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Sex Differences in the Associations Among Sickness Behavior, Immune Status, and Immune Function in an Adolescent Murine Model

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Abstract

Animals exhibit familiar behavioral changes in response to infection including anorexia, adipsia, hypersomnia, and reduced social interaction. The coordinated, non-specific behavioral changes associated with infection are collectively known as "sickness behavior". Experimental evidence suggests that sickness behavior is not a passive consequence of infection but rather an adaptive strategy that complements physiological and immunological responses to infection. Although the immune system changes over the course of the lifespan, there is currently no research investigating sickness behavior during adolescence, a critical period of development.

The purpose of this thesis was to investigate the association between sickness behavior, immune status, and immune function in periadolescent (PN 28-42) C57BL/6J mice infected with herpes simplex virus (HSV)-1. Food consumption, water intake, and body weight were measured for male (n=19) and female (n=20) C57BL/6J mice over eleven days. On day 6, all mice were infected with HSV-1 in both rear foot pads. On day 11, mice were sacrificed and popliteal lymph nodes were removed to observe immune status (lymphocyte production of interferon (IFN)-γ) and function (HSV-1 specific T-lymphocyte lysis) in response to HSV-1 infection.

It was hypothesized that there would be positive correlations between measures of sickness behavior and measures of immune status and function. Largely, these hypotheses were not supported by the analyses. There was a significant correlation between decrease in liquid consumption and immune function in female mice. A discussion of these results indicates limitations of this analysis and possible future directions for this line of research.

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List of Abbreviations

^{51}Cr = Chromium 51 radioisotope
ANOVA = analysis of variance
IL = interleukin
IFN-γ = interferon-gamma
HPA-axis = hypothalamic-pituitary-adrenal axis
HSV = herpes simplex virus
LPS = lipopolysaccharide
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
NK = natural killer cell
TNF-α = tumor necrosis factor-alpha
PN = post natal day

Introduction

Animals exhibit familiar behavioral changes in response to infection including anorexia, adipsia, hypersomnia, and reduced social interaction. The coordinated, non-specific behavioral changes associated with infection are collectively known as "sickness behavior." Sickness behavior is mediated by proinflammatory cytokines including interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α). These cytokines and others including interferon-gamma (IFN-γ) are produced by activated immune cells in response to pathogens and help to coordinate multiple other aspects of immunity, including inflammation, fever, and the acute-phase response. Proinflammatory cytokines promote changes in brain physiology which lead to a psychological change in motivational state which prioritizes infection response and recovery over other needs. In this way, sickness behavior represents biologically-important communication from the immune system to the brain. Originally, sickness behavior was understood as general weakness that occurred when an animal diverted energy from other metabolic activities to fight an infection. Further investigation has revealed that sickness behavior is a highly adaptive reaction to infection which complements physiological and metabolic responses. This introduction will discuss the biological mechanisms responsible for sickness behavior, present evidence for the hypothesis that sickness behavior is an adaptive response to infection, and give a brief overview of the purpose and significance of this thesis.

Sickness Behavior is Ubiquitous Among Vertebrates

Since antiquity, it has been recognized that sick animals behave differently than their healthy counterparts. Aristotle notes in *The History of Animals* that "Sickness in birds may be diagnosed from their plumage, which is ruffled when they are sickly instead of lying smooth as when they are well" (Aristotle, Thompson, 1942, p. 601). In addition to reduced grooming, sick animals express a subset of the following symptoms which characterize sickness behavior: anorexia (decreased appetite), adipsia (decreased thirst), hypersomnia (excessive sleepiness), decreased activity, decreased reproductive behavior, and decreased social interaction. Humans exhibit the same behavioral changes as animals when sick. One nineteenth-century naturalist noted that "a sick dog is in many ways like a sick child" (Anon., 1874, p. 46). Humans also report psychological changes associated with sickness including depression, malaise, and the inability to concentrate. These behavioral and psychological changes are nonspecific and can be triggered by infections from a variety of pathogenic agents (Hart, 1988). Sickness behavior is often although not always accompanied by fever. Sickness behavior and fever appear to be ubiquitous among vertebrates (Wingfield, 2003).

Sickness Behavior is Mediated by Proinflammatory Cytokines

Because behavior is ultimately regulated by the brain, the central nervous system must somehow detect infectious pathogens to elicit sickness behavior. Generally, the brain cannot directly detect infectious agents because most pathogens are unable to cross the blood-brain barrier and neurons do not have receptors to detect bacteria or viruses. Rather, the innate immune system detects pathogens and communicates with the brain to

cause sickness behavior. The immune system communicates with the nervous system via a subset of small signaling proteins known as cytokines.

Multiple lines of evidence suggest that sickness behavior is mediated by proinflammatory cytokines released in response to infection. Krueger and colleagues (1984) found that rabbits intravenously injected with "endogenous pyrogen," a fever-causing factor now recognized as the proinflammatory cytokine IL-1β, exhibited increased sleep compared to controls. Experiments by Tazi and colleagues (1988) demonstrated that male Sprague-Dawley rats injected peripherally or centrally with physiological levels of recombinant IL-1β demonstrated anorexia and decreased social exploration. Similar behavioral changes were observed after injecting other proinflammatory cytokines, including IL-6 and TNF-α, into adult rodent models (Johnson, 2002).

The hypothesis that sickness behavior is mediated by proinflammatory cytokines is further supported by evidence suggesting that behavioral changes associated with infection could be prevented by blocking cytokine action. For example, C3H/HeJ mice have a mutation that renders their activated lymphocytes unable to produce cytokines. As a result, adult male C3H/HeJ mice do not exhibit sickness behavior after being injected with lipopolysaccharide (LPS), an antigenic component of gram-negative bacterial walls (Johnson, Gheusi, Segreti, Dantzer, & Kelley, 1997). When injected with recombinant IL-1β, the mutant mice exhibited typical sickness behavior. Similarly, some of the sickness behavior associated with the injection of LPS can be attenuated by the administration of IL-1 receptor antagonists in adult male albino Wistar rats (Bluthe, Dantzer, & Kelley, 1992).

Evidence from animal experiments is complemented by clinical trials of recombinant cytokines as potential chemotherapeutic agents. Administration of proinflammatory cytokines in humans caused a number of undesirable side effects, including malaise, fatigue, and weakness. These side effects were identical to the typical non-specific symptoms of illness in humans and consistent with the sickness behavior observed in animals (Dantzer & Kelley, 1989).

Sickness behavior is the result of molecular and neuronal communication between the immune and nervous systems. Following the phagocytosis of particulate antigens, macrophages and monocytes become activated and secrete a variety of factors involved in the inflammatory response, fever, and sickness behavior (Goldsby, Kindt, Osborne, & Kuby, 2003). The primary factors responsible for mediating sickness behavior are a subset of the proinflammatory cytokines including interleukin 1 (IL-1), interleukin 6 (IL-6), and tumor necrosis factor alpha (TNF-α) (Dantzer, *et al.*, 1998). These secreted cytokines enter circulation and eventually reach the central nervous system. Because proinflammatory cytokines are large hydrophilic peptides they are regarded as being unable to passively cross the blood brain barrier (Exton, 1997). A number of mechanisms have been proposed to explain how these cytokines ultimately transmit their signal to the brain.

One possibility is that circulating cytokines enter the brain and exert their influence directly. Cytokines may enter the brain at the circumventricular organs where the blood brain barrier is incomplete and less selective (Maier, 2003). There also is experimental evidence that suggests IL-1α may cross the blood-brain barrier via active transport (Maier & Watkins, 2003). A second possibility is that peripherally-synthesized

cytokines do not enter the brain directly, but rather induce the expression of proinflammatory cytokines within the brain through an unknown mechanism. For example, rats injected peripherally with endotoxin exhibited increased IL-1β, IL-6, and TNF-α transcription by microglial cells in discrete brain areas (van Dam, Brouns, Louisse, & Berkenbosch, 1992).

Whether cytokines are imported into or synthesized within the brain, evidence suggests cytokines bind to endothelial receptors within the brain which activate prostaglandin E_2 (PGE_2) synthesis. PGE_2 and neurotransmitters act as secondary messengers and ultimately activate neuronal projections which communicate the signal to multiple brain areas (Exton, 1997).

An alternate mechanism of cytokine action posits that the vagus nerve carries signals about the presence of proinflammatory cytokines directly from the periphery to the brain. Multiple regions of the vagus nerve contain IL-1 binding sites in rats (Goehler, et al., 1997). Intravenous injection of IL-1 increases the activity of the hepatic branch of the vagus in adult male Wistar rats (Niijima, 1992). Furthermore, adult male Winstar rats receiving vagotomies display attenuated sickness behavior following exogenous proinflammatory cytokine administration (Kelley, et al., 2003).

The various mechanisms of cytokine signal transduction to the brain are not mutually exclusive and are likely complimentary (Johnson, 2002). It has been suggested that neural signals via the vagus represent a rapid transmission pathway that sensitize appropriate brain regions for receiving signals via the slow transmission pathway as the cytokines cross the blood brain barrier or induce cytokine synthesis within the brain (Dantzer, 2001).

Although the mechanisms by which the immune system communicates with the brain via proinflammatory cytokines are well-elucidated, there still is no consensus as to how the message carried by cytokines ultimately causes sickness behavior within the brain (Dantzer R. , 2006). Recent research has implicated multiple transcription factors in the process, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Li & Qin, 2005). The molecular and neural mechanisms which ultimately cause psychological changes are likely complex and require further investigation.

Sickness Behavior is a Motivational State

When humans are very sick, they typically prioritize recovery over other needs including food, grooming, or sexual activity. Similar behavior is observed in animals. Prior to the 1960's, it was assumed that sickness behavior was the result of physical weakness as the organism battled the infection (Aubert, 1999). Within this paradigm, sickness behavior was a collection of passive and deleterious symptoms exhibited by a compromised animal. Neal Miller, a famous American psychologist, was the first to challenge this hypothesis by suggesting that sickness behavior represents an adaptive change in motivational state that prioritized infection response and recovery.

