T ratio and area under the curve (AUC) for all animals by sex and nicotine treatment group (adjusted means ± SEM).

<table>
<thead>
<tr>
<th>Male</th>
<th>3.12:1</th>
<th>6.25:1</th>
<th>12.5:1</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=19)</td>
<td>1.70 ± 1.09</td>
<td>3.11 ± 1.09</td>
<td>6.79 ± 1.59</td>
<td>2.54 ± 0.94</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>2.68 ± 1.10</td>
<td>2.52 ± 1.09</td>
<td>5.28 ± 1.59</td>
<td>1.89 ± 0.92</td>
</tr>
<tr>
<td>High (n=20)</td>
<td>2.33 ± 1.07</td>
<td>3.23 ± 1.06</td>
<td>5.64 ± 1.55</td>
<td>0.86 ± 0.92</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>2.24 ± 0.63</td>
<td>2.95 ± 0.62</td>
<td>5.90 ± 0.91</td>
<td>1.76 ± 0.54</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Female</th>
<th>4.31:1</th>
<th>6.25:1</th>
<th>12.5:1</th>
<th>AUC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=20)</td>
<td>3.13 ± 1.07</td>
<td>5.48 ± 1.06</td>
<td>7.64 ± 1.55</td>
<td>1.80 ± 0.94</td>
</tr>
<tr>
<td>Low (n=19)</td>
<td>1.60 ± 1.09</td>
<td>1.84 ± 1.09</td>
<td>5.55 ± 1.59</td>
<td>2.23 ± 0.97</td>
</tr>
<tr>
<td>High (n=19)</td>
<td>1.33 ± 1.09</td>
<td>2.95 ± 1.09</td>
<td>7.19 ± 1.59</td>
<td>0.63 ± 0.94</td>
</tr>
<tr>
<td>Total (n=58)</td>
<td>2.02 ± 0.63</td>
<td>3.42 ± 0.62</td>
<td>6.79 ± 0.91</td>
<td>1.55 ± 0.54</td>
</tr>
</tbody>
</table>

Figure 9 presents the %$^{51}$Cr release from the peptide-pulsed target cells as a function of the E:T ratios for all animals by sex and nicotine treatment group. Figures 10 through 13 present the %$^{51}$Cr release at each E:T ratio and the AUC for the peptide-pulsed and MOCK-
pulsed target cells together for all animals by sex and nicotine treatment group. For each
animal, % $^{51}$Cr release at each E:T ratio and AUC was compared between the peptide-pulsed
and the MOCK-pulsed target cells. Using paired t-tests, there was a significantly greater %
$^{51}$Cr release by the peptide-pulsed target cells as compared to the MOCK-pulsed target cells
at each E:T ratio [$t$’s (115) > 15.22, $p$’s < 0.05] and for AUC [$t$(115) = 14.12, $p$<0.05]. The
data were split by sex and nicotine treatment group. Paired t-tests were re-run and revealed
that % $^{51}$Cr release was greater by the peptide-pulsed target cells than the MOCK-pulsed
target cells regardless of sex or nicotine treatment group [$t$’s(115) > 4.41, $p$’s < 0.05].

The following partial correlations were conducted to understand how % $^{51}$Cr release
by both peptide-pulsed and MOCK-pulsed target cells may be related to other dependent
variables measured (e.g., nicotine consumption (mg/kg), number of lymphocytes isolated).
These analyses especially from the MOCK-pulsed target cells may increase understanding of
how nicotine treatment or sex affects the functioning of HSV-1 specific T lymphocytes in an
environment that lacks HSV-1 labeled target cells; therefore, increasing the understanding of
the ability of the T lymphocytes to discriminate between HSV-1 labeled and non-labeled
target cells. Using partial correlations, % $^{51}$Cr release at each E:T ratio and AUC for the
peptide-pulsed target cells positively correlated with one another [$r$’s(109) > +0.76,$p$’s<0.05]. The % $^{51}$Cr release at each E:T ratio and AUC for the peptide-pulsed target cells
was not correlated with serum corticosterone (ng/mL) or corticosterone adjusted for body
weight (ng/mL/g), number of lymphocytes isolated adjusted for body weight, or IFN-γ levels.
The % $^{51}$Cr release at each E:T ratio and AUC for the peptide-pulsed target cells for nicotine-
treated animals was not correlated with self-administered nicotine dosage (mg/kg) on day 12.
or total nicotine intake (mg/kg) across the 12 days or with serum cotinine (ng/mL) or cotinine adjusted for body weight (ng/mL/g).

The % $^{51}$Cr release at each E:T ratio and AUC for the MOCK-pulsed target cells was positively correlated with one another [$r’s(109) > +0.46$, $p’s < 0.05$]. However, the % $^{51}$Cr release at E:T ratio and AUC did not correlate between the peptide-pulsed and MOCK-pulsed target cells [$r’s(109) < +0.13$, $p’s > 0.05$], which was not expected. The % $^{51}$Cr release at each E:T ratio and AUC for the MOCK-pulsed target cells for all animals was positively correlated with IFN-$\gamma$ levels [$r’s(109) > +0.21$, $p’s < 0.05$].

Due to the significant sex and nicotine treatment effects in IFN-$\gamma$ production, data were split by sex and partial correlations re-run. Next, data were split by nicotine treatment and partial correlations were conducted. For females, the % $^{51}$Cr release at each E:T ratio for the MOCK-pulsed target cells was positively correlated with IFN-$\gamma$ levels [$r’s (51) > +0.30$, $p’s < 0.05$] and trended toward significance for AUC [$r (51) = +0.26$, $p=0.06$]. For males, the % $^{51}$Cr release at the 12.5:1 E:T ratio for the MOCK-pulsed target cells was positively correlated with IFN-$\gamma$ levels [$r (51) = +0.31$, $p<0.05$], but the % $^{51}$Cr release at the other two E:T ratios or AUC for the MOCK-pulsed target cells were not correlated [$r’s(51) < 0.14$, $p’s > 0.05$]. For the CONRTOL group, the % $^{51}$Cr release at the 3.12:1 E:T ratio and AUC for the MOCK-pulsed target cells were positively correlated with IFN-$\gamma$ levels [$r’s(32) > +0.34$, $p’s<0.05$], but the % $^{51}$Cr release at the other two E:T ratios for the MOCK-pulsed target cells were not correlated [$r’s(32) < 0.30$, $p’s >0.05$]. For the LOW treatment group, % $^{51}$Cr release at the 12.5:1 E:T ratio for the MOCK-pulsed target cells was positively correlated with IFN-$\gamma$ levels [$r(31) = +0.37$, $p<0.05$], but the % $^{51}$Cr release at the other two E:T ratios or AUC for the MOCK-pulsed target cells were not significant [$r’s(31) < 0.13$, $p’s >0.05$].
For the HIGH treatment group, the % $^{51}$Cr release at the 3.12:1 and 6.25:1 E:T ratios for the MOCK-pulsed target cells were positively correlated with IFN-$\gamma$ levels [$r$’s(32) > +0.38, $p$<0.05], but the % $^{51}$Cr release at the 12.5:1 E:T ratio or AUC for the MOCK-pulsed target cells trended towards significance [$r$’s(32) < +0.32, $p$’s = 0.06].

The % $^{51}$Cr release at each E:T ratio and AUC for the MOCK-pulsed target cells for all animals was not correlated with serum corticosterone (ng/mL) or corticosterone adjusted for body weight (ng/mL/g) or number of lymphocytes isolated adjusted for body weight. The % $^{51}$Cr release at each E:T ratio and AUC for the MOCK-pulsed target cells for nicotine exposed animals was not correlated with self-administered nicotine dosage (mg/kg) on day 12 or total nicotine intake (mg/kg) across the 12 days or with serum cotinine (ng/mL) or cotinine adjusted for body weight (ng/mL/g).

Table 10: Summary of statistically significant and statistically non-significant nicotine treatment and sex findings on measures of immune function.

<table>
<thead>
<tr>
<th>Immune Measure</th>
<th>Statistically Significant</th>
<th>Statistically Non-significant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lymphocyte Cell Number Adjusted for Body Weight</td>
<td>Females &gt; Males</td>
<td>No main effect for nicotine treatment; No correlation with nicotine exposure, corticosterone, or cotinine</td>
</tr>
<tr>
<td>IFN-$\gamma$ levels from Supernatant</td>
<td>Females &gt; Males</td>
<td>HIGH group not different than CONTROL or LOW; No correlation with nicotine exposure, corticosterone, cotinine, or number of lymphocytes</td>
</tr>
<tr>
<td></td>
<td>Control &gt; LOW</td>
<td></td>
</tr>
<tr>
<td>$^{51}$Cr Release Assay</td>
<td>Peptide-pulsed &gt; MOCK-pulsed</td>
<td>No main effects for sex or nicotine treatment; No correlation with nicotine exposure, corticosterone, cotinine, IFN-$\gamma$, or number of lymphocytes</td>
</tr>
<tr>
<td>% lysis peptide-pulsed target cells</td>
<td>% lysis at E:T ratios + correlated with one another and % lysis of AUC</td>
<td>No correlation with nicotine exposure, corticosterone, cotinine, IFN-$\gamma$, or number of lymphocytes</td>
</tr>
<tr>
<td>% lysis MOCK-pulsed target cells</td>
<td>% lysis at E:T ratios and AUC + correlated with IFN-$\gamma$ levels and one another</td>
<td>No correlation with nicotine exposure, corticosterone, cotinine, or number of lymphocytes</td>
</tr>
</tbody>
</table>
Figure 9. Percent $^{51}$Cr release from peptide-pulsed target cells as a function of the E:T ratios for all animals by sex (58 males; 58 females) and nicotine treatment groups [39 control; 38 low nicotine; 39 high nicotine (adjusted means ± SEM)].
Figure 10. Percent $^{51}$Cr release of the 3.12:1 E:T ratio for the peptide-pulsed and MOCK-pulsed target cells for all animals by sex (58 males; 58 females) and nicotine treatment groups [39 control; 38 low nicotine; 39 high nicotine (adjusted means ± SEM)].
Figure 11. Percent $^{51}$Cr release of the 6.25:1 E:T ratio for the peptide-pulsed and MOCK-pulsed target cells for all animals by sex (58 males; 58 females) and nicotine treatment groups [39 control; 38 low nicotine; 39 high nicotine (adjusted means ± SEM)].

$^1 p<0.05$, Peptide>MOCK
Figure 12. Percent $^{51}$Cr release of the 12.5:1 E:T ratio for the peptide-pulsed and MOCK-pulsed target cells for all animals by (58 males; 58 females) and nicotine treatment groups [39 control; 38 low nicotine; 39 high nicotine (adjusted means ± SEM)].
Figure 13. Percent $^{51}$Cr release of the AUC for the peptide-pulsed and MOCK-pulsed target cells for all animals by sex (58 males; 58 females) and nicotine treatment groups [39 control; 38 low nicotine; 39 high nicotine (adjusted means ± SEM)].
**Confirmation of Hypotheses**

**Hypothesis 1: Confirmation of Nicotine Manipulation**

The confirmatory hypothesis that nicotine exposure would result in a significant dose-dependent elevation in cotinine levels was **supported**.

**Hypothesis 2: HPA-axis stimulation via Nicotine Exposure**

It was hypothesized that nicotine treatment would result in significant dose-dependent stimulation of the HPA-axis, as indexed by serum corticosterone levels at necropsy. This hypothesis was **not supported**. There were no differences between the nicotine treatment groups in serum corticosterone levels.

**Hypothesis 3: Nicotine Exposure and Lymphocyte Proliferation 5 Days Post-Infection**

The hypothesis that nicotine exposure would result in a dose-dependent reduction in the number of lymphocytes that were isolated from the popliteal lymph nodes, regardless of sex, was **not supported**. There were no differences between the nicotine treatment groups in number of lymphocytes isolated from the popliteal lymph node.

It was further hypothesized that females would demonstrate a greater reduction in the number of isolated lymphocytes from the popliteal lymph node compared to their male counterparts due to nicotine exposure. This hypothesis was **not supported**. However, the data revealed that female mice mounted a greater anti-viral response compared to their male counterparts which was demonstrated by having a greater number of lymphocytes isolated from the popliteal lymph node per gram of body weight.

**Hypothesis 4: Nicotine Exposure and T-Lymphocytes Activation**

The hypothesis that the production of IFN-γ by T lymphocytes during the cell culture incubation would be reduced in a dose-dependent manner by nicotine treatment, regardless of
sex, was **partially supported**. In females only, the LOW group produced significantly less IFN-γ compared to the CONTROL group; however, the HIGH group produced similar IFN-γ levels to both the CONTROL and the LOW group. There was no significant nicotine effect in the males.

Further, it was hypothesized that nicotine exposure would have a greater reduction in IFN-γ production for female mice compared to their male counterparts’ T lymphocytes. This hypothesis was **partially supported**. However, the data revealed that the T lymphocytes from only CONTROL female mice produced a greater amount of IFN-γ compared to their male counterparts. Therefore, nicotine exposure in the LOW and HIGH groups removed the significant increase in the anti-viral response, indexed by IFN-γ production, between male and female mice.

**Hypothesis 5: Nicotine Exposure and HSV-1 Specific T-Lymphocyte Activity**

The hypothesis that nicotine exposure would result in a dose-dependent reduction in HSV-specific T-lymphocyte functionality, regardless of sex, was **not supported**. There were no differences between the nicotine treatment groups in % $^{51}$Cr release by the HSV-1 specific T lymphocyte.

Further, it was hypothesized that among nicotine-exposed mice, female HSV-1 specific T lymphocyte would display less % $^{51}$Cr release compared to males. This hypothesis was **not supported**. There were no differences in % $^{51}$Cr release by the HSV-1 specific T lymphocyte between the sexes.
Chapter IV: Discussion

Overview

Tobacco use continues to be the most preventable cause of illness and death in the United States (USDHHS, 2001). Unfortunately, approximately 1.5 million adolescents still begin to smoke every year, with more than 25% becoming daily cigarette smokers (SAMHSA, 2008). On average, adults who smoke cigarettes die 14 years earlier than do nonsmokers (CDC, 2002; Doll, Peto, Boreham, & Sutherland, 2004). Lung cancer, heart disease, and chronic lung diseases such as emphysema, bronchitis, and chronic airway obstruction are responsible for the largest number of smoking-related deaths (CDC, 2005). While these diseases develop over the course of 10-20+ years in the life of an adult smoker, smokers also have an increased susceptibility to viral infection (Friedman, Pross, & Klein, 2006). Therefore, there is a need to understand the mechanisms through which nicotine, the primary addictive ingredient in tobacco, or other components of tobacco smoke affect the functioning of the immune system.

The purpose of this dissertation was to investigate the dose-dependent effects of nicotine on the anti-viral immune response to HSV-1 rear footpad infection in adolescent male and female C57BL/J6 mice. Excitingly, nicotine exposure significantly decreased IFN-\(\gamma\) production during the 3-day T-lymphocyte incubation in the LOW nicotine exposure group compared to the CONTROL, but not in the HIGH nicotine exposure group. The reduction in IFN-\(\gamma\) levels was a result of less activated T lymphocytes across the 3-day incubation period. This experiment was the first to investigate how nicotine modulates the pathogenesis of HSV-1 in vivo, as opposed to in vitro challenges to lymphocytes isolated from nicotine exposed animals or lymphocytes exposed to nicotine in vitro and then challenged.
Additionally, this is the first report of sex differences in the anti-viral immune response to HSV-1 indexed by an increased number of lymphocytes isolated at necropsy and increased IFN-\(\gamma\) production in female mice compared to their male counterparts.

The section will review findings from this dissertation and offer some theoretical answers to the questions raised by the results. It concludes with a discussion of the necessary next steps to help move the field closer to understanding the effect of nicotine on the anti-viral response.

**Forced Oral Nicotine Administration in Adolescent Mice**

Since the early 1980’s, the inverse relationship between nicotine and body weight has been observed in humans and adults rats (e.g., Grunberg, 1982, 1985; Grunberg, Bowen, & Morse, 1984; Grunberg, Popp, & Winders, 1988; Saah, Raygada, & Grunberg, 1994) and adolescent rats (Klein, 2001; Faraday, Elliott, & Grunberg, 2001). However, research has failed to reveal the inverse relationship in adolescent mice (e.g., Adriani et al., 2004, Adriani, Macri, Pacifici, & Laviola, 2002; Klein et al., 2004). In the present experiment, the HIGH nicotine treatment group lost a significant amount of weight upon initiation of nicotine treatment in males and females compared to the LOW nicotine treatment group. Until the 8\(^{th}\) day of nicotine administration, the significant nicotine affect on body weight continued although the HIGH nicotine treatment group appeared to be catching up to growth observed in the LOW nicotine treatment group. Neither the HIGH nor the LOW nicotine treatment group’s body weight differed compared to the CONTROL throughout the 12-day forced nicotine paradigm. Therefore, it is difficult to tell if the LOW nicotine group had accelerated growth or if the HIGH nicotine group had decelerated growth or a combination of both.
Using the nicotine oral self-administration, Adriani, Macri, Pacifici, and Laviola (2002) reported that oral nicotine intake concentrations similar to the LOW nicotine treatment group in this experiment resulted in significant weight gained compared to the no nicotine treatment group starting on PN 24. Adriani et al. (2002) did not have a group exposed to oral nicotine concentrations similar to the HIGH nicotine treatment group as in the present experiment; therefore, the body weight suppression observed in the HIGH nicotine treatment group in the present experiment suggests that there is a threshold of nicotine exposure that must be met before weight suppression occurs. Klein and colleagues (2004) did not find a significant effect of nicotine administration on body weight with water nicotine concentrations ranging from 10 µg/mL to 200 µg/mL. However, choice nicotine self-administration did not begin until PN 35 in the Klein et al. (2004) paper. In the present experiment, nicotine self-administration was forced and the nicotine administration began on PN 30. Therefore, the differences in the paradigm of oral nicotine self-administration (choice vs. forced) and post-natal day of initiation of nicotine exposure could explain why the present experiment found significant differences on body weight differences between the HIGH and LOW nicotine treatment groups because the forced paradigm resulted in greater nicotine exposure compared to the choice self-administered nicotine volumes in Adriani et al. (2002) and Klein et al. (2004) which resulted in lower blood nicotine and cotinine levels.

