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THE IMPACT OF ENVIRONMENTAL ENRICHMENT ON HERPES SIMPLEX VIRUS-SPECIFIC IMMUNITY AND PATHOGENESIS

A Thesis in

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ABSTRACT

The *Guide for the Care and Use of Laboratory Animals*[27] states, "It is desirable that social animals be housed in groups; however, when they must be housed alone, other forms of enrichment should be provided….". Environmental enrichment (EE) has been introduced at increasing levels into laboratory animal programs in recent years and is being applied to species (e.g. rodents) other than non-human primates, for which it is currently required by law. EE is defined as any modification in the environment of captive animals that seeks to enhance their physical and psychological well-being by providing stimuli that meet their species-specific needs.

The initial research use of EE was to understand how spatial learning molds the brain, but has since changed to its current purpose which is to improve the welfare of laboratory animals. Environmental conditions such as housing and husbandry have a major impact on laboratory animals throughout their life and could thereby influence the outcome of animal-based experiments. Exactly what type of impact has yet to be determined because only a few objective studies have been conducted to determine the effects of EE on the overall well-being of an animal. The rationale for this thesis is based on the fact that EE is being increasingly introduced into animal housing without sufficient objective data regarding its impact on a variety of psychological and physiological parameters.

This study objectively evaluated the effect of EE on the immune response to and pathogenicity of herpes simplex virus type 1 (HSV-1) in both group- and singly-housed mice. The long-term goal of this project is to expand our knowledge of the relationship between EE and physiological parameters associated with the bi-directional communication that exists among the nervous, endocrine, and immune systems.

Our hypothesis was that environmental enrichment (EE) decreases stress and enhances the murine immune response to herpes simplex virus (HSV) infection, which together, in turn, decreases the HSV-associated pathogenesis in both the periphery and the central nervous system.

The overall objective of this study was to determine how EE modulates both immune function and susceptibility to HSV-1 infection in mice subjected to either group- or singly-housed conditions. This study utilized 8-12-week old C57BL/6 male mice intranasally infected with HSV-1. Measures of serum corticosterone were determined in EE and non-enriched (NE) mice both group- and singly-housed as well as both pre- and post-HSV-1 infection. Measures of HSV-specific immune function were evaluated at 7 days post-infection, the time of the peak of the immune response in this model of HSV infection.

We concluded that although enrichment alone does not have any significant effect on corticosterone (CORT) levels either pre- or post-infection, the HSV-1 infection itself reduces serum CORT levels. This reduction in CORT may possibly be due to the infection itself down-regulating corticosterone levels in order to allow for a stronger overall immune response. Also, since none of the CORT levels were outside of the normal baseline range for non-stressed mice, we confirmed that EE can be added to the mouse's cage without causing stress. A common trend was noted in the immune responses of both the group-housed mice and singly-housed mice. In the group-housed mice, there was a decreased immune response in the superficial cervical lymph nodes (CLNs) and mediastinal lymph nodes (MLNs) of EE mice as compared to the NE singly-housed mice. In the singly-housed mice, there was trend seen indicating an increased immune response in the CLNs and MLNs of EE mice as compared to NE mice. These observations support the overall conclusion that EE does not cause stress nor

significantly alters certain immune parameters except for in the MLNs in the group-housed mice where there was a significant decrease in $CD8^+$ $gB_{498-505}{}^+$ cells.

Information regarding usefulness, advantages, disadvantages, and current literature should be collected about a specific enrichment device before it is added to a cage as it may be deemed an extra variable in an experimental study. However, its use should not be excluded from normal husbandry because it may have many benefits and provide an excellent means by which to mimic species-specific behavior.

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LIST OF ABBREVIATIONS

APC	allophycocyanin
CLNs	superficial cervical lymph nodes
Con A	concanavalin A
CORT	corticosterone
CRH	corticotropin releasing hormone
CTL	cytotoxic T lymphocytes
DHEA	dehydroepiandrosterone
DMSO	dimethyl sulfoxide
EE	environmental enrichment or environmentally enriched
FITC	fluorescein isothiocyanate
HHV	human herpesvirus
HPA axis	hypothalamic-pituitary-adrenal axis
HSV	herpes simplex virus
IL	interleukin
IMDM	Iscove's-modified Dulbecco's media
IP	intraperitoneal
MHC	major histocompatibility complex
ml	milliliter
MLNs	mediastinal lymph nodes
NK	natural killer cells
NE	non-enriched
PBS	phosphate-buffered saline
PE	phycoerythrin
PFU	plaque forming units

RIA	radioimmunoassay
SAM	sympathetic adrenal medullary
SNS	sympathetic nervous system
TCR	T cell receptor
TGF	tumor-derived growth factor
TH	tyrosine hydroxylase
Th	helper T cells

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Chapter 1

INTRODUCTION

I. Environmental Enrichment (EE)

A. Definitions

EE is any modification in the environment of captive animals that seeks to enhance their physical and psychological well-being by providing stimuli that meet their species-specific needs[6,46]. EE is the practice of providing animals under managed care with environmental stimuli. The Enrichment Working Group of the Behavior and Husbandry Advisory Group for the American Zoo and Aquarium defines EE as a dynamic process in which changes to structures and husbandry practices are made with the goal of increasing behavioral choices available to animals and drawing out their species-appropriate behaviors and abilities, thus enhancing animal welfare[9]. Sheperdson[53] called EE a concept which describes how the environments of captive animals can be changed for their benefit.

B. Initial Purpose

In 1949, Donald O. Hebb, a psychologist and the father of neuropsychology and neural networks, by accident found that animals from enriched housing outperformed those that were in non-enriched housing in learning and memory tests (e.g. Hebb-Williams maze test)[8,30]. This incidental finding sparked scientists to use EE as a research tool to understand how spatial learning molds the brain. This initial purpose has since changed to now include the promotion of animal welfare and the enhancement of their environment.

C. Goals of EE

There are five principle goals of EE as has been described by Chamove and Shepherdson[15,54]. These goals have been established to ensure adequate implementation of EE and to express the potential benefits of EE for laboratory animals and their living conditions.

The first goal is to promote species-specific behavior/positive natural behaviors. If animals are not able to carry out the behaviors that are innate to them, their welfare will be compromised[20,21,34]. This, in turn, may cause negative behavior thereby increasing stress levels. Murine species-specific behaviors typically include exploring, hiding, foraging, gnawing, and nesting[35]. Providing nesting material alone has been shown to improve mouse welfare by allowing the performance of important species-specific behaviors such as nest-building as well as providing shelter for hiding[47]. This results in an increased control of the environment which reduces stress[65]. Nevison et al.[45] found that the most common behavior of laboratory mice, who are nocturnal in the wild, during the dark and supposedly "active" period was sleeping. He pointed out that this change in normal behavior could be a welfare problem if it is caused by a lack of stimulation as is the case in a non-enriched environment. EE is thought to reinforce the natural behaviors of mice such as being active at night.

The prevention of abnormal/stereotypical behavior is the second goal of EE. An example of such behaviors in mice is defined as unvariable repetitions of a behavior shown out of context and with no obvious function. Examples include bar-mouthing, jumping, and back-flipping[14]. Other abnormal behaviors that are seen in laboratory mice are excessive grooming (barbering), hyperactivity, fighting, aggression, and biting. Each of these behaviors can have deleterious effects on the results obtained from a scientific study. These abnormal behaviors may also result in a waste of animals due to the fact

that they may become unsuitable to use for a certain project because they may need to be euthanized for humane reasons (e.g. fighting mice). EE has been shown to prevent or reduce abnormal/stereotypical behavior. Mice in a non-enriched environment may be left with an excess of time when all of their physical needs are taken care of; therefore suffering from boredom[62]. A recent study by Kaiser demonstrated the ability of EE to reduce agonistic and stereotypical behaviors in female mice. Their play and sociopositive behavior (e.g. grooming) towards their cage mates were enhanced[38]. Therefore, by keeping mice active, there is less opportunity for them to become bored and potentially exhibit abnormal behaviors.

Ambrose[2] and Jones[37] showed that decreasing the familiarity of the cage significantly reduced aggression in male mice. The addition of novel objects into the cage can enhance the need for exploration thereby decreasing "boredom" in the mice. Others have also shown that EE can significantly reduce post-cage-cleaning aggression as compared with mice housed in a non-enriched environment. This decrease in aggression can, in turn, decrease the need for separating fighting animals and, thereby, the need to euthanize injured animals. The provision of enrichment devices (e.g. mouse huts, igloos, tunnels) can also offer subordinate animals cover and escape routes[4] from a cage aggressor. Giving the weaker animal a place to hide can decrease stress and injury to that weaker animal.

Third, EE can increase an animal's positive utilization of their environment (i.e. the cage). A restricted environment offers few opportunities to acquire information or to interact with the environment[50,64]. Enrichment allows the animal a chance to interact with these devices and other cage mates. The use of different types of cage inserts facilitates such structuring of the cages and increases the usable space of the cage. EE allows mice to climb and make better use of the three-dimensional space of the cage[50], thereby increasing positive use of their environment. Mice can also use sheltered areas

(i.e. an igloo) for resting and retreat. Such a device can promote the feeling of security and comfort in their environment thereby decreasing feelings of stress and anxiety. Structures that provide shelter along with an opportunity for exploration are expected to improve welfare by providing an opportunity to perform important behaviors and increase control over their environment[47]. EE has the ability to make the cage a more comfortable and provide a positive habitat for the animal.

The fourth goal of EE is to increase the animal's ability to cope with challenges in a more normal way. On a daily basis, mice are subjected to different types of stimuli whether it is an animal care worker changing cages or a maintenance worker carrying out routine repairs within the animal facility. Animals who are subject to an aversive stimulus elicit a stress reaction if the animal lacks control, whereas there may be no physiological reaction to the same stimulus if that stimulus is predictable and controllable[65]. Animals in an enriched environment tend to react with less anxiety to both routine and non-routine procedures[31]. This decreased anxiety aids in creating a less stressful situation for not only mice when they are being handled but also for the animal caretakers and/or researchers. Some believe this less stressful situation will allow an animal to maintain homeostasis in response to environmental challenges[24], thereby decreasing adverse behaviors and increasing the welfare of the animals.

The fifth goal of EE is to complement experimental outcomes by decreasing variability and increasing post-procedural recovery, both of which are of paramount importance. A guiding principle of the laboratory animal medicine community is that animal welfare promotes better science. Chronic stress, as a result of adverse housing conditions, can affect the animals and, in turn, affect the research. Providing EE can possibly prevent this adverse effect. One way that the effects of stress on mice have been examined is through physiological parameters. These parameters have typically included body weight, hormone levels, and organ weights. These measures can provide

information about changes in the mice physiology which may affect the outcome of scientific experiments[47]. Previous studies have shown that there is no significant change in physiological parameters due to EE[58,59]. Coviello-Mclaughlin[20] showed that there was a positive effect of EE on the psychological well being of surgically manipulated mice. For example, when animals bearing wound clips were exposed to the preferred enrichment, premature wound clip removal decreased, suggesting a positive effect. Many reports of both objective investigations and anecdotal information suggest that EE decreases stress and a non-stressed animal allows for less confounding variables in research data.

D. Types of EE

In addition to the goals of EE, it is important to take into account the variety of enrichment available. There are five types of enrichment[11]. They are as follows: (1) social enrichment via group housing or pair housing, as well as by human contact provided by veterinarians, researchers, and animal care technicians; (2) occupational enrichment, consisting of exercise (e.g. mechanical devices, running) and psychological devices (e.g. puzzles); (3) physical enrichment, such as the enclosure (space and divisions in the cage) and accessories such as toys, hanging objects, ropes, and bars; (4) sensory enrichment, whether it be visual or auditory; and (5) nutritional enrichment in the form of treats and supplements, especially as a component of postoperative care. All of these different forms of enrichment can modulate species-specific behavior in all laboratory species. As was discussed earlier, the species-specific needs and housing situation are pertinent in selecting the type of enrichment used.

E. Conflicting Viewpoints

Even though there are numerous goals that EE intends to meet, there are still many conflicting viewpoints as to the actual benefit and necessity of EE. Some researchers[26] feel that EE is not essential to animal welfare and that EE may interfere with experimental outcome and increase the variability in their data. These same researchers also feel that EE may compromise the validity of experiments by hampering environmental standardization. Others have shown that EE increases aggressive behavior in male mice[5] even though the exact opposite has been shown as well[2]. This is only one example of how there is a lack of and, therefore, a need for objective data to test EE significance. Historically, a purpose for EE has been based mainly on behavioral data, not physiological parameters. However within the past decade, many researchers have shown that EE does indeed benefit laboratory animal medicine and research[2,4,5,7,26]. These studies have shown that EE decreases aggression, anxiety, fear, and excitability as well as enhances learning performance and brain function. There is no reason why mice should not be provided with nesting material as no harmful effects have been found and a number of preference experiments suggest that easily available materials such as tissue or paper towels fulfill the requirements of mice[4,26,59,60]. These enrichment devices will encourage animals to perform species-specific behavior which, in turn, will decrease stress and improve animal welfare.

II. Stress

A. Introduction

Stress is the condition that results when any agent or demand is placed upon the body that leads to the individual perceiving a discrepancy, whether real or not, between the demands of a situation and the resources of the individuals biological, psychological, or social systems. Stress is the biological response of an organism to a perceived threat of its homeostasis[43]. A stressor is defined as a stimulus or event that provokes a stress response in an organism. Stress can be categorized as either acute or chronic, and as external or internal to the organism.