Miller designed a set of eloquent operant conditioning experiments in which he demonstrated that adult male rats injected with *E. coli* endotoxin altered their behavior in order to minimize energy expenditure (1964). Rats injected with endotoxin stopped pressing a bar to receive water. Yet the mice would drink freely if water was provided to them without pressing a bar. Similar results were seen for a food reward. Furthermore, when the same mice were placed in a revolving drum they would readily press a bar to

stop the drum so they could rest. These experiments suggested that the mice were responding appropriately and flexibly to minimize total energy expenditure. The mice were motivated to be inactive. Further experiments have demonstrated that sickness behavior prioritizes heat retention and consumption of a diet enriched for carbohydrates in addition to inactivity (Dantzer, 2001).

Sickness Behavior Is an Adaptive Response

Sickness behavior is not the result of general weakness or debilitation from infection. Rather, it is considered an adaptive motivational state that reorganizes behavior to battle infection (Hart, 1988). These changes in behavior compliment metabolic and physiological changes in response to infection. Yet some sickness behaviors, particularly anorexia and adipsia, appear to be counter-productive to an animal's survival.

To understand the adaptive value of sickness behavior I first will consider fever and plasma iron reduction, two physiological responses to infection. These responses also represent seemingly counter-intuitive strategies against infection which share many biochemical pathways with sickness behavior. Importantly, both physiological changes and sickness behavior are cytokine-mediated "emergency responses" that work together in order to prioritize short-term survival in the face of infection.

Physiological Responses to Infection

Like sickness behavior, the fever response begins with the release of proinflammatory cytokines including IL-1, IL-6, and TNF-α by activated monocytes and macrophages (Mackowiak, 1992). Circulating cytokines cross the blood-brain barrier at the circumventricular organs and bind to endothelial receptors which ultimately activate prostaglandin E_2 (PGE_2) synthesis (Dinarello, et al., 1984). PGE_2 is bound by prostaglandin E receptor 3 (EP_3) in the preoptic area of the brain, which innervates the hypothalamus and other brain regions. Among its many functions, the hypothalamus acts as a thermostat for the body, and signals from the PGE_2 pathway effectively raise the thermostat. The hypothalamus responds through endocrine signals which promote thermogenesis and deters heat loss via vasoconstriction. The combined effect is the marked rise in body temperature which defines fever.

The fever response has several significant costs and risks associated with it. First, the fever response is very costly from a metabolic perspective. While the fever response is variable among individuals, studies have estimated each 1°C increase in temperature causes a 13% increase in metabolism in humans (Kluger, 1979). Fever has a similar metabolic cost in mice (Blatteis, 2003). Secondly, the fever response may accelerate potentially deleterious responses to infection such as muscle proteolysis (Goldberg, Kettelhut, Furuno, Fagan, & Baracos, 1988). Finally, the fever response can become overactive and maladaptive in response to certain infections. Sustained fevers over 41°C (105.8 °F) in humans can cause neuronal and cardiac tissue damage and are considered medical emergencies in humans (McGugan, 2001). The significant costs and risks of fever combined with its high degree of evolutionary conservation among all vertebrates

suggests that fever has adaptive value and increases survivability (Hart, 1988). This hypothesis is supported by experimental and clinical evidence.

Multiple experiments have demonstrated that disrupting or preventing the febrile response is deleterious to infected animals. Rabbits subjected to bacterial infection display reduced survivability when given a fever-reducing drug (Kluger & Vaughn, 1978). Ferrets prevented from developing fever displayed significantly higher viral loads than controls when infected with the influenza virus (Husseini, Swat, Collie, & Smith, 1982). Interestingly, similar positive correlations between fever and infection survival are observed in ectotherms as well (Moltz, 1993).

Clinical observations also support the hypothesis that fever battles pathogens in some way. In 1927 Julius Wagner-Jauregg was awarded a Nobel Prize in Medicine for the development of fever therapy. Wagner-Jauregg successfully treated neurosyphilis patients by infecting them with fever-inducing malaria, which could later be controlled with quinine (see Raju, 2006, for review). Fever therapy was widely used for the treatment of gonorrhea and other temperature-sensitive pathogens until the advent of antibiotics. More recently, retrospective clinical analyses have consistently demonstrated that patients who develop fever are more likely to survive a severe bacterial infection than those who do not (Moltz, 1993). Given fever's benefit in battling infection, there is growing concern that antipyretics are overused, particularly in children (Walsh, 2006).

Two main mechanisms have been proposed to explain the correlation between the febrile response and infection outcomes (Hart, 1988). The first posits a positive correlation between temperature and multiple aspects of innate and adaptive immune function. The second mechanism notes that a febrile temperature is above the optimum

range for replication in many pathogens. These hypotheses have been supported by multiple *in vitro* studies (Blatteis, 1986). There is, however, a lack of convincing *in vivo* evidence to confirm the validity of these proposed mechanisms (Moltz, 1993). This has lead to an ongoing debate about the adaptive value and mechanism of fever in different animals and in response to different pathogens (Blatteis, 2003).

In addition to the febrile response, many animals also exhibit a drop in plasma iron and zinc concentrations when subjected to infection (Weinberg, 1984). This drop is an effective physiological strategy since iron is required for the growth and replication of many bacterial pathogens. Low plasma iron acts synergistically with the febrile response to limit bacterial growth (Kluger & Rothernburg, 1979). The proinflammatory cytokine IL-1 mediates this physiologic response in addition to its role in sickness behavior the febrile response (Hart, 1988).

Sickness Behavior Complements Physiological Responses to Infection

Many aspects of sickness behavior are counter-intuitive. Perhaps the best example is anorexia. One might predict a sick animal would exhibit an increased appetite to meet the increased metabolic demands from the febrile response. The adaptive value of sickness behavior can only be understood by examining the complementary interactions between sickness behavior and immunological and physiologic responses to infection.

Anorexia and Adipsia. Although infection-induced anorexia seems counter-intuitive, experimental evidence strongly suggests its adaptive value (Exton, 1997). For example, adult female DBA/2 mice subjected to *Listeria monocytogenes* infection exhibited markedly higher survival rates if they had been subjected to a forced-starvation

protocol immediately prior to infection (Wing & Young, 1980). Alternatively, adult Swiss-Webster mice (sex not indicated) placed on a forced-feeding protocol suffered much higher mortality rates than controls allowed to develop anorexia during the course of a *Listeria* infection (Murray & Murray, 1979).

Multiple hypotheses seek to explain how anorexia benefits a sick animal. The first hypothesis notes that prolonged anorexia lowers plasma iron concentration and likely complements infection-induced iron sequestration. Food-restriction for at least a week dramatically reduces plasma iron concentration in a variety of animals, including hamsters, rats, and humans (Exton, 1997). Even short-term food restriction significantly reduces plasma iron concentration in humans and rabbits (White, 1980; Schumann & Haen, 1988). Unsurprisingly, anorexia is more effective at reducing plasma iron concentrations in carnivores and omnivores than herbivores.

Short-term feeding restriction experiments suggest that anorexia promotes immunocompetence. Fasting humans and mice subjected to forced-starvation protocols lasting less than one week exhibit improved immune status (measured *in vivo*) and immune function (measured *in vitro*) (Exton, 1997). For example, acute starvation increases macrophage activation, natural killer (NK) cell activity, and T-cell proliferation in multiple strains of male (Nakamura, et al., 1990; Boissonneault & Harrison, 1994) and female (Wing & Young, 1980) mice. Similarly, macrophages isolated from obese humans subjected to a fasting protocol exhibited increased ability to destroy bacteria and tumor-cells *in vitro* (Wing, Stanko, Winkelstein, & Adibi, 1983).

Finally, anorexia also encourages behavioral changes which are beneficial to a sick animal. Anorexic animals are less inclined to seek food and are therefore less

susceptible to predation when they are more vulnerable due to their illness (Hart, 1990). Anorexic animals are also more likely to adopt a resting posture, which aids in heat retention and complements the physiological changes which cause the febrile response (Exton, 1997).

The functionality of adipsia is less-well understood than anorexia. Adipsia reduces water-seeking behavior and may reduce the risk of predation. Alternatively, adipsia may be a secondary effect of proinflammatory cytokines with no adaptive value. Either way, several complementary physiological and behavioral changes typically accompany adipsia, reducing its potential harm. LPS administration significantly increases the blood osmolarity at which a dog seeks water (Szczepanska-Sadowska, Sobocinska, & Kozloska, 1979). Rats injected with pro-inflammatory cytokines exhibit increased sodium excretion, which counteracts the hypertonicity caused by reduced liquid consumption (Beasley, Cannon, & Dinarello, 1987). Behavioral changes include reduced oral grooming in rodents, which significantly reduces water loss (Bolles, 1960).

Hypersomnia. As previously mentioned, observations that IL-1 administration cause hypersomnia in rabbits first suggested that proinflammatory cytokines mediated sickness behavior. The benefits of hypersomnia for a sick animal are two-fold. First, sleep is necessary for repairing cellular damage caused by the invading pathogen and for maintaining a properly functioning immune system (Zager, Andersen, Ruiz, Antunes, & Tufik, 2007). Sleeping animals also assume postures which encourage heat retention, thus decreasing the metabolic demands of the febrile response (Hart, 1988).

Sickness Behavior as a Non-Adaptive Response: Cytokine Theory of Depression

When properly regulated, cytokine-induced sickness behavior is an adaptive response to infection which complements metabolic, physiologic, and immunological changes. This adaptive response suggests that sickness behavior that is improperly regulated or inappropriate to its stimulus may cause maladaptive behavioral changes. The macrophage theory of depression posits that chronic overproduction and dysregulation of proinflammatory cytokines induces or contributes to clinical depression in humans (Smith, 1991). There is also evidence that infection with the Borna virus is related to the etiology of some cases of depression in humans (Bode and Ludwig, 2003). Both theories are still controversial and await convincing causal evidence. There are, however, several interesting links between depression, cytokines, and inflammation.