The adolescent mice varied greatly in food and fluid consumption across the 12-day nicotine treatment. Food consumption seemed to increase days 1-3 and then decrease days 4-6. On day 7, the pattern appears to begin with food consumption increasing again; however, the HSV-1 injection on day 8 caused a statistically significant drop in food consumption or a premature decrease. Starting on day 9, mice continually increased their food consumption
for three days and then on day 12 the decreasing portion of the pattern begins again. Not only was there a decreased in food consumption during the 24-hrs following the HSV-1 injection, fluid intake decreased as well.

Fluid intake also varied daily regardless of nicotine treatment. In an effort to pinpoint what may have caused this fluctuation in consumption behavior, a review of the protocol does not provide any suggestions as to what might be the cause. For example, there were no change cages throughout the treatment period which can cause a shift in the mice’s behavior due to the novelty of the cage. However, the fluid was replaced with regularity throughout the study. Refreshing the liquid may have systemically inflated intake volume on a particular day. The bottles for the CONTROL group were refreshed on days 3 and 10. Upon reviewing the fluid consumption data, on day 3 for the CONTROL group there may have an inflation of the fluid intake volume; however, on day 10, the data clearly do not support inflation in fluid intake volume. Nicotine bottles were always refreshed on the same days which were usually days 4, 7, 9, and 12. For the nicotine treatment groups, it appears that there may have been an inflation of fluid intake on days 9 and 12, but not on days 4 and 7. An important fact to recall is that the HSV-1 injection occurred on day 8; therefore the inflation observed on days 9 and 12 may have more to do with recovery from the injection than the refreshing of the liquid. This variability in day to day consumption does not appear to be related to study protocol (e.g., specific to days were the liquid was refreshed), but perhaps it may have to do with the drastic growth that occurs during the periadolescent period in mice and/or recovery from a viral injection. Additional studies are necessary to determine what in the protocol caused the observed effects on the food and fluid consumption behaviors of periadolescent mice.
Future studies examining the relationship between nicotine and body weight in adolescent mice should include multiple nicotine concentrations in forced and choice oral intake paradigms, along with blood measures of nicotine and cotinine to determine the nicotine concentrations where the effect of nicotine on body weight goes from increasing body weight to suppressing it. In addition, the initiation of nicotine treatment should be tested as a critical difference between this present experiment and Adriani and colleagues (2002) compared to the null findings in Klein and colleagues (2004). It may be that nicotine exposure must occur at or prior to PN 30 in adolescent mice to capture a significant effect on body weight.

In the present experiment, the observed nicotine effect on body weight ended after the HSV-1 infection on day 8. As mentioned above, the body weight data from the HIGH nicotine treatment group appeared to be “catching up” to the LOW nicotine treatment group suggesting that, regardless of the HSV-1 infection, the body weight difference may have disappeared. However, the HSV-1 infection resulted in a significant reduction in fluid intake (mL), food consumption (g), and nicotine consumption (mg/kg) in the 24 hr period following the injections compared to the 24 hr period preceding the injection. These drastic behavior changes suggest that the HSV-1 injection procedure and initial infection was an acute stressor because it is the only day when both food and fluid consumption decreased together. This acute stressor modified food and fluid consumption which in turn appears to have retarded adolescent growth such that the observable nicotine effect on body weight disappeared following the HSV-1 injection.

The HSV-1 infection caused an unforeseen complication, a significant disruption in daily behavior during the 24-hr period following the injection as compared to the previous
24-hr period, when analyzing the changes across daily body weight (g), food consumption (g), fluid intake (mL), and nicotine consumption (mg/kg) such that a repeated measures ANCOVA was unable to truly capture differences in these food and fluid consumption behaviors before and after the injection. Therefore, more sophisticated statistical analyses may reveal fine grained results about how body weight and food and fluid consumption behaviors were affected by the injection on day 8 in concert with the pre-programmed adolescent growth. For example, using PROC MIX in SAS would allow for comparison of body weight changes day to day while also allowing for changes observed prior to and after HSV-1 injection. These analyses will be conducted in the future to extend the findings of the present experiment.

**Unanticipated Corticosterone Results**

There are two interesting findings with regard to the serum corticosterone data from this dissertation. First, sacrifice order had an effect on total serum corticosterone levels at necropsy, such that when a mouse was sacrificed in one of the first four of twenty spots, their corticosterone levels were elevated compared to those sacrificed in positions 5 through 20. Second, nicotine exposure had no effect on the total serum corticosterone at necropsy, even with removal of the first four mice at each necropsy from the statistical analyses. These unexpected corticosterone results initially were concerning because all animals were handled similarly by the researchers and nicotine should have lead to the activation of the hypothalamic-pituitary-adrenal (HPA)-axis; however, upon further investigation several explanations may shed light on the corticosterone results.

Like humans, mice have a robust diurnal adrenal glucocorticoid rhythm (Cheifetz, 1971; Krieger, 1975). The peak of corticosterone occurs at the onset of the active period,
which is the dark phase (or night time) for mice. Corticosterone levels decrease throughout
the dark phase and reach its nadir after the inactive or light phase of the day has begun.
Therefore, it is possible that the first sacrifice group (first four mice) of each necropsy
session were sacrificed while corticosterone levels still were decreasing and had not reached
the nadir of the diurnal rhythm. The first sacrifice group was euthanized 40 – 60 minutes
after the onset of the light cycle. The remaining sacrifice groups were sacrificed within
roughly 20 minutes of the preceding group (e.g., the 2nd sacrifice group was euthanized
within 80 minutes of the start of the light cycle, the 3rd sacrifice group was euthanized with
100 minutes into the light cycle). If the corticosterone nadir for mice occurs after the onset
of the light cycle, it is reasonable to expect that the earlier a mouse is euthanized, the more
elevated their corticosterone level would be. In future studies, a delay in necropsy time may
aid in reducing the diurnal rhythmicity effects on the corticosterone level. Based on data
from this experiment, delaying the start of necropsy until 1 hour after the onset of the light
cycle may reduce the chance of finding elevated corticosterone levels in the earlier sacrificed
mice. Unfortunately, delaying the sacrifice also could result in reduced serum cotinine
levels, which also is an important dependent measure in oral nicotine intake studies. This
reduction is because of the short half-life of nicotine and cotinine and that mice typically fall
asleep, and thus stop drinking, about 60 – 90 minutes after the onset of the light cycle.

Another methodological factor may have resulted in an increase of corticosterone
levels among the first sacrifice group of mice in each cohort. All mice were weighed (i.e.,
handled) prior to sacrifice. Among mice, handling results in stimulation of the HPA axis
even if the mice have experienced prior handling (Smolensky, Halberg, Harter, His, &
Nelson, 1978). Mice naturally do not prefer to be handled; therefore, removal from their
home cage to be weighed could be considered an acute stressor that results in an elevation of corticosterone. As necropsy progressed, the time from being handled to the time sacrificed increased thereby allowing time for the corticosterone levels to drop. Although I did not document the time mice were handled for weighing, I do know that the first mouse was euthanized approximately within 20 minutes of being handled. It took roughly an hour to weigh all the mice and their food and water bottles. Therefore, the last euthanized mouse was handled about 1 hour after the first mouse was weighed; however, the last mouse was euthanized 2 hours later than the first mouse. The difference in time since being handled is a reasonable suggestion for the difference in corticosterone levels between the first sacrifice group and the rest of animals. The longer the time gap between handling and euthanizing the more likely the corticosterone levels of the mouse would return to the basal inactive corticosterone level. In addition, the timing of the handling within the diurnal rhythm also enhanced the chance of observing an effect of handling on the corticosterone levels. That is, because corticosterone levels are at the lowest levels of the day, any disturbance induced increase most likely will be detected. In fact, human laboratory studies of HPA-axis response to stress typically are conducted in the afternoon when cortisol levels are decreasing so that stress-Induces cortisol levels are more readily detected (e.g., Kirschbaum, Pirke, & Hellhammer, 1993). In future studies, an attempt to keep the timing similar between the handling and euthanizing of the mice must be made across the necropsy.

The lack of an effect of nicotine exposure on corticosterone levels is surprising in light of reports that nicotine stimulates the HPA axis (Balfour, 1989; Matta et al., 1988; Seyler et al., 1986) and that human smokers have elevated basal cortisol levels compared to non-smokers (Steptoe & Ussher, 2006; Rohleder & Kirschbaum, 2006; Badrick, Kirschbaum,
However, chronic nicotine exposure, whether injection, infusion, or oral administration, leads to tolerance of this acute nicotine induced corticosterone stimulation of the central nervous system (Balfour, 1980; Collins, Bhat, Pauly, & Marks, 1990; Marks, Grady, & Collins, 1993). In mice, an upregulation of neuronal nAChRs occurs under chronic exposure of nicotine which simultaneously occurs with onset of tolerance (Robinson, Grun, Pauly, & Collins, 1996). In fact, corticosterone is instrumental in the desensitization to nicotine in the brain (Caggiula, et al., 1998; Martin & Wehner, 1988; Pauly, Grun, & Collins, 1992). More recently, Tapper and colleagues (2004) have shown that activation of the α4 subunit of the nAChRs is critical to the development tolerance to the behavioral and physiological effects of nicotine in α4* nAChR knock-in mice starting at 4 weeks old. Therefore, chronic 12-day administration of oral nicotine was long enough to result in desensitization of nAChRs in the parvocellular paraventricular nucleus of the hypothalamus and other areas of the brain such as the locus coeruleus. This desensitization most likely led to reduced HPA-axis stimulation by nicotine.

Another possibility is the introduction of the HSV-1 into the experimental design. Numerous studies have documented bi-directional communication between the brain and the immune system (for review see Wrona, 2006). Viral infections are potent stimulators of the HPA-axis via cytokines such as IL-1β (Bailey, Engler, Hunzeker, & Sheridan, 2003; Webster & Sternberg, 2004). Therefore, the introduction of HSV-1 into nicotine-exposed mice may have masked or modified any differences in corticosterone levels that could be attributed to nicotine exposure alone. One way to test whether or not there was a nicotine effect on corticosterone levels would be to collect blood prior to the HSV-1 injection, as well as at sacrifice. Collecting serum at both time points would aid in the determination of how
nicotine exposure and HSV-1 infection dependently and/or independently affect the HPA-axis function.

Although there is no relationship between cotinine and corticosterone levels, it appears that there are two satisfactory conclusions as to why nicotine administration did not stimulate the HPA-axis in a dose-dependent manner. Desensitization of the nAChRs in the hypothalamus and throughout other areas of the brain, as well as the addition of a viral infection, could explain the null corticosterone results.

**Modulation of the Anti-Viral Response**

This experiment was the first to directly test sex differences in the anti-viral response to HSV-1 via rear foot pad injections. Previous work has examined male and female adult mice in separate studies depending on the route of infection and the overall research question. For example, female mice were used to study the effect of stress on modulation of the anti-viral response to HSV-2 infection via vaginal route of infection or dam-to-offspring protective immunity (e.g., Ashcraft & Bonneau, 2008; Yorty & Bonneau, 2004b; Yorty et al., 2004; Zahwa et al., 2008). However, the majority of research has used adult male mice only (e.g., Anglen et al., 2003; Bonneau, 1996; Bonneau, et al., 1998; Freeman et al., 2007; Kusnecov et al., 1992; Ortiz et al., 2003; Truckenmiller et al., 2005). There was no directional hypothesis on how sex may affect modulation of the anti-viral response to HSV-1 during adolescence because of this lack of prior research.

Results suggest that sex is critical when comparing the anti-viral response as indexed by the number of isolated lymphocytes at necropsy and the production of IFN-γ during the 3-day incubation period. Overall, female mice mounted a greater anti-viral response compared to the males. There also was a greater number of lymphocytes isolated adjusted for body
weight and a greater production of IFN-γ by the lymphocytes in vitro among the females compared males. Therefore, it is absolutely imperative to study both males and females when investigating the modulation of the anti-viral response.

All mice received the same HSV-1 dose, 1 x 10⁶ PFU/foot pad. However, there were statistically significant body weight differences on day 8 between the sexes and nicotine treatment groups. Recall that males weighed more than did females and that the LOW nicotine treatment group weighed more than did the HIGH nicotine treatment group. One might wonder if this significant sex weight difference might have led to the significant modulation of the anti-viral response to HSV-1. Therefore, a viral dosage was calculated and a two-way ANCOVA revealed a significant difference between the sexes, where females received a greater viral dosage than did the males \( F(1,106) = 279.31, p<0.05 \). There was neither a statistically significant difference among the nicotine treatment groups nor was there a sex X nicotine treatment interaction. Thus, an empirical question arises: could HSV-1 viral dosage affect the anti-viral response?

Based on the available literature, the answer to the question proposed has not been addressed. However, one study by Hubalek (1980) concluded that a higher viral dose increased severity of the disease but did not affect the height of the virus neutralization antibody titer in male and female mice infected with Tahyna virus. When comparing sexes, more male mice had a greater virus neutralization antibody titer than did females, suggesting that male mice were more susceptible to this virus. Hubalek (1980) used two doses of the virus; the higher dose was 100 times as concentrated as the lower dose. Looking at the data from the present experiment, even though the viral dosage is significantly different, the actual dosage for the females was 1.2 times that of the males, which may or may not be
biologically significant. Prior to continuing the sex differences research, a study should be conducted to control for viral dosage between the sexes to ensure that the greater anti-viral response observed in females is not an artifact of study design and favors the increase viral exposure in females.

Unlike psychopharmacology studies where it is critical to maintain a constant drug level across subjects, during a viral challenge or infection, the immune system is activated regardless of the amount of virus present once a threshold is reached. Assuming that the slight increase in viral dosage among females is not biologically significant or the underlying cause for the significant increase in the anti-viral response, the greater lymphocyte proliferation and activation *in vitro* observed in females needs to be carefully considered. In a recent editorial by Holdcroft (2007) and reviewed by Taylor, Klein, and colleagues (2000) and in a book commissioned by the Institute of Medicine “Exploring the Biological Contributions to Human Health: Does Sex Matter?” (2001), although major steps have been taken to include females in research since the 1970s, researchers themselves choose to exclude females citing lack of physiological data, repeat of a previous study that only used males, and economic costs associated with including women in research. The present experiment adds to the evidence that males and females must be studied equally because there is obviously something innately different between the sexes’ anti-viral response even during adolescence.

Sexual dimorphic immune responses have been observed in both animal and human populations (e.g., Barna, Komatsu, Bi, & Reiss, 1996; Gabrilova & Marotti, 2000; Gourdy et al., 2005; Kennedy et al., 2009; Ma, Guzman, Muller, Walker, & Owen, 2007; Mitchell, 1999; Schneider, Schwacha, & Chaudry, 2006). However, given the extensive amount of
data on the immune system and its function, there is a relatively small number that can be
identified as studying sex differences of the immune system (Cook, 2008). The majority of
sex differences research on immunity surrounds the significantly greater prevalence of
autoimmune diseases suffered by females compared to males (e.g., Fairweather, Frisancho-
Kiss, & Rose, 2008; Lang, Nguyen, Papadimitriou, & Via, 2003; Lockshin, 2006).

Approximately 14 – 22 million of the U.S. population has some type of an autoimmune
disease and 78% of those who suffer from an autoimmune disease are female (Jacobson,
Gange, Rose, & Graham, 1997; National Institutes of Health Autoimmune Disease
Coordinating Committee Report, 2000; USDHHS, 2010). Most research points to the effects
of gonadal hormones for the dimorphic immune responses.

Estrogen and progesterone levels have been linked to the observed increase in
immune function among women (e.g., Ahmed, Hissong, Verthelyi, Donner, Becker, &
Karpuzoglu-Sahin, 1999; Beagley & Gockel, 2003; De Leon-Nava et al., 2009; Han,
Lundberg, Tanamachi, Openshaw, Longmate, & Cantin, 2001; Karpuzoglu-Sahin, Zhi-Jun,
Lengi, Sriranganathan, & Ahmed, 2001; Rider & Abdou, 2001). In addition, androgen and
estrogen receptors are found on immature immune cells in the thymus and bone marrow
(e.g., Rosen, Tran, Lackey, & Viselli, 1999). Sarvetnick and Fox (1990) discuss the role of
IFN-\(\gamma\) in sexual dimorphism of the immune system including a modulation of this key
cytokine X sex steroid interaction as the leading cause of sex differences in autoimmunity
prevalence. The results from the present experiment partially support Sarvetnick & Fox’s
(1990) conclusion as the lymphocytes from female mice produced more IFN-\(\gamma\) than did the
male lymphocytes. Interestingly, Whetzel, Corwin & Klein (2007) found elevated IFN-\(\gamma\)
levels and a shift towards Th\(_1\) systemic cytokine environment among healthy young adult
female smokers compared to male smokers and male and female non-smokers, which suggests that female smokers may be primed to mount a greater anti-viral response in humans. In addition, smoking may increase Th1 autoimmune disease vulnerability such as multiple sclerosis among women (Nguyen et al., 2003; Whetzel, Corwin, & Klein, 2007). However, the present experiment was conducted on male and female mice during periadolescence which is prior to when the dramatic change in gonadal hormones occurs during puberty. Therefore, sex hormones were not elevated in these periadolescent mice and cannot be the cause for the sex difference observed in anti-viral response.