B. Stress Syndrome (General Adaptation Syndrome)

Hans Selye, a pioneer on stress research, defined a paradigm based on his model on stress, called the general adaptation syndrome[52]. This syndrome consists of three stages: (1) The alarm stage is the initial response when the threat or stressor has been identified and is represented by the bodily expression of a generalized call to arms of the defensive forces in the organism. The cells of adrenal cortex discharge their hormone containing granules of secretion into the bloodstream (corticosterone). The blood then becomes concentrated and there is a marked loss of body weight and rapid decrease in size of thymus, spleen, lymph glands, and liver; (2) The resistance stage, if the stressor persists, becomes necessary to attempt some means of coping with the stress. The adrenal glands are greatly enlarged and the cortex accumulates an abundant reserve of secretory granules. The blood is diluted and the body weight returns toward normal; (3) The exhaustion stage occurs when all of the body's resources are depleted and the body is unable to maintain normal function.

C. Physiologic Basis of the Stress Response

1. Introduction

The limbic system and the autonomic nervous system are two parts of the nervous system that are especially significant in mediating the stress response. The autonomic nervous system consists of two parts that function in opposition to each other. They are the sympathetic nervous system and the parasympathetic nervous system.

2. The Limbic System

The physiologic basis of the stress response starts with the limbic system[52]. The limbic system is a set of brain structures consisting mainly of the hypothalamus, amygdala, and hippocampus. Other structures that comprise the limbic system include the cingulated gyrus, fornix, and thalamus. The hypothalamus is a small part of the brain located just below the thalamus on both sides of the third ventricle and is mainly involved with regulation of homeostasis. The hypothalamus regulates hunger, thirst, response to pain, levels of pleasure, anger, and aggressive behavior. The hippocampus consists of two horns that curve back from the amygdala. This structure is important in converting short-term thoughts into long-term memory. The amygdala is comprised of two almond-shaped masses of neurons on either side of the thalamus at the lower end of the hippocampus. The amygdala is responsible for anger and aggression when stimulated. The limbic system is primarily responsible for emotions, behavior, and long-term memory, and operates by influencing the endocrine system and the autonomic nervous system.

3. Sympathetic Nervous System

The sympathetic nervous system (SNS) is a branch of the autonomic nervous system[48,52]. The SNS is always active at a basal level and becomes more active during times of stress. It is the actions of the SNS during the stress response that comprise the fight-or-flight response. The adrenal medulla secretes acetylcholine, which activates the secretion of catecholamines epinephrine (adrenaline) and norepinephrine (noradrenaline). These compounds act primarily on the cardiovascular system. For example, epinephrine (adrenaline) increases the heart rate and the force of heart contractions, facilitates blood flow to the muscles and brain, causes relaxation of smooth muscles, and helps with conversion of glycogen to glucose in the liver. Norepinephrine (noradrenaline) causes vasoconstrictive effects, thus increasing blood pressure. Norepinephrine mediates the conversion of tyrosine to dopamine and modulates a range of immune functions such as cell proliferation, cytokine and antibody production, cytolytic activity, and cell trafficking. Norepinephrine also signals the hippocampus to store an emotional experience in the long-term memory. Lastly, norepinephrine suppresses activity in parts of the brain associated with short-tem memory, concentration, and rational thinking.

4. Hypothalamic-pituitary-adrenal (HPA) axis

The hypothalamic-pituitary-adrenal (HPA) axis is a complex set of direct influences and feedback interactions among the hypothalamus, the pituitary gland, and the adrenal glands[48,52,56]. This axis is a major part of the neuroendocrine system that controls reactions to stress and regulates various body processes including digestion,

the immune system, mood and sexuality, and energy usage. The hypothalamus is a hollow funnel-shaped part of the brain which contains neuroendocrine neurons that synthesize and secrete vasopressin and corticotrophin-releasing hormone (CRH) from the anterior lobe of the pituitary gland, a pea-shaped structure located below the hypothalamus. The corticotropes in the pituitary release adrenocorticotropic hormone (ACTH). ACTH is transported by the blood to the cortex of the adrenal gland. The adrenal glands, small paired pyramidal organs, are endocrine glands located immediately anterior to the kidneys and consist of a cortex and a medulla. The cortex (outer region) produces mineralocorticoids which regulate electrolyte balance. It consists of the zona glomerulosa which produces aldosterone. Aldosterone inhibits the level of sodium excreted into the urine and is responsible for maintaining blood volume and blood pressure. The cortex also synthesizes glucocorticoids to regulate carbohydrate metabolism and androgen precursors. The zona fasciculate produces cortisol which functions to suppress inflammatory reactions, as was discussed earlier. An acute production of glucocorticoid hormones stimulates the metabolism of glucose to provide for energy to flee or combat an immediate threat[52]. The zona reticularis produces testosterone, dehydroepiandrosterone (DHEA), and androstenedione, all sex steroids. The adrenal glands rapidly stimulate biosynthesis of corticosteroids such as cortisol (humans) or corticosterone (mice). Corticosterone (CORT) is the principal glucocorticoid produced by mice and a sensitive and reliable index of stress[63]. CORT regulates metabolism (glucocorticoids) and electrolytes (mineralocorticoids). CORT also suppresses inflammatory reactions in the body and increases to down-regulate the immune system.

D. Impact of Stress on Organ Systems

1. Non-immune Systems

Stress has various impacts on the different organ systems of the body. The effects of stress on the gastrointestinal system include the ability to increase gastric motility, diarrhea, constipation, and bloating, as well as possibly causing irritable bowel syndrome and abnormal weight loss[41]. Regarding the reproductive system, stress affects fertility by increasing levels of cortisol in the blood which inhibit release of gonadotropin releasing hormone (GnRH), the primary hormone that signals a cascade of hormones that direct reproduction and sexual activity, and luteinizing hormone (LH), prompts ovulation and sperm release[25]. Stress can also intensify the chronic pain associated with arthritis and other joint disorders of the musculoskeletal system. Studies have shown that increased work-related stressors are related to musculoskeletal pain[22,57]. The cardiovascular system is affected by increased heart rate and blood pressure. The impact of stress causes the arteries to narrow, which may block the flow of blood to the heart. Stress can cause the release of extra clotting factors into the blood which increases the risk of clot formation and artery blockage. Stress can also trigger the release of fat into the bloodstream and cause the spleen to release more blood cells intro the circulation[19].

2. Immune System

Stress has a significant impact on the immune response to an antigenic stimulus. As was discussed earlier, the two main pathways by which the immune system is modulated by psychological stress include the HPA- and SAM-axes. The glucocorticoid hormones and the catecholamines are the two major groups of mediators of stress and

which exert an impact on the immune response. Psychological stress can influence immune function and alter the pathophysiology of infection. Chronic activation at the HPA axis causes deterioration in general health and aggravates existing diseases[48]. If the immune system is already impaired or weakened, stress can increase one's risk of becoming ill. The changes in the immune response that have been linked to psychological stress include innate immunity (e.g. NK cells) and specific T and B lymphocyte functions including specific reactions against infectious agents[13,33,55]. Previous studies have indicated that stressed animals have a higher proliferative response to Con A, a T lymphocyte mitogen, than do non-stressed mice[39]. This increased response to Con A demonstrates that there is indeed an impact caused by stress on the immune response in laboratory mice. Stress triggers an increased cytokine production (e.g. IL-6 and TNF-α) and thereby causes the body to mount an immune response.

The thymus is a pinkish-gray soft structure located in the upper anterior portion in the chest cavity just behind the sternum. Lymphocytic stem cell precursors from the bone marrow migrate to the thymus where they mature into T lymphocytes. These T lymphocytes leave the thymus and become important in the adaptive immune response. The thymus weight has been shown to decrease under the conditions of stress. This decrease is caused by an increase in apoptosis of thymus cells whereas tyrosine hydroxylase (TH) activity rises under stressful conditions reflecting increased activity of the HPA axis[40].

E. Impact of EE on Stress

The impact of EE on stress has been examined in only a few studies. The main index used to measure stress levels in mice is corticosterone. Meijer[42] examined the

effects of EE and handling on the acute physiologic stress response caused by short periods of restraint. For example, heart rate and body temperature were measured by radiotelemetry. The plasma corticosterone levels were measured and postmortem thymus weight and tyrosine hydroxylase (TH) activity were also assessed. The results of Meijer's study indicated that EE and handling increased the stress response but after facilitated recovery of the plasma CORT values; thus measured 90 min after restraint were lower in enriched groups. Also, baseline heart rate was higher in non-enriched groups, suggesting that chronic stress was greater for mice that lacked enrichment items in the cage. Several other investigators have shown that EE attenuates or decreases stress as evidenced by decreased CORT levels[7,35,39].

Chamove[16] demonstrated that the presence of vertical partitions in the cages of mice reduced the stressfulness of caging and also led to mice being less reactive to being manipulated outside of their cages. Also, when reared in these more complex cages, the animals gained more weight and were observed to be more active. Hennesy[31] observed that if mice were placed in a novel enrichment condition with metal foil or cardboard being available, they chewed these materials. These animals had lower plasma corticosterone levels than mice in the same environment without access to these chewable items. Van Loo[61] reported that long term provision of nesting material and its transfer during cage cleaning influenced several stress-related physiological parameters. For example, mice housed in cages enriched with nesting material had lower urine corticosterone levels and heavier thymuses, and consumed less food and water than NE mice. Van Loo concluded that the long-term provision of nesting material, including the transfer of nesting material during cage cleaning, reduces stress and thereby enhances the welfare of laboratory mice. Since animals under chronic stress are considered to be inappropriate research subjects, the studies described in this thesis

investigated if the presence of EE decreases signs of stress in mice as measured by serum CORT levels.

F. Relationship Among EE, Stress, and the Immune Response

As is outlined in the previous section, EE can have a significant impact on stress, such as decreased CORT levels. EE also has an impact on the immune response, such as increased immune function. Enrichment has been shown to decrease or attenuate stress in mice. EE may prevent the elevation of corticosterone levels in response to simple procedures such as handling, which is a common task performed on mice. This reduction in the signs of stress can potentially result in an increased level of immune function or at least prevent a diminished immune response. Long-term stress that is provoked by restrictive housing conditions combined with short-term stress from husbandry and experimental procedures such as restraint, cage changes, and injections may lead to physiological changes, which interfere with normal biological functions such as those of the immune system[43]. This thesis was designed to rigorously test the hypothesis that EE can reduce stress by objectively measuring various aspects of immune function; an innate response highly influenced by stress.

III. Immune System

A. Introduction

The immune system is a collection of highly complex, coordinated mechanisms within an organism that, among other functions, protects against disease by identifying and killing pathogens and tumor cells[56]. This system detects a wide variety of agents

and distinguishes them from the healthy cells of the organism. Physical barriers are the first line of defense against pathogens such as bacteria and viruses from entering the body. If a pathogen breaks through these barriers, the innate component of the immune system provides an immediate response. This component is found in all plants and animals and is the dominant system of host defense.

B. Innate Immunity

Innate immunity is responsible for inflammation which is one of the first responses of the immune system to infection[56]. Symptoms of inflammation include redness and swelling and the production of eicosanoids and cytokines which are released by injured or infected cells. Innate immunity includes the complement proteins, phagocytes, and natural killer (NK) cells. The complement system is a cascade that culminates in the attack of the surface of foreign cells. Complement is the major humoral component of the innate immune response and consists of three modes of activation: classical, alternative, and lectin activation pathway. The classical pathway is triggered by the activation of the C1 complex; either by the binding of C1q to antibodies from the IgM or IgG isotypes, complexed with antigens, or by the binding of C1q to the surface of the pathogen. The alternative pathway is triggered by C3 hydrolysis directly in the surface of a pathogen. This pathway does not rely on a pathogen-binding protein like the other pathways. The lectin activation pathway is homologous to the classical pathway, but with opsonin, mannan-binding lectin (MBL) and ficolins, instead of C1q.

Phagocytosis is a cellular process of engulfing solid particles by the cell membrane to form an internal phagosome and is a major mechanism used to remove pathogens and cellular debris. Phagocytes generally patrol the body searching for pathogens, but can also be recruited to specific locations by cytokines. Once a

pathogen has been engulfed by one of these phagocytes it becomes trapped in an intracellular vesicle called a lysosome to form a phagolysosome. The pathogen is killed by the activity of digestive enzymes. Macrophages and neutrophils travel throughout the body in pursuit of invading pathogens. Macrophages are cells within the tissues that originate from monocytes. These cells phagocytose cellular debris and pathogens and present pathogen-derived antigens to corresponding helper T cell therefore initiating an immune response. These cells also stimulate lymphocytes and other immune cells to respond to the pathogen and produce enzymes, complement-associated proteins, and IL-1 and carry receptors for lymphokines. Neutrophils are the hallmark of acute inflammation. These cells are capable of ingesting microorganisms or particles as well as internalizing and killing many microbes resulting in formation of a phagosome into which reactive oxygen species and hydrolytic enzymes are secreted.

NK cells are leukocytes that attach to and destroy tumor cells. These cells play an important role in host resistance to viral infections and cancer development. These cells kill by releasing small cytoplasmic granules of proteins called perforin and granzyme that cause the target cell to die by apoptosis. This mechanism is sensitive to stress manipulations[7].

C. Adaptive Immune Response

The adaptive immune response is antigen-specific and requires the recognition of specific “non-self” antigens during a process called antigen presentation. The cells of the adaptive immune system are called lymphocytes, specifically B cells and T cells. These cells are derived from hematopoietic stem cells in the bone marrow. B cells are primarily involved in the humoral immune response and T cells are primarily involved in cell-mediated immune response.

1. Humoral Response

Humoral immunity is mediated primarily by B cells. These cells originate from the bone marrow. A B cell identifies pathogens when antibodies on its surface bind to a specific foreign antigen. To produce antibodies, B cells must first be activated. Activation of a naive B cell requires two signals. The first is the clustering of B cell receptors and their associated signaling molecules. The second signal is called the co-stimulatory signal which is provided by helper T cells (T-cell dependent activation). These B cells then become plasma cells or memory cells. Once antibodies are secreted, they bind to antigens on the surface of invading microbes. This binding can lead to T helper cell 2 activation and cytokine production which produces the following cytokines: IL-4, IL-10, and TGF-ß.

