Environmental Enrichment, Stress, and Immune Response to HSV-1 Infection in C57BL/6 Mice

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Citation

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Abstract

Environmental enrichment (EE) is being introduced at increasing levels into laboratory animal programs and is being applied to species (e.g. rodents) other than non-human primates, for which it is currently required by law. However, there has yet to be sufficient objective data regarding the impact of EE on various psychological and physiological parameters. The studies described herein investigated the effect of EE on the immune response to and pathogenicity of herpes simplex virus type 1 (HSV-1) infection in both group- and single-housed mice. C57BL/6 male mice were intranasally infected with HSV-1. Levels of serum corticosterone (CORT) were determined in EE and non-enriched (NE) mice that were either group- or single-housed, both pre- and post-HSV-1 infection. HSV-specific CD8+ T cell number and function was evaluated at 7 days post-infection. We determined that, at least in our model, EE did not have a significant effect on the levels of serum CORT in either single- or group-housed mice. However, the impact of EE on the number and function of HSV-specific CD8+ T cells varied depending on how the mice were housed and the lymph nodes in which the immune response was assessed. Overall, these studies support the hypothesis that enrichment devices may serve as an additional variable that must still be considered in the design of an experimental study. However, the use of enrichment devices should not be automatically excluded from routine animal husbandry because of the many benefits of providing an animal with an environment which allows them to exhibit species-specific behavior.

List of acronyms and nonstandard abbreviations used in the manuscript and their definitions:

CLN superficial cervical lymph nodes

CORT corticosterone

CTL cytotoxic T lymphocytes

EE environmental enrichment or environmentally enriched

HSV herpes simplex virus

MLN mediastinal lymph nodes

NE non-enriched

INTRODUCTION

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. In 1949, Donald O. Hebb, a psychologist and the father of neuropsychology and neural networks, discovered by accident that animals which experience enriched housing outperform, in learning and memory tests (e.g. Hebb-Williams maze test)[8,30], those animals that are in non-enriched housing. Based on this finding, EE has been used as a research tool to understand how spatial learning molds the brain. EE has since evolved to now include its use to promote animal welfare and the enhancement of their environment.

There are five principle goals of EE as described by

Chamove and Shepherdson[15,54]. These goals are to: (1) promote species-specific positive behavior[20,21,34], (2) prevent abnormal or stereotypical behaviors[2,37], (3) increase an animal's positive utilization of its environment[47,50,64], (4) increase an animal's ability to cope with challenges in a more normal way[24,31,65], and (5) complement experimental outcomes by decreasing variability and increasing post-procedural recovery[20,58,59]. Despite the many reports of the benefits of EE, there are still some conflicting viewpoints on the necessity of enrichment. For example, some researchers feel that EE is not essential to animal welfare and that it may compromise the validity of experiments by hampering environmental standardization, interfering with experimental outcome, increasing the variability of the data[26], as well as increasing aggressive behavior in male mice[5]. However within the past decade, EE has been shown to benefit laboratory animal medicine and research by decreasing aggression, anxiety, fear, and excitability as well as enhancing learning performance and brain function[2,4,5,7,26,33] and post-operative recovery[20]. Others have shown that enrichment has no harmful effects. A number of preference experiments suggest that readily available materials such as tissue or paper towels fulfill the requirements of mice[4,26,59,60]. These enrichment devices encourage animals to perform species-specific behavior which, in turn, decreases stress and improves animal welfare.

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 individual's biological, psychological, or social systems[52]. Psychological stress can influence immune function and alter the pathophysiology of infection. Chronic activation of the hypothalamic-pituitary-adrenal (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. natural killer cells) and specific T and B lymphocyte functions including specific reactions against infectious agents[13,33,55]. Chronic stress, as a result of adverse housing conditions, can affect the animals and, in turn, affect the research. Reports from both objective investigations as well as from anecdotal information suggest that EE decreases stress and that the use of a non-stressed animal results in less confounding variables in research data.