There could be a genetic or an epigenetic modification between the sexes that has a significant effect without the abundant presence of gonadal hormones. Rosen, Tran, Lackey, and Viselli (1999) found splenocytes’ mitogenic [phorbol 12-myristate 13-acetate (PMA) + ionomycin (ION)] response was greater among 3 week old C57BL/6 male mice than among female mice. However, by 4 weeks old and continuing until 6 weeks, females had a greater response than did the males. No sex difference was found using a T-cell receptor dependent challenge as such ConA. Therefore, it was hypothesized that the sex difference in PMA + ION (cell surface independent) stimulation was due to a greater response in B lymphocytes in 3 week old males and 4-6 week old females (Rosen et al., 1999). However, there were no data collected that could explain the reversal in the sex difference response to PMA + ION.

In more recent studies, examination of sex differences in the immune system has focused on serological or antibody responses to vaccination. Surprisingly, being male is a risk factor for a reduced protection or sero-negative responses to the many routine vaccinations regardless of age (e.g., Kennedy et al., 2009). However, this reduction in serological response is dependent on type of vaccine (Cook, 2008). For example, men show
an increased response to pneumococcal, meningococcal, yellow fever, and Venezulean equine encephalitis vaccines (e.g., Edwards, Burns, Adkins, Carroll, Drayson, & Ring, 2008; Monath et al., 2002; Pittman, Makuch, Mangiafico, Cannon, Gibbs, & Peter, 1996; Sankilampi, Honkanen, Bloigu, Herva & Leinonen, 1996); where as women have a greater serological response to influenza, hepatitis A & B, tetanus, and brucella vaccines (e.g., Edwards et al., 2007; Goronzy, Fulbright, Crowson, Poland, O’Fallon, & Weyand, 2001; Reutter, Bart, Francioli, Safety, & Frei, 1998; Rhodes, Scott, Markham, & Monk-Jones, 1969). However, there are inconsistent results between the sexes for other vaccinations (e.g., Briggs et al., 2000; Lau & Sisson, 2002; Mark, Carlsson, & Granstorm, 1999; Moore et al., 2006). In general, it appears that males have a greater response to bacterial vaccination which points to a stronger innate immune while females’ adaptive immune response appears to provide the stronger serological response.

Using pre-pubertal, pubertal, and post-pubertal C57BL/6 male and female mice, Lamason and colleagues (2006) found that puberty results in the sexual dimorphism in expression of the innate and adaptive immune genes. Following puberty, a cluster of genes indicative of activation of the adaptive immune system had increased expression in females while in males the active expression increased in a cluster of genes for the innate immune system (Lamason et al., 2006). There was no statistically significant difference in the expression of the gene cluster between pre-pubertal male and female mice. Further, Lamason and colleagues (2006) found that estrogen influences immunoglobulin levels in post-pubertal female mice. Although Lamason and colleagues’ (2006) work is invaluable, it simply concludes that estrogen is the reason why the immune system is sexually dimorphic, which suggests that, in the current study, the mice went into early puberty (prior to 6 weeks
of age) or that there are additional gene clusters that must be examined. The increase in nicotine metabolism observed in women as compared to men has been linked to the presence of estrogen (Benowitz, Hukkanen, & Jacob, 2009). The testing gonadal hormone levels in the remaining serum from the mice might help answer whether or not the mice were pre-pubertal in the present study. Therefore, conclusions may be drawn about whether or not gondal hormones may have influenced the observed immune differences and nicotine consumption in this present study.

In addition to sex differences in serological responses to vaccinations, tobacco use and/or exposure to 2nd hand smoke has a significant detrimental effect on the serological response to vaccines in children and adults (e.g., Baynam et al., 2007; Hagedorn et al., 2010; Lin et al., 2008). This reduced response to vaccination as well as increased risk of infection in tobacco/nicotine exposed individuals (e.g., Baynam et al., 2007; Friedman, Pross, & Klein, 2006; Hagedorn et al., 2010; Lin et al., 2008) is hypothesized to be a result of nicotine modulating the immune system (e.g., Sopori et al., 1998; Wang et al., 2003; Wang et al., 2004). Using in vitro and in vivo experimental designs, nicotine was shown to suppress activation of T lymphocytes, dendritic cells, monocytes, neutrophils, and inhibits class switching of B lymphocytes (e.g., Grando et al., 2003; Kawashima et al., 2007; Nouri-Shirazi & Guinet, 2003; Saeed et al., 2005; Singh et al., 2000; Skok et al., 2005; Sopori et al., 1998; Wang et al., 2003; Wang et al., 2004). However, none of the studies have tested the effects of nicotine on the immune system in vivo during an immunological challenge such as a primary viral infection.

Infecting mice with HSV-1 allows for investigating the “big picture” or overall functioning and harmony of both branches of the immune system. As the data from the
present experiment demonstrate, it is difficult to modulate the anti-viral response of the immune system. However, a nicotine treatment effect was observed on IFN-γ levels. Lymphocytes from the LOW nicotine treatment group produced significantly less IFN-γ compared to lymphocytes from the control group. IFN-γ production during the 3-day incubation is a measure of T-lymphocyte activity and function in vitro. Therefore, the significant reduction in the LOW nicotine treatment group suggests there was a decrease in T-lymphocyte activity across the 3-day incubation period. Nicotine can trigger T-lymphocytes into a state of anergy, which makes them non-responsive to the presentation of antigen and unable to be activated (Singh et al., 2000). If the T-lymphocytes were not activated in vivo, then the production of IFN-γ would be suppressed.

As mentioned above, T lymphocytes from female mice produced significantly more IFN-γ than did the T lymphocytes from male mice. When the data were split by nicotine treatment group, the sex effect was only statistically for the CONTROL group. This finding suggests that nicotine treatment significantly reduced the INF-γ production in the females so production was more similar to lymphocytes from male mice. In addition, the suppression of IFN-γ production observed in the LOW nicotine group only was statistically significant for the females when the data were split by sex. Again, the data suggest that nicotine more adversely affected the females’ anti-viral response compared to the males’ response. The data did not reveal a sex difference in nicotine consumption (mg/kg) for the HIGH nicotine treatment group until day 7 of nicotine treatment, which was the 24 hr period prior to the injection. Following the injection, females in both nicotine treatment groups consumed more nicotine (mg/kg) than did males on days 9, 11, and 12. This difference in nicotine dosage could explain the varying nicotine effects observed between the sexes and the loss of
increased IFN-γ production observed in the CONTROL females compared to the CONTROL males.

An unexpected result from this experiment was that the reduction IFN-γ only occurred in the LOW nicotine treated mice, but not in the HIGH nicotine treated mice. Across the 12-day forced nicotine treatment period, the HIGH group consumed significantly more nicotine (mg/kg) compared to the LOW group. Therefore, one would think that greater nicotine consumption (mg/kg) would lead to greater immune suppression. However, just as the nAChR in the brain desensitize to nicotine (Balfour, 1980; Collins et al., 1990; Robinson et al., 1996), the nAChRs on the immune cells could also modify their ability to bind nicotine based on exposure to nicotine. To date, there are no studies investigating the response of nAChRs on immune cells to the chronic exposure of nicotine. The field still is defining the role nAChRs on immune cells and how stimulation affects the activity of the immune cells. A future step must include studying nAChR regulation on immune cells in response to chronic exposure to nicotine.

Aside from the possibility of desensitization of nAChRs on immune cells, during the 24 hr period post-injection, the drop in nicotine consumption (mg/kg) was greater in the HIGH nicotine treatment group than in the LOW nicotine treatment group. This significant change in nicotine consumption (mg/kg) in the HIGH nicotine treatment group during the first 24 hours after the infection might have been enough to dampen observable nicotine effects. One way to determine whether or not the decrease in nicotine consumption (mg/kg) caused the null nicotine effect for the HIGH treatment group would be to use passive administration such as injecting the nicotine, an osmotic mini-pump, or administering nicotine via tobacco smoke exposure. The reduction in nicotine consumption following the
HSV-1 injection by the mice does mirror reports in humans. Humans reduce the number of cigarettes they smoke or attempt to quit at the onset of illness, especially tobacco-related health events (Boudreaux, Baumann, Camargo, O’Hea, & Ziedonis, 2007). Corwin and Klein (2003) hypothesized that when a smoker gets sick the increase in cytokine production due to illness masks the increase in cytokine activity, which resembles “sickness behavior,” due to the decrease in nicotine exposure (Meliska et al., 1995). The end result of masking the cytokine increase due to nicotine absentience may allow for a more successful quit attempt in smokers (Corwin & Klein, 2003). Although 24-hr abstinence did not result in an increase in cytokine production, the CRP levels of a sub-group of smokers increased, as well as an increase in their depressed mood (Corwin & Klein, 2003).

Given the significant sex and nicotine effects observed in lymphocyte proliferation and activation measured by IFN-γ, the lack of significant differences in 51Cr release is surprising. The number of lymphocytes isolated from the lymph nodes is the least HSV-1 specific measure of immune activity. The lymphocytes are counted using trypan blue exclusion (if the cell is blue, it means the cell is dead); however, using a microscope, one can not differentiate a T lymphocyte from a B lymphocyte. Therefore, the lymphocyte isolation counts include T lymphocytes, B lymphocytes, macrophages, and dendritic cells (Kindt et al., 2007). During a primary viral infection, macrophages and dendritic cells are responsible for presenting the viral antigen and viral epitope markers to the B lymphocytes and T lymphocytes (Kindt et al., 2007). Once the B and T lymphocytes are activated, clonal expansion begins in the lymph node (Kindt et al., 2007). However, the CD8+ T-lymphocytes are the only cells able to develop HSV-1 specific cytotoxic ability to lyse the peptide-pulsed target cells during the 51Cr release assay. Theoretically, the significant increase in
lymphocytes observed among female mice could be a result in greater proliferation of B lymphocytes and T helper cells (CD4+) which do not necessarily equate to greater $^{51}$Cr lysis if it did not lead to greater CD8+ proliferation. Rosen and colleagues (1999) did hypothesize that the adolescent sex difference they observed was due to a greater response from B lymphocytes. A more sophisticated analysis using flow cytometry could have involved identifying a representative sample of lymphocytes isolated from each mouse to determine what lymphoid cells were actually present. Identifying the actual types of lymphocytes present might have provided insight into why there are no significant sex or nicotine differences in ability to lysis labeled target cells.

IFN-$\gamma$ analysis is a more specific immune activation measure than lymphocyte isolations for identifying cytotoxic T lymphocyte activation. IFN-$\gamma$ is produced by CD4+ and CD8+ T lymphocytes. IFN-$\gamma$ is responsible for supporting the classic cell mediated function, such as, activation of CD8+ T lymphocytes (Kindt et al., 2007). Therefore, significant differences in the cytokine environment should result in significant differences in CD8+ T lymphocytes activation. Theoretically, the significant reduction in IFN-$\gamma$ levels observed in the LOW nicotine treatment group should have correlated with a reduction in percent of $^{51}$Cr release during the assay. In addition, the significant increase in IFN-$\gamma$ production observed by female lymphocytes should have resulted in a greater percentage of $^{51}$Cr release during the assay. However, there are additional cytokine levels that could be examined. For example, IL-2 and TNF-\(\beta\) also are produced by CD4+ T lymphocytes to support the cell-mediated functions of the immune system. Analyzing these cytokine levels may provide additional insight as to why there were no differences in the percent of $^{51}$Cr release during the assay. In addition, the supernatant could be analyzed for cytokines IL-4,
IL-5, IL-6, and IL-10, which support B-lymphocyte activation and are produced by CD4+ lymphocytes. Whetzel, Corwin, and Klein (2007) found an elevated IFN-γ:IL-10 systemic cytokine ratio in female smokers compared to male smokers and male and female non-smokers, suggests that measuring IL-10 in addition to IFN-γ may have provided insight to the cytokine environment (Th1 vs. Th2) of the lymphocytes during the anti-viral response in vivo. Therefore, the addition of these cytokine assays may shed light on the increased lymphocyte counts but lack of differences in the HSV-1 specific results.

The $^{51}$Cr release assay itself also may have contributed to the lack of significant differences in $^{51}$Cr release between the experimental groups. One critical variable in the $^{51}$Cr release assay is pre-determination of the proper E:T ratios to capture functioning of HSV-1 specific CD8+ T lymphocytes. There are age-related changes in the functioning of the immune system. For example, Rosen and colleagues (1999) found that, starting at 6 weeks of age, the thymus and spleen mass decrease as well as the number of CD4+CD8+ double positive T lymphocytes in the thymus compared to mice of 3-4 weeks old. This age-related change in mass suggests that, during puberty, primary immune organs begin an involution that continues throughout adulthood, therefore, reducing the number of immature and naïve T lymphocytes available to be “trained” to fight new infections. This change also suggests that, prior to puberty, the immune system is somewhat immature and may not efficiently “train” T lymphocytes as effectively as adult mice. Hence, the following E:T ratios were selected in the present study: 100:1, 50:1, 25:1, 12.5:1, 6.25:1, and 3.12:1. In consultation with Dr. Bonneau, these E:T ratios were selected based on previous research in adults (Bonneau et al., 1991; Bonneau, 1996) and modified slightly higher to mirror the E:T ratios from the previous
$^{51}$Cr release assays in neonate (e.g., Zahwa, Yorty, & Bonneau, 2008) because it was the first time the $^{51}$Cr release was being performed using effector cells from adolescent mice.

Ideally, one would expect that the greater the number of effector cells the greater the ability to lyse all the target cells for a maximum release of $^{51}$Cr. Data for the three highest E:T ratios resulted in $^{51}$Cr release levels that leveled off or reached a plateau. The plateau in percent $^{51}$Cr lysis is not uncommon because the percent $^{51}$Cr lysis usually follows a 3rd or 4th order polynomial instead of a linear line. This plateau occurs because of a phenomenon known as steric hindrance. Steric hindrance occurs when there are too many cells present and the effector cells are unable to “find” the target cells to lyse. The effector cells can make contact with one another or other lymphocytes (e.g., B cells or $T_H$ cells) instead of the target cells. Therefore, these three higher E:T ratios (100:1, 50:1, and 25:1) were not included in analyses because change in percent $^{51}$Cr lysis did not occur. Inclusion of the lowest three E:T ratios (12.5:1, 6.25:1, and 3.12:1) provided significant changes in percent $^{51}$Cr lysis for all mice, yet, the plateau on the lower end of percent $^{51}$Cr lysis was not reached; therefore, including lower E:T ratios, such as 1.5:1 and 0.75:1, may have revealed differences in $^{51}$Cr release between the experimental groups.

Another methodological issue could have resulted from the type of target cell labeling used in the $^{51}$Cr release assay. Based on previously published report, use of synthetic peptide gB$_{498-505}$ provides reliable and valid results in HSV-1 specific lysis compared to infecting the target cells with HSV-1 (Bonneau, 1996). Consequently, I decided to use the synthetic peptide to label the target cells instead of infecting the target cells with HSV-1 to mimic the numerous published studies investigating the modulation of HSV-1 pathogenesis by stress (e.g., Bonneau, 1996; Bonneau, et al., 1998). However, the gB$_{498-505}$ synthetic peptide
represents only one epitope to which the C57BL/6 mouse can generate during the immune response. The majority of the previous work was done in adult males; therefore, the age of the mice and/or the exposure to nicotine may have had unanticipated consequences on the epitope composition that the immune cells from the mice created \textit{in vivo}.

When infecting the target cells with HSV-1, there is no control over the composition of the epitope that the target cells express which limits control. Another common synthetic epitope, RRI\textsubscript{822-829} (Bonneau, 1996), has been used to study HSV-1 specific T lymphocyte lysis activity. Therefore, it would be valuable to test target cells labeled with different epitopes or via infection of the target cells. However, in adolescents, it would be difficult because the number of lymphocytes isolated at necropsy is lower than that found among adults. Thus, the inclusion of one known epitope may have limited the ability to detect overall differences in HSV-1 specific lysis as the IFN-\(\gamma\) data suggest. Female mice may have had more activated CD8+ T lymphocytes with epitopes more closely related to RRI\textsubscript{822-829} synthetic epitope that were not able to effectively lyse target cells with the gB\textsubscript{498-505} synthetic epitope. All mice may have generated a similar number of active CD8+ T lymphocytes that expressed the gB\textsubscript{498-505}, yet, the decrease in IFN-\(\gamma\) production by the LOW nicotine treatment group could have been a result of no additional CD8+ T lymphocytes expressing another epitope. In hindsight, with the number of modifications to the sample population (i.e., age and drug exposure) compared to published studies, infecting the target cells with HSV-1 may have provided a better platform to observe changes in T lymphocyte cytotoxic activity, but it would also increase the biosafety issues due to the increase in virus handling.

Previous research investigating the effect of stress on the HSV anti-viral response included adult mice and 12-day old neonatal mice using the same design (e.g., rear footpad
injections with sacrifice 5 post infection). Comparing the present periadolescent data to the adult data, the number of lymphocytes isolated in adult control male mice was $2.15 \times 10^7$ (Bonneau et al., 1991), which is greater than $1.6 \times 10^7$ cells isolated from the male control mice from this present study. This difference, however, can almost be explained by body weight. The periadolescent mice weighed about 20.5 g and 7-8 week old adult mice weighed 24.5 g. Adjusting the number of lymphocytes isolated by body weight decreases the difference from 5.5 million cells to 97,000 cells, which is relatively similar; however, the adults still had a greater number of lymphocytes. Bonneau and colleagues (1991) reported the $\% {^{51}}$Cr release at E:T ratios that paralleled the data from this study. The $\% {^{51}}$Cr release was very similar among the adults and periadolescents male mice. For the adult males at 12.5:1 E:T ratio, there was 47% $^{51}$Cr release and for the 6.25 E:T ratio, there was 40% $^{51}$Cr release. For the periadolescent males at the 12.5:1 E:T ratio, there was 45% $^{51}$Cr release and for the 6.25 E:T ratio, there was 38% $^{51}$Cr release. Due to the similarity in values of $^{51}$Cr release, one could conclude that the $^{51}$Cr assay was run effectively in the present study and that adolescent and adult control male mice mounted relatively similar HSV-1 anti-viral immune responses.