B cells, which originate in the bone marrow, rearrange the gene segments that encode their heavy and light chain proteins, and display two classes of antibody molecules: IgM and IgD. Once a B cell is activated it is ready to produce IgM antibodies; first antibodies to appear in response to an initial exposure to an antigen. However, a B cell also has the opportunity to change the class of antibodies. IgG neutralizes toxins, viruses and bacteria in the blood and extracellular fluids and also opsonizes them for phagocytosis. IgA fights ingested antigens and prevents attachment of virus and bacteria to skin and intestinal lining surfaces. IgE is responsible for allergic reactions.

2. Cell-Mediated Immunity

a) T cell Function

T cell-mediated immunity is an immune response that involves the activation of macrophages, natural killer cells, antigen-specific cytotoxic T lymphocytes, and the release of various cytokines in response to an antigen. This immune response is most effective in destroying virus-infected cells and consists of cytotoxic T cells (CTL), helper T cells, and regulatory T cells.

CTL function to induce the apoptosis of cells displaying epitopes of foreign antigens on their surface; for example, virus-infected cells, cells with intracellular bacteria, and cancer cells displaying tumor antigens. For activation, these CTL require the presence of a T cell receptor (TCR) on their cell surface. Their TCR must bind to this specific antigen in a complex with the MHC class I receptor of another cell. Recognition of this MHC:antigen complex is aided by a co-receptor on the T cell, called CD8. Once a CTL is activated, it proliferates rapidly and leaves the lymph node, enters the blood, and searches for the area of the body where the infected cells are located and kills them. CTL produce perforin which is a protein that can bind to and punch holes in cellular membranes. Along with granzyme B, the target cell reacts and encloses the granzyme and perforin in a vesicle. Once inside the target cell, the perforin molecules make holes in the vesicle, allowing the granzyme B to escape into the cytoplasm. Granzyme B then triggers an enzymatic chain reaction that results in the apoptosis of the target cell.

Helper T cells regulate both the innate and adaptive immune responses. The primary role of helper T cells (Th), once activated, is to secrete small proteins called cytokines. Cytokines regulate the immune response by promoting T cell and

macrophage migration to the site of infection. These small proteins also activate those cells stimulating them to produce more cytokines. When virgin helper T cells are first activated, they secrete IL-2 which induces proliferation of T cells.

Regulatory T cells are crucial for maintenance of immunological tolerance. Their major role is to shut down T cell-mediated immunity toward the end of an immune reaction and to suppress auto-reactive T cells that escaped the process of negative selection in the thymus.

b) T cell Activation

T cell activation of $CD4^+$ T cells occurs through the combining of TCR and CD28 on the T cell by the major histocompatibility complex (MHC) peptide and B7 family members of the antigen presenting cell (APC). The first signal is binding of TCR to a short peptide presented by MHC on another cell such as a dendritic cell or B cell that has digested an Ag. The peptide is presented to $CD8^+$ cells by MHC class I. The peptide is presented to $CD4^+$ cells by MHC class II. The second signal is co-stimulation and allows the T cell to respond to an antigen.

IV. Impact of EE on Immune Response

Benaroya-Milshtein[7] observed that EE mediated a statistically significant improvement of the outcome of immunotherapy in an experimental model of B cell lymphoma as well as enhanced NK cell activity. This paper concluded that EE has a beneficial effect on anxiety-like behavior (grip-strength test, staircase and elevated plus maze test), stress response (CORT levels) and NK cell activity (cytotoxicity assay). Non-enriched animals also have been shown to have a higher proliferative response to

Con A, a lymphocyte mitogen, than do enriched mice[39]. The author of this study suggested that enrichment and housing density were important factors influencing immune responses and in response to exercise stress. However, few objective studies are available to determine the effects of EE on the immune system even though EE is being increasingly introduced into animal housing without sufficient data. EE may promote the immune response in murine animals thereby improving animal welfare. The basis of this thesis project was an objective analysis of the impact of EE on the immune response in mice.

V. Herpes Simplex Virus (HSV)

A. Structure

HSV is comprised of a single molecule of linear, double-stranded DNA approximately 152 kbp. HSV is 100 nM in diameter and is constructed of 162 capsomers. The genome circularizes upon infection and encodes nearly 100 transcripts and is comprised of more than 70 open translational reading frames. There are four major components of the virion: (1) a lipid-rich bilayer envelope studded with surface glycoprotein, (2) a tegument of amorphous material located between the capsid and the envelope and containing approximately 20 virally encoded proteins that are unique to herpes virus, (3) an icosahedral nucleocapsid surrounding the core of the DNA genome/protein, and (4) protein spool on which the DNA is wrapped.

B. Cellular Entry

The virus particle is covered by an envelope which, when bound to specific receptors on the cell surface, fuses with the cell membrane and creates an opening through which the virus enters the host cell. The viral capsid is transported to the cell nucleus into which it ejects its DNA contents via the capsid portal. The DNA then exits the capsid in a single linear segment.

C. Host Range

A wide host range exists for herpes viruses. Man is the natural host but animals can also be infected. Eight human herpesviruses are known to exist. Non-human herpesviruses affect the following species: pigs, primates, cattle, chickens, horses, frogs, and fish. HSV has the capacity to establish latent infections in the nervous system of infected humans/animals.

D. Infection

The virus particle is covered by an envelope which, when bound to specific receptors on the cell surface, fuses with the cell membrane and create an opening, or pore, through which the virus enters the host cell. The entry of HSV into the host cell involves interactions of several viral glycoproteins with cell surface receptors. These interactions bring the membrane surface into mutual proximity and allow for other surface glycoproteins to interact. After the viral capsid enters the cellular cytoplasm, it is transported to the cell nucleus. Once attached to the nucleus at a nuclear entry pore, the

capsid ejects its DNA contents via the capsid portal. The DNA exits the capsid in a single linear segment.

E. Propagation, Quantification, and Detection

HSV-1 and HSV-2 form plaques on many cell types making it a relatively easy virus to quantify. The virus also grows rapidly in cells in culture and has a relatively short replication time. The virus also efficiently destroys infected cells.

F. Types of HSV Diseases

1. HSV-1

Herpes simplex virus type 1 (HSV-1) is usually associated with infections of the lips, mouth, and face. It is the most common type of herpes simplex virus and which many people acquire in their first two decades of life. HSV-1 often causes lesions inside the mouth, such as cold sores (fever blisters) and is transmitted by contact with infected saliva. By adulthood, up to 90% of people will have antibodies to HSV-1. Latency location of this type occurs in the trigeminal ganglion.

2. HSV-2

Herpes simplex virus 2 (HSV-2) is sexually transmitted. Symptoms include genital and oral ulcers or sores. HSV-2 can also lead to complications such as infection of the lining of the brain and the brain itself. This complication is termed meningoencephalitis, which is associated with headache and increasing confusion. Keratoconjunctivitis, infection of the eye, can also occur, especially the conjunctiva and cornea. However, some people that have HSV-2 do not show symptoms. Up to 30% of

U.S. adults have antibodies against HSV-2. Latency location of genital HSV-2 occurs in the spinal dorsal root ganglia.

3. Severe HSV infection

In neonatally-acquired herpes simplex virus infection, the neonate may develop symptoms of herpes infection before he/she is born or within the first 6 weeks of life. This type of infection is mainly confined to the skin, eye, and mouth. A mother who is infected with HSV may transmit the virus to her newborn during vaginal delivery, especially if the mother has an active infection at the time of delivery.

G. Latency

During primary HSV infection, the virus is replicated to high levels in the epithelial cells at peripheral sites of infection. These infections eventually resolve and the virus is cleared. The virus then enters sensory neurons and travels by retrograde axonal transport involving microtubules to the sensory nerve ganglion and enters the nucleus. The productive infection is generally suppressed in the neuron and a period of acute infection in the ganglion occurs. The infection resolves, a few neurons die, and latency establishes itself in these infected neurons. Reactivation can occur but an intact neuron is needed. The frequency of this occurrence decreases with time since the time of the primary infection. The frequency of reactivation leading to recurrent lesions is related to the severity of lesions caused by the first infection and can often appear at the same peripheral site as the original infection. Reactivation is often precipitated by particular stimuli such as stress, extreme sunlight, fever, local skin trauma, and menstruation[49].

H. Diagnostics

Several diagnostic methods are used in detecting HSV (both 1 and 2) infection. An identifiable characteristic lesion such as a thin-walled blister on an inflamed base of skin may sometimes be observed and indicative of an infection. A Tzanck test, which is a microscopic examination of tissue scrapings, may be also be used. If characteristic lesions are present, a viral culture of the lesion may be performed. If no lesions are present, then a type-specific blood test for HSV can be utilized.

I. Transmission

For an infection to occur, the herpes simplex viruses (both HSV-1 and HSV-2) must access the body through broken skin or a mucous membrane such as inside the mouth or on the genital area. Each virus can be carried in bodily fluids (such saliva, semen, vaginal fluids, and shedded skin in pelvic region from eczema) or in fluid from herpes sores. The risk for infection is highest with direct contact of blisters or sores during an outbreak. Infection may also be transmitted during childbirth, as previously discussed.

J. Treatment

Oral (e.g. Acyclovir, Famcyclovir, and Valacyclovir) and topical (e.g. Penciclovir, Acyclovir, Docosanol) treatments, sometimes in combination with nutritional supplements (e.g. L-Lysine) are commonly used to suppress symptoms and decrease viral shedding for both oral and genital infections.

K. Relationship Among Stress, HSV, and the Immune Response

After the initial infection, HSV lies dormant in the body, but can be reactivated by various stimuli such as stress. The virus escapes the usual immune response by penetrating nerve fibers. As the immune system moves in to control the infection via phagocytosis and T cell mediated immunity, the virus hides itself within nerve cells. The virus is carried to the nerve cell body within a ganglion located close to the spinal cord (genital infection) or in the trigeminal nerve (oral infection). When the virus reaches the cell body, the viral DNA is added alongside the nerve cell's own DNA in the nucleus. The

virus remains there, hidden within the nerve cell and in an inactive state, for the lifetime of the infected individual[48].

VI. Relationship Among EE, Stress, and Immune Response to HSV Infection

Stress is known to decrease host-resistance to pathogenic microorganisms[17]. Studies have demonstrated that housing conditions, stress, and enrichment conditions can both enhance and suppress various immune responses[39]. Kingston showed that EE reduces the large oscillations in immune reactivity and/or shortens the period of recovery from acute stress. Endocrinological stress responses have immunosuppressive effects. This effect may lead to increased susceptibility to disease[63]. Therefore, the present investigation is particularly important for laboratory animal medicine in that it will add to our understanding of the influence of environment on the susceptibility and pathogenesis of a variety of rodent pathogens that are immunologically resisted. To date, the use of EE has been based on trial-and-error, general observations, anecdotes, and published best practices. Thus, the recommendations provided are often conflicting. The impact of EE on an immune response to infectious pathogens such as HSV-1 has yet to be determined and forms the basis for this thesis.

Chapter 2

MATERIALS AND METHODS

I. Animals

A total of 72 five- to six-week old C57BL/6 male mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice were quarantined for five days after which they were used for experimental procedures (see Figures 1 and 7).

II. Housing and Choice of Enrichment Devices

Mice were housed either singly or in groups of four in standard 29 x 19 x 13 cm polycarbonate cages containing either an enriched or non-enriched environment. These cages were placed in isolation cubicles in a biohazard facility. Animals were allowed to acclimate to these conditions for seven days before experimental manipulations were performed. All mice were housed in a controlled-temperature room (22-25 °C) with a 12:12 hour light / dark cycle (lights on 0700-1900). Standard Harlan Teklad rodent diet (#2018 Indianapolis, IN) and water were made available ad libitum. All animals were handled in accordance with the Guide for the Care and Use of Laboratory Animals and approved by the Penn State Hershey Institutional Animal Care and Use Committee (IACUC). Environmental enrichment (EE) was provided for both group and singly-housed mice by red-tinted transparent polycarbonate certified mouse igloos (2 1/4" tall x 4 1/8" diameter, Bio-Serv, Frenchtown, NJ) and tunnels (3 7/8" long, 2" inside diameter, Bio-Serv). The tunnels were suspended with stainless steel hangers to increase usable floor space. Standard Harlan Teklad 1/4" size corncob bedding (#7092 Indianapolis, IN) was also included in all cages.

III. Virus

HSV-1 strain McIntyre was prepared by infection of Vero cells at a multiplicity of infection of 0.01. Viral titers were assessed by plaque assay on Vero cells and viral stocks were stored at -70°C.

IV. Retro-orbital Bleeding

Mice were anesthetized with isoflurane. When the mice were no longer moving voluntarily, the forefinger of the operator's non-dominant hand was used to pull the facial skin taut and cause the eyes to protrude slightly while the skin at the back of the neck was grasped by the thumb and remaining fingers to restrain. Breathing was monitored throughout the procedure to ensure that the restraint did not compromise the airway. Using the dominant hand, the tip of a 200 µl capillary tube was gently inserted below the eye at approximately a 45 degree angle into the space between the globe and the lower eyelid. When the tip of the pipette contacted the boney floor of the orbit it was gently twisted between thumb and forefinger to rupture the capillary plexus/sinus. Blood was allowed to flow by capillary action into the pipette. At the conclusion of the blood withdrawal, tension on the animal was released and a gauze pad was gently pressed over the eye for a few seconds until the bleeding had stopped. Respiration was confirmed and the animal was returned to its cage for recovery. Blood was transferred to Eppendorf tubes and put on ice for at least 30 minutes to allow clotting to occur. All

blood samples were centrifuged at 16,000 x *g* for ten minutes and serum was collected and stored at -70°C. Mice were bled at 0900, 2 hours after lights-on to eliminate the confounding variable of circadian rhythm.