Depression is a common comorbidity for chronic inflammatory diseases, such as coronary heart disease, type 2 diabetes, and rheumatoid arthritis (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Conversely, patients suffering from major depression exhibit a chronically activated inflammatory response, with elevated levels of circulating IL-6 and other proinflammatory cytokines (Dantzer R. , 2006).

As previously mentioned, the administration of recombinant proinflammatory cytokines as chemotherapeutic agents is associated with the development of cytokine sickness, which is characterized by sensitivity to pain, malaise, and ahedonia. There is a strong similarity between the symptoms of cytokine sickness and major depressive disorder (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Interestingly, the symptoms of cytokine sickness can be attenuated by the administration of anti-depressants.

The chronic activation or dysregulation of the immune system may lead to the overproduction of depression-promoting proinflammatory cytokines. A depressed individual may be in a constant state of cytokine sickness. While causal evidence is lacking, this hypothesis suggests a plausible mechanism for the relationship between cytokines and depression. Proinflammatory cytokines are already known to affect the HPA-axis (i.e., stress response) and modulate serotonin and noradrenaline receptors (O'Brien, Scott, & Dinan, 2004). Further research may suggest new immune targets for the pharmacological treatment of depression, which can be related to sickness behavior.

Unanswered Research Questions: The Effects of Age and Sex on Sickness Behavior

The physiological mechanisms of sickness behavior have been well-elucidated primarily by examining adult rodent models. Although evidence suggests that age modulates the intensity of sickness behavior in mice (Godbout, et al., 2005; Moltz, 1993), there is no known research examining sickness behavior during adolescence. This gap is somewhat surprising given that adolescence is a critical period in the development of the immune system (West, 2002). Furthermore, most studies of sickness behavior have examined only one sex, usually males. Research comparing sickness behavior in males and females would be useful given the many influences of sex on the immune system (Goldsby, Kindt, Osborne, & Kuby, 2003).

Introduction to the Experiment

The purpose of this thesis was to explore the association between sickness behavior, immune status, and immune function in an adolescent murine model. The

central research question was whether adolescent male and female mice that exhibit more pronounced sickness behavior also have heightened immune status and function. A positive correlation among these three variables would support the hypothesis that, similar to adults, sickness behavior is an adaptive response to an invading pathogen rather than a secondary consequence of infection.

In this experiment, sickness behavior was quantified using three measures: body weight, food consumption, and water intake. These variables were measured over the course of the experiment and tracked three of the most prominent aspects of sickness behavior: cachexia, anorexia, and adipsia. Immune status was measured by lymphocyte cell number adjusted for body weight and IFN-γ serum levels at the time of sacrifice. Lymphocytes are immune cells which produce proinflammatory cytokines and other cytokines such as IFN-γ which coordinate sickness behavior and other aspects of the immune response. Although IFN-γ is not classified as a proinflammatory cytokine, it is released in concert with IL-1, IL-6 and TNF-α and can be used as a marker of immune status. Taken together, lymphocyte count and IFN-γ levels represented innate immune status. Antibody-dependent cell-mediated cytotoxicity, an aspect of immune function, was measured using a ^{51}Cr release assay. This assay tests the ability of HSV-1 specific cytotoxic T cells from the *in vivo* HSV-1 infected mice to lyse HSV-1 target cells. These measures of sickness behavior, immune status, and immune function were correlated using the statistical analyses described in the methods section.

This experiment is significant because, to the author's knowledge, it is the first investigation of the relationship between sickness behavior and immune function in an adolescent mouse model. Research comparing adult and aged mice suggests that age may

significantly influence the severity of sickness behavior and associated neuroinflammation (Godbout, et al., 2005; Moltz, 1993). This experiment also is important because it examines sex effects on sickness behavior and its associations with immune status and function. Sex is known to impact many aspects of the immune system, including vaccine response, resilience in response to stress, and risk for developing autoimmunity (Goldsby, Kindt, Osborne, & Kuby, 2003; Chrousos, 2010). These findings suggest that sex may affect sickness behavior as well, a hypothesis that has not been examined before. This thesis serves as a preliminary investigation into the link between sickness behavior, proinflammatory cytokines, and immune function in male and female adolescent mice.

Methods

Thirty-nine periadolescent C57BL/6J mice (Jackson Laboratory; Bar Harbor, ME), nineteen males and twenty females, were divided into six cohorts which arrived a week apart to allow time for data collection. Mice were housed individually in standard shoebox-style plexiglass cages with filter tops and ¼ inch bedding (Bed-o' Cobs; The Andersons Agriservices, Inc., Maumee, OH). Mice were maintained in climate-controlled rooms (21 ± 2 ℃ and 51.2% relative humidity) on a 12-hour light-dark cycle. Throughout the experiment mice had *ad libitum* access to standard rodent chow (LabDiet 5001 Rodent Diet; PMI Nutrition International, Brentwood, MO) and tap water.

All mice arrived on post-natal (PN) day 25 and were given three days to acclimate to their new environment. Beginning on PN 28, mice were handled daily for the remainder of the experiment to obtain their body weight, food consumption, and water intake. On PN 37, mice were anesthetized via 5% isoflurane inhalation for two minutes and HSV-1 Patton (1×10^6 plaque-forming units, pfu; donated by Dr. Robert Bonneau, Penn State Hershey Medical Center) was injected subcutaneously into each footpad in a volume of 30 μL. See Appendix A for complete HSV-1 preparation and injection protocols. On PN 42, mice were euthanized via cervical dislocation at the onset of the light cycle. Blood and tissue samples were collected immediately following euthanization. An overview of the experimental timeline appears in Figure 1. All procedures were reviewed and approved by the Pennsylvania State University Institutional Animal Care and Use Committee (IACUC #31606; see Appendix B for approval letter) and the Pennsylvania State University Institutional Biosafety Committee (IBC #31626).

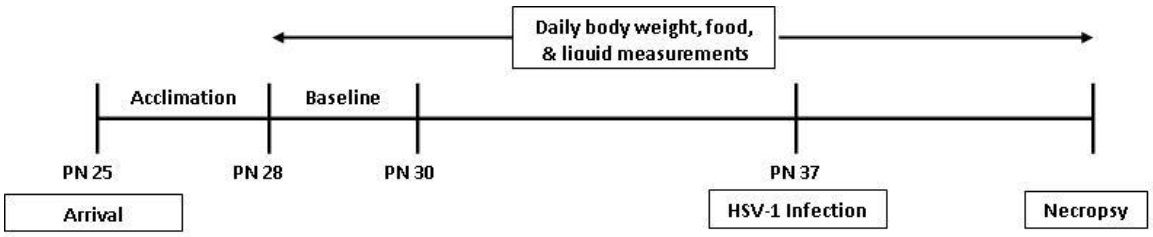

Figure 1: Experimental Timeline

Immediately following euthanization, blood was collected by cardiac puncture and 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 and serum was stored at -70 °C for later assessment of biomarkers reported elsewhere (Bennett, 2010). Popliteal lymph nodes also were removed from the mice and the viability of single cell suspensions was determined by trypan blue dye exclusion. See Appendix C for the popliteal lymph node removal protocol. Total cell count was determined using a Coulter counter. The lymphocytes were plated in 12-well tissue culture plates in 0.8 mL of supplemented IMDM at a density of 4 x 10^6 cells/mL, and maintained at 37 °C in 5% CO_2. Cell-mediated cytotoxicity was determined by ^{51}Cr release assay. This assay tests the ability of HSV-1 specific T_C cells (effector cells) from the HSV-1 infected mice to lyse HSV-1 specific-pulsed and MOCK-pulsed target cells. See appendix D for ^{51}Cr release assay protocol. The target cells were cultured B6/WT-3 fibroblast cells. Additionally, the supernatant from the 3-day lymphocyte culture was analyzed for IFN-γ levels by commercially available enzyme immunoassay kits (R&D Systems; Minneapolis, MN). See appendix E for IFN-γ assay protocol. All assays were conducted

by Dr. Jeanette Bennett in the Biobehavioral Health Biomarker Core Laboratory under the direction of Dr. Laura Klein.

Treatment of Data and Statistical Analyses

Data were entered into Statistical Program for the Social Sciences (SPSS; Chicago, IL) for statistical analyses. Supernatant IFN-γ levels were adjusted by natural logarithmic transformation to achieve a normal distribution. All tests were two-tailed, and statistical significance was accepted at an $\alpha = 0.05$.

Results

Sickness Behavior

Body Weight

Figure 2 presents average body weight by sex over the course of the experiment. All animals gained weight over the 10-day course of the experiment [time effect: $F(1,9) = 62.70$, $p<0.05$]. Males weighed more than did females [sex effect: $F(1,1) = 108.60$, $p<0.05$] and gained weight at a faster rate than did females [time X sex interaction: $F(1,9) = 3.74$, $p<0.05$].

During the 4-day pre-injection phase, all mice gained weight [$F(1,4) = 13.60$, $p<0.05$], males weighed more than did females [$F(1,1) = 13.55$, $p<0.05$], and males gained weight at a faster rate than did females [$F(1,4) = 4.50$, $p<0.05$].

Similar to the pre-injection phase, all mice continued to gain weight [$F(1,4) = 2311.80$, $p<0.05$], males weighed more than did females [$F(1,1) = 70.98$, $p<0.05$], and males gained weight at a faster rate than did females [$F(1,4) = 30.04$, $p<0.05$] during the 4-day post-injection phase.

A body weight change score also was calculated for each animal by subtracting the body weight on the first day from the body weight on the last day of each time period. Figure 3 compares pre- and post-infection body weight change scores by sex. Paired, two-tailed t-tests of the pre- and post-injection body weight change scores indicate that males gained weight more slowly post-injection [$t(18) = 3.27$, $p<.0.05$] while females exhibited a non-significant acceleration in weight gain post-injection [$t(19) = -1.85$, n.s.].