In a study by Zahwa, Yorty, and Bonneau (2008), 12-day old neonates were infected with HSV-1 by rear footpad injections and then sacrificed on day 17. Due to the size of the popliteal lymph nodes that were dissected, the cell isolations were pooled among 2-3 animals, therefore, data was not provided for number of cell isolated per individual mouse. However, the lymphocytes were incubated at a similar concentration for 3 days after sacrifice. The IFN-$\gamma$ production of the neonates was about one-fifth of the production in periadolescent mice (9 ng/mL vs. 47 ng/mL, respectively). This difference suggests that the
immature immune system of the neonate was not able to produce a similar anti-viral immune response as compared to the periadolescent mice. However, the % $^{51}$Cr release results suggest otherwise. For the neonates, the % $^{51}$Cr release for the 12.5:1, 6.25:1, and 3.12:1 E:T ratios were the following: 58%, 35%, and 25%, respectively. For the periadolescent mice, the % $^{51}$Cr release for the 12.5:1, 6.25:1, and 3.12:1 E:T ratios were the following: 45%, 39%, and 25%, respectively. The % $^{51}$Cr release data suggest that the neonates were able to produce just as effective HSV-1 specific CD8+ T lymphocytes as the periadolescent male mice maybe even greater at the highest E:T ratio (58% vs. 45%). The comparison of periadolescent to adult and neonate data suggest that there are some age related difference; however, using the $^{51}$Cr release assay provides similar data across the different age populations.

Although there was no significant modulation of HSV-1 specific immunity by sex or nicotine treatment group, the significant effect of sex on the anti-viral lymphocyte proliferation and IFN-$\gamma$ production adds to the literature of females mounting greater immune responses to a variety of vaccinations and females being a greater risk of developing an autoimmune disease. The significant effect of nicotine on IFN-$\gamma$ production supports current research findings that nicotine suppresses T lymphocyte activation, which ultimately adds to the literature that links exposure to tobacco products/nicotine to decrease in serological responses to vaccinations and an increase risk in susceptibility to infection. However, neither the effect of sex nor nicotine was found to modulate the anti-viral response to HSV-1 in adolescent C57BL/6 mice. This in vivo immunologic challenge in the presence of chronic nicotine exposure demonstrates that the immune system is a complex, adaptable set of cells
whose overall outcome – developing lymphocytes effective at removing specific infected cells – is difficult to modify.

**Clinical Relevance**

The use of the HSV-1 mouse model has allowed for the first investigation of the concurrent effects of nicotine exposure and exposure to a sexually transmitted virus during adolescence. A link between adolescents who abuse drugs and initiation of early risky sexual behaviors has been reported (e.g., Porter, Oakley, Ronis, & Neal, 1996; for a review, see Irwin & Millstein, 1992). Initiation of risky sexual behaviors increases the likelihood of contracting a sexually transmitted disease. Therefore, the effect of drugs like nicotine (via tobacco smoking) on the immune system during an anti-viral response must be studied during adolescence due to the possibility of simultaneous exposure to nicotine and viruses.

As the present data demonstrate, the anti-viral response to HSV-1 exposure differs dramatically by sex during periadolescence regardless of nicotine exposure. Therefore, it is critical to study the effects of childhood vaccinations or environmental factors on immune responses in both males and females before and during adolescence. Although previous reports suggest that sex differences in immune function are the result of sex hormones [e.g., estrogen, progesterone (Ahmed et al., 1999; Beagley & Gockel, 2003; De Leon-Nava et al., 2009; Han et al., 2001; Karpuzoglu-Sahin et al., 2001; Rider & Abdou, 2001)], the finding that periadolescent females displayed an increase in number of lymphocytes isolated from the popliteal lymph node and elevated IFN-γ levels as compared to males prior to the onset of puberty when sex steroids are high suggests a genetic or epi-genetic underlying difference between the sexes.
In addition to these sex differences in the anti-viral response, the statistically significant difference in viral exposure (i.e., viral load) could have important clinical implications. According to the CDC (2006), current childhood vaccination protocols do not adjust the dosage of the vaccine for the child’s body weight. Rather, the vaccine dose (not dosage), route of administration, and needle gauge and length is based on the child’s age, which approximates weight. The CDC has established guidelines for vaccine administration to pediatric and adult patients (CDC, 2006); however, none of the guidelines discuss age- or sex-related differences in body weight observed throughout the life span. On average, males weigh more than do females. Therefore, on average, females of all ages receive a greater dosage of most vaccines compared to males. Could this difference in viral load be an underlying cause for the finding that females tend to have a greater response to viral vaccinations compared to males (e.g., Edwards et al., 2007; Goronzy et al., 2001; Kennedy et al., 2009)?

Based on the literature, this increase in viral load is clinically significant for the immune system at a 100-fold difference in viral exposure (Hubalek, 1980). However, Hubalek (1980) only used two levels of viral exposure were used. Incremental increases in viral load would be invaluable to help determine at what point an increase in viral exposure becomes clinically significant. At this time, any reported significant differences in the immune response between the sexes have not been connected to sex differences in viral load. In addition, there are no studies to suggest that body weight is or is not a significant factor in the immune response to viral or vaccine exposure. It is critical to determine whether the body weight difference which leads to a statistical difference in viral load has any clinical significance, particularly for children.
Age of sample population also is critical. It is important not to generalize findings in adult populations to pediatric or geriatric populations. It also is important not to generalize from adolescent to adult. The present data suggest suppression of the anti-viral response in LOW nicotine exposed mice and an increase of the anti-viral response observed in periadolescent females did not result in modulation of HSV-1 specific immunity during periadolescence. Therefore, the immune system is quite capable of mounting a robust pathogen specific (e.g., HSV-1) immunity during periadolescence even if the more generic anti-viral response is affected by environmental factors, which previous adult and neonatal studies do report when studying restraint stress or glucocorticoid exposure (e.g., Bonneau, 1996; Bonneau et al., 1991; Zahwa et al., 2008). Thus, conducting this experimental protocol in adult or neonatal mice may yield different results due to the vulnerability of the immune system that varies with age.

Neonatal mice rely primarily on passive immunity from their mother because their immune system is immature (e.g., Yorty & Bonneau, 2004a; Yorty & Bonneau, 2004b; Yorty et al., 2004; Zahwa et al., 2008). This neonatal paradigm may allow for an observable effect of nicotine on the HSV-specific response. Starting at onset of puberty, the mouse’s thymus and spleen decrease in size as age increases (e.g., Rosen et al., 1999). This decrease in volume of the immune organs and beginning of immunosenescence may weaken the immune system as a whole and be more vulnerable to the effects of nicotine exposure. Therefore, the conclusion that nicotine does not modulate the specific T lymphocyte anti-viral response cannot be made for all age groups. This protocol must be conducted in all age populations before proper conclusions can be drawn about the effect of nicotine on the anti-viral response in neonates and adult mice.
**Future Directions**

The present experiment has provided many more questions than concrete answers concerning the effect of nicotine on the anti-viral response to HSV-1, an *in vivo* immunologic challenge. As it turns out, the response of the immune system to HSV-1 was robust and difficult to modulate with chronic nicotine exposure during adolescence. There are many additional questions that could be asked or paradigms that could be used to test how chronic nicotine exposure could affect the functioning of the immune system. For example, one could investigate the impact of chronic nicotine exposure earlier in life such as pre- or neonatal on anti-viral immunity during adolescence or even later in adulthood. Or, the next step could be to study the effect of chronic nicotine exposure during adolescence on anti-viral immunity in adulthood. Both of these future studies mimic more closely the chronic tobacco use or second hand smoke exposure that humans experience.

Another research direction would be to administer tobacco smoke extracts or tar exudates as opposed to just nicotine. Although nicotine clearly is responsible for behavioral addiction (Clarke & Pert, 1985; Clarke et al., 1985; Koob & Bloom, 1988; Pontieri et al., 1996) and able to interact with the immune system (e.g., Grando et al., 2003; Kawashima et al., 2007; Nouri-Shirazi & Guinet, 2003; Saeed et al., 2005; Singh et al., 2000; Skok et al., 2005; Sopori et al., 1998; Wang et al., 2003; Wang et al., 2004), there are over 4000 chemicals that are inhaled into the body along with nicotine. The design of the present study clearly removes these other chemicals from the equation. Inhaling smoke or just air pollution increases immune system activity in the lungs and causes chronic inflammatory problems such as asthma and chronic obstructive pulmonary disease (e.g., Pujades-Rodriguez, Lewis, McKeever, Britton, & Venn, 2009; Silverman & Ito, 2010; Suwanwaiphathana, Ruangdej,
tobacco smoking causes an increase in lung inflammation, but suppresses the production of cytokines in the peripheral immune system via nicotine exposure – a counter intuitive relationship that research has yet to thoroughly address. One step would be to use smoke extracts or tar exudates, which contain nicotine, to investigate how additional chemicals inhaled through cigarette smoke may impact the immune system. Is it possible that, similar to lung inflammation, ingested smoke extracts or injected tar exudates would lead to inflammation in the lining of the stomach or esophagus or in the skin surrounding the injection? It is difficult to predict which chemical or component of the smoke extract or tar exudate is actually modulating the immune effect. However, understanding how the immune system is compromised by the smoke extract or tar exudate might be worth the ambiguity, which will only lead to further research.

The α7 nAChR knockout mouse has been used in previous nicotine-immune studies investigating the effects of nicotine on sepsis and/or toxic shock (Ghia et al., 2006; Guarini et al., 2003; Guarini et al., 2004; Huston et al., 2006; Luyer et al., 2005; Orr-Urtreger et al., 1997; van Westerloo et al., 2004; Wang et al., 2004). The use of α7 nAChR knockout mice to study the immune system will offer a unique insight into how the α7 nAChR relate to the proper functioning of the immune system. The α7 nAChR knockout mouse is viable and generally exhibits normal behaviors (Morley & Rodriguez-Sierra, 2004). However, the estrous cycle of female α7 nAChR knockout mice is asynchronous and there is a reduction in the number of surviving pups compared to wild-type mice (Morley & Rodriguez-Sierra, 2004). Naylor, Quarta, Fernandes, and Stolerman (2005) found that α7 nAChR knockout mice still developed a tolerance to nicotine administration. In addition, Salas, Main,
Gangitano, and de Biasti (2007) found a reduction in nicotine withdrawal symptoms. Therefore, it is known that the $\alpha 7$ nAChR knockout mice will self-administer nicotine, but less is known regarding how the immune system functions in an environment without $\alpha 7$ nAChR.

To date, there is only one published study on total serum and anti-ovalbumin antibodies and cytokine production in stimulated splenic cells in the $\alpha 7$ nAChR knockout male and female mice 10 – 15 wks old compared to age and sex matched wild-type C57BL/6J mice. Fujii and colleagues (2007) found elevated IgG1, total and anti-ovalbumin specific, in the $\alpha 7$ nAChR knockout mice, but not IgM between the two groups. In addition, splenic cells from the $\alpha 7$ nAChR knockout mice produced significantly more TNF-$\alpha$, IFN-$\gamma$, and IL-6 compared to the wild-type (Fujii et al., 2007). Therefore, it appears the $\alpha 7$ nAChR knockout mouse’s immune system does function when primed with an antigen; however, the antigen was not an active, virulent invader.

An experiment designed to study the HSV-1 anti-viral response in the $\alpha 7$ nAChR knockout male and female adult mice might make for an important next step. If it is assumed that the $\alpha 7$ nAChR is critical to the effect of nicotine on the immune system (Wang et al., 2004; Wang et al., 2003), then determining whether or not the immune system has a “normal” response without the $\alpha 7$ nAChR to a viral stimulus could aid the field in understanding how chronic nicotine exposure may affect the anti-viral response. As mentioned above, research has shown that chronic nicotine exposure quickly leads to desensitization of the neuronal nAChRs (Balfour, 1980; Collins et al., 1990; Robinson et al., 1996). However, current research has not explored desensitization of nAChRs on immune cells. This concept is one that must be thoroughly investigated. If $\alpha 7$ nAChR are solely
responsible for the effect of nicotine and they desensitize to the effects of nicotine on
immune cells, then all the in vitro acute nicotine research will have a completely different
interpretation.

Because the immune system is an adaptive, biological system, it seems reasonable to
conclude that desensitization of the $\alpha 7$ nAChR would not prevent the immune system from
functioning, yet when, how, and among whom (e.g., males vs. females) the immune system
adapts to nicotine exposure is completely unexplored. The addition of $\alpha 7$ nAChR knockout
mice to an experiment investigating the effects of chronic nicotine exposure on the anti-viral
response could provide answers to several questions. Theoretically, the $\alpha 7$ nAChR knockout
mice would represent the complete desensitization of $\alpha 7$ nAChR and its effects on the anti-
viral response. The $\alpha 7$ nAChR knockout mice exposed to nicotine would determine if the $\alpha 7$
nAChR was the only nAChR responsible for the impairment of the immune system. The
wild-type mouse would be used to confirm the results of this experiment and provide as a
control for the $\alpha 7$ nAChR knockout mouse. Assuming that the lack of the $\alpha 7$ nAChR would
represent complete desensitization, it is an important piece in determining whether or not the
immune system is adaptive to the loss of stimulation from $\alpha 7$ nAChR.

As interesting as it might be to study the anti-viral response in $\alpha 7$ nAChR knockout
mice with or without nicotine present, data from this dissertation create questions that only a
truly interdisciplinary team can answer. A research team of behavioral scientists,
immunologists, and molecular geneticists would provide the necessary expertise, as well as
the equipment and techniques required for the thorough investigation of relationship between
nicotine exposure and the anti-viral immune response. The use of HSV-1 as an
immunological challenge to the immune system is absolutely critical to understanding how
the overall immune system works instead of singling out one type of immune cell. This next experiment also should include male and female C57BL/6 mice because the data suggest that female mice may be more sensitive to the effect of nicotine on the functioning of the immune system. The mice would be adolescent; however, the need to study the effect of nicotine on the anti-viral response in adulthood or post-puberty also should be done.

To ensure that the decrease in nicotine consumption (mg/kg) after the HSV-1 injection would not occur in one group, nicotine administration method should be modified. In the nicotine treated mice, there could be two daily injections of nicotine (adjusted for body weight) roughly 12 hrs apart. These injections would ensure that the nicotine dosage in the injection-only mice would have a consistent nicotine exposure throughout the study. Including this nicotine injection-only group only provides a platform to understand the effect of a constant nicotine exposure level and not supporting generalizability because the half-life of nicotine is extremely short and very few human smokers only smoke two times a day separated by 12 hours. There could be an injection plus 3-bottle (2 nicotine concentrations and 1 water) choice group to allow for free-choice of nicotine intake. There also would be a group where saline injections occur daily until the day of HSV-1 infection and then be switched to a nicotine injection until sacrifice. This group would not have access to nicotine water and would approximate the effect of acute nicotine exposure at time of HSV-1 infection. Appropriate control groups should be used such that the controls would get injections of saline instead of nicotine. The control groups would aid in the explanation of the effect of 2 daily injections on nicotine consumption in the 3-bottle choice group and how the stress of the injections may affect the anti-viral response to HSV-1. Similar to the design of this dissertation and prior studies (Bonneau, 1996; Bonneau et al., 1991; Bonneau et al.,
1998), mice should be injected with nicotine or saline and have access to the nicotine water 7 days prior to infection with HSV-1 via rear footpad injections. All mice would be sacrificed 5 days after the infection.

At necropsy, the popliteal lymph nodes would be removed as well as the spleen. The popliteal lymph nodes would be handled in a similar manner as the present study; however, additional and more sophisticated immune assays should be conducted. Once the lymphocytes from the popliteal lymph node were isolated, a sample of the isolation would be used to quantify the type of lymphocytes present. For example, the amount of CD3+, CD4+, CD8+, and CD20+ lymphocytes would be measured. CD3+ lymphocytes include T cells and NK cells (Kindt et al., 2007). CD4+ lymphocytes are TH cells (Kindt et al., 2007). CD8+ lymphocytes are TC cells (Kindt et al., 2007). CD20+ lymphocytes are B cells (Kindt et al., 2007). Quantifying the lymphocytes would allow for greater interpretation of the sex difference that was observed in this dissertation. This additional outcome measure could reveal a nicotine effect that the current experiment was unable to reveal. The lymphocytes would be incubated and the $^{51}$Cr release assay performed similar to the current experiment. Additionally, a subset of lymphocytes would be used to detect gB$_{498-505}$ epitope-specific CD8+ T lymphocytes following necropsy which may provide insight to activation levels measured by IFN-$\gamma$ levels and the outcome of the $^{51}$Cr release assay. Adding the quantification of lymphocytes isolated and HSV-1 specific T lymphocytes would allow for a greater explanation and/or clarification of the results from the current experiment.

Removal of the spleen would allow for investigation of how chronic nicotine exposure affects the rest of the immune system outside of the HSV-1 anti-viral response. Using the spleen lymphocytes, quantification of the lymphocytes and challenge assays could
be performed. Using LPS or ConA to stimulate the splenocytes would more closely resemble current nicotine and adolescent published reports. In addition, the splenocytes could be used to look at expression of the α7 nAChRs without taking away from HSV-1 specific lymphocytes from the popliteal lymph node. To determine if desensitization of the α7 nAChR on the immune cells has occurred, the LPS or ConA challenges could be done in the presence of nicotine. Thus, there would be splenocytes from chronically nicotine exposed mice and nicotine naïve mice which would be stimulated in the presence of nicotine. The results would provide insight into the desensitization of α7 nAChR on immune cells. In addition, the expression of α7 nAChR could be analyzed using reverse transcription polymerase chain reaction (RT-PCR), a nuclease protection assay, flow cytometry, and western blotting using membrane proteins (e.g., De Rosa, Dionisio, Agriello, Bouzat, & Esandi Mdel, 2009; Plummer, Dhar, & Schuller, 2005) on the splenic lymphocytes to determine if nicotine exposure leads to modification of the α7 nAChRs. If there are enough lymphocytes from the popliteal lymph node following the 51Cr release assay prep, it would be interesting to look at the expression of α7 nAChR on HSV-1 specific lymphocytes from the popliteal lymph node as compared to non-HSV-1 specific lymphocytes from the spleen. The findings from this more thorough immune study may provide some answers to the questions that the current experiment has created.