V. Quantification of Corticosterone

The levels of serum corticosterone were determined using a radioimmunoassay (RIA) kit (MP Biomedicals, Costa Mesa, Ca). These levels were calculated using a standard curve generated from standards containing 0-1000 ng/ml of corticosterone.

VI. Intranasal Infection

Six- to eight-week old male mice were anesthetized with isoflurane following 7 days of acclimation in either an enriched or non-enriched environment. All mice were infected with $1x10^7$ plaque forming units (PFU) of HSV-1 McIntyre in media. A volume of 23 µl was administered in both nostrils alternating until full volume was given.

VII. Collection of Nasal Washes to Determine the Extent of HSV Colonization and Virus Replication

Following euthanasia, jaw muscles were severed and the mouse was secured to a dissection board with pins by the jowls. A 10-gauge scalpel blade was used to cut the

palate along the tooth line. The palate was grasped with forceps and peeled away from the incisors toward the molars. Approximately 200 µL PBS/1%FBS were pipetted in and out of nasal cavity 10 times of all mice. This step was repeated four times for a total of 1 ml which was then placed in Eppendorf tubes. Samples were freeze/thawed three times in a 95% ethanol/dry ice slurry then sonicated and centrifuged at 16,000 x *g* for ten minutes. Samples were then transferred to new Eppendorf tubes. The levels of HSV in these samples were determined using plaque assays. Plaque assays are the most widely used technique for virus isolation and purification, and to determine viral titers. The basis of the technique is to measure the ability of a single infectious virus to form a plaque on a confluent monolayer culture of HSV-susceptible cells. Vero cells in growth media were added to 6-well tissue culture dishes. The plates were incubated until the cells were 90-100% confluent. The virus and a 10^{-2} dilution of the virus in PBS were added to the wells in duplicate letting the virus flow gently onto the cell monolayer. The virus-cell mixture was incubated at 37°C for one hour. At the end of this one hour period, methylcellulose overlay media was added to each well. The cells were then allowed to incubate at 37°C for 4 days. Cells were then stained with 0.5% crystal violet/ 5% formaldehyde in order to visualize the plaques for counting. The plaques were counted and the concentration of the initial viral suspension was calculated in terms of pfu/ml.

VIII. Isolation of Cells from Lymph Nodes

Mice were anesthetized seven days post-infection with sodium pentobarbital 50-80 mg/kg i.p. The mice were then exsanguinated via cardiac puncture. The superficial cervical (CLNs) and mediastinal (MLNs) lymph nodes were removed and placed in GIBCO™ Iscove's-modified Dulbecco's media (IMDM) (Invitrogen, Carlsbad, CA)

supplemented with 10% (v/v) fetal bovine serum (FBS), 50 μM 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, and 100 ug/ml streptomycin sulfate. Lymph nodes were mechanically dissociated by passage through a 70 μm nylon cell strainer (BD Biosciences, San Jose, CA) and the resulting cell suspension was washed with supplemented IMDM. The number of viable cells was determined by trypan blue dye exclusion.

IX. Collection of Brains to Determine the Extent of HSV Colonization and Replication

The brains were collected in 5 ml snap cap tubes and stored at -70°C.

Purification of total DNA from the brain was performed using a DNeasy Blood & Tissue Kit (Qiagen). The samples were lysed using proteinase K. Buffering conditions were adjusted to provide optimal DNA-binding conditions and the lysate was loaded onto the DNeasy MINI spin column. During centrifugation, DNA is selectively bound to the DNeasy membrane as contaminants pass through. The remaining contaminants and enzyme inhibitors were removed in two efficient wash steps and DNA was then eluted in buffer. The extent of DNA amplification was then viewed via real-time PCR.

X. Synthetic Peptides

Synthetic peptides corresponding to the HSV-1 CTL recognition epitope $gB_{498–505}$ (SSIEFARL)[12] and ovalbumin amino acid residues 257-264 ($OVA_{257-264}$; SIINFEKL) were synthesized at the Macromolecular Core Facility of the Milton S. Hershey Medical Center by FMoc chemistry using an automated peptide synthesizer (9050) MilliGen PepSynthesizer. The purity and amino acid composition of each peptide

was determined by HPLC tracing (Waters, a division of MilliGen). Peptide stock solutions were prepared by solubilizing the lyophilized peptides in dimethyl sulfoxide (DMSO) and adjusting the concentration to 1 mM with non-supplemented RPMI 1640 medium.

XI. Quantification of Lymphoid Cell Subsets by Cell Surface Marker Expression and Tetramer Binding Specificity

Flow cytometric analysis of cell surface markers was determined as described previously[3,44,66] with slight modifications. Briefly, CD16/CD32 Fcγ receptors on isolated mononuclear cells were blocked with antibody in a 2.4G2 hybridoma cell culture supernatant supplemented with 20% mouse serum (Sigma)[3]. Cell surface expression of CD8 was detected using anti-CD8 APC antibody (clone 53-6.7; eBioscience). For the detection and quantification of $gB_{498-505}$ epitope-specific T lymphocytes, cells were incubated with a PE-labeled tetramer that was prepared as described previously[1] and was provided by the National Institute of Health tetramer facility. This tetramer detects the H-$2K^b$-restricted, $gB_{498-505}$-specific T cell receptor complex[10], which has previously been described as the immunodominant epitope in C57BL/6 mice[12,29]. Following washes with FACS buffer (PBS supplemented with 1% [v/v] FBS, 0.02% [w/v] sodium azide), cells were resuspended in 2% (w/v) paraformaldehyde (prepared in PBS) prior to analysis by flow cytometry.

XII. Intracellular Cytokine Staining for IFN-γ

The lymph node-derived lymphocytes were isolated as described above.

These cells were resuspended in supplemented IMDM and incubated with 1 μM of $gB_{498-505}$ peptide for 2 hours at 37ºC. Cells were treated with brefeldin A (Sigma) (final concentration of 5 μg/ml) to prevent the secretion of cytokines and incubated for an additional 4 hours at 37ºC. Cells were then washed twice with FACS buffer and the CD16/CD32 Fcγ receptors blocked with 2.4G2 cell culture supernatant supplemented with mouse serum as described above. To identify $CD8^+$ T lymphocytes, cells were incubated with anti-CD8 APC antibody. Following staining for CD8, cells were fixed in 2% paraformaldehyde and incubated at room temperature for 20 minutes in the dark. Cells were then washed twice with FACS buffer and incubated with anti-IFN-γ FITC antibody (clone XMG1.2; eBioscience) diluted in 0.5% (w/v) saponin (Sigma) prepared in FACS buffer. Subsequently, cells were washed twice in 0.5% saponin, resuspended in 2% paraformaldehyde, and analyzed by flow cytometry.

XIII. Flow Cytometry Analysis

Flow cytometric analysis was conducted using a FACSCalibur and FACSCanto flow cytometer (Becton Dickinson, San Diego, CA). Using forward-angle light scatter and 90° light scatter profiles, electronic gates were set around the live cells and at least 50,000 events were collected per sample. Dot plots and histograms were analyzed using FlowJo Software (TreeStar, Inc.; Ashland, OR). The total number of cells per sample was determined as follows: [percentage of specific cell type in sample] x [number of viable cells in sample].

XIV. Data Analysis

Statistical significance was determined by unpaired *t*-test using StatView 5.0.1 software (SAS Institute Inc, Cary, NC). Comparisons between groups were performed and p values < 0.05 were considered significant.

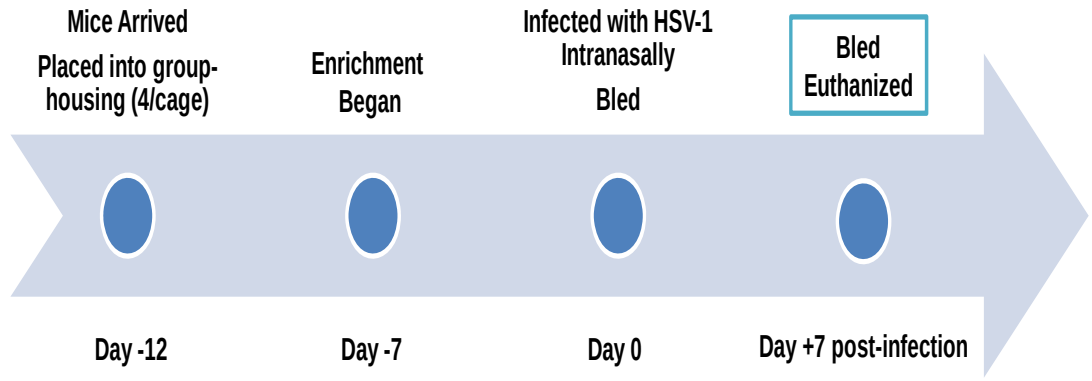
Chapter 3

RESULTS

1. Experimental Design (group-housed)

We developed a model to determine the impact of EE on murine stress levels and their immune response to HSV infection (Figure 1). At 5 days after mice were placed into group-housing (4/cage), EE was added to half of the cages. EE consisted of both a mouse igloo and a suspended tunnel. On day 0, mice were bled retro-orbitally and infected intranasally with HSV-1. At 7 days post-infection mice were bled again and euthanized via sodium pentobarbital overdose. The brains, nasal washes, CLNs and MLNs were collected.

EXPERIMENTAL DESIGN (Group-housed)



Group-housed
•3 cages of 4 mice = 12 mice/group
•2 cohorts: enriched and non-enriched

Collect brains and nasal washes
•Quantify infectious HSV-1 by plaque assays
•Quantify HSV DNA by real-time PCR

Collect blood
•Quantify corticosterone

Collect draining CLN's and MLN's
•Assess immune responses:
•Flow cytometry
•Tetramer and ICS assays

Figure 1. Experimental Design (group-housed). This figure depicts the timeline followed to determine, in group-housed mice, the impact of EE on the levels of CORT and the $CD8^+$ T cell-mediated immune response to HSV-1 infection.

2. Serum corticosterone levels of EE and NE group-housed mice pre- and post-HSV-1 infection.

Given that CORT is the principle indicator of stress in mice[61], we measured CORT levels in an attempt to determine the impact of EE on the degree of stress exhibited by mice in group-housed conditions, both pre- and post-HSV-1 infection. A pre- and post- infection CORT level was measured to determine if EE had an effect on CORT levels as compared to the NE group. We hypothesized that EE would decrease levels of stress in mice. This study showed no significant difference between EE and NE group both pre- and post-HSV infection (Figure 2). However EE mice had a significantly lower level of VORT post-infection as compared to the CORT level pre-infection. There was a trend of decreasing CORT levels post-infection in EE and NE groups, as compared to pre-infected, possibly due to the infection itself down-regulating CORT levels in order to allow for a stronger immune response. The range of CORT levels observed was within the normal baseline range of non-stressed mice.

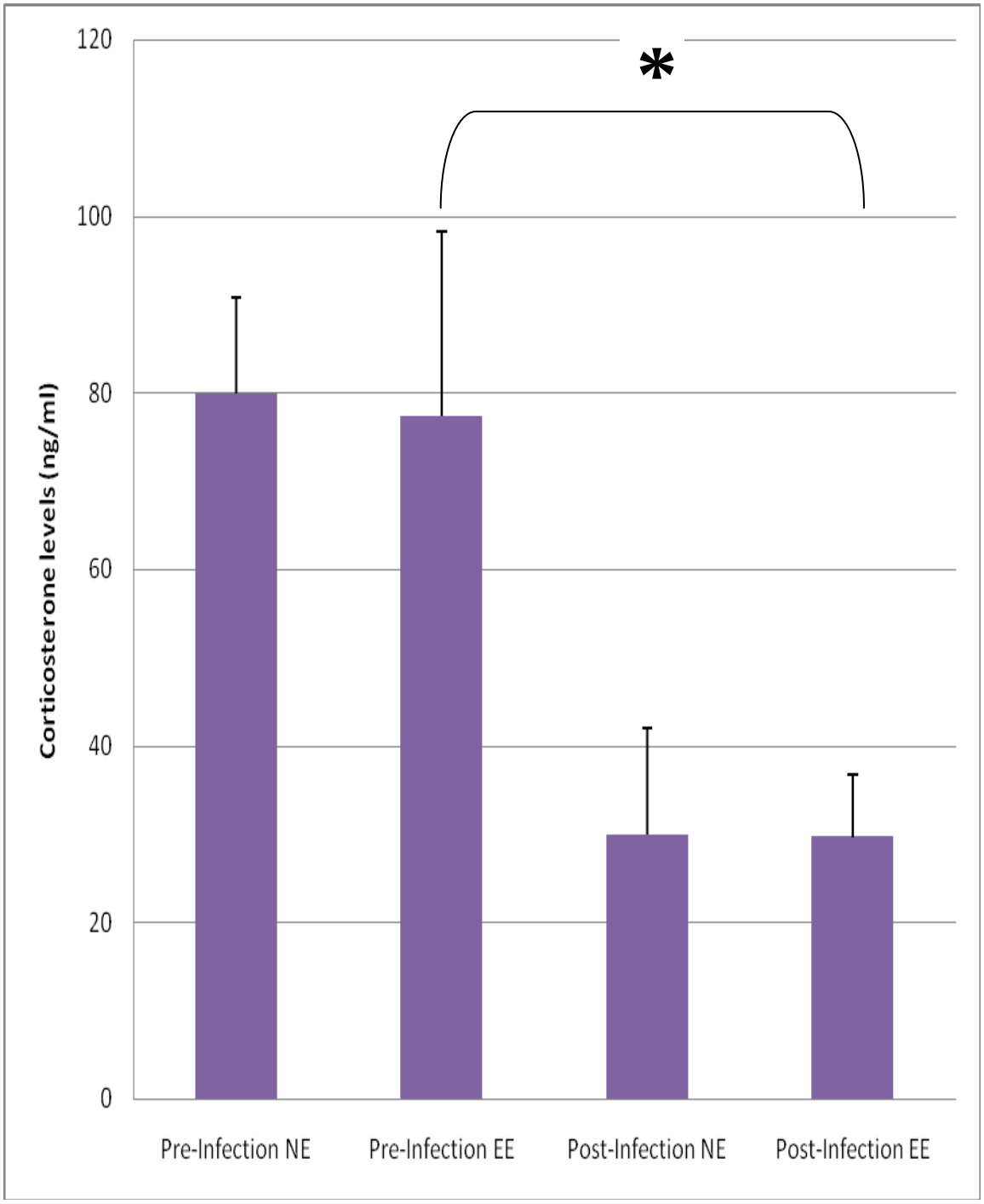


Figure 2. Effects of EE on serum corticosterone levels in group-housed mice pre- and post- HSV-1 infection. EE mice had a significantly lower level of CORT post-infection as compared to the CORT level pre-infection. *p<0.05 (unpaired *t*-test)

3. Percentage of $CD8^+$ $gB_{498\text{-}505}^+$ cells in the CLNs of EE and NE group-housed HSV-1 infected mice.

In rodents, the immune response to intranasal pathogens occurs within the nasopharyngeal-associated lymphoid tissue (NALT) and the lymph nodes that drain the upper and lower respiratory tract[32,59]. Specifically, the CLNs drain the upper respiratory tract and nasal cavity, and the MLNs drain the lower respiratory tract. The vast majority of the CTL generated in response to HSV-1 infection in C57BL/6 mice recognize the $gB_{498\text{-}505}$ immunodominant peptide in the context of the $H\text{-}2K^b$ MHC class I molecule[12].