Food Consumption

Figure 4 presents the average change in food consumption by sex over the course of the experiment. For males, average food consumption was 4.84 g ± 0.07 pre-injection and 4.71 g ± 0.08 post-injection. For females, average food consumption was 4.23 ± 0.06 pre-injection and 4.36 ± 0.07 post-injection. Figure 5 compares average pre- and post-infection food consumption by sex. Paired, two-tailed t-tests revealed there was not a statistically significant difference between average food consumption during the pre- and post-injection time periods among males [$t(19) = 1.67$, n.s.] or females [$t(20) = -1.57$, n.s.]. However, there was a statistically significant difference between food consumption on the day before and day of injection. This relationship was true for both males [$F(1,1) = 12.37$, $p<0.05$] and females [$F(1,1) = 10.18$, $p<0.05$]. Food consumption between any other two consecutive days did not differ.

Water Intake

Figure 6 presents the average change in water intake by sex over the course of the experiment. For males, average water intake was 7.30 mL ± 0.11 pre-injection and 6.71 mL ± 0.12 post-injection. A single female post-injection water consumption data point was eliminated as an outlier. For females, average water intake was 6.61 mL ± 0.10 pre-injection and 6.21 mL ± 0.12 post-injection. Figure 7 compares pre- and post-infection body weight change scores by sex. Paired, two-tailed t-tests revealed males exhibited a significant decrease in average water intake between the pre- and post-injection period [$t(19) = 4.11$, $p<0.05$]. Females also exhibited a significant decrease in water intake between the two time periods [$t(19) = 3.02$, $p<0.05$].

There was a significant drop in between water intake between the day before and of injection. This drop was observed both males [$F(1,1) = 34.18$, $p<0.05$] and females [$F(1,1) = 16.10$, $p<0.05$]. There was also a significant drop in water intake between the first and second day of the experiment for both males [$F(1,1) = 40.33$, $p<0.05$] and females [$F(1,1) = 42.98$, $p<0.05$]. This drop in water consumption likely was the result of the animals adjusting to their new environment. The difference in water consumption between any other two consecutive days was not significant.

Immune Status and Function

The average number of isolated T-cells per isolate lymph node adjusted for body weight was statistically higher in males (M = 15.92, SEM = 0.07) than females (M = 15.73, SEM = 0.04) [$t(37) = 2.39$, $p<0.05$]. Average serum IFN-γ levels were also calculated for males (M = 47.51 ng/mL, SEM = 15.46) and females (M = 64.29 ng/mL, SEM = 9.93). Average integrated area under the curve from the ^{51}Cr release assay was also determined for males (M = 16.27, SEM = 2.47) and females (M = 18.67 ng/mL, SEM = 2.12). There was no statistically-significant sex difference in INF levels [$t(37) = 0.92$, n.s.] or ^{51}Cr release assay values [$t(37) = 0.74$, n.s.].

Sickness Behavior and Immune Status

Overview of Sickness Behavior Change Scores

Sickness behavior was quantified by a series of change scores that were correlated with immunological measures. An average pre-injection body weight change score was determined for males (M = 1.63 g, SEM = 0.22) and females (M = 0.69 g, SE M= 0.13)

by subtracting body weight on the first day of the period from body weight on the last day of the period. The average body weight change score for the post-injection time period was 1.63 g ± 0.23 and 0.69 g ± 0.14 for males and females, respectively.

A change score for each animal was calculated by subtracting food consumption on the day before injection from food consumption on the day of injection. The average food consumption change score was -0.44 g ± 0.12 for males and -0.45 g ± 0.14 for females. A water intake change score was calculated for each animal in the same manner as the food consumption change score. The water intake change score was -0.87 mL ± 0.15 for males and -0.70 mL ± 0.18 for females.

Analyses

These measures of sickness behavior (body weight change score, food consumption change score, and water intake change score) were correlated with two measures of immune function (natural log transformed supernatant IFN-γ levels and isolated T-cells per isolate lymph node adjusted for body weight). The results of the two-tailed bivariate correlations separated by sex appear in Table 1a and 1b. None of the six correlations were statistically significant.

Sickness Behavior and Immune Function

Three measures of sickness behavior (body weight change score, food consumption change score, and water intake change score) were correlated to immune function as measured by the integrated area under the curve from the ^{51}Cr release assay. The results of the two-tailed bivariate correlations separated by sex appear in Table 2.

Among females, there was a statistically significant positive correlation between water intake and immune function. Thus, immune function decreased as water consumption went down following infection.

Discussion

Introduction

All animals exhibit sickness behavior in response to infection, including anorexia, adipsia, and reduced social interaction. Research suggests that sickness behavior is not a passive consequence of infection but rather a coordinated change in motivational state that prioritizes infection response over other needs. Sickness behavior represents proinflammatory cytokine-mediated communication from the immune system to the brain. Despite the known effects of age and sex on the immune system, sickness behavior is well-studied only in adult male rodent models. Further, although the immune system changes over the course of the lifespan, there is currently no research investigating sickness behavior during adolescence, a critical period of development. The purpose of this experiment was to investigate the associations among sickness behavior, immune status, and immune function in adolescent male and female mice. This discussion reviews the results of this thesis, provides possible interpretations of these findings, and concludes with directions for future research.

Sickness Behavior

As expected, all infected mice exhibited sickness behavior to some degree. On average, adolescent mice exhibited anorexia and adipsia following infection with HSV-1. The mice did not exhibit weight loss but rather a slowing in the rate of growth following infection. This slowing in weight gain as opposed to weight loss is reasonable, given that adolescence is a time of rapid body growth and it would take a major physiological challenge (i.e., severe illness or infection) to stop growth. Interestingly, only males exhibited a statistically significant decrease in the rate of weight gain post-infection. This

result suggests that male and female mice may experience sickness behavior differently. This finding is consistent with the literature that notes that adult male mice exhibit a more severe behavioral reaction to immune challenge by vaccination (Kind, 1958), bacterial infection (Komukai et al., 1999) and viral infection (Kozak et al., 1997). This sex difference could be caused in part from the effects of sex hormones on the immune system. The hypothesis that sex alters the severity of sickness behavior, particularly weight loss, in adolescent mice could be tested by designing an experiment with sham-injected control mice. This experiment would allow the comparison of control and experimental animals within each sex and affirm that differences observed in the present experiment were sex X treatment effects and not simply sex effects. It would also be interesting to expand this experiment to include different aged mice to examine the severity of sickness behavior between sexes and across the lifespan (e.g., adolescent, young adult, old).

Average food and water consumption were not significantly different during the pre- and post-injection phases. Rather, average food and water intake were statistically different on the days before and of injection. Taken together, these results suggest that sickness behavior in adolescent mice may be most intense immediately following infection and transient. Again, a sham injection-controlled sample could confirm that observed reduction in food and water consumption were the result of sickness behavior and not simply the stress of injection and associated handling. There were no sex differences in average food or water consumption.

Correlations Between Sickness Behavior, Immune Status, and Immune Function

The primary hypothesis of this thesis predicted a positive correlation between measures of sickness behavior, immune status, and immune function in the adolescent mice. This hypothesis is consistent with the existing literature (e.g., Hart, 1988; Exton, 1997) but not the results of this thesis. There were no statistically-significant correlations between any measure of sickness behavior and immune status in either males or females. Furthermore, with one exception, there were no statistically significant correlations between sickness behavior and immune function. These results must be interpreted in light of a number of differences between this experiment and other investigations of sickness behavior.

First, and perhaps most importantly, this experiment examined sickness behavior in adolescent rather than adult mice. As previously mentioned, sickness behavior is more severe in aged mice compared to adults (Godbout, 2005). It is conceivable that adolescent mice exhibit attenuated sickness response compared to adult mice, which may make it more difficult to observe correlations between sickness behavior and immune function. This hypothesis could be investigated by increasing the sample size to detect more subtle changes in sickness behavior or immune system change and by comparing sickness behavior across age groups.

Secondly, this experiment induced sickness behavior with HSV-1 (i.e., viral) pathogen rather than a bacterial pathogen, LPS, or an endogenous cytokine. Even though all sickness behavior appears to be mediated by the same immunological and physiological pathways, different pathogens and antigens can elicit variable intensity of sickness behavior (Hart, 1988). It is possible that HSV-1 elicits mild sickness behavior

compared to other pathogens, making it more difficult to examine correlations between sickness behavior and immune function in this thesis. The American Social Health Association estimates that 50-90% of American adults have antibodies to HSV-1 (2009). The high prevalence of HSV-1 infection in human populations suggests that it may still be important to understand the potential clinical effects of HSV-1 infection on behavior. Some evidence suggests that latent HSV-1 reactivation may affect behavior in mice by altering IL-6 synthesis (Baker, 1999). Data on the effects of acute and chronic HSV-1 infection on sickness behavior are currently limited and require further investigation.

Finally, this experiment used wild-type C57BL/6J mice. This strain of mice has not been extensively used for sickness behavior research. It is possible that C57BL/6J mice may differ from other strains in the intensity, duration, and other aspects of sickness behavior. This possibility is supported by human and animal data which demonstrate that genetic polymorphisms of cytokine-related genes are related to differences in the severity of sickness behavior (Ute, 2008). The hypothesis that C57BL/6J display characteristic sickness behavior could be examined by quantifying the sickness behavior of multiple strains of mice subjected to the same protocol. Considered together, the unique aspects of this experiment may partially explain the disconnect between the results of this thesis and other studies.

In females, there was a statistically significant positive correlation between adipsia (quantified by the liquid consumption change score), and immune function (measured by the ^{51}Cr release assay). That is, the females with lower water intake had lower immune function. This finding is contradictory to other studies which demonstrate that adipsia and sickness behavior in general improves survivability in mice (Johnson,

2002). Although unexpected, this result may suggest a sex difference in the relationship between certain sickness behaviors and immune function mediated by sex hormones.