As discussed, there are many directions in which this nicotine/anti-viral immune response can go. However, the possibility of α7 nAChR desensitization on the immune cells must be understood before research on the effects of nicotine on the immune system continues. In addition, studying the effects of nicotine on the immune system without the context of an in vivo immune challenge really limits the field’s advancement in
understanding how adaptable the whole immune system might be in the presence of chronic exposure to nicotine or other exogenous compounds (e.g., additional drugs of abuse).

**Summary**

This dissertation was the first step in investigating the effects of nicotine exposure on the anti-viral immune response in adolescent mice exposed to HSV-1. The significant sex difference in the anti-viral response has demonstrated the importance of studying both male and female mice. The single significant nicotine effect on IFN-γ production by T lymphocytes supports the current literature that nicotine exposure suppresses the activation of T lymphocytes; however, the lack of suppression in the HIGH nicotine group may suggest that desensitization of the α7 nAChR occurs on immune cells. On the day of infection, nicotine consumption (mg/kg) decreased to a level where nicotine may not have had a biologically relevant effect during the anti-viral response due to the desensitization of α7 nAChRs that might have occurred in the presence of high nicotine levels. Therefore, the *in vivo* viral infection appears to result in an extremely robust anti-viral immune response that 7 days of pre-treatment of nicotine and 5 days post-infection nicotine treatment could not substantially modulate (i.e., decrease HSV-1 specific immunity) during adolescence. This dissertation has opened the door to future research to understand the intricate details associated with the modulation of the immune system by nicotine across the life span and males and females.
References


Silverman, R. A., & Ito K. (2010). Age-related association of fine particles and ozone with severe acute asthma in New York City. *Journal of Allergy and Clinical Immunology, 125*, 367-373.


Appendix A: Nicotine Calculation and Solution Preparation
Nicotine Calculations and Solution Preparation

I. 50 μg nic/ml solution

Step 1: Make 10-fold concentrated nicotine solution (1L)
   Add 198 μL freebase nicotine to 400 mL of tap water

Calculation
   500 μg freebase nicotine/mL solution × 400 mL = 200,000 μg nicotine → 200 mg → 0.2 g
   0.2 g nicotine/1.01 g/mL (density of nicotine) = 0.198 mL freebase nicotine → 198 μL freebase nicotine

Step 2: Make 1L 50 μg/ml solution
   100 mL 10-fold 50 μg/mL NIC solution + 900 mL of tap water

II. 200 μg nic/ml solution

Step 1: Make 10-fold concentrated nicotine solution (1L)
   Add 792 μL freebase nicotine to 400 mL H₂O

Calculation
   2000 μg freebase nicotine/mL solution × 400 mL = 800,000 μg nicotine → 800 mg → 0.8 g
   0.8 g nicotine/1.01 g/mL (density of nicotine) = 0.792 mL freebase nicotine → 792 μL freebase nicotine

Step 2: Make 1L 100 μg/ml solution
   100 mL 10-fold 200 μg/mL NIC solution + 900 mL of tap water
Appendix B: HSV-1 Preparation and Infection Protocol
HSV-1 Preparation and Infection Protocol

Preparation of HSV-1 for Injections

Items needed:
- 200 μL pipettor
- 5 mL serological pipette
- 5 mL snap-cap tube
- aliquots of HSV-1
- 1% fetal bovine serum in PBS
- 70% alcohol
- latex gloves
- automatic pipettor
- ice bucket

Procedure:

1) Set up laminar flow hood:
   a. Wipe down inner surface of hood with 70% EtOH.
   b. Label 5 mL snap tube with HSV-1
   c. Set up automatic pipettor and 200 μL pipettor in flow hood.
   d. Place tips and serological pipette in flow hood.
   e. When working in hood, always work behind the air barrier at front of hood, and never touch sterile pipettes/tools to anything in the hood. If a pipette is contaminated because it touches something, throw it out and use a new clean one.

2) With gloved hands, thaw 2 aliquots of 250 μL of HSV-1 Patton strain virus in 37°C water bath. Swirl regularly to ensure even thaw. Place on ice.

3) Take to flow hood.

4) Put 3.668 mL of 1% fetal bovine serum in PBS into 5 mL snap top tube.

5) Put a total of 332 μL of HSV-1 Patton strain into the 5 mL snap top tube. Invert multiple times to ensure proper mixing. Put on ice.

6) Clean up supplies and wipe down flow hood with 70% EtOH.

7) Transport to CBL for injections.

Calculation to obtain 1 X 10^6 PFU/30 μL

Starting concentration = 4 X 10^5 PFU/μL
Needed concentration = 3.3 X 10^4 PFU/μL

0.083 μL of 4 X 10^5 PFU/μL + 0.917 μL of 1% fetal bovine serum in PBS = 1 μL of 3.3 X 10^4 PFU/μL

Make a total of 4 mL or 4000 μL

332 μL of 4 X 10^5 PFU/μL + 3668 μL of 1% fetal bovine serum in PBS
**HSV-1 Injections at CBL**

Items needed:
- ice bucket with ice and rack
- 6 - 1 ml syringe
- 6 – 30-gauge needle
- latex gloves
- isoflurane and vaporizer (at CBL)
- disposable bench top covers
- Prescription of Valtrex in case of needle stick

Procedure:

1. Set up injection area in flow hood in the animal room:
   a. Place disposable, absorbent bench top cover in hood.
   b. Place 5 mL snap top tube containing virus in wire rack.
   c. Place 1 mL syringes and 30 gauge needles in hood.
2. Ensure the proper amount of isoflurane is in the vaporizer.
3. Turn on fan, start the oxygen tank, and set isoflurane to 5%. Ensure nose cone is receiving isoflurane and put into flow hood.
4. With gloved hands, prepare first syringe by pulling 0.6 mL of virus.
5. Remove 27 ½-gauge needle and put in biohazard box. Put a 30-gauge needle on syringe.
6. On bench top cover, anesthetize the mouse with 5% isoflurane until immobile and not responsive to rear foot pad squeeze.
7. Inject 30 μL of virus into each rear foot pad.
8. Remove nose cone after 2 mins of isoflurane exposure (often occurs during 2nd foot pad injection).
9. After injections are complete, place mouse back in home cage.
11. Place used syringes in biohazard box.
12. After injections complete, dispose of bench top cover in biohazard bag and disinfect flow hood and nose cone with quatricide.
13. Turn off isoflurane vaporizer, oxygen, and fan, and prepare for storage.
14. Observe mouse for 10 to 15 minutes after injections to ensure that mice can access food and water without impairment.
Appendix C: Cotinine Enzyme Immunoassay
COTININE DIRECT ELISA KIT

Ver: 01/2004

Immunalysis Corporation:
Catalog Number: 217-0096 1 x 96 well plates
217-0480 5 x 96 well plates

THE IMMUNALYSIS COTININE DIRECT ELISA KIT IS INTENDED FOR RESEARCH USE ONLY.

EXPLANATION OF THE TEST

The Immunalysis Cotinine Direct ELISA Kit is a specific and sensitive in-vitro test to detect the presence of cotinine in serum and urine. Exposure to tobacco smoke can be detected by measuring nicotine and its metabolites. Nicotine has a short half life and is not used as a marker for tobacco smoke exposure. Cotinine due to its longer half life has been used in research as a reliable marker for smoking status and smoking cessation studies.

PRINCIPLES OF THE PROCEDURE

The Immunalysis Cotinine Direct ELISA Kit is based upon the competitive binding to antibody of enzyme labeled antigen and unlabeled antigen, in proportion to their concentration in the reaction mixture.

A 10 µL aliquot of a diluted unknown specimen is incubated with a 100 µL dilution of enzyme (Horseradish peroxidase) labeled Cotinine derivative in micro-plate wells, coated with fixed amounts of oriented high affinity purified polyclonal antibody. The wells are washed thoroughly and a chromogenic substrate added. The color produced is stopped using a dilute acid stop solution and the wells read at 450 nm. The intensity of the color developed is inversely proportional to the concentration of drug in the sample. The technique is sensitive to 1 ng/ml.

REAGENTS

Immunalysis COTININE Direct ELISA Kit Contents

<table>
<thead>
<tr>
<th>Component</th>
<th>96 test Kit</th>
<th>480 test Kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>96 well Micro-plate</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>Cotinine Conjugate</td>
<td>15 mL</td>
<td>60 mL</td>
</tr>
<tr>
<td>Neg Serum Std</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 5 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 10 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 25 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 50 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Cotinine 100 ng/mL</td>
<td>1 mL</td>
<td>2 x 1 mL</td>
</tr>
<tr>
<td>Neg Urine Std</td>
<td>2 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>Cotinine Pos Std</td>
<td>2 mL</td>
<td>5 mL</td>
</tr>
<tr>
<td>TMB Substrate</td>
<td>30 mL</td>
<td>2 x 30 mL</td>
</tr>
<tr>
<td>Stop Reagent</td>
<td>25 mL</td>
<td>55 mL</td>
</tr>
</tbody>
</table>

96 well micro-plate. The micro-plate is coated with polyclonal antibody to Cotinine via a spacer chain to provide optimally oriented binding sites. The plates are sealed in a moisture and air barrier pouch with a desiccant.

Cotinine-Enzyme Conjugate. The conjugate solution contains a Cotinine derivative labeled with horseradish peroxidase in a buffered, protein solution with stabilizers, pH 7.5 containing non azide preservatives.

Negative Serum Standard. This bottle contains drug free rabbit serum containing azide free preservatives.

Cat# 217 ELISA Cotinine Serum 1-04
Serum Cotinine Standards. These bottles contains 5, 10, 25, 50 and 100 ng/mL of Cotinine dissolved in a rabbit serum non azide preservatives.

Negative Urine Standard. This bottle contains drug free synthetic urine matrix containing azide free preservatives.

Urine Cotinine Positive Standard. This bottle contains 500 ng/mL of cotinine in a synthetic urine matrix containing azide free preservatives.

TMB chromogenic substrate. The color reagent contains 3,3',5,5' tetramethylbenzidine and urea peroxidase in buffer.

Stop Reagent. This contains 1 N hydrochloric acid.

MATERIALS AND EQUIPMENT

Materials and equipment required but not supplied with the Immunoasys Cotinine Direct ELISA Kit are itemized below
12x75 mm Disposable Glass or Plastic Culture Tubes to predilute samples (if required).
Manual or electronic micropipets (single channel or multichannel) or automated pipetting stations.
Refrigerator (for kit storage).
Interval Timer.
Wash bottle or Plate Washer.
Microplate reader capable of reading at 450 nm. And 650 nm.

Precautions
1. Do not use internal or External Use in Humans or Animals.
2. There should be no eating or drinking within work area.
3. Always wear gloves and a protective lab coat.
4. No pipetting should be done by mouth. Handle all specimens and reagents as potentially infectious and biohazardous.
5. Do not add sodium azide to samples as preservative.
6. Do not use external controls containing sodium azide.
7. Use disposable pipet tips to avoid contaminating chromogenic substrate reagent. Discard reagent if it turns blue.
8. Do not pour chromogenic substrate back into container after use.
9. Do not freeze reagents.
10. Do not mix reagents from different kit lot numbers.
11. Keep reagents out of direct sunlight.
12. Handle stop reagent with care, since it is corrosive.
13. Bring all reagents to room temperature.
14. Viscous forensic samples should always be diluted in phosphate buffered saline or distilled water prior to pipetting.
15. Ensure the bag containing the micro-plate strips and desiccant is well sealed if only a few strips are used.

General. Precise pipetting is the essence of successful immunocassay. It is critical to pipet right at the center and bottom of each well to ensure good replicates and coefficients of variation. Microtiter plates supplied by "Eppendorf" or "Smit" with disposable tips are excellent when used carefully according to instructions to insure the necessary accuracy. Now automatic dispensers improve reliable delivery.

Storage. The expiration date of the kit is stated on the label. The kit can be expected to perform satisfactorily until the expiration date if stored in the refrigerator at 2 – 8°C.

Indications of Deterioration. A drop of greater than 50% in the A4 (zero standard absorbance reading) for a constant incubation time indicates deterioration of the antibody plate, enzyme conjugate or chromogenic substrate. A significant shift of the standard curve to the right would result from deterioration of the standards. Development of blue color in the chromogenic substrate without the addition of enzyme conjugate indicates contamination of the substrate.

SPECIMEN COLLECTION

Precautions
The Immunoasys Cotinine Direct ELISA Kit is to be used with human urine or serum. Immunoasys has not tested all possible applications of this assay. Cutoff criteria are important in deciding the sample dilution.
Additives
Specimens to which sodium azide has been added affect the assay.

DETAILS OF THE PROCEDURE.
All reagents must be brought to room temperature (20-25°C) before use.

The procedure as described below may be followed in sequence using manual pipettes. Alternatively all reagents may be added using an automated pipette. Use urine calibrators for urine and serum calibrators for serum. Depending on the cutoffs a sample dilution may be required for urine applications.

1. Add 10 μl of calibrators and standards to each well in duplicate.
2. Add 10 μl of the specimens in duplicate (recommended) to each well.
3. Add 100 μl of the Enzyme Conjugate to each well. Tap the sides of the plate holder to ensure proper mixing.
4. Incubate for 60 minutes at room temperature (20-25°C) preferably in the dark, after addition of enzyme conjugate to the last well.
5. Wash the wells 6 times with 350 μl distilled water using either a suitable plate washer or wash bottle taking care not to cross contaminate wells.
6. Invert wells and vigorously slap dry on absorbent paper to ensure all residual moisture is removed. This step is critical to ensure that residual enzyme conjugate does not skew results. If using an automated system, ensure that the final aspiration on the wash cycle aspirates from either side of the well.
7. Add 100 μl of Substrate reagent to each well and tap sides of plate holder to ensure proper mixing.
8. Incubate for 30 minutes at room temperature, preferably in the dark.
9. Add 100 μl of Stop Solution to each well, to change the blue color to yellow.
10. Measure the absorbance at a dual wavelength of 450 nm and 550 nm.
11. Wells should be read within 1 hour of yellow color development.

The following data represent a typical dose/response serum cotinine curve.

<table>
<thead>
<tr>
<th>Cotinine (ng/ml)</th>
<th>Absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.759</td>
</tr>
<tr>
<td>5</td>
<td>1.075</td>
</tr>
<tr>
<td>10</td>
<td>0.865</td>
</tr>
<tr>
<td>25</td>
<td>0.691</td>
</tr>
<tr>
<td>50</td>
<td>0.495</td>
</tr>
<tr>
<td>100</td>
<td>0.419</td>
</tr>
</tbody>
</table>

The dose/response curve shown above should not be used in assay calculations. It is recommended that at least one in-house positive quality control sample be included with every assay run. A dose response curve or a cutoff calibrator should be run with every plate.

RESULTS
If the average sample absorbance is equal to or less than the average absorbance of the laboratory positive reference standard the sample is POSITIVE for Cotinine. If the average sample absorbance is greater than the average absorbance of the laboratory positive reference standard the sample is called NEGATIVE for Cotinine.

Alternatively a dose response curve can be established by plotting standard concentration (abscissa) against corresponding absorbance (ordinate). Values for unknown samples are obtained by interpolation from the curve.

SPECIFIC PERFORMANCE CHARACTERISTICS

Accuracy
20 urine samples from non smokers were screened with the Immulynosis Cotinine ELISA method. All 20 samples screened negative with the ELISA method. 15 samples from smokers which contained various amounts of Cotinine were screened with the Immulynosis Cotinine Direct ELISA Kit. All 15 samples showed a presence of cotinine at a level greater than 500 ng/ml.

Cat# 217 ELISA Cotinine Serum 1-04
Three urine samples submitted by individuals exposed to passive inhalation for over 30 days all showed levels of 5 to 10 mg/mL of cotinine when extrapolated of a dose response curve.

**Sensitivity**
Assay sensitivity based on the minimum Cotinine concentration required to produce a three standard deviation from assay A0 is 1 ng/mL.

**Specificity**
The specificity of the Immunoassay Cotinine ELISA was determined by generating inhibition curves for each of the compounds listed below. The antisera cross-reactivities are listed below.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Approx. ng/mL equivalent to 100 ng Cotinine</th>
<th>Cross-reactivities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cotinine</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>Nicotine</td>
<td>&gt;10000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Nicotinamide</td>
<td>&gt;10000</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Nicotinic Acid</td>
<td>&gt;10000</td>
<td>&lt; 1</td>
</tr>
</tbody>
</table>

**Cross-Reactivity with Unrelated Drugs**
All lots of a human urine matrix were spiked with the following compounds at a concentration of 50,000 ng/mL. None of these compounds gave values in the assay that were equal to or greater than the assay sensitivity level.

Acetaminophen, Acetylsalicylic acid, Amphetamine, Aminopyrine, Ampicillin, Amobarbital, Ascorbic acid, Atropine, Barbitral, Butalbarbital, Caffeine, Cocaine, Carbamazepine, Codeine, Chloroquine, Chlorpromazine, Carbromal, Desipramine, Dextromethorphan, Dextropropoxyphene, 5,5-Diphenylhydantoin, 10,11-Dihydrocarbamazepine, Diazepam, Ethosuximide, Estradiol, Estrone, Estraadiol, Ethanol, Glutethimide, Hexobarbital, Ibuprofen, Imipramine, Lidocaine, LSD, Methadone, Methadone-primary metabolites, Methaqualone, Methamphetamine, Metharbital, Meprynydrox, Meprobamate, Methyl PEMA, Metaxaemic, 4-Methylprazepam, Morphine, Meperidone, Nicotinamide, Norethindrone, N-Normethaeximide, Phenobarbital, Phenaximide, PEMA, Primidone, Phenycyclidine, Pentobarbital, Phenothiazine, Phenylpropanolamine, Procaine, Quinine, Seconobarbital, Tetracycline, Tetrahydrozoline

**IMMUNALYSIS CORPORATION**
Pomona, CA, 91767, USA
(909) 482-0840
Appendix D: Corticosterone Enzyme Immunoassay
DetectX™

Corticosterone

Enzyme Immunoassay Kit
Catalog Number K014-H1

Species Independent
Sample Types Validated:
Dried Feces, Serum, EDTA and Heparin Plasmas and Tissue Culture Media

Please read this insert completely prior to using the product.