We wanted to determine the impact of EE on the frequency of lymph-node-derived $CD8^+$ $gB_{498\text{-}505}^+$ cells in the CLNs. CLNs drain the upper respiratory tract and nasal cavity. Using the experimental design illustrated in Figure 1, CLNs were stained with anti-CD8 APC antibody and APC-conjugated $gB_{498\text{-}505}$ tetramer and analyzed using flow cytometry. Comparisons were made to the NE group (control group) which was arbitrarily designed a value of 100%. Mice that were provided EE had a slightly lower percentage of $CD8^+$ $gB_{498\text{-}505}^+$ cells as compared to mice in the NE group (Figure 3). However, this difference was not significant. These findings indicate that EE does not alter the ability to generate HSV-1-specific CTL that are specific for the HSV-encoded immunodominant epitope, $gB_{498\text{-}505}$.

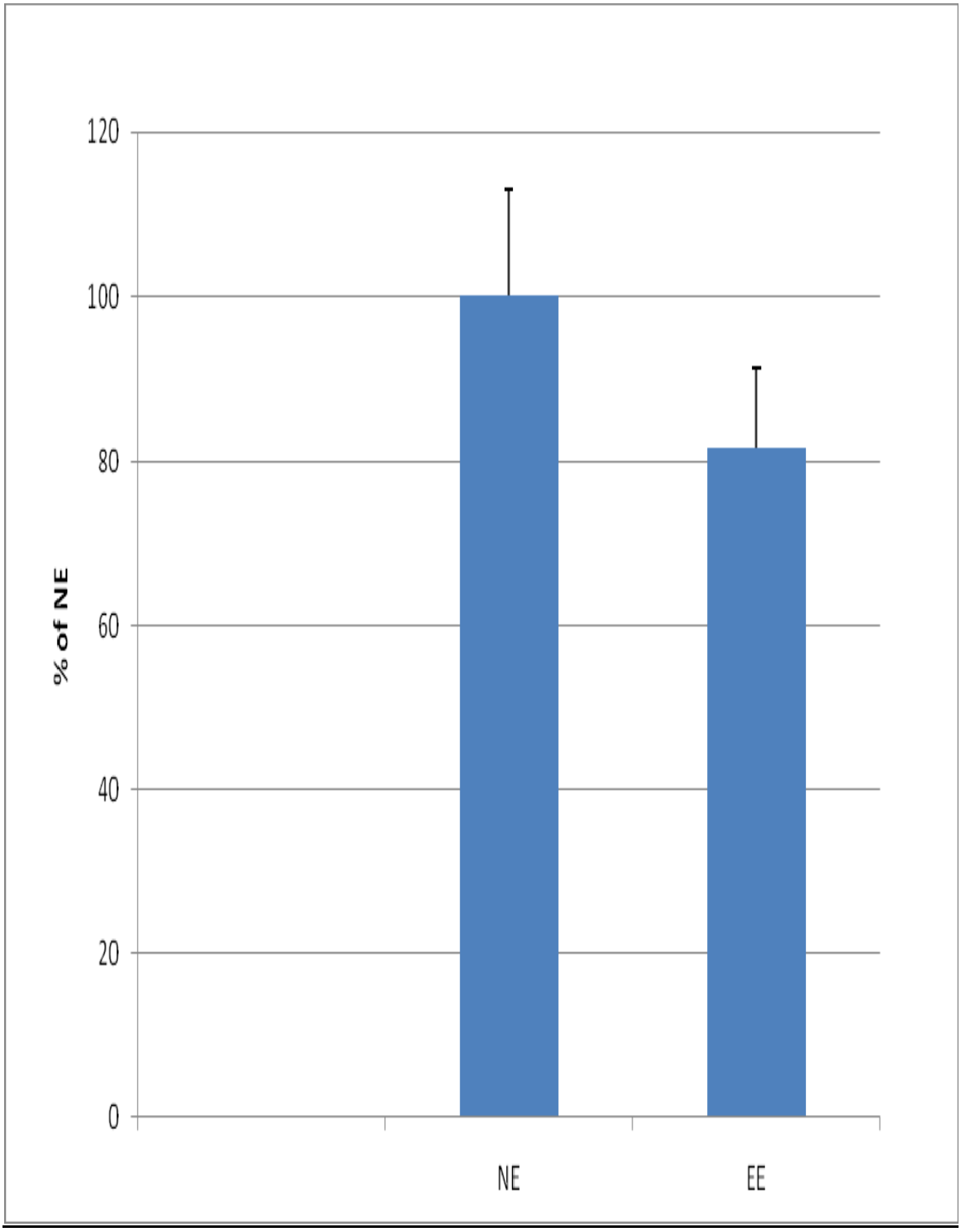


Figure 3. Percentage of $CD8^+$ $gB_{498\text{-}505}{}^+$cells in the CLNs of EE and NE group-housed HSV-1 infected mice.

4. Percentage of $CD8^+$ $gB_{498\text{-}505}^+$ cells in the MLNs of EE and NE group-housed HSV-1 infected mice.

We wanted to determine the impact of EE on the frequency of lymph-node-derived $CD8^+$ $gB_{498\text{-}505}^+$ cells in the MLNs. MLNs drain the lower respiratory tract. Although we are delivering the virus into the upper respiratory tract, the virus spreads to the lower respiratory tract. Hence, this is why the MLNs were collected and analyzed. Using the experimental design illustrated in Figure 1, MLNs were stained with anti-CD8 APC antibody and APC-conjugated $gB_{498\text{-}505}$ tetramer and analyzed using flow cytometry. As is illustrated in Figure 4, mice exposed to EE demonstrated significantly fewer $CD8^+$ $gB_{498\text{-}505}^+$-specific cells than did the mice in the NE environment.

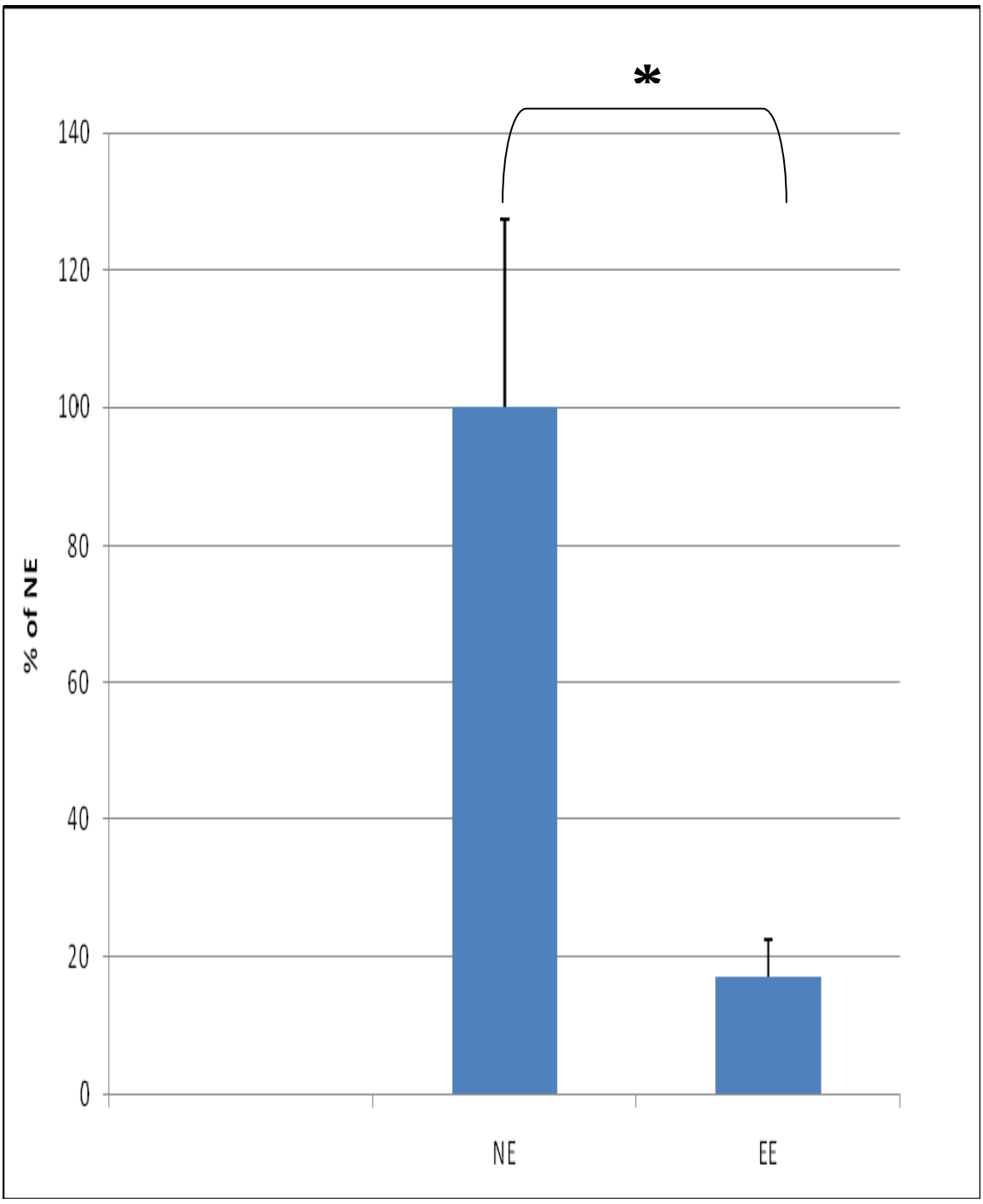


Figure 4. Percentage of $CD8^+$ $gB_{498\text{-}505}{}^+$ cells in the MLNs of EE and NE group-housed HSV-1 infected mice. EE mice had a significantly lower percentage of $CD8^+$ $gB_{498\text{-}505}{}^+$ cells as compared to the NE . *, $p<0.05$ (unpaired *t*-test).

5. Percentage of $CD8^+$ IFN-γ^+ cells in the CLNs of EE and NE group-housed HSV-1 infected mice.

To further delineate the effects of EE on the HSV-specific cellular immune response, we determined percentage of $CD8^+$ IFN-γ^+ cells in the lymphocytes of the CLNs. IFN-γ can play a number of roles in the overall immune response and defense against HSV infection. An intracellular cytokine assay (ICS) for IFN-γ was used. ICS is a flow cytometry-based method to assess CTL function as measured by the level of IFN-γ produced in response to antigen-specific stimulation. The tetramer analysis that shown previously (Figures 3 and 4) is limited to depicting the number of cells only. Knowing the number of cells is important. However, knowing if the cells are functional or not is even more essential. EE mice demonstrated a trend of reduced percentages (*p* value = 0.2676) of $CD8^+$IFN-γ^+ cells in the CLNs as compared to the NE mice (Figure 5). However, there was no significant difference between these EE and NE groups. These findings show the percentage of HSV-specific $CD8^+$ cells in the CLNs that are able to produce IFN-γ is not affected by housing conditions.