Limitations

This thesis has some limitations which must be considered when interpreting the results of the analyses. Most notably, this thesis is a *post hoc* analysis of the data collected for Dr. Jeanette Bennett's doctoral dissertation (2010), which constrained my analyses in a number of ways. First, because this thesis relied on the control group of the parent study, the sample size may have been too small to test the primary hypotheses.

Secondly, the variables used to quantify sickness behavior, immune status, and immune function may not have been the most direct. The markers of immune status (INF-γ) and immune function (^{51}Cr release) were collected on sacrifice day and, thus, could not show changes in the immune system around the time of initial infection. Further, immune status was quantified in part using INF-γ rather than a proinflammatory cytokine such as IL-1, IL-6, or TNF-α. While INF-γ and proinflammatory cytokines are released in response to the same stimuli, proinflammatory cytokines are responsible for invoking sickness behavior. Therefore, INF-γ may not have been the best biomarker for the purposes of this thesis. Proinflammatory cytokines such as IL-1, IL-6, or TNF-α may have been more appropriate markers.

Future Directions

Although this thesis was a preliminary analysis limited by several constraints, it does suggest a number of directions for future research. First, the sex and time differences suggested by this thesis could be supported or challenged by a well-designed prospective experiment. Increasing the sample size would give the study more power to detect smaller behavioral effects between sexes. Including different aged mice would allow for comparison of sickness behavior across the lifespan. For example, infant mice may exhibit different sickness behaviors in order to attract the attention of their mothers.

A control group for each age could be established by injecting a subset of the mice with saline solution or rather than HSV-1. The control group could confirm that observed changes were the effect of sickness behavior and not the stress of injection. A control group would also limit concerns that growth rate changes observed in adolescents were not simply part of normal development.

The scope of this thesis could also be expanded by including more behavioral and immune measures. It would be interesting to examine other aspects of sickness behavior such as activity level, grooming, and environment exploration. Perhaps some of these behaviors are better predictors of immune function or overall survivability. Other proinflammatory cytokines such as IL-1, IL-6, and TNF-α could be included as additional markers of immune status. Because cytokines are partially redundant but still exert different influences, it is possible that some proinflammatory cytokines are better predictors of sickness behavior or immune function than others in this adolescent mouse model.

The basic experimental model could be expanded to include different infectious agents. Bacterial pathogens such as *Salmonella enteritidis* or the bacterial antigen LPS could be used. Proinflammatory cytokines may be a predictor of immune function for only a subset of pathogens.

Finally, it would be interesting to explore the link between antidepressants and sickness behavior in a mouse model. As mentioned, antidepressive pharmaceuticals are partially effective at attenuating cytokine sickness in human cancer patients taking chemotherapeutic proinflammatory cytokines. Similar effects would be expected in a mouse model. Perhaps sickness behavior caused by an infection (not an exogenous cytokine) could also be diminished by the administration of an antidepressant. Would lessening sickness behavior with an antidepressant medication affect a mouse's immune function or overall survivability? Based on the differences between human adult and adolescent responses to antidepressives, it is possible that adolescent and adult mice may exhibit different changes in sickness behavior or immune response as well.

Conclusions

In this thesis, sickness behavior modestly was correlated to immune status and immune function in adolescent male and female mice. While most correlations were not statistically significant, two interesting sex differences emerged: males grew more slowly following infection and females who drank more following infection exhibited higher immune function *in vitro*. The results of this thesis do not support those of other studies of sickness behavior, perhaps because this study examined adolescent rather than adult

mice. Further research is needed to examine the impact of sex and age on the relationship between sickness behavior and the immune system.

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Tables

Table 1a: R- and p-values for two-tailed bivariate correlations between measures of sickness behavior and immune status in males (N=19).

		Immune Status			
		ln-transformed IFN-γ level		T cells/lymph node adjusted for body weight	
		r-value	p-value	r-value	p-value
Sickness Behavior	Body Weight Change Score	0.08	0.76	0.11	0.64
	Food Consumption Change Score	0.10	0.69	0.31	0.31
	Liquid Consumption Change Score	-0.36	0.13	0.25	0.31

Table 1b: R- and p-values for two-tailed bivariatie correlations between measures of sickness behavior and immune status in females (N=20).

		Immune Status			
		ln-transformed IFN-γ level		T cells/lymph node adjusted for body weight	
		r-value	p-value	r-value	p-value
Sickness Behavior	Body Weight Change Score	-0.18	0.44	-0.18	0.46
	Food Consumption Change Score	0.19	0.42	0.25	0.29
	Liquid Consumption Change Score	-0.01	0.97	0.00	0.99

Table 2: R- and p-values for two-tailed bivariate correlations between measures of sickness behavior and immune function in males (N=19) and females (N=20).

		Immune Function: ^{51}Cr release Assay AUC			
		Males		Females	
		r-value	p-value	r-value	p-value
Sickness Behavior	Body Weight Change Score	-0.28	0.25	-0.52	0.83
	Food Consumption Change Score	-0.12	0.62	0.16	0.48
	Liquid Consumption Change Score	0.14	0.56	0.53	0.02 (*)

* Denotes statistical significance

Figures

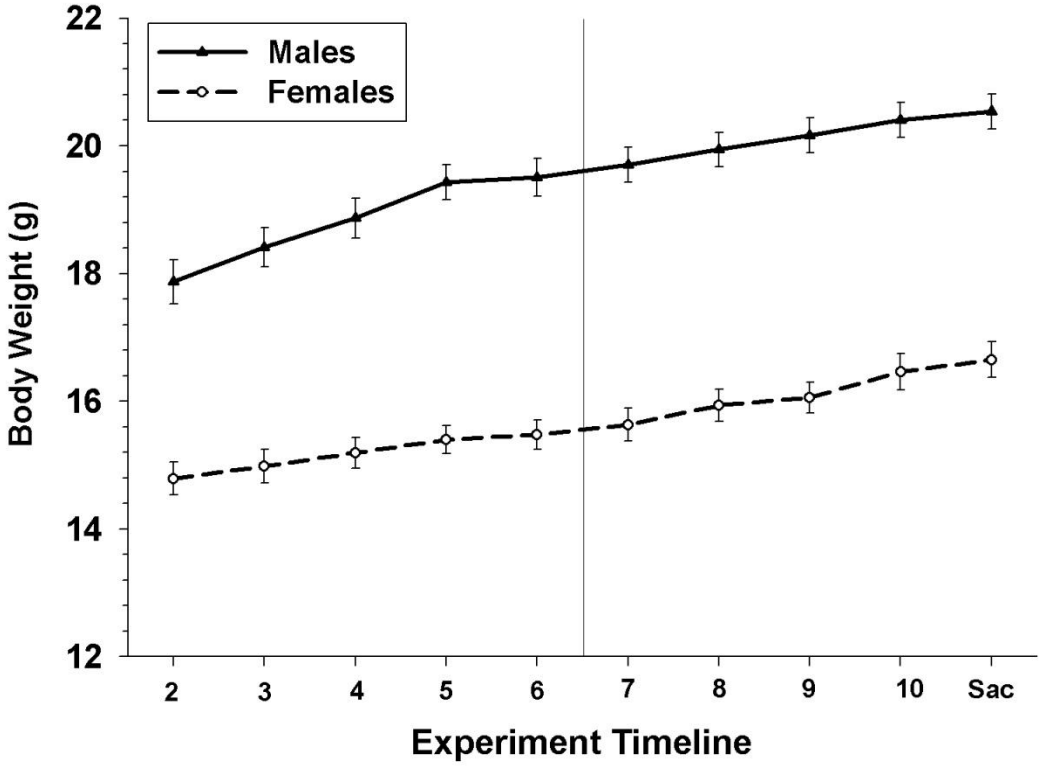

Figure 2: Averaged body weight (g) for all animals (N=39) by sex over the course of the experiment (adjusted means + SEM). HSV-1 injection day indicated by the vertical line.

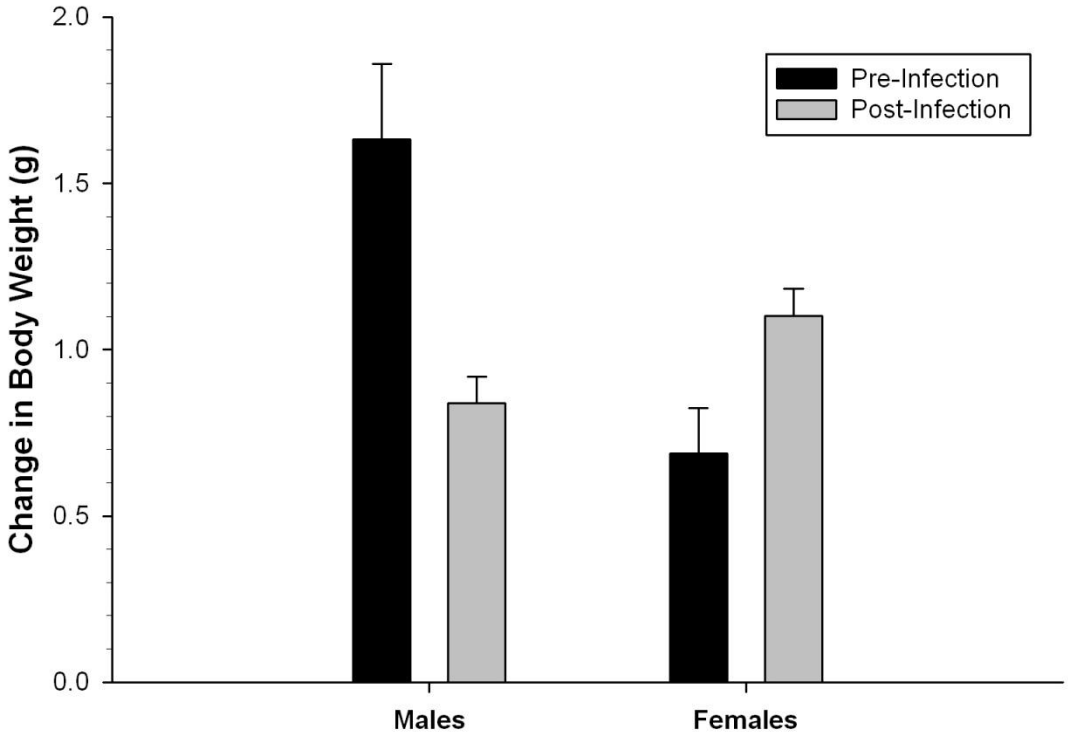

Figure 3: Averaged pre- and post-injection body weight change score (g) for all animals (N=39) by sex (adjusted means + SEM).