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROTOCOLS
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INTRODUCTION

Corticosterone (C_{21}H_{30}O_{6}, Kendall’s Compound ‘B’) is a glucocorticoid secreted by the cortex of the adrenal gland. Corticosterone is produced in response to stimulation of the adrenal cortex by ACTH and is the precursor of aldosterone. Corticosterone is a major indicator of stress and is the major stress steroid produced in non-human mammals. Studies involving corticosterone and levels of stress include impairment of long term memory retrieval, chronic corticosterone elevation due to dietary restrictions and in response to burn injuries. In addition to stress levels, corticosterone is believed to play a decisive role in sleep-wake patterns.

![](image)

Corticosterone

THE ARBOR ASSAYS DetectX™ CORTICOSTERONE IMMUNOASSAY KIT

Assay Principle
The DetectX™ Corticosterone Immunoassay kit is designed to quantitatively measure Corticosterone present in extracted dried fecal samples, serum, plasma and tissue culture media samples. Please read the complete kit insert before performing this assay. This kit measures total corticosterone in serum and plasma and in extracted fecal samples. A corticosterone standard is provided to generate a standard curve for the assay and all samples should be read off the standard curve. Standards or diluted samples are pipetted into a clear microtiter plate coated with an antibody to capture sheep antibodies. A corticosterone-peroxidase conjugate is added to the standards and samples in the wells. The binding reaction is initiated by the addition of a polyclonal antibody to corticosterone to each well. After an hour incubation the plate is washed and substrate is added. The substrate reacts with the bound corticosterone-peroxidase conjugate. After a short incubation, the reaction is stopped and the intensity of the generated color is detected in a microtiter plate reader capable of measuring 450nm wavelength. The concentration of the corticosterone in the sample is calculated, after making suitable correction for the dilution of the sample, using software available with most plate readers.

Related Products
DetectX™ Cortisol Immunoassay Kits
K003-H1, K003-H5, K003-H1W, K003-H5W, and K003-B1
DetectX™ Colorimetric and Fluorescent Glutathione Detection Kits
K006-H1, K006-F1, and K006-F5
DetectX™ Fluorescent Glutathione S-Transferase Activity Kit K008-F1
DetectX™ Fluorescent Glutathione Reductase Activity Kit K009-F1
AbX™ Glutathione Monoclonal Antibody A001-50UG
AbX™ Cysteine Monoclonal Antibody A002-50UG
Supplied Components

**Coated Clear Microtiter Plate**
96 well Catalog Number X061-1EA
A clear plastic microplate coated with donkey anti-sheep IgG.

**Corticosterone Standard**
125 μL Catalog Number C043-125UL
A stock solution of corticosterone at 50,000 pg/mL.

**DetectX™**

**Corticosterone Antibody**
3 mL Catalog Number C044-3ML
A sheep polyclonal antibody specific for corticosterone.

**DetectX™**

**Corticosterone Conjugate**
3 mL Catalog Number C045-3ML
A corticosterone-peroxidase conjugate in a special stabilizing solution.

**Assay Buffer**
28 mL Catalog Number X059-28ML

**Dissociation Reagent**
1 mL Catalog Number X058-1ML

**Wash Buffer Concentrate**
30 mL Catalog Number X007-30ML
A 20X concentrate that should be diluted with deionized or distilled water.

**TMB Substrate**
11 mL Catalog Number X019-11ML

**Stop Solution**
5 mL Catalog Number X020-5ML
A 1N hydrochloric acid solution. **Caustic.**

**Plate Sealer**
1 each Catalog Number X002-1EA

**Storage Instructions**
All components of this kit should be stored at 4 °C until the expiration date of the kit.
Other Materials Required
A microplate shaker and washer.
A supply of distilled or deionized water.
Colorimetric 96 well microplate reader capable of reading optical density at 450 nm, preferably with correction between 570 and 590 nm.
Software for converting raw relative optical density readings from the plate reader and carrying out four parameter logistic curve (4PLC) fitting. Contact your plate reader manufacturer for details.

Precautions
As with all such products, this kit should only be used by qualified personnel who have had laboratory safety instruction. The complete insert should be read and understood before attempting to use the product.

The Stop Solution is acid. The solution should not come in contact with skin or eyes. Take appropriate precautions when handling this reagent.

Sample Types
This assay has been validated for extracts of a wide variety of mammalian fecal samples, and for diluted mammalian serum, EDTA and heparin plasmas including human, rat, dog, pig, monkey and mouse samples, and for tissue culture samples. Samples containing visible particulate should be centrifuged prior to using. Moderate to severely hemolyzed samples should not be used in this kit.

Corticosterone is identical across all species and we expect this kit may measure corticosterone from sources other than those listed above. The end user should evaluate recoveries of corticosterone in other samples being tested.
Sample Preparation

Dried Fecal Samples
We have a detailed Extraction Protocol available on our website at: http://www.ArborAssays.com/resources/lit.asp. Briefly we recommend weighing about 0.2 gm of dried crushed feces into a clean 4 mL vial. 2 mL (1 mL/100 mg) of ethanol is added to the vial and it vigorously mixed for 30 minutes. Centrifuge samples at 5,000 rpm for 15 minutes. Transfer a known volume of the supernatant to a clean vial. Dry in a SpeedVac or under nitrogen. Reconstitute the dried sample with 100 μL of ethanol and dilute with 400 μL of kit Assay Buffer. Dilute the sample with Assay Buffer prior to running in the assay. Final concentration of ethanol must be ≤ 5% in the well.

See page 14 for measured corticosterone values for samples.

Samples extracted in this manner typically give extraction efficiencies of approximately 100%. To determine the efficiency, add a known amount of corticosterone standard to an aliquot of the sample prior to ethanol extraction and compare the value obtained in the spiked sample with an unspiked sample.

Serum and Plasma Samples
Serum and plasma samples should be diluted with an equal volume of the supplied Dissociation Reagent. Addition of this reagent will yield the total corticosterone concentration in serum or plasma. The Dissociation Reagent treated samples should then be further diluted ≥1:25 for plasma and ≥ 1:50 for serum with the supplied Assay Buffer prior running in the assay.

Tissue Culture Media
For measuring corticosterone in tissue culture media (TCM), samples should be read off a standard curve generated in TCM. Samples may need to be diluted further in TCM. We have validated the assay using RPMI-1640.
Standard Preparation

Label seven test tubes as #1 through #7. Pipet 450 µL of Assay Buffer into tube #1 and 250 µL into tubes #2 to #7. Carefully add 50 µL of the corticosterone stock solution to tube #1 and vortex completely. Take 250 µL of the corticosterone solution in tube #1 and add it to tube #2 and vortex completely. Repeat the serial dilutions for tubes #3 through #7. The concentration of corticosterone in tubes 1 through 7 will be 5,000, 2,500, 1,250, 625, 312.5, 156.25 and 78.125 pg/mL.

Use all Standards within 2 hours of preparation.

Wash Buffer Preparation

Prepare the Wash Buffer by diluting it 1:20 with distilled or deionized water. Once diluted it is stable for 3 months at room temperature.

Assay Protocol

Allow the kit reagents to come to room temperature for 30 minutes. The recommended format is 1 hr at room temperature with shaking. Incubating at room temperature for 1 hr without shaking, results in an approximate 45% reduction in bound signal. We recommend that all standards and samples be run in duplicate to allow the end user to accurately determine Corticosterone concentrations. Ensure that all samples have reached room temperature and have been diluted as appropriate prior to running them in the kit.
**Assay Protocol**

1. Use the plate layout sheet on the back cover to aid in proper sample and standard identification. Determine the number of wells to be used and return unused wells to foil pouch with desiccant. Seal the ziploc plate bag and store at 4°C.

2. Pipet 50 μL of samples or standards into wells in the plate. Pipet 75 μL of Assay Buffer into the non-specific binding (NSB) wells. Pipet 50 μL of Assay Buffer into wells to act as maximum binding wells (80).

3. Add 25 μL of the DetectX™ Corticosterone-Peroxidase conjugate to each well, using a repeater or multichannel pipet.

4. Add 25 μL of the DetectX™ Corticosterone Antibody solution to each well, except the NSB wells, using a repeater or a multichannel pipet.

5. Gently tap the sides of the plate to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and shake at room temperature for 1 hour.

6. Aspirate the plate and wash 4 times with the wash buffer. Tap the plate dry on absorbent towels.

7. Add 100 μL of the TMB Substrate to each well, using a repeater or a multichannel pipet.

8. Incubate the plate at room temperature for 30 minutes without shaking.

9. Add 50 μL of the Stop Solution to each well, using a repeater or a multichannel pipet.

10. Read the optical density generated from each well in a plate reader capable of reading at 450 nm. Please contact your plate manufacturer for details.

11. Use the plate reader's built-in 4PLC software capabilities to calculate corticosterone concentration for each sample.
Calculation of Results
Average the duplicate OD readings for each standard and sample. Create a standard curve by reducing the data using the 4PLC fitting routine on the plate reader, after subtracting the mean OD's for the NSB. The sample concentrations obtained, calculated from the %B/Bo curve, should be multiplied by the dilution factor to obtain neat sample values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Mean OD</th>
<th>Net OD</th>
<th>%B/Bo</th>
<th>Concentration pg/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB</td>
<td>0.047</td>
<td>0</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Std 1</td>
<td>0.270</td>
<td>0.224</td>
<td>18.4</td>
<td>3,200</td>
</tr>
<tr>
<td>Std 2</td>
<td>0.380</td>
<td>0.333</td>
<td>27.5</td>
<td>1,600</td>
</tr>
<tr>
<td>Std 3</td>
<td>0.529</td>
<td>0.482</td>
<td>39.8</td>
<td>800</td>
</tr>
<tr>
<td>Std 4</td>
<td>0.689</td>
<td>0.642</td>
<td>53.0</td>
<td>400</td>
</tr>
<tr>
<td>Std 5</td>
<td>0.882</td>
<td>0.836</td>
<td>68.9</td>
<td>200</td>
</tr>
<tr>
<td>Std 6</td>
<td>0.993</td>
<td>0.947</td>
<td>78.1</td>
<td>100</td>
</tr>
<tr>
<td>Std 7</td>
<td>1.094</td>
<td>1.047</td>
<td>86.4</td>
<td>50</td>
</tr>
<tr>
<td>Bo</td>
<td>1.259</td>
<td>1.213</td>
<td>100.0</td>
<td>0</td>
</tr>
<tr>
<td>Sample 1</td>
<td>0.686</td>
<td>0.340</td>
<td>28.0</td>
<td>2,387.9</td>
</tr>
<tr>
<td>Sample 2</td>
<td>1.034</td>
<td>0.988</td>
<td>81.4</td>
<td>127.2</td>
</tr>
</tbody>
</table>

Always run your own standard curve for calculation of results. Do not use this data.

Conversion Factor: 1 pg/mL of Corticosterone is equivalent to 2.886 pM.
Typical Standard Curve

Always run your own standard curve for calculation of results.
Do not use these data.

**Sensitivity**
Sensitivity was calculated by comparing the OD’s for twenty wells run for each of the Bo and standard #7. The detection limit was determined at two (2) standard deviations from the Bo along the standard curve.

**Sensitivity was determined as 18.6 pg/mL.**

**Limit of Detection**
The Limit of Detection for the assay was determined in a similar manner by comparing the OD’s for twenty wells for each of the zero standard and a low concentration human sample.

**Limit of Detection was determined as 16.9 pg/mL.**
**Linearity**

Linearity was determined by taking two serum samples treated with Dissociation Reagent and diluted 1:50 with Assay Buffer, one with a low diluted corticosterone level of 104.6 pg/mL and one with a higher diluted level of 2,890.5 pg/mL, and mixing them in the ratios given below. The measured concentrations were compared to the expected values based on the ratios used.

<table>
<thead>
<tr>
<th>Low Serum</th>
<th>High Serum</th>
<th>Observed Conc. (pg/mL)</th>
<th>Expected Conc. (pg/mL)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>100%</td>
<td>0%</td>
<td>104.6</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>80%</td>
<td>20%</td>
<td>654.0</td>
<td>661.8</td>
<td>96.4</td>
</tr>
<tr>
<td>60%</td>
<td>40%</td>
<td>1,232.3</td>
<td>1,219.0</td>
<td>99.3</td>
</tr>
<tr>
<td>40%</td>
<td>60%</td>
<td>1,763.9</td>
<td>1,776.1</td>
<td>101.1</td>
</tr>
<tr>
<td>20%</td>
<td>80%</td>
<td>2,249.5</td>
<td>2,333.3</td>
<td>98.8</td>
</tr>
<tr>
<td>0%</td>
<td>100%</td>
<td>2,890.5</td>
<td>---</td>
<td>---</td>
</tr>
</tbody>
</table>

**Mean Recovery** 98.9%

![Corticosterone Sample Linearity](chart.png)

\[
y = 0.9545x + 45.521 \\
R^2 = 0.9985
\]
Intra Assay Precision

Four human samples were diluted with Assay Buffer and run in replicates of 20 in an assay. The mean and precision of the calculated Cortisol concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cortisol Conc. (pg/mL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,460.6</td>
<td>6.3</td>
</tr>
<tr>
<td>2</td>
<td>601.5</td>
<td>6.5</td>
</tr>
<tr>
<td>3</td>
<td>371.6</td>
<td>3.1</td>
</tr>
<tr>
<td>4</td>
<td>259.0</td>
<td>4.8</td>
</tr>
</tbody>
</table>

Inter Assay Precision

Three human samples were diluted with Assay Buffer and run in duplicates in fourteen assays run over multiple days by four operators. The mean and standard deviation of the calculated Cortisol concentrations were:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cortisol Conc. (pg/mL)</th>
<th>%CV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2,618.3</td>
<td>7.5</td>
</tr>
<tr>
<td>2</td>
<td>630.1</td>
<td>6.4</td>
</tr>
<tr>
<td>3</td>
<td>267.9</td>
<td>9.9</td>
</tr>
</tbody>
</table>
**Sample Values**

Six random mammalian serum and plasma samples were tested in the assay. Neat sample values ranged from 0.87 to 38.5 µg/dL with an average for the human samples of 1.56 µg/dL. The normal reference range for serum corticosterone is 0.13–2.3 µg/dL. Dried fecal samples were processed as described on page 7 and the reconstituted extracts were tested in the assay. The following results were obtained:

**Samples kindly donated by Dr. J. Williams, Indianapolis Zoo.**

<table>
<thead>
<tr>
<th>Animal</th>
<th>pg Corticosterone/ mg Sample</th>
<th>Animal</th>
<th>pg Corticosterone/ mg Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Addoro Gazelle</td>
<td>20.70</td>
<td>Lion</td>
<td>81.60</td>
</tr>
<tr>
<td>Amur Tiger</td>
<td>28.40</td>
<td>Reeves Muntjac</td>
<td>13.90</td>
</tr>
<tr>
<td>Giraffe</td>
<td>13.80</td>
<td>White Handed Gibbon</td>
<td>8.03</td>
</tr>
<tr>
<td>Kudu</td>
<td>15.67</td>
<td>White Rhino</td>
<td>7.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zebra</td>
<td>8.80</td>
</tr>
</tbody>
</table>

**Cross Reactivity**

The following cross reactants were tested in the assay and calculated at the 50% binding point.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Cross Reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>100%</td>
</tr>
<tr>
<td>Desoxycorticosterone</td>
<td>12.30%</td>
</tr>
<tr>
<td>Tetrahydrocorticosterone</td>
<td>0.76%</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.62%</td>
</tr>
<tr>
<td>Cortisol</td>
<td>0.38%</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.24%</td>
</tr>
<tr>
<td>Cortisone</td>
<td>&lt; 0.03%</td>
</tr>
<tr>
<td>Estradiol</td>
<td>&lt; 0.03%</td>
</tr>
</tbody>
</table>
LIMITED WARRANTY

Arbor Assays warrants that at the time of shipment this product is free from defects in materials and workmanship. This warranty is in lieu of any other warranty expressed or implied, including but not limited to, any implied warranty of merchantability or fitness for a particular purpose.

We must be notified of any breach of this warranty within 48 hours of receipt of the product. No claim shall be honored if we are not notified within this time period, or if the product has been stored in any way other than outlined in this publication. The sole and exclusive remedy of the customer for any liability based upon this warranty is limited to the replacement of the product, or refund of the invoice price of the goods.