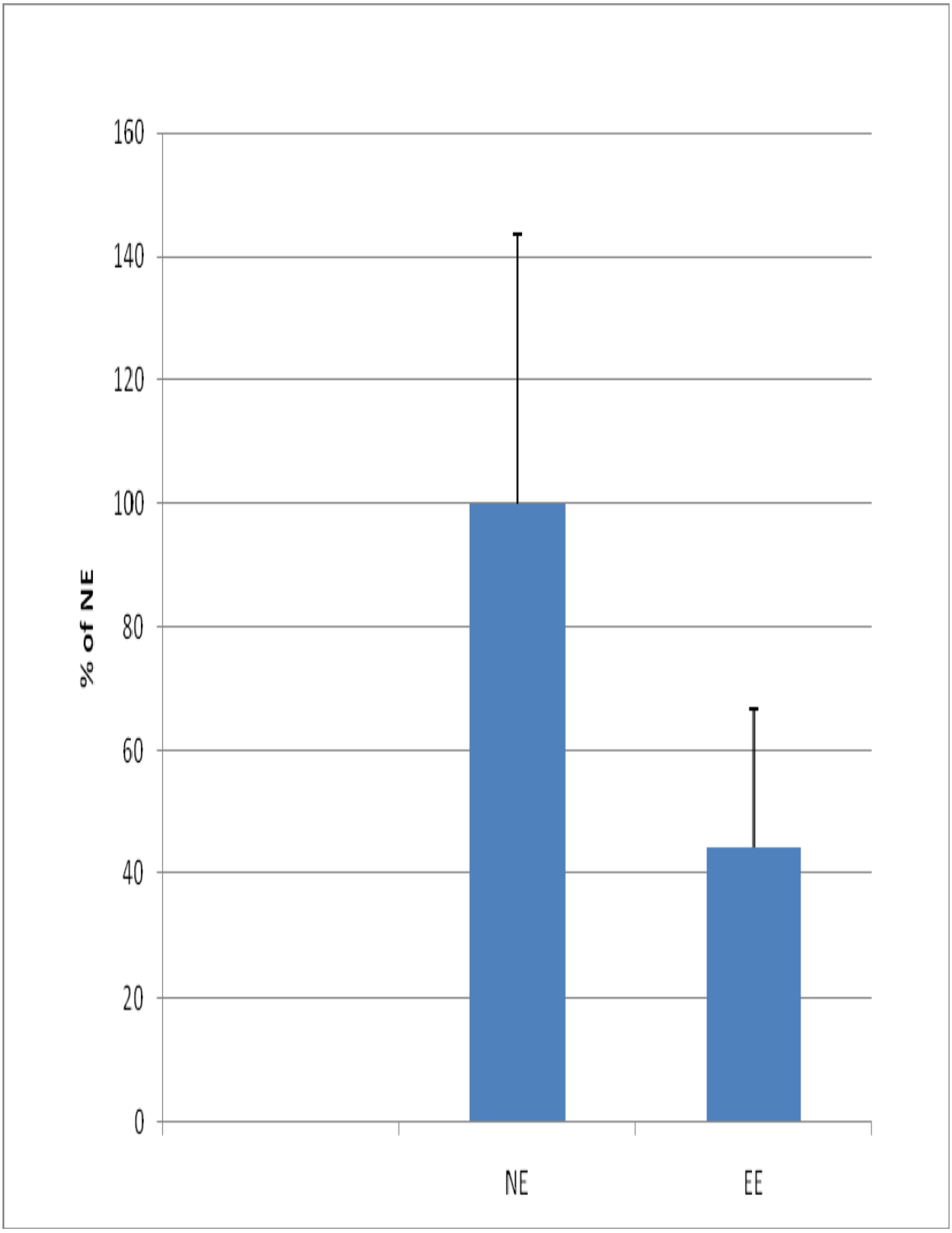


Figure 5. Percentage of $CD8^{+}IFN\text{-}\gamma^{+}$ cells in the CLNs of EE and NE group-housed HSV-1 infected mice.

6. Percentage of $CD8^+$ IFN-γ^+ cells in the MLNs of EE and NE group-housed HSV-1 infected mice.

Although EE mice demonstrated a trend toward reduced percentages of $CD8^+$IFN-γ^+ cells in the MLNs as compared to NE mice, this difference was not significant (Figure 6). These findings demonstrate that both EE and NE have no significant effect on the percentage of HSV-specific $CD8^+$ cells that are able to produce IFN-γ in both MLNs and CLNs.

A trend was observed in all group-housed mice regarding their immune responses. The EE mice had a decreased immune response as compared to the NE mice. This decreased response may be due to a decreased viral replication in the lower respiratory tract due to the increased immune based protection. Alternatively, these mice may be able to mount an early or delayed immune response due to EE.

7. Extent of HSV colonization and virus replication (Group-housed)

Plaque assays were performed to determine the titers of HSV in nasal washes. Group-housed mice, both EE and NE, showed no infectious virus in nasal washes. This finding suggests that either the infection is possibly cleared by day 7 or the assay, due to its relatively low level of sensitivity (1%), was unable to detect low levels of infectious virus.

Using the brain samples that were collected, real-time PCR was used to view the extent of DNA amplification. Group-housed mice, both EE and NE, did not show the presence of any viral genomes. This finding is an indication of the virus not being present in the brain and the mice mounting an adequate local (intranasal) immune response to viral infection. The sensitivity of this latter assay was approximately 320-640 pfu equivalents/brain.

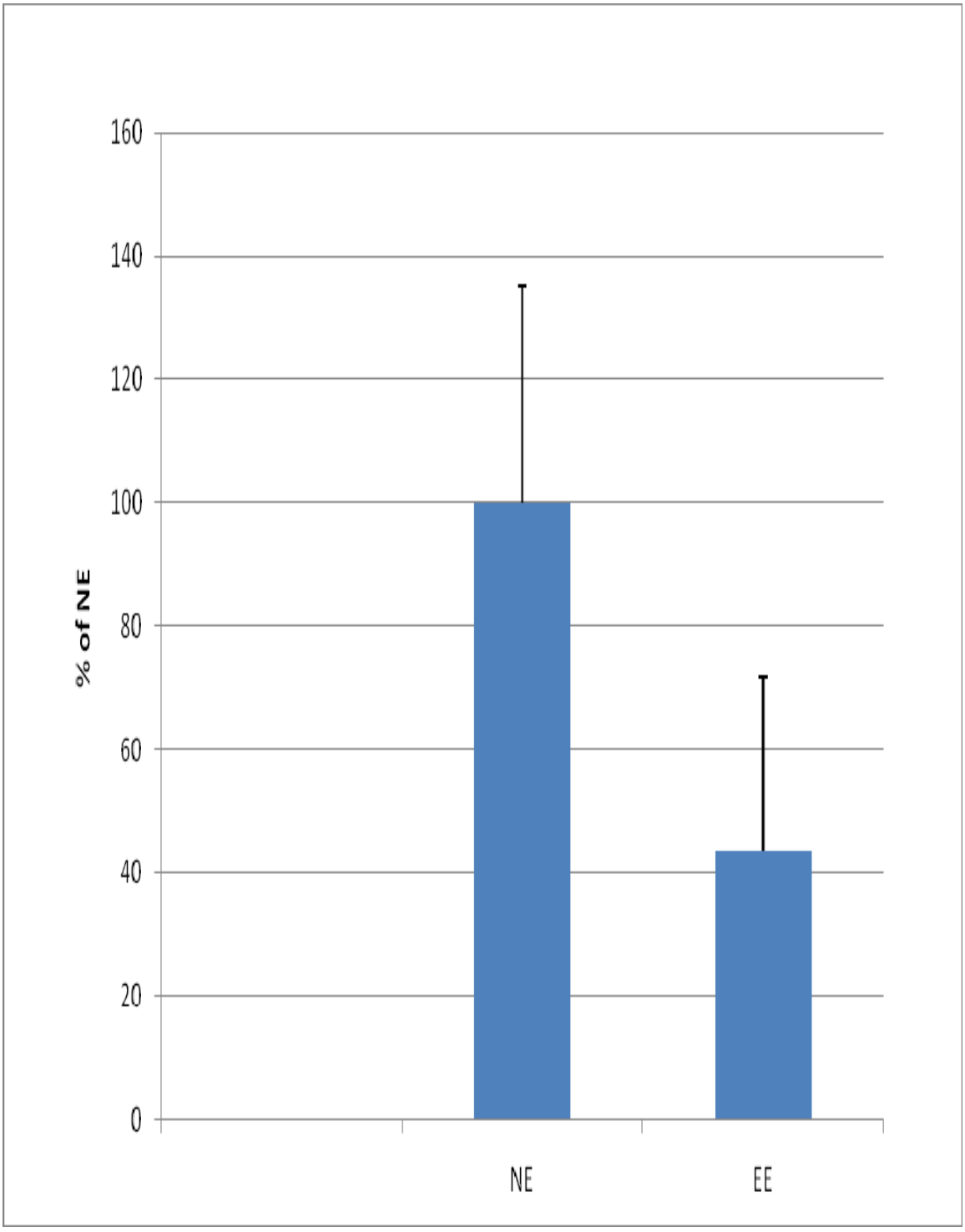


Figure 6. Percentage of $CD8^{+}IFN\text{-}\gamma^{+}$ cells in the MLNs of EE and NE group-housed HSV-1 infected mice.

8. Experiment Design (singly-housed)

The *Guide for the Care and Use of Laboratory Animals*[27] states, "It is desirable that social animals be housed in groups; however, when they must be housed alone, other forms of enrichment should be provided to compensate for the absence of other animals." Since group-housing is considered a form of enrichment, we wanted to determine if the lack of this form of EE would alter stress levels (as measured by CORT) or decrease the immune response in HSV-1 infected mice. We also wanted to determine if the chosen enrichment devices actually did provide enrichment. At 5 days after mice were placed into single housing, EE was added to half of the cages. At day 0, mice were bled retro-orbitally and infected intranasally with HSV-1. At 7 days post-infection mice were euthanized via sodium pentobarbital overdose and brains, nasal washes, CLNs and MLNs were collected.

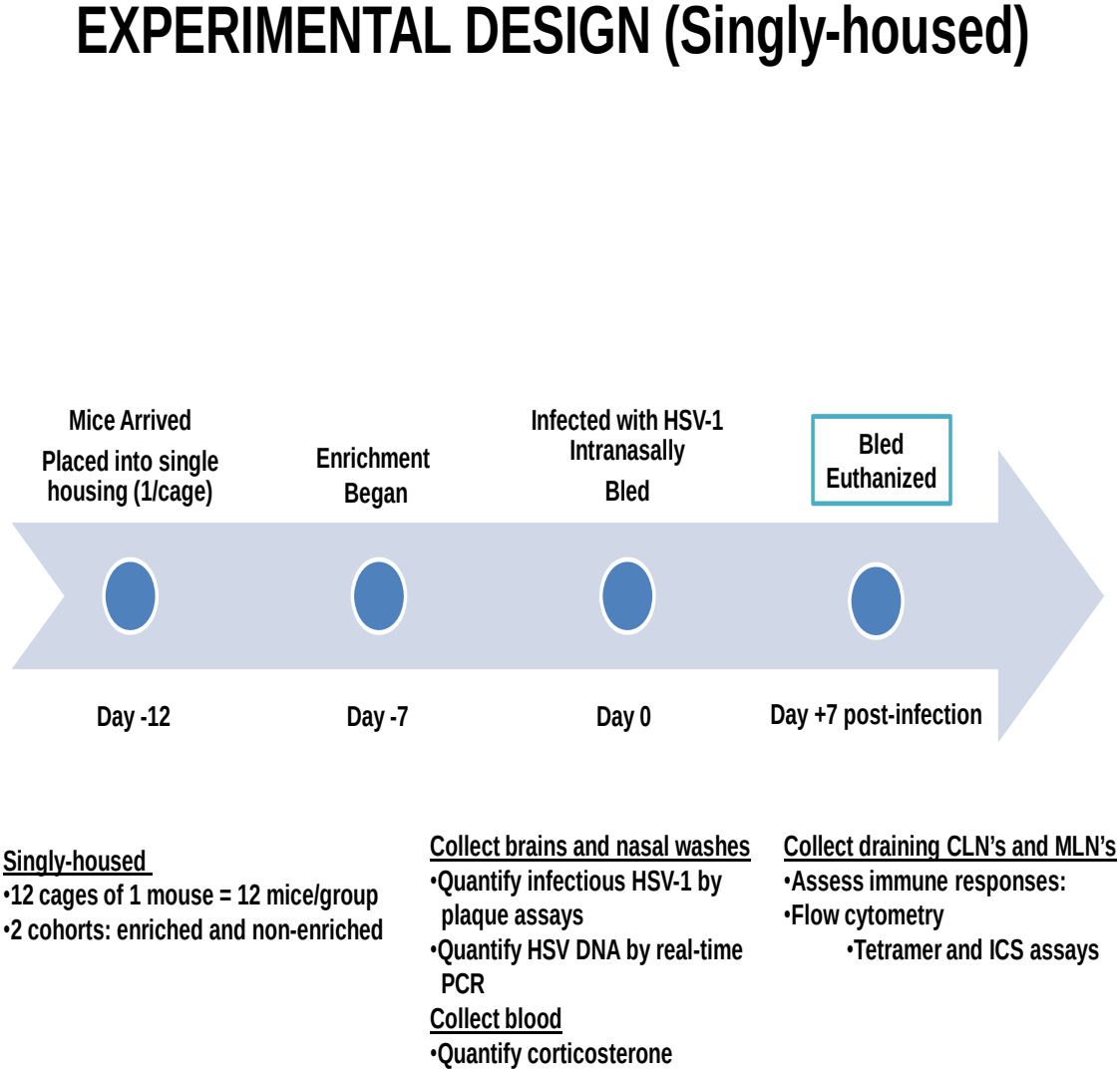


Figure 7. Experimental Design (singly-housed)

This figure depicts the timeline followed to determine, in singly-housed mice, the impact of EE on the levels of CORT and the $CD8^+$ T cell-mediated immune response to HSV-1 infection.

9. Serum corticosterone levels of EE and NE singly-housed mice pre- and post-HSV infection.

As is stated earlier, group housing alone is considered to be a form of enrichment. Therefore, we wanted to determine if the lack of this type of EE had any impact on stress levels as measured by levels of serum CORT. Therefore, we analyzed the impact of EE on singly-housed mice. We also wanted to determine if a similar decrease that was observed in the group-housed mice post-HSV-1-infection was also seen in post-HSV-1 infected singly-housed mice. A pre- and post-infection CORT level was observed to determine if EE had a decreasing effect on CORT levels as compared to the NE group. We hypothesized that EE would decrease levels of stress in individually housed mice. This study showed no significant difference between EE and NE group post HSV infection. There was a decrease in CORT (as also seen in Fig 2) post-infection possibly due to the infection itself down-regulating CORT levels in order to allow for a stronger immune response. The range of CORT observed was within the normal baseline range of non-stressed mice and not very different from that obtained in the group-housed mice.