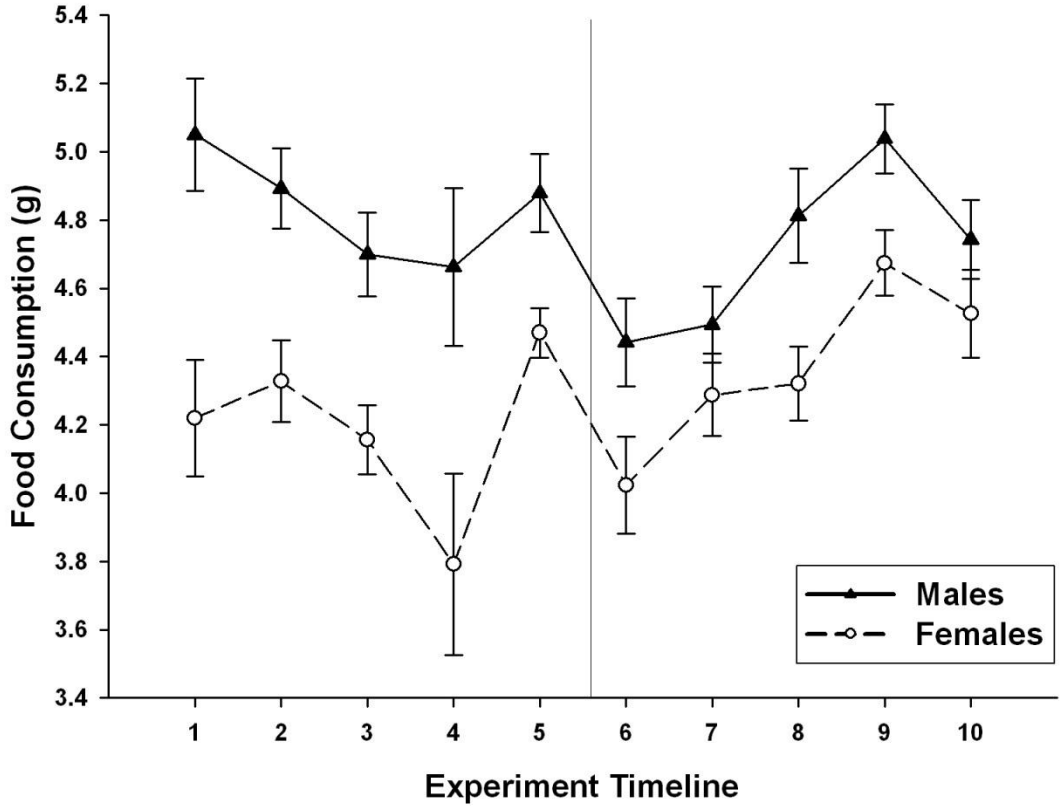

Figure 4: Averaged food consumption (g) for all animals (N=39) by sex over the course of the experiment (adjusted means + SEM). HSV-1 injection day indicated by the vertical line.

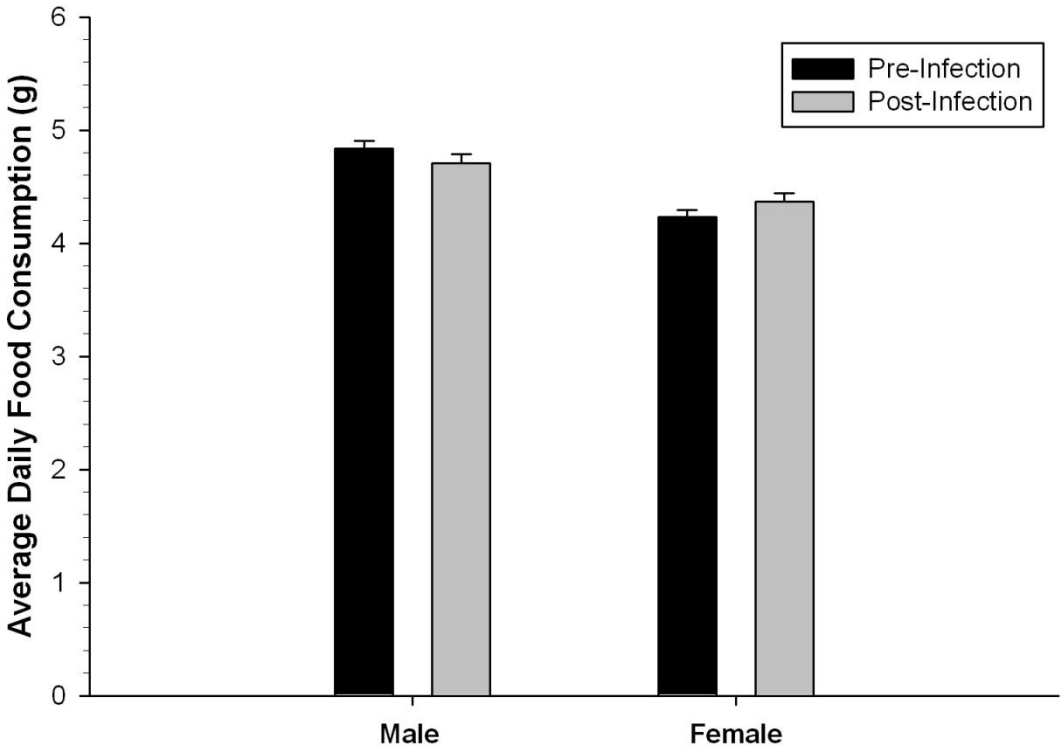

Figure 5: Averaged pre- and post-injection food consumption (g) for all animals (N=39) by sex (adjusted means + SEM).

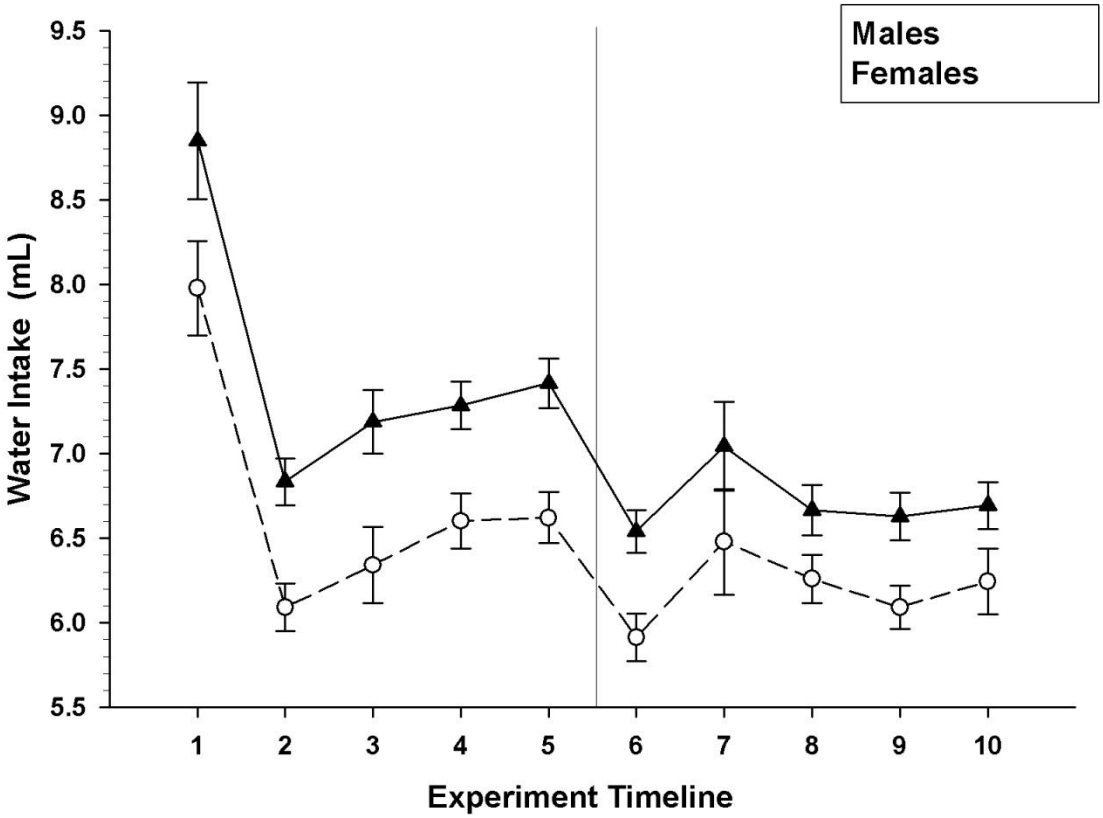

Figure 6: Averaged water intake (mL) for all animals (N=39) by sex over the course of the experiment (adjusted means + SEM). HSV-1 injection day indicated by the vertical line.