CONTACT INFORMATION

For details concerning this kit or to order any of our products please contact us:

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Appendix E: Popliteal Lymph Node Removal and Lymphocyte Isolation
**Popliteal Lymph Node Removal and Lymphocyte Isolation**

**Equipment & Supplies needed:**

**For lymph node extraction:**
- a. Necropsy tools (Small scissors, small rat-toothed forceps & small forceps, ‘pins'/needles, cork board, 70% EtOH in glass vial to soak tools between each mouse)
- b. Snap-cap tubes with 4mL of complete Iscove’s media for lymphocytes (1/mouse)
- c. Ice in bucket
- d. Disposable bench top cover

**For lymph node processing/incubation:**
- a. CO2 Incubator (37°C, 95% humidity, 5% CO2)
- b. Nylon Tissue Sieves (one/mouse; 70 gauge mesh; catalog no. XXX)
- c. 50 mL conical tube with 5 mL of complete Iscove’s media for lymphocytes (1/mouse)
- d. Automatic Pipettor
- e. Serological Pipettes -- 5 mL
- f. Hemacytometer
- g. Pipette (200 μL)
- h. Sterile Pipette tips (200 μL)
- i. Complete Iscove’s media for lymphocytes
- j. 5 syringes plungers; 1/mouse
- k. Conical centrifuge tubes (15 mL & 50 mL, 1 of each tube/mouse)
- l. Trypan blue dye – aliquotted in 200 μL (see Rob Bonneau)
- m. 70% EtOH – in squirt bottle
- n. Tube racks – snap top tubes, 15 mL and 50 mL conical tubes
- o. Centrifuge bucket inserts for 15 mL centrifuge tubes
- p. 12-Well Petri dishes
- q. One rack to house Petri dishes from BSC II to incubator

**Several days prior to popliteal node removal:**

1) **Calibrate CO2 incubator:** Turn incubator on and set to 37°C, with 95% humidity, 5% CO2 and water in the tray at bottom at least 2 days before calibration. Once temperature and humidity have stabilized over several days, turn off the source of CO2. When the CO2 is purged from the incubator (which should happen in a few hours), the CO2 readout should read 0.0%. If not, then adjustments must be made. Using the screwdriver concealed in one of the control knobs, turn the small inset screw labeled ‘zero’ below the CO2 readout panel until the panel reads 0.0. See incubator instructions for more information on calibration process.

2) **Prepare the following items:**
   - a. Complete the Iscove’s media (see media protocol) for lymphocytes
   - b. Place 200 μL of trypan blue in eppendorf tubes
   - c. Place 4 mL of Iscove’s complete media for lymphocytes in 5 mL snap tubes
   - d. Place 5mL of Iscove’s complete media for lymphocytes in 50 mL conical tubes
Day of popliteal node removal and processing:

2) Items to bring to CBL:
   a. 5 mL snap top tubes with media on ice (1/mouse)
   b. Necropsy tools
   c. 70% EtOH
   d. Disposable bench top cover

3) Set up popliteal node pulling work station:
   a. Put snap tube labeled with ID of mouse in rack in laminar flow hood.
   b. Place all surgical tools in beaker of 70% EtOH.
   c. Cover work surface with disposable cover and place cork board on cover.

4) Remove popliteal nodes:
   a. Sacrifice mouse via cervical dislocation and perform heart stick.
   b. Pin dead mouse, back-side down, to cork board using push pins. Pin back legs at 90° angle from body (i.e. splayed to either side) and soak both legs with 70% EtOH.
   c. Lift and clip a large piece of skin behind rat knee to expose the muscle and then cut into muscle behind knee to expose the popliteal node within the muscle layer. You should see a fat pad behind the knee – pull on this fat pad and hopefully the popliteal node will come out with the fat. Be careful not to damage the popliteal node. The popliteal node will be within the fat pad at the junction of vasculature and will be pinkish and about 2-3mm in diameter. Remove extra tissue from node – e.g. fat – and drop node into Iscove’s media and place tube back on ice. If you harvested fat instead of lymph node, the tissue will float in the media; a lymph node will sink. Using the same procedure, pull popliteal from other side, and place into same snap tube and place on ice.
   d. Pass off mouse for liver removal and weighing.
   e. Process all mice in sacrifice order using the same method.

5) Clean up necropsy work area.

6) Transport lymph nodes in ice-cold Iscove’s media to the laminar flow hood area in S. 38 Henderson.

7) Set up laminar flow hood: (The following work should all be aseptic.)
   a. Wipe down inner surface of hood with 70% EtOH.
   b. Plug in automatic pipette, have Complete Iscove’s media for T-lymphocytes, and racks for snap top tubes, 50 mL conical tubes, and 15 mL conical tubes.
   c. On cart next to laminar flow hood, place all necessary supplies for isolation – pre-filled 50 mL conical tubes labeled, 15 mL conical tubes labeled, disposable nylon screens, 5 mL and 10 mL serological pipettes, ice bucket w/ lymph nodes from necropsy, gloves, 70% ethanol squirt bottle, sterile pipette tips, 200 μL pipette, and eppendorf tubes with 200 μL of trypan blue.
   d. When working in hood, always work behind the air barrier at front of hood, and never touch sterile pipettes/tools to anything in the hood. If a pipette is contaminated because it touches something, throw it out and use a new clean one.
8) Break up popliteal cells:
   a. Get correctly labeled 50 mL conical tube with 5 mL of media and disposable
      nylon screen (open nylon screen such that little handle on the lip faces the
      opening of the bag to reduce contamination).
   b. Place disposable nylon screen in top of 50 mL conical tube, dump lymph
      nodes and 4 mL into screen (make sure two lymph nodes are in mesh screen).
   c. Move lymph nodes into middle of screen and using the rubber end of a 5 mL
      sterile syringe plunger, smash and push the lymph node cells through the
      nylon screen.
   d. Using left hand, hold plunger and nylon screen (prior to lifting off 50 mL tube
      – slightly tilt nylon screen as to not lose any media or cells), suck up the 5ml
      of media from 50 mL tube and rinse off plunger over screen.
   e. Using left hand, hold plunger and nylon screen (prior to lifting off 50 mL tube
      – slightly tilt nylon screen as to not lose any media or cells), suck up the 5ml
      of media from 50 mL tube and rinse the nylon screen. (Rub the tube against
      the screen to make a scratchy noise and can feel vibrations.)
   f. Change pipette and pull 5ml of Iscove’s from the clean source bottle and rinse
      again the screen and plunger.
   g. Pipette up all the media and cells in the 50 mL conical tube (volume should 14
      mL) and transfer to correctly labeled 15mL centrifuge tube. Place 15 mL tube
      on ice.
   h. Repeat for all samples.
   i. Centrifuge 15 mL conical tubes at 1000 rpm for 5 mins to pellet cells. (Make
      sure centrifuge is balanced.)
   j. Pour off supernatant.
   k. Re-suspend in 5 mL of Complete Iscove’s media for T lymphocytes.

9) Count cell concentration:
   a. Sterilely, remove 200 μL of cell/media solution and add to the prepared 200
      μL of trypan blue aliquots in an eppendorf tube.
   b. Mix dye and cells with pipette tip then put small volume onto a
      hemacytometer and allow one minute for the cells to migrate to bottom of
      slide. Using the shortest objective, count the number of cells visible across all
      25 squares in the center of the hemacytometer if greater than 30 cells.
      Otherwise, count four corners of the greater 9 squares and then take average.
      Cells will have white highlighted outline.
   c. Pull up excel spreadsheet (Cell isolation counts) to calculate total number of
      cells. Multiple the number of cells counted on 25 square grid by 2 (dilution
      factor). Then multiple by the total volume (ml) of liquid in the centrifuge tube
      that contains the popliteal cells. Then multiply by $1 \times 10^4$. This gives you the
      total number of cells in the centrifuge tube. For example, if you count 60 cells
      across all 25 squares of the hemacytometer, and you pulled these cells from a
      centrifuge tube with 12ml of Iscove’s, your total cell count would be:

      \[
      60 \times 2 \times 12 \times 1 \times 10^4 = 1.44 \times 10^7
      \]
d. Calculate this number for each centrifuge tube of cells, and write down the total number of cells in each of the centrifuge tubes – make sure to keep track of the number of cells in each tube.

10) Re-suspend lymph node-derived cells at 4 x 10^6 cells/ml:
   a. Centrifuge all the 15ml centrifuge tubes with cells for 5min at 1000rpm at RT.
   b. Look for the pellet of popliteal cells at the bottom of each tube, and then pour off supernatant without pouring out the cells.
   c. Re-suspend cells with the appropriate amount of complete Iscove’s to bring them up to a 4x10^6 concentration. From the example above, if you calculated that there are 1.44x10^7 cells in your centrifuge tube, then add 3.6ml of complete Iscove’s to the tube.

11) Plate the cells:
   a. Place 0.8 mL of cell solution into 12-well plates. Label plate lid with animal number corresponding to number of wells filled with each animal’s lymphocytes.
   b. After all plates are pipetted, place them into a plastic tray with lid.
   c. Place plates into CO2 incubator.
   d. Clean up the laminar flow hood area.
Appendix F: $^{51}$Cr Release Assay Protocol
**51Cr Release Assay Protocol**

**Must wear dosimeter, gloves, lab coat, pants and close-toe shoes**

1. Peptide pulsing and 51Cr labeling of WT-3 cells
   a. Take T-25 flask containing WT-3 cells remove excess media using a 5 mL serological pipet.
   b. Add 0.5 mL trypsin/versene to dissociate cells at 37°C for 1-2 minutes. Be sure not to over-trypsinize! Alternatively, you can used pre-warmed (37°C) aliquot trypsin-versene and therefore not worry about the need for a 37°C incubator for doing this step.
   c. Add 4.5 mL of WT-3 media and transfer the dissociated cells to 15 mL conical tube and mix well.
   d. Perform cell count by placing 200 μL of cell solution into 200 μL of trypan blue.
   e. Centrifuge at 1000 RPM for 5 mins. Carefully pour off supernatant and save pellet.
   f. Use Excel spreadsheet Cell counts for 51Cr assay to calculate.
   g. Re-suspend cells at 1 x 10⁶ cells/ml of complete Iscove’s media for WT-3 cells.
   h. Place 1.5 mL of the WT-3 cell solution into two 15 mL conical tubes (one labeled MOCK and one labeled gB).
   i. Peptide pulse WT-3 Cells in “gB” labeled 15 mL conical tube.
      i. Thaw one vial of gB 498-505 peptide in 37°C water bath or at room temperature.
      ii. Pipet 100 μL of the gB 498-505 peptide into 15 mL conical tube; vortex. Be sure to only place the pipet tip into the tube; in other words, you do not want to contaminate the barrel of the pipettor with the peptide. Add 100 μL of tissue culture media to the "MOCK" tube.
   j. 51Cr label WT-3 Cells
      i. Using 1 mL syringe pull up 250 μCi (behind lead bricks)
         Calculated: Date of 1000 μCi = 
         Today’s Date = Days Past = 
         μCi today = 
         (250 μCi)/(μCi today) = μL 51Cr(Na₂CrO₄)
      ii. Add to 15 mL conical tube of peptide-pulsed WT-3 Cells and MOCK (behind lead bricks – always handle MOCK first). Be sure that the 51Cr gets into the small volume of media containing the cells at the bottom of the tube.
   iii. Place in 37°C water bath for 2 hours; swirl every 15 minutes
      Swirl #1 Swirl #5
      Swirl #2 Swirl #6
      Swirl #3 Swirl #7
      Swirl #4 Swirl #8
   iv. Add 10 ml of media to each tube
   v. Centrifuge 15 mL tubes at 1000 RPM x 3 minutes (this step is NOT considered to be a "wash").
   vi. Remove tubes from centrifuge; gently pour off supernatant into liquid
51Cr waste

vii. Re-suspend pellets in residual media by tapping bottom of tube
viii. Add 10 mL 5 x 1 media to each tube; cap tightly and invert to mix thoroughly
ix. Centrifuge vials at 1000 RPM x 3 minutes (Wash #1)
x. Remove tubes from centrifuge; **gently** pour off supernatant into liquid 51Cr waste
xi. Re-suspend pellets in residual media by tapping bottom of tube
xii. Add 10 mL 5 x 1 media to each tube; cap tightly and invert to mix thoroughly
xiii. Centrifuge vial at 1000 RPM x 3 minutes (Wash #2)
xiv. Remove tubes from centrifuge; **gently** pour off supernatant into liquid 51Cr waste
xv. Re-suspend pellets in residual media by tapping bottom of tube
xvi. Add 10 mL 5 x 1 media to each tube; cap tightly and invert to mix thoroughly
xvii. Centrifuge vial at 1000 RPM x 3 minutes (Wash #3)
xviii. Remove tubes from centrifuge; **gently** pour off supernatant into liquid 51Cr waste
xix. Re-suspend in 5 mL of complete Iscove’s media for T-lymphocytes.
xx. The final concentration of cells for the assay is 2 x 10^4 cells/ml. Therefore, if you start with 1 x 10^6 cells, you can make 50 ml of ready-to-use target cells. If you don't need 50 ml of target cells (50 ml will make enough for 500 wells of a 96-well plate), prepare amount necessary for assay – for example 2.5 mL of target cells into 22.5 mL of complete Iscove’s media for T-lymphocytes or 5 mL of target cells into 45 mL of complete Iscove’s media for T-lymphocytes.

2. Harvesting CTL Effector Cell
   a. Observe via inverted microscope; determine if CTL matured.
   b. Transfer cells to 15 mL conical tube using a 5 ml serological pipet.
   c. Centrifuge at 1000 RPM for 5 mins. Pour off supernatant and save pellet.
   d. Add 0.75 mL per well of plated cells. This will give a starting effector-to-target cell ratio of 100:1
   e. Vortex sample prior to plating.

3. Measuring Effector Lytic Activity
   a. Label all 96-well "v"-bottom plates according to template ** *** Run each E:T ratio in triplicate ***
   b. Fill all wells of diluted effector ratios with 100 μL of complete Iscove’s media for T lymphocytes.
   c. Put 200 μL of effector cells in highest E:T ratio wells
   d. Serially dilute by removing 100 μL from row A into row B. Pump liquid 10 times by pushing to only the "first stop". On the last of the 10 pumps, go all the way to the 2nd stop. Remove the pipet tips from the liquid and then carefully remove 100 μL from well and transfer to the next well. Then move 100 μL to
row C and continue until all ratios completed. Discard the leftover 100 μL of effector cells. *** AVOID BUBBLES ***
e. Add 100 μL of each gB peptide-pulsed 51Cr-labelled target cells using a multi-channel pipet, holding the tips above the wells, and depressing the plunger all the way to the 2nd stop quickly to "shoot" the cells into the wells.
f. Run 6 wells of Spontaneous (SPON) and 6 wells of Maximum (MAX) for each target.
   i. SPON = 100 μL Iscove's Media + 100 μL gB peptide-pulsed 51Cr-labelled target cells using a multi-channel pipet, holding the tips above the wells, and depressing the plunger quickly.
   ii. MAX = 100 μL SDS + 100 μL Target gB peptide/mock-pulsed 51Cr-labeled target cells.
g. Final volume in each well should equal 200 μL.
h. Repeat process with MOCK labeled target cells.
i. Open new plate lids or clean an appropriate number of plate lids with ethanol.
j. Cover plates with lids; place plates on balance; use water to equilibrate.
k. Centrifuge plates at 600 RPM x 3 minutes (BRAKE OFF!!!).
l. Remove plates from centrifuge.
m. Incubate at 37°C on bottom shelf in radioactive tray for 4 - 5 hours.

4. Harvest samples:
   a. Transfer 100 μL of each sample to corresponding collection tube.
   b. Use multi-channel pipet; pipet slowly; avoid the pellet by placing the pipet tips at a 45° angle.
   c. Begin with lowest E:T ratio of a particular target cell and work your way up to the highest E:T ratio. There is no need to switch pipet tips between any of the effector-to-target cell ratios...as long as you start with the lowest E:T ratio (e.g. 3.12:1) and work your way up to the higher E:T ratio (e.g. 100:1).
   d. Transfer SPON, then MAX last (reduce chance of contamination of other wells with SDS).
   e. Repeat for each Target (MOCK & gB).
   f. Place Q-Tip in each collection tube.
   g. Samples may be stored on bench top behind lead bricks until gamma counter analysis.

5. Analyze samples on the gamma counter
   a. Package samples and flash drive for transportation to Noll laboratory.
   b. In 117 Noll/GCRC, prep gamma counter for 51Cr counting – select run #2.
   c. Find clip with barcode 02 and begin to fill with 6 SPON and 6 MAX followed by unknowns for peptide (gB) plate. Ensure 2nd and subsequent clips have no barcode.
   d. Fill MOCK-pulsed target cells in behind peptide-pulsed target cells start with 6 SPON and 6 MAX, then unknowns.
e. Once all samples are loaded, use the STOP STOP barcoded clip to stop gamma counter.
f. After run complete, go to Klein folder on desktop and copy data to flash drive.
g. Package up all samples return to 30 S. Henderson for disposal.
h. Do not throw samples away until data reviewed.

6. Analyze Data using excel to calculate % lysis.

7. Using Excel, calculate the best fit polynomial equation for each mouse based on calculated % lysis.

8. Using SolveMyMath website’s definite integral calculator integrate the polynomial equation to obtain the area under the curve for each mouse.
Appendix G: Interferon-γ Enzyme Immunoassay
Quantikine®

Mouse IFN-γ Immunoassay

Catalog Number MIF00
    SMIF00
    PMIF00

For the quantitative determination of mouse interferon gamma (IFN-γ) concentrations in cell culture supernates and mouse serum.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.
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INTRODUCTION

Interferon gamma (IFN-γ, also known as Type II interferon) was originally identified as an anti-viral activity produced by mitogen-activated T lymphocytes (1). The protein shares no significant homology with IFN-β or the various IFN-α family proteins. Besides its anti-viral activity, IFN-γ has been shown to play a key role in host defense by exerting antiproliferative, immunoregulatory, and proinflammatory activities. IFN-γ induces the production of cytokines and upregulates the expression of class I and II MHC antigens, Fc receptor, and leukocyte adhesion molecules. IFN-γ modulates macrophage effector functions, influences isotype switching, and potentiates the secretion of immunoglobulins by B cells (2, 3). IFN-γ has also been shown to augment IL-12-induced Th1 development (4).

IFN-γ is produced primarily by T lymphocytes and natural killer cells. The production of IFN-γ is induced by antigenic challenge and by cytokines such as IL-12 (6). Mouse IFN-γ encodes a 155 amino acid (aa) residue precursor protein with a hydrophobic signal peptide that is cleaved to generate the 133 aa residue mature protein (6). In solution, IFN-γ has been shown to exist as a non-covalently associated homodimer with topological similarity to IL-10 (2, 7). Mouse IFN-γ shows approximately 40% aa sequence identity to human IFN-γ and there is no cross-reactivity across species (2, 3).