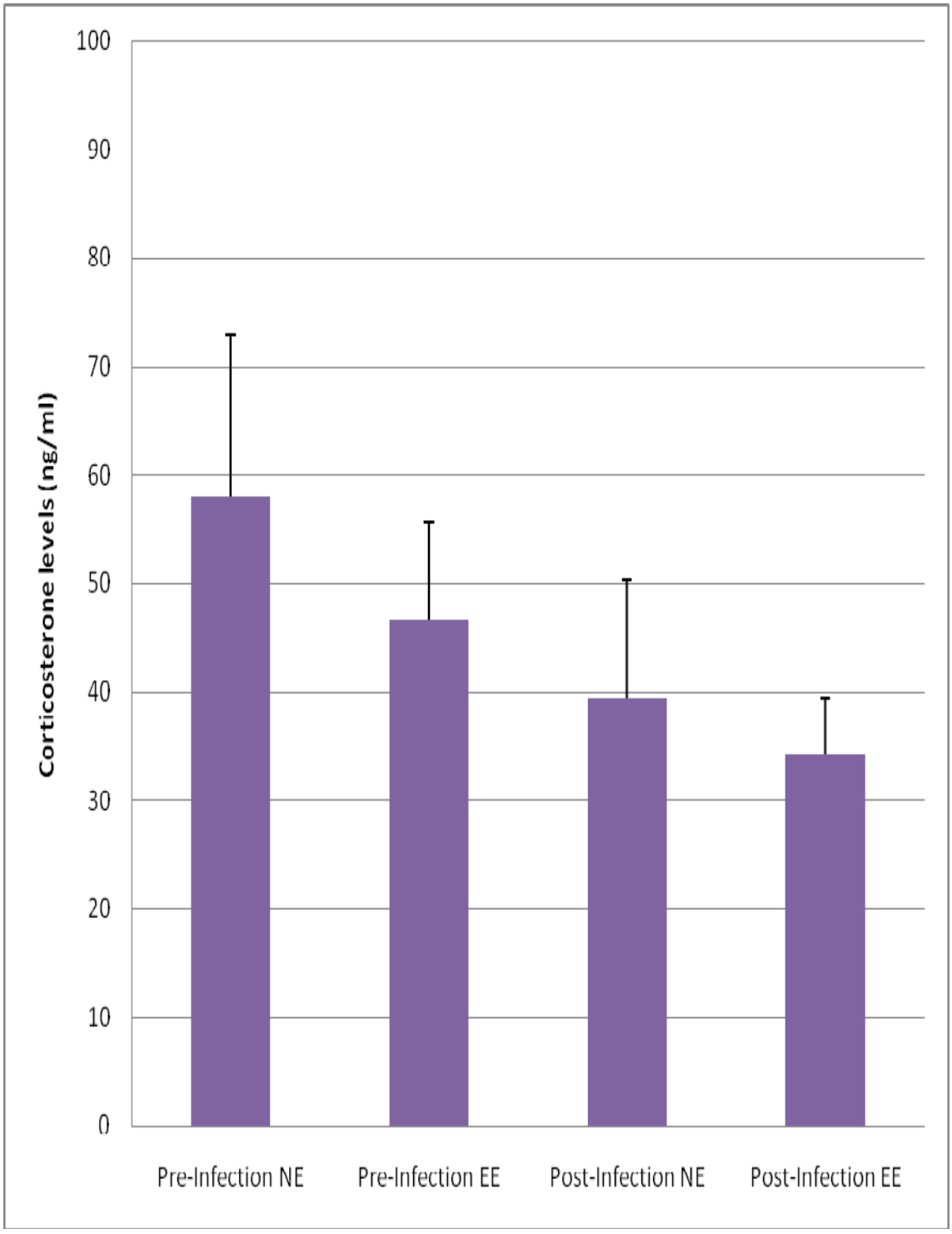


Figure 8. Effects of EE on serum corticosterone levels in singly-housed mice pre-and post- HSV-1 infection.

10. Percentage of CD8$^+$ gB$_{498\text{-}505}^+$ cells in the CLNs of EE and NE singly-housed HSV-1 infected mice.

We wanted to determine if the lack of EE in singly-housed mice had an effect on the CD8$^+$ T cell immune response in HSV infected mice. Using the experimental design described in Section 8, cells from CLNs were stained with anti-CD8 APC antibody and APC-conjugated gB$_{498\text{-}505}$ tetramer and analyzed using flow cytometry. EE mice had a trend of slightly higher percentages of CD8$^+$ gB$_{498\text{-}505}^+$ cells as compared to the NE mice. However, there was no significant difference between EE and NE groups. The findings were not significant and indicated that there was no significant difference between EE and NE groups ability to generate HSV-1 specific CTL that are specific for HSV-encoded immunodominant epitope.

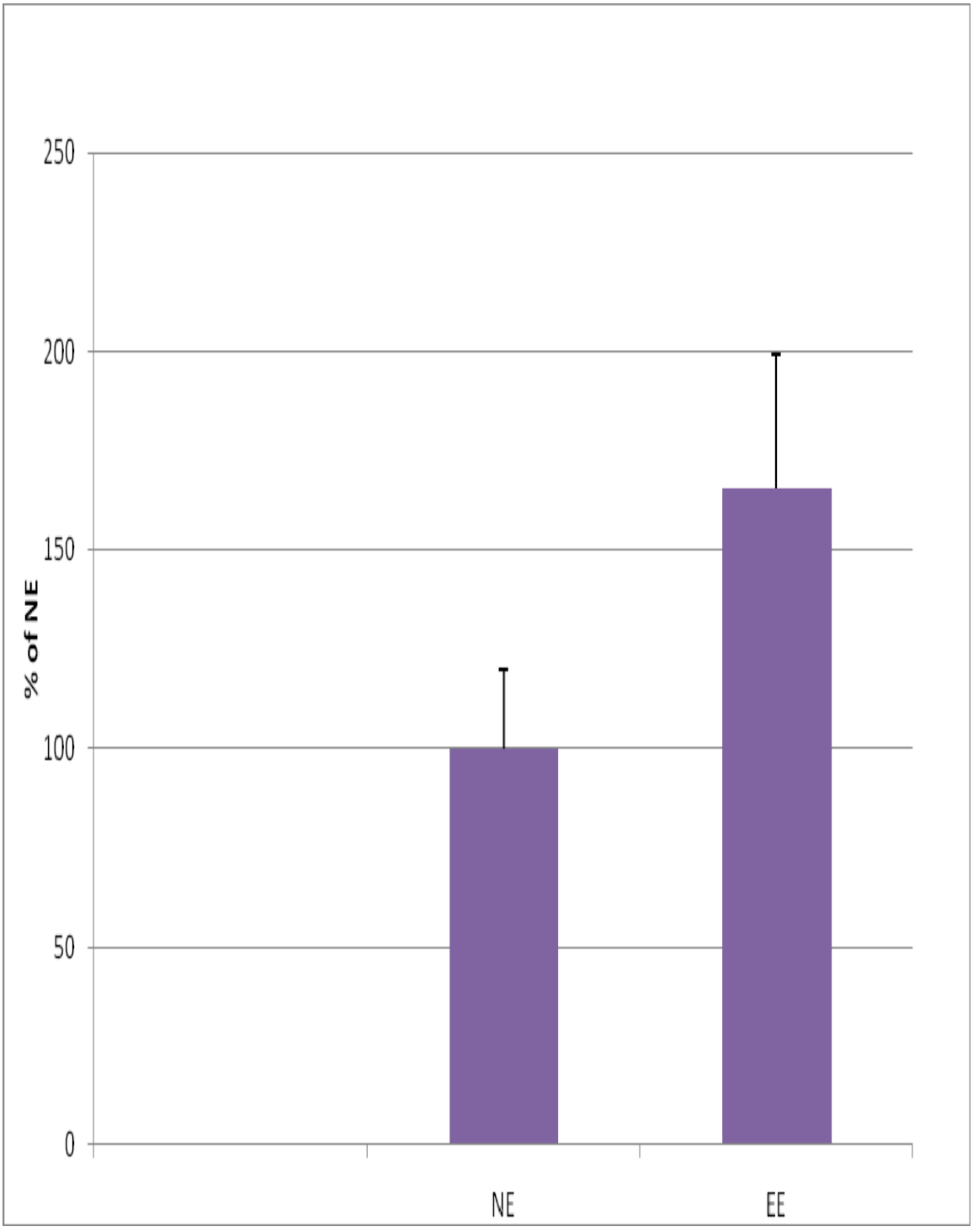


Figure 9. Percentage of $CD8^+$ $gB_{498\text{-}505}{}^+$cells in the CLNs of EE and NE singly-housed HSV-1 infected mice.

11. Percentage of $CD8^+$ $gB_{498-505}{}^+$cells in the MLNs of EE and NE singly-housed HSV-1 infected mice.

Using the experimental design in Section 5, MLNs were stained with APC-conjugated $gB_{498-505}$ tetramer and analyzed using flow cytometry. EE mice had a trend of higher percentages of $CD8^+$ $gB_{498-505}{}^+$ cells as compared to the NE. These results were opposite of those found in group-housed mice as seen in figure 4. However, there was no significant difference between EE and NE groups. These findings indicate that there was no significant difference between EE and NE groups ability to generate HSV-1 specific CTL that are specific for HSV-encoded immunodominant epitope.

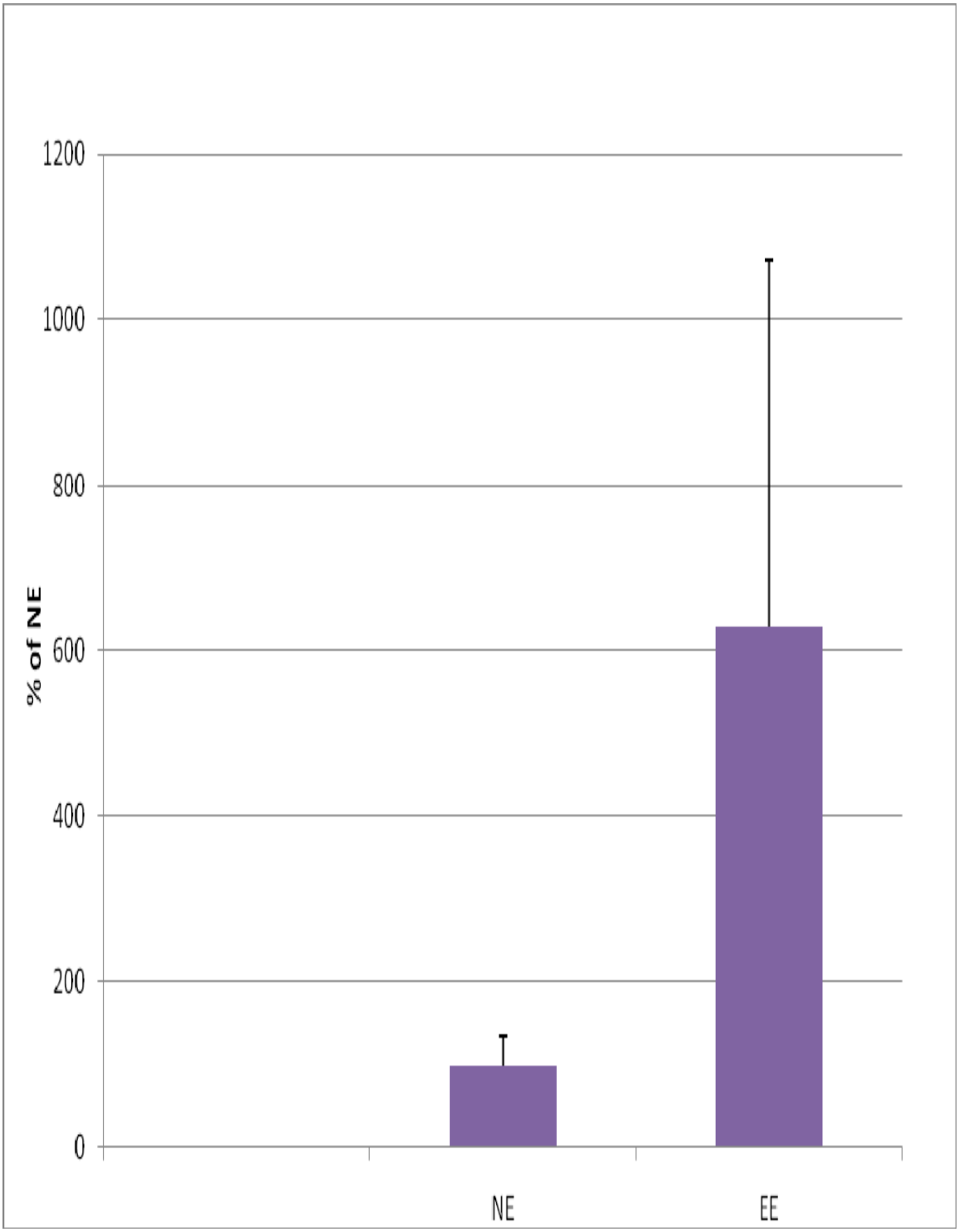


Figure 10. Percentage of $CD8^+$ $gB_{498\text{-}505}{}^+$ cells in the MLNs of EE and NE singly-housed HSV-1 infected mice.

12. Percentage of $CD8^+IFN\text{-}\gamma^+$ cells in the CLNs of EE and NE singly-housed HSV-1 infected mice.

EE mice demonstrated a trend of reduced percentage of $CD8^+IFN\text{-}\gamma^+$ cells in the CLNs. However, there was no significant difference between EE and NE groups. These findings demonstrate that both EE and NE have no significant effect on the percentage of HSV-specific $CD8^+$ cells that are able to produce IFN-γ.