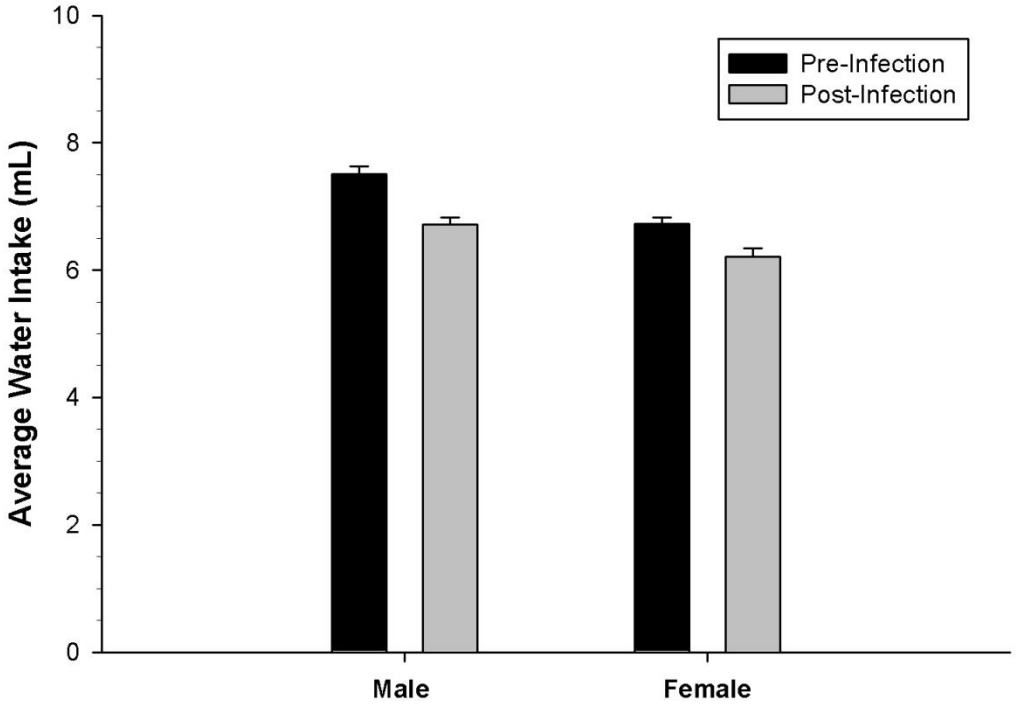

Figure 7: Averaged pre- and post-injection water intake (mL) for all animals (N=39) by sex (adjusted means + SEM).

Appendix A: 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^{o}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.
10) Prepare new syringe after every 5th mouse. See steps 4 & 5.
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 B: IACUC Approval

PENNSTATE
Vice President for Research
Office for Research Protections
The Pennsylvania State University
The 330 Building, Suite 205
University Park, PA 16802-3301
(814) 865-1775
Fax: (814) 863-8699
www.research.psu.edu/orp/

Date: July 9, 2010

From: William G. Greer, Assistant Director, Animal Care, Biosafety and Radiation Programs

To: Laura C. Klein

Subject: Results of IACUC Protocol Review – Annual Review **(IACUC# 31606)**

Approval Expiration Date: July 5, 2011

"Nicotine Modulation of Adolescent Antiviral Immunity"

The Annual Project Review form for your protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) for the continued use of animals in your research. **This approval has been granted for a one-year period.**

Approval for the use of animals in this research project is given for a period covering one year from the date of this memo. **If your study extends beyond this approval period, you must contact this office to request an annual review of this research.**

This Institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance number is A 3141-01. The Pennsylvania State University is also registered with the US Department of Agriculture (Certificate No. 23-R-0021). As of February 13, 2001, The Pennsylvania State University was awarded Full Accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

The IACUC does not require the principal investigator to provide copies of permits (e.g., PA Game Commission, Bird Banding, US Fish and Wildlife Service) prior to approval. However, if your research mandates a permit requirement, it is your responsibility to acquire such permits prior to conducting the research described in your IACUC protocol.

By accepting this decision, you agree to notify the Office for Research Protections of (1) any additions or procedural changes and (2) any unanticipated study results that impact the animals. Prior approval must be obtained for any planned changes to the approved protocol. Any unanticipated pain or distress, morbidity or mortality must be reported to the attending veterinarian and the IACUC.

On behalf of the IACUC and the University, I thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of animals.

If you are interested in subscribing or being removed from ORP listserv, send an email to: L-ORP-Research-L-subscribe-request@lists.psu.edu to subscribe or L-ORP-Research-L-unsubscribe-request@lists.psu.edu to unsubscribe. There is no need to add any text in the subject line or in the message body of the email.

WGG/ci
Attachment

cc: Jeanette M. Bennett
Robert H. Bonneau

To the Investigator:

Please forward the enclosed original approval letter to your funding agency, if applicable. This approval is effective for one year. During this time, you should notify this office of any changes in the protocol that will affect the care and use of the approved animals or that will result in the use of additional animals.

In a continuing effort to comply with federal regulations, this office reviews IACUC approvals on an annual basis. On the anniversary of this approval, you should expect to receive a letter soliciting your request for an "annual review" by the IACUC. It is my hope that this process aids researchers in maintaining active IACUC approvals and avoids the use of animals without the proper approval.

Also, in order for records of your animal usage at ARP and ORP to remain current, please review the information below. If you feel there is any discrepancy between this information and your request, please contact our office (ORP) immediately at 865-1775. Thank you.

IACUC#: 31606 **Approved:** July 9, 2010

PI: Laura C. Klein

Title: "Nicotine Modulation of Adolescent Antiviral Immunity"

Species Used	Total # Approved	# Used to Date	# Not Yet
Mouse: Unspecified	172	124	48

Appendix C: Popliteal Lymph Node Removal and Lymphocyte Isolation Protocol

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. CO_2 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 C02. 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
e. Label snap tubes, 50 mL conical tubes, and eppendorf tubes with animal IDs

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 $1x10^4$. 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:

$$\underset{\text{Cells}}{60} \times \underset{\text{dilution}}{2} \times \underset{\text{volume}}{12} \times \underset{\text{hemacytometer factor}}{1x10^4} = 1.44x10^7$$

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 $4x10^6$ 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 CO_2 incubator.
d. Clean up the laminar flow hood area.

Appendix D: ^{51}Cr Release Assay Protocol

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

1. Peptide pulsing and ^{51}Cr 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 typrsin/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 ^{51}Cr assay to calculate.
    g. Re-suspend cells at 1 x 10^6 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. ^{51}Cr 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 $^{51}Cr(Na_2CrO_4)$
        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 ^{51}Cr 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 175
^{51}Cr 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 ^{51}Cr 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 ^{51}Cr 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)
xii. Remove tubes from centrifuge; ***gently*** pour off supernatant into liquid ^{51}Cr waste
xiii. Re-suspend in 5 mL of complete Iscove's media for T-lymphocytes.
xiv. 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 readyto-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 ^{51}Cr-labelled target cells using a multichannel 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 ^{51}Cr-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 ^{51}Crlabeled
    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
n. Prepare collection tubes in blue racks in a pattern identical to each plate
o. Remove plates from incubator
p. Centrifuge plates at 1000 RPM x 3 minutes (**BRAKE OFF!!!**) to pellet cells

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 ^{51}Cr 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 E: INF-γ Assay Protocol

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 (5). 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

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

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

Unopened Kit	Store at 2 - 8° C. Do not use beyond kit expiration date.	
Opened/ Reconstituted Reagents	Diluted Wash Buffer	May be stored for up to 1 month at 2 - 8° C.*
	Stop Solution	
	Calibrator Diluent RD5Y	
	Calibrator Diluent RD6-12	
	Assay Diluent RD1-21	
	Conjugate	
	Unmixed Color Reagent A	
	Unmixed Color Reagent B	
	Mouse IFN-γ Standard	Use a new Standard and Control for each assay.
	Mouse IFN-γ Control	
	Microplate Wells	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.*

*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.

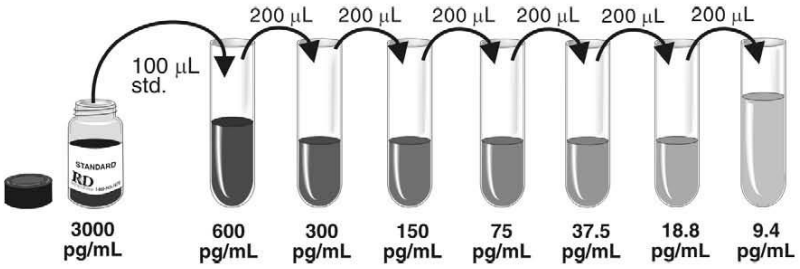
Mouse IFN-γ Kit Control - Reconstitute the Kit Control with 1.0 mL deionized or distilled water. Assay the Control undiluted.

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.

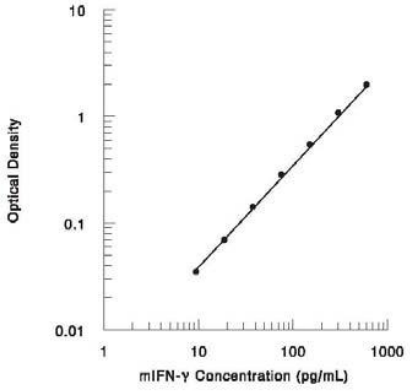
To determine the IFN-γ concentration of each sample, first find the absorbance value on the y-axis and extend a horizontal line to the standard curve. At the point of intersection, extend a vertical line to the x-axis and read the corresponding IFN-γ concentration.

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

TYPICAL DATA

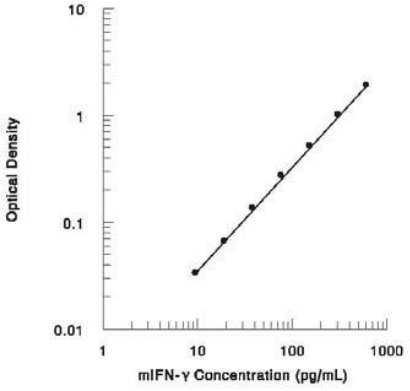
These standard curves are provided for demonstration only. A standard curve should be generated for each set of samples assayed.