The IFN-γ receptor consists of two subunits (8). The α subunit binds IFN-γ with high-affinity and species-specificity in the absence or presence of the β subunit. The β subunit (also referred to as the accessory factor 1, AF-1) interacts with the α subunit in a species-specific manner and is required for signal transduction. Both the α and β subunits are members of the type II cytokine receptor family that also includes the IL-10 receptor (9). The presence of a naturally occurring soluble form of the ligand binding IFN-γ receptor has been reported in normal human urine (10).

The Quantikine Mouse IFN-γ immunoassay is a 4.5 hour solid phase ELISA designed to measure mouse IFN-γ in cell culture supernates and mouse serum. It contains E. coli-expressed mouse IFN-γ and antibodies raised against the recombinant factor. This immunoassay has been shown to quantitate recombinant mouse IFN-γ accurately. Results obtained using natural mouse IFN-γ showed dose-response curves that were parallel to the standard curves obtained using the recombinant kit standards. These results indicate that the Quantikine Mouse IFN-γ immunoassay kit can be used to determine relative mass values for natural mouse IFN-γ.
PRINCIPLE OF THE ASSAY
This assay employs the quantitative sandwich enzyme immunoassay technique. Monoclonal antibody specific for mouse IFN-γ has been pre-coated onto a microplate. Standards, Control, and samples are pipetted into the wells and any mouse IFN-γ present is bound by the immobilized antibody. After washing away any unbound substances, an enzyme-linked polyclonal antibody specific for mouse IFN-γ is added to the wells. Following a wash to remove any unbound antibody-enzyme reagent, a substrate solution is added to the wells. The enzyme reaction yields a blue product that turns yellow when the Stop Solution is added. The intensity of the color measured is in proportion to the amount of mouse IFN-γ bound in the initial step. The sample values are then read off the standard curve.

LIMITATIONS OF THE PROCEDURE
- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- If samples generate values higher than the highest standard, dilute the samples with the appropriate Calibrator Diluent and repeat the assay.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
- This assay is designed to eliminate interference by soluble receptors, binding proteins, and other factors present in biological samples. Until all factors have been tested in the Quantikine Immunoassay, the possibility of interference cannot be excluded.

PRECAUTION
The Stop Solution provided with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

TECHNICAL HINTS
- When mixing or reconstituting protein solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.
- For best results, pipette reagents and samples into the center of each well.
- It is recommended that the samples be pipetted within 15 minutes.
- To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary.
- Substrate Solution should remain colorless until added to the plate. Keep Substrate Solution protected from light. Substrate Solution should change from colorless to gradations of blue. The color developed in the wells will turn from blue to yellow upon addition of the Stop Solution.
- Stop Solution should be added to the plate in the same order as the Substrate Solution.
## MATERIALS PROVIDED

<table>
<thead>
<tr>
<th>Description</th>
<th>Part #</th>
<th>Cat. # MIF00</th>
<th>Cat. # SMIF00</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mouse IFN-γ Microplates</strong> - 96 well polystyrene microplates (12 strips of 8 wells) coated with a monoclonal antibody specific for mouse IFN-γ.</td>
<td>890475</td>
<td>2 plates</td>
<td>6 plates</td>
</tr>
<tr>
<td><strong>Mouse IFN-γ Conjugate</strong> - 23 mL/vial of a polyclonal antibody against mouse IFN-γ conjugated to horseradish peroxidase with preservatives.</td>
<td>892666</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Mouse IFN-γ Standard</strong> - 6 ng/vial of recombinant mouse IFN-γ in a buffered protein base with preservatives; lyophilized.</td>
<td>890477</td>
<td>3 vials</td>
<td>9 vials</td>
</tr>
<tr>
<td><strong>Mouse IFN-γ Control</strong> - Recombinant mouse IFN-γ in a buffered protein base with preservatives; lyophilized. The concentration range of mouse IFN-γ after reconstitution is shown on the vial label. The assay value of the Control should be within the range specified on the label.</td>
<td>890478</td>
<td>3 vials</td>
<td>9 vials</td>
</tr>
<tr>
<td><strong>Assay Diluent RD1-21</strong> - 12.5 mL/vial of a buffered protein solution with preservatives.</td>
<td>895215</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Calibrator Diluent RD5Y</strong> - 21 mL/vial of a buffered protein solution with preservatives. <em>For cell culture supernate samples.</em></td>
<td>895201</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Calibrator Diluent RD6-12</strong> - 21 mL/vial of a buffered protein solution with preservatives. <em>For serum samples.</em></td>
<td>895214</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Wash Buffer Concentrate</strong> - 50 mL/vial of a 25-fold concentrated solution of a buffered surfactant with preservative.</td>
<td>895024</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Color Reagent A</strong> - 12.5 mL/vial of stabilized hydrogen peroxide.</td>
<td>895000</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Color Reagent B</strong> - 12.5 mL/vial of stabilized chromogen (tetramethylbenzidine).</td>
<td>895001</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Stop Solution</strong> - 23 mL/vial of a diluted hydrochloric acid solution.</td>
<td>895174</td>
<td>1 vial</td>
<td>3 vials</td>
</tr>
<tr>
<td><strong>Plate Covers</strong> - Adhesive strips.</td>
<td>————</td>
<td>8 strips</td>
<td>24 strips</td>
</tr>
</tbody>
</table>

MIF00 contains sufficient materials to run ELISAs on two 96 well plates. SMIF00 (SixPak) contains sufficient materials to run ELISAs on six 96 well plates.

This kit is also available in a PharmPak (R&D Systems, Catalog # PMIF00). PharmPaks contain sufficient materials to run ELISAs on 50 microplates. Specific vial counts of each component may vary. Please refer to the literature accompanying your order for specific vial counts.
STORAGE

<table>
<thead>
<tr>
<th>Unopened Kit</th>
<th>Diluted Wash Buffer</th>
<th>Stop Solution</th>
<th>Calibrator Diluent RD6Y</th>
<th>Calibrator Diluent RD6-12</th>
<th>Assay Diluent RD1-21</th>
<th>Conjugate</th>
<th>Unmixed Color Reagent A</th>
<th>Unmixed Color Reagent B</th>
<th>May be stored for up to 1 month at 2 - 8° C.*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Opened/Reconstituted Reagents</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Use a new Standard and Control for each assay.</td>
</tr>
<tr>
<td></td>
<td>Mouse IFN-γ Standard</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Return unused wells to the foil pouch containing the desiccant pack, reseal along entire edge of zip-seal. May be stored for up to 1 month at 2 - 8° C.*</td>
</tr>
</tbody>
</table>

*Provided this is within the expiration date of the kit.

OTHER SUPPLIES REQUIRED

- Microplate reader capable of measuring absorbance at 450 nm, with the correction wavelength set at 540 nm or 570 nm.
- Pipettes and pipette tips.
- 1000 mL graduated cylinder.
- Deionized or distilled water.
- Squirt bottle, manifold dispenser, or automated microplate washer.
- Polypropylene tubes.

SAMPLE COLLECTION AND STORAGE

Cell Culture Supernates - Remove particulates by centrifugation and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Serum - Allow blood samples to clot for 2 hours at room temperature or overnight at 2 - 8° C before centrifuging for 20 minutes at 2000 x g. Remove serum and assay immediately or aliquot and store samples at ≤ -20° C. Avoid repeated freeze-thaw cycles.

Note: Grossly hemolyzed or lipemic samples may not be suitable for measurement of mouse IFN-γ with this assay.
REAGENT PREPARATION

Bring all reagents to room temperature before use.

**Mouse IFN-γ Kit Control** - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

**Wash Buffer** - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare enough Wash Buffer for one plate, add 25 mL Wash Buffer Concentrate into deionized or distilled water to prepare 625 mL of Wash Buffer.

**Substrate Solution** - Color Reagents A and B should be mixed together in equal volumes within 15 minutes of use. Protect from light. 100 μL of the resultant mixture is required per well.

**Mouse IFN-γ Standard** - Reconstitute the mouse IFN-γ Standard with 2.0 mL of Calibrator Diluent RD5Y (for cell culture supernate samples) or Calibrator Diluent RD6-12 (for serum samples). Do not substitute other diluents. This reconstitution produces a stock solution of 3000 pg/mL. Allow the standard to sit for a minimum of 5 minutes with gentle mixing prior to making dilutions.

**Use polypropylene tubes.** Pipette 400 μL of the appropriate Calibrator Diluent (Calibrator Diluent RD5Y for cell culture supernate samples or Calibrator Diluent RD6-12 for serum samples) into the 600 pg/mL tube. Pipette 200 μL of the appropriate Calibrator Diluent in the remaining tubes. Use the stock solution to produce a dilution series (below). Mix each tube thoroughly before the next transfer. The 600 pg/mL standard serves as the high standard. The appropriate Calibrator Diluent serves as the zero standard (0 pg/mL).
ASSAY PROCEDURE

Bring all reagents and samples to room temperature before use. It is recommended that all samples, standards, and control be assayed in duplicate.

1. Prepare reagents, samples, and standards as directed by the previous sections.

2. Remove excess microplate strips from the plate frame, return them to the foil pouch containing the desiccant pack, and reseal.

3. Add 50 µL of Assay Diluent RD1-21 to each well.

4. Add 50 µL of Standard, Control, or sample to each well. Mix by gently tapping the plate frame for 1 minute. Cover with the adhesive strip provided. Incubate for 2 hours at room temperature. Plate layouts are provided as a record of samples and standards assayed.

5. Aspirate each well and wash, repeating the process four times for a total of five washes. Wash by filling each well with Wash Buffer (400 µL) using a squirt bottle, manifold dispenser, or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.

6. Add 100 µL of mouse IFN-γ Conjugate to each well. Cover with a new adhesive strip. Incubate for 2 hours at room temperature.

7. Repeat the aspiration/wash as in step 5.

8. Add 100 µL of Substrate Solution to each well. Incubate for 30 minutes at room temperature. Protect from light.

9. Add 100 µL of Stop Solution to each well. Gently tap the plate to ensure thorough mixing.

10. Determine the optical density of each well within 30 minutes, using a microplate reader set to 450 nm. If wavelength correction is available, set to 540 nm or 570 nm. If wavelength correction is not available, subtract readings at 540 nm or 570 nm from the readings at 450 nm. This subtraction will correct for optical imperfections in the plate. Readings made directly at 450 nm without correction may be higher and less accurate.
PROCEDURE SUMMARY AND CHECKLIST

1. □ Bring all reagents to room temperature.
□ Prepare reagents and samples as instructed.
□ Return unused components to storage temperature as indicated in the instructions.

2. □ Add 50 μL Assay Diluent to each well.

3. □ Add 50 μL Standard, Control, or sample to each well.
□ Tap plate gently for one minute.
□ Cover the plate and incubate for 2 hours at room temperature.

4. □ Aspirate and wash each well five times.

5. □ Add 100 μL Conjugate to each well.
□ Cover the plate and incubate 2 hours at room temperature.

6. □ Aspirate and wash each well five times.

7. □ Add 100 μL Substrate Solution to each well. Incubate 30 minutes at room temperature. Protect from light.

8. □ Add 100 μL Stop Solution to each well.

9. □ Read Optical Density at 450 nm (correction wavelength set at 540 nm or 570 nm).
CALCULATION OF RESULTS

Average the duplicate readings for each standard, control, and sample and subtract the average zero standard optical density.

Plot the optical density for the standards versus the concentration of the standards and draw the best curve. The data can be linearized by using log/log paper and regression analysis may be applied to the log transformation.

To determine the IFN-γ concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding IFN-γ concentration.

If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

TYPICAL DATA

These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.
**PRECISION**

**Intra-assay Precision** (Precision within an assay)
Three samples of known concentration were tested twenty times on one plate to assess intra-assay precision.

**Inter-assay Precision** (Precision between assays)
Three samples of known concentration were tested in twenty assays to assess inter-assay precision.

### Cell Culture Supernate Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-assay Precision</th>
<th>Inter-assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean (pg/mL)</td>
<td>44.8</td>
<td>156</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.2</td>
<td>5.0</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.9</td>
<td>3.2</td>
</tr>
</tbody>
</table>

### Serum Assay

<table>
<thead>
<tr>
<th>Sample</th>
<th>Intra-assay Precision</th>
<th>Inter-assay Precision</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>n</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>Mean (pg/mL)</td>
<td>45.1</td>
<td>143</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>2.1</td>
<td>4.4</td>
</tr>
<tr>
<td>CV (%)</td>
<td>4.7</td>
<td>3.1</td>
</tr>
</tbody>
</table>
RECOVERY
The recovery of mouse IFN-γ spiked to three levels throughout the range of the assay in various matrices was evaluated.

<table>
<thead>
<tr>
<th>Sample Type</th>
<th>Average % Recovery</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell culture supernates (n = 7)</td>
<td>105</td>
<td>98 - 115%</td>
</tr>
<tr>
<td>Mouse serum (n = 5)</td>
<td>97</td>
<td>91 - 106%</td>
</tr>
</tbody>
</table>

LINEARITY
To assess the linearity of the assay, five or more samples containing and/or spiked with various concentrations of mouse IFN-γ in each matrix were diluted with the appropriate Calibrator Diluent and then assayed.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Dilution</th>
<th>Observed (pg/mL)</th>
<th>Expected (pg/mL)</th>
<th>Observed x 100 Expected</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Culture</td>
<td>neat</td>
<td>477</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Supernates</td>
<td>1/2</td>
<td>252</td>
<td>238</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td>1/4</td>
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SENSITIVITY
The minimum detectable dose of mouse IFN-γ is typically less than 2 pg/mL.
The minimum detectable dose was determined by adding two standard deviations to the mean
optical density value of twenty zero standard replicates and calculating the corresponding
concentration.

CALIBRATION
This immunoassay is calibrated against a highly purified E. coli-expressed recombinant mouse
IFN-γ produced at R&D Systems. This 136 amino acid residue recombinant mouse IFN-γ
contains three additional amino-terminal residues (cys-tyr-cys), and has a predicted molecular
mass of 16 kDa.

Based on total amino acid analysis, the absorbance of a 1 mg/mL solution of the
E. coli-expressed recombinant mouse IFN-γ at 280 nm was determined to be 1.12 A.U.

The NIH reference preparation mouse IFN-γ Gg02-901-533 which was intended as a bioassay
standard, was evaluated in this kit. Each ampule contains a nominal 1 μg of natural mouse
IFN-γ and was assigned an arbitrary unitage of 1000 Units/ampule.

NIH Gg02-901-533: 1 Unit of standard = 230 pg of Quantikine Mouse IFN-γ

SAMPLE VALUES
Serum - Forty individual mouse serum samples were evaluated for the presence of mouse
IFN-γ in this assay. Thirty-eight samples measured less than the lowest mouse IFN-γ standard,
9.4 pg/mL. Two samples read 24 pg/mL and 60 pg/mL, respectively.

Cell Culture Supernates -
Mouse splenocytes (2 x 10⁶ cells/mL) were cultured for 3 days in RPMI plus 10% fetal calf
serum and stimulated with 10 μg/mL PHA. The cell culture supernate was assayed for mouse
IFN-γ and measured 32 ng/mL.

Mouse thymoma cells (EL-4; 9 x 10⁵ cells/mL) were cultured for 2 days in DMEM plus
10% fetal calf serum and stimulated with 10 μg/mL PHA and 10 ng/mL PMA. The cell culture
supernate was assayed for mouse IFN-γ and measured 4 ng/mL.

Mouse lung conditioned media (1 lung, 1 - 2 mm pieces in 10 mL of medium) was collected
after culturing for 5 days in RPMI plus 10% fetal calf serum. The cell culture supernate was
assayed for mouse IFN-γ and measured 198 pg/mL.
SPECIFICITY

This assay recognizes both recombinant and natural mouse IFN-γ. The factors listed below were prepared at 50 ng/mL in Calibrator Diluent and assayed for cross-reactivity. Preparations of the following factors prepared at 50 ng/mL in a mid-range mouse IFN-γ control were assayed for interference. No significant cross-reactivity or interference was observed.

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</table>

A sample containing 66 ng/mL of recombinant rat IFN-γ measured 27 pg/mL in this assay (0.04% cross-reactivity). Upon dilution, the dose-curve of the rrIFN-γ was parallel to the mouse IFN-γ standard curve.

REFERENCES

PLATE LAYOUTS
Use these plate layouts as a record of standards and samples assayed.
Curriculum Vitae
Jeanette Marie Bennett

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Email: jmbennett@psu.edu

Education

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Professional and Research Experience

2005 – 2010  Graduate Research Assistant, Department of Biobehavioral Health (BBH), The Pennsylvania State University (PSU), Advisor: Dr. Laura Cousino Klein.
2004 – 2005  Supervisor, Department of Anatomic Pathology Operations, Covance Laboratories, Vienna, Virginia
2003 – 2004  Research Assistant, Institute of Pathology, Case Western Reserve University School of Medicine, Cleveland, Ohio (Professor: Dr. Thomas Pretlow)

Grants and Awards

2009 – 2010  Kligman Graduate Fellowship (Kligman Graduate Fellowship Endowment and the College of Health and Human Development, PSU)
2009 – 2010  Hintz Graduate Education Enhancement Fellowship (BBH, PSU)
2009        Howard F. Martin Graduate Assistant Outstanding Teaching Award (Graduate School and the Office of the Vice President and Dean for Undergraduate Education, PSU)
2009        Biobehavioral Health Outstanding Graduate Teaching Award (BBH, PSU)
2008 – 2009  Hintz Graduate Education Enhancement Fellowship (BBH, PSU)
2005 – 2006  Hintz Graduate Education Enhancement Fellowship (BBH, PSU)
2002        Honors Scholar, magna cum laude (Gannon University)
2002        Joe Luckey Award for Service to the University (Gannon University)

Scientific Publications in Refereed Journals