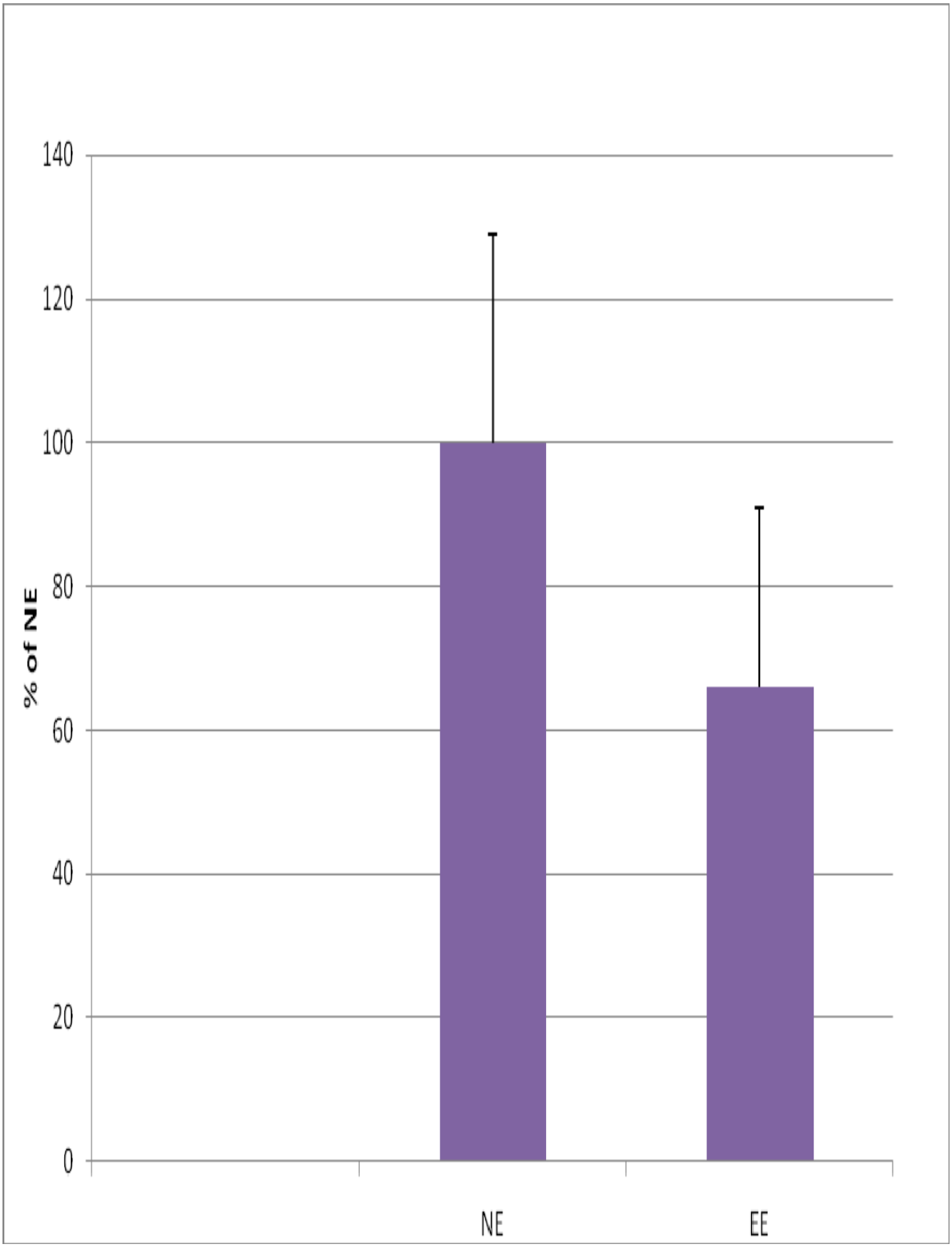


Figure 11. Percentage of $CD8^{+}IFN\text{-}\gamma^{+}$ cells in the CLNs of EE and NE singly-housed HSV-1 infected mice.

13. Percentage of $CD8^+IFN\text{-}\gamma^+$ cells in the MLNs of EE and NE singly-housed HSV-1 infected mice.

EE mice demonstrated an increased percentage of $CD8^+IFN\text{-}\gamma^+$ cells in the MLNs. However, there was no significant difference between EE and NE groups. These findings demonstrate that both EE and NE have no significant effect on the percentage of HSV-specific $CD8^+$ cells that are able to produce IFN-γ.

A common trend was observed in all singly-housed mice immune response specifically in the MLNs. The EE mice had a trend of an increased immune response as compared to the NE mice. This may be potentially due to a decreased viral replication in the lower respiratory tract due to an increase in immune based protection. The EE mice may require a stronger immune response due to the lack of EE or there was a rapid spread of the virus.

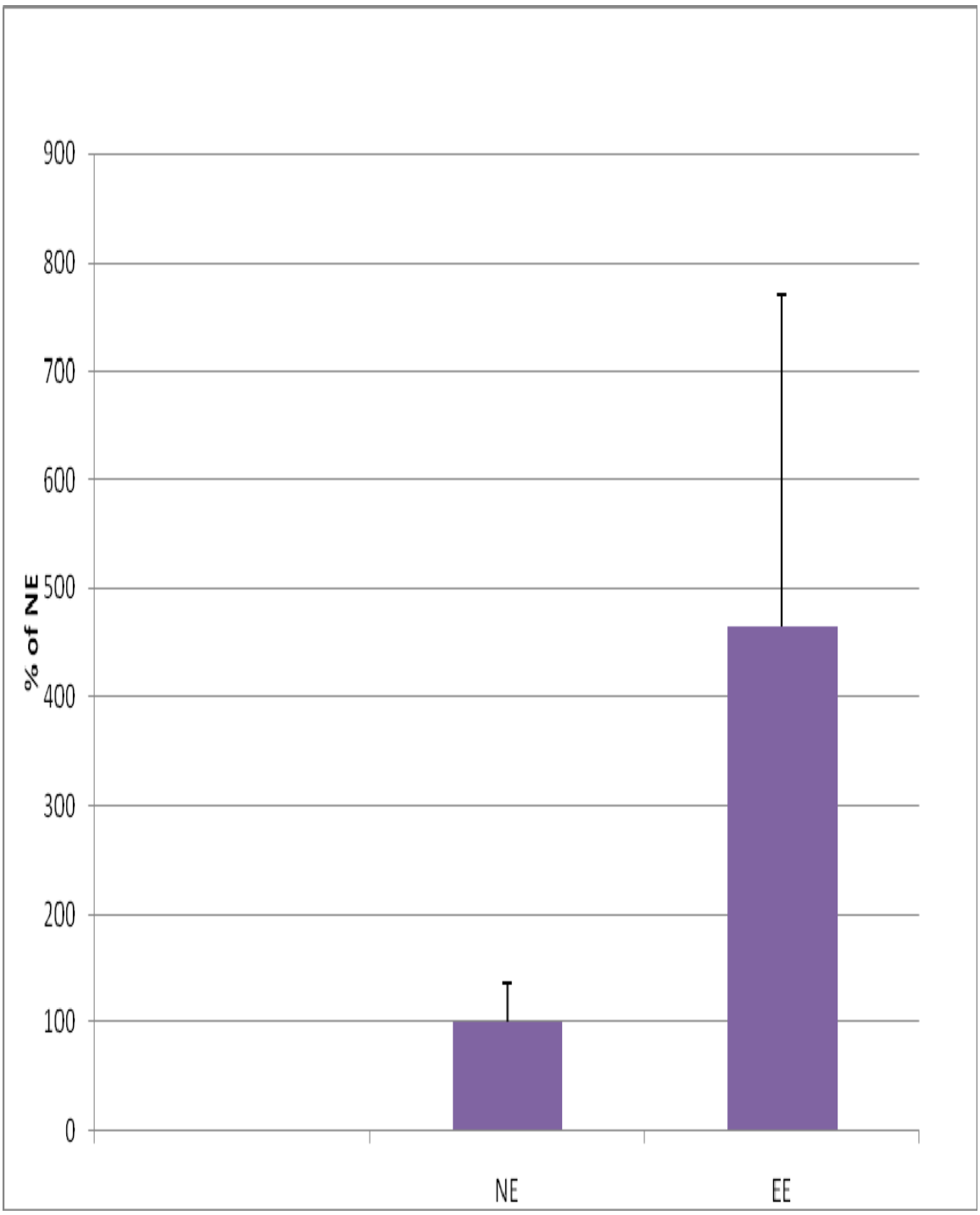


Figure 12. Percentage of $CD8^{+}IFN\text{-}\gamma^{+}$ cells in the MLNs of EE and NE singly-housed HSV-1 infected mice.

14. Extent of HSV colonization and replication (singly-housed)

Plaque assays were performed to determine titers of HSV in nasal washes. Singly-housed mice, both EE and NE, showed no infectious virus in nasal washes. This is an indication that the infection possibly cleared by day 7 or the assay was unable to detect the low levels of infectious virus due to the relatively low level of sensitivity (1%).

Using the brain samples that were collected, real-time PCR was used to view the extent of DNA amplification. Singly-housed mice, both EE and NE, showed the presence of some viral genomes but the levels were not significant.

Chapter 4

DISCUSSION

To date, the use of EE has been based on trial-and-error, general observations, anecdotes, or recently published practices. Thus, the recommendations provided are often conflicting. The studies described in this thesis have objectively evaluated the effect of EE on stress levels, immune function, and susceptibility to HSV-1 infections in both group- and singly-housed mice under carefully controlled experimental conditions. These studies are particularly important for laboratory animal medicine and add to our understanding of the influence of environment on the susceptibility and pathogenesis of a variety of rodent pathogens that are immunologically resisted.

Potential concerns we had regarding the addition or absence of EE were that the mice housed in enriched cages would fight over the use of the EE devices leading to aggression and stress and therefore increasing CORT levels. We also thought those NE mice would be stressed due to the lack of EE therefore resulting in higher CORT levels as compared to the EE mice. As we and others have previously demonstrated[15,31,61], EE does not increase levels of psychological stress. Many other scientists have reported a similar decrease in mice housed in EE[7,30,34]. This decrease in CORT levels may be attributed to the addition of EE to the group-housed setting which reduced stress levels; alteration in dominance hierarchy; or decrease in aggression. At no time were the corticosterone levels outside of the normal baseline ranges (<200 ng/ml) of non-stressed mice. This proves that the introduction of EE is not stressful to the animals and may possibly reduce the incidence of stress occurring due to the decrease in CORT levels observed post-infection. However, we did see a common trend that the post-infected mice, both EE and NE, exhibited a decreased CORT level. This may possibly be due to the infection itself down-regulating corticosterone levels in order to allow for a stronger immune response.

Group-housed EE mice showed a significant decrease observed in the immune response, specifically the MLNs. This may be due to decreased viral replication in the lower respiratory tract due to an increase in immune based protection. The EE mice may also have been able to mount an immune response earlier than the 7-day interval typically seen in NE mice to EE or conversely a delayed response as a result of EE. No virus was found in nasal washes via plaque assays. This is an indication that either infection was possibly cleared by day 7 post-infection or the efficiency of the plaque assay technique was too low. No DNA amplification of the virus was detected to have spread to the brain via real-time PCR. This is an indication of virus not being present in the brain and suggests that the mice mounted an adequate local (intranasal) immune response to viral infection. Immune response was increased in the MLNs of EE in singly-housed mice but was not significant. This increase may be due to an enhanced viral replication in the lower respiratory tract and rapid spread of the virus. Also a stronger immune response may have been needed due to the lack of EE (e.g. group housing).

Singly-housed EE mice had a robust immune response. This may be due to an enhanced viral replication in the lower respiratory tract. An intriguing explanation for this finding is due to the lack of social contact in singly-housed mice, which itself is considered a form of enrichment; this required the mice to mount a stronger immune response. Also there may have been more of a rapid spread of the virus. This rapid spread may explain why DNA amplification of the virus was found in some of the brain tissues of singly-housed mice.

Together, these findings demonstrate that EE may have a significant effect on CORT levels in group-housed mice post-HSV-1 infection. However, EE does not have any significant effect on CORT levels pre- or post-infection in singly-housed mice. The HSV-1 infection itself may be responsible for the tendency of the corticosterone levels to

be reduced post-infection. In this model of HSV-1 infection, these findings also indicate that EE modulates a magnitude of immune responses in various lymphoid tissues whether it is the enrichment device or the choice of group or singly- housed mice. In group-housed mice there was a decrease immune response in the EE mice specifically the MLNs. The opposite was observed in singly-housed mice where the EE mice showed an increase in immune response as compared to the NE mice. Additional objective data is necessary to determine whether or not EE influences other infectious pathogens in a similar way.

Some thought should be taken before adding any type of enrichment device as it is an extra variable. However, an attempt to mimic species-specific behavior such as gnawing, nesting and hiding, and foraging should be made whenever possible. EE has been successful in mimicking these particular behaviors. EE has been proven to be beneficial in reducing aggression[2, 33] and in increasing post-operative recovery[20]. Further studies need to be conducted to determine EE effects on all aspects of the overall welfare of laboratory mice.

In the future, we would like to conduct a more extensive kinetic analysis of the HSV-specific immune response. We would also like to examine the impact of EE on the development and function of HSV-specific memory CTL – a lymphoid population that we have extensive evidence of playing a critical role in limiting the extent of recurrent HSV infection. It would also be interesting to determine if cage-based enrichment can counter the effects of stressors (e.g. noise) that are inherent to many laboratory animal facilities. There may also be gender-related and strain-related differences in their response to enriched/non-enriched environments and immune function that will need to be evaluated as well.

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