Calibrator Diluent RD5Y



(pg/mL)	O.D.	Average	Corrected
0	0.042 0.043	0.042	—
9.4	0.077 0.077	0.077	0.035
18.8	0.113 0.112	0.112	0.070
37.5	0.183 0.186	0.184	0.142
75	0.327 0.329	0.328	0.286
150	0.580 0.594	0.587	0.545
300	1.130 1.124	1.127	1.085
600	2.020 2.042	2.031	1.989

Calibrator Diluent RD6-12



(pg/mL)	O.D.	Average	Corrected
0	0.035 0.037	0.036	—
9.4	0.069 0.071	0.070	0.034
18.8	0.099 0.107	0.103	0.067
37.5	0.170 0.179	0.174	0.138
75	0.309 0.318	0.314	0.278
150	0.551 0.576	0.564	0.528
300	1.053 1.081	1.067	1.031
600	1.950 2.005	1.978	1.942

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

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	44.8	156	518	43.3	144	509
Standard deviation	2.2	5.0	14.2	3.6	14.7	48.6
CV (%)	4.9	3.2	2.7	8.3	10.2	9.5

Serum Assay

	Intra-assay Precision			Inter-assay Precision		
Sample	1	2	3	1	2	3
n	20	20	20	20	20	20
Mean (pg/mL)	45.1	143	528	42.8	139	518
Standard deviation	2.1	4.4	11.8	3.6	13.6	46.5
CV (%)	4.7	3.1	2.2	8.4	9.8	9.0

RECOVERY

The recovery of mouse IFN-γ spiked to three levels throughout the range of the assay in various matrices was evaluated.

Sample Type	Average % Recovery	Range
Cell culture supernates (n = 7)	105	98 - 115%
Mouse serum (n = 5)	97	91 - 106%

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.

Sample	Dilution	Observed (pg/mL)	Expected (pg/mL)	Observed / Expected x 100
Cell Culture Supernates	neat	477		
	1/2	252	238	106
	1/4	122	119	103
	1/8	61	60	102
	1/16	31	30	103
Mouse Serum	spiked	475		
	1/2	230	238	97
	1/4	116	119	97
	1/8	58	60	97
	1/16	29	30	97

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 10^6 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 10^5 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.

Recombinant mouse:			**Recombinant human:**
C10	IL-6	LIF	IFN-γ
G-CSF	IL-7	M-CSF	
GM-CSF	IL-9	MIP-1α	
IL-1α	IL-10	MIP-1β	
IL-1β	IL-10 sR	MIP-2	
IL-2	IL-12	SCF	
IL-3	IL-13	TNF-α	
IL-4	JE	Tpo	
IL-5	KC	VEGF	

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.

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3. Gray, P.W. (1994) in *Guidebook to Cytokines and their Receptors*, N.A. Nicola ed., Oxford University Press, New York, p. 118.
4. Wenner, C.A. *et al.* (1996) J. Immunol. **156**:1442.
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6. Gray, P.W. and D.V. Goeddel (1983) Proc. Natl. Acad. Sci. USA **80**:5842.
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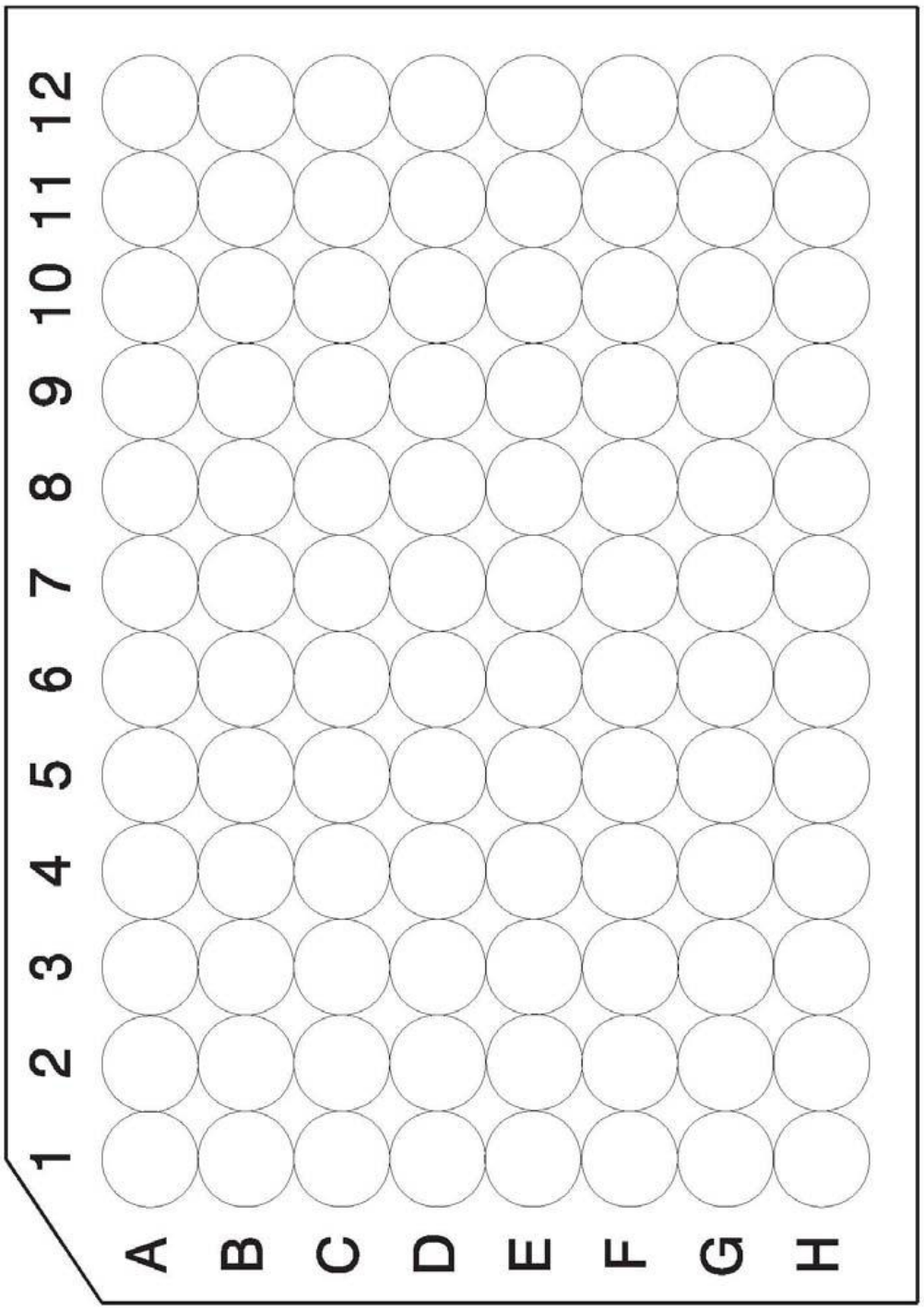
PLATE LAYOUTS

Use these plate layouts as a record of standards and samples assayed.



Curriculum Vitae
Patrick Buckley

EDUCATION

The Pennsylvania State University — **University Park, PA**
- Bachelor of Science, Biology – Schreyer Honors College Scholar — December 2010

RESEARCH EXPERIENCE

Klein Laboratory, Pennsylvania State University — **University Park, PA**
Research Assistant — August 2009–December 2010
- Assisted in two mouse studies and two longitudinal human studies examining various biobehavioral aspects of stress and/or nicotine addiction
- Investigated the association between immune-related sickness behavior and immune function in adolescent mouse model for honors thesis

Zentrum für Chronobiologie, University of Munich (LMU) — **Munich, Germany**
Research Intern — Summer 2009
- Successfully genetically engineered *Neurospora* fungi to express luciferase fusion proteins in order to monitor circadian gene expression *in vivo*.

Bartell Laboratory, Pennsylvania State University — **University Park, PA**
Research Assistant & Independent Studies — January 2008–May 2009
- Investigated role of circadian cycles on oviposition and gene expression in *Coturnix japonica*

EMPLOYMENT

IMS Health Management Consulting — **Wyomissing, PA**
Intern, Competitive Intelligence — Summer 2010
- Collected, analyzed, and synthesized primary and secondary business intelligence in the pharmaceutical and biotechnology sectors within a management consulting context
- Conducted interviews with key opinion leaders in the healthcare sector at the 2010 American Diabetes Association conference in Orlando; Covered FDA's Avandia advisory committee meetings

The Pennsylvania State University — **University Park, PA**
Organic Chemistry Tutor — Spring 2009 & Fall 2010
Introductory Biology Teaching Assistant — August 2009–May 2010

COMMUNITY INVOLVEMENT

ASERA Health Care Agency & Berks Heim County Nursing Home — **State College & Leesport, PA**
Hospice Volunteer — May 2006–Spring 2010
- Provide companionship for hospice patients in a nursing home on a weekly basis

Children's Health Center — **West Reading, PA**
Reach Out and Read Program — Summer 2008
- Working with a local physician, helped to found a Reach Out and Read site at local clinic for underserved populations; program provides free books to at-risk children during well visits
- Wrote startup grant funded for $12,000

Schreyer Signature Speaker Series — **University Park, PA**
Committee Member — August 2008–December 2009

HONORS AND AWARDS

- Student Marshal, Eberly College of Science, Fall 2010 Commencement
- 1st Place, Penn State/*New York Times* Civic Engagement Public Speaking Competition, Spring 2008
- 3rd Place, Penn State Undergraduate Research Exhibition, Spring 2009
- Grants
  - Penn State College of Agriculture Undergraduate Research Grants, 2008 & 2009
  - Eberly College of Science Travel Grant, Summer 2009
  - Schreyer Honors College Summer Internship Grant, Summer 2009
  - Deutscher Akademischer Austausch Dienst (DAAD) RISE Grant, Summer 2009
- Scholarships
  - Morrow Family Endowed Prize, 2010
  - Schreyer Honors College Academic Excellence Scholarship, 2007–2010
  - National Merit Scholarship, 2007