The main index that is used to measure stress levels in mice is plasma/serum corticosterone (CORT). Meijer[42] examined the effects of EE and handling on the acute physiologic stress response caused by short periods of restraint. The results of these studies indicate that EE and handling increases the stress response but facilitates the recovery of plasma corticosterone as CORT levels measured 90 min after restraint were lower in enriched groups than in non-enriched groups. Several other investigators have shown that EE attenuates or decreases stress as evidenced by decreased CORT levels[7,35,39]. Chamove[16], Hennesy[31], and Van Loo[61] all demonstrated that the addition of EE to a mouse cage resulted in a decrease in stress. Since animals under chronic stress are generally considered to be inappropriate research subjects, the studies described in this paper investigated if the presence of EE decreases signs of stress in mice as measured by serum CORT levels. Benaroya-Milshtein[7] observed that EE has a beneficial effect on anxiety-like behavior (grip-strength test, staircase and elevated plus maze test), stress response (CORT levels) and natural killer cell activity (cytotoxicity assay). They concluded that EE mediated a significant improvement in the outcome of immunotherapy in their experimental model of B cell lymphoma. Non-enriched animals also have been shown to have a higher proliferative response to concanavalin A, a lymphocyte mitogen, than do enriched mice[39]. The author of this latter study suggested that enrichment and housing density were important factors that influence immune responses and in response to exercise stress.

Stress is known to decrease host-resistance to pathogenic microorganisms[17]. An established murine model system of herpes simplex virus (HSV) infection has been used to demonstrate that stress decreases cellular immune response to HSV infection and increases viral pathogenicity[12,13,44]. These studies have defined the mechanisms underlying stress-associated neuroendocrine interactions with the immune system and how these interactions may contribute to the pathogenesis of HSV infection. These studies focused on the stress-associated activation of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system and the role of both in modulating the activation and function of both primary and memory HSV-specific cytotoxic T lymphocytes (CTL). Other studies have demonstrated that housing conditions, stress, and enrichment conditions can both enhance and suppress various immune responses[39]. For example, 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 been shown to have immunosuppressive effects and thus may lead to increased susceptibility to disease[63]. Therefore, the studies described herein are particularly important for laboratory animal medicine in that it will add to our understanding of the influence of environment on the susceptibility to and pathogenesis of a variety of rodent pathogens that are immunologically resisted.

The implementation of EE as a part of routine husbandry to this point has been based primarily, on trial-and-error, general observations, anecdotes, and published best practices. Thus, the recommendations provided are often conflicting and often provided with little thought given to how the EE may influence the experimental outcome. In particular, few objective studies have been conducted to determine the effects of EE on the immune system. The ability of environmental enrichment to reduce stress may enhance the immune response to a pathogen.

The objective of the present investigation was to determine the effect of EE on the immune response to, and the pathogenicity of HSV-1 infection in both group- and single-housed mice with enriched or non-enriched environments. Our hypothesis was that environmental enrichment (EE) would decrease stress, thereby enhancing the murine immune response to HSV infection and, in turn, decreasing the HSV-associated pathogenesis in both the peripheral lymphoid tissues and central nervous system.

MATERIALS AND METHODS

Animals. A total of 72 five- to six-week old C57BL/6 male mice (The Jackson Laboratory, Bar Harbor, ME) were used for the study. Following their arrival at Penn State Hershey, mice were allowed to recover from the transport-associated stress for five days after which time they were assigned to the experimental groups as described below. Mice were housed either singly or in groups of four in standard 29 cm x 19 cm x 13 cm polycarbonate cages containing either an enriched or non-enriched environment as described below. These cages were placed in isolation cubicles in a biohazard facility. Due to the low level of both investigator and animal caretaker traffic that is associated with this particular facility, the mice were provided with a relatively low-stress environment throughout the duration of the study. Mice were allowed to acclimate to these conditions for seven days before any experimental procedures were performed. All mice were housed in a temperature-controlled environment (21 ± 0.5°C) with a 12 hour:12 hour light:dark cycle with no twilight (lights on at 0700-1900; lights off at 1900-0700). Food (2018 Global Rodent Diet, Harlan Teklad, Indianapolis, IN) and water were provided ad libitum. Corncob bedding (¼") (7092 Harlan Teklad) was used as contact bedding in all cages. Environmental enrichment was provided for both group- and single-housed mice by red-tinted transparent polycarbonate 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 from the lid of the cage using stainless steel hangers so as not to decrease the amount of usable floor space. 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).

HSV-1 Preparation and Infection of Mice. 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. 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 1 x 107 plaque forming units (PFU) of HSV-1 McIntyre. A volume of 23 µl was administered into the nostrils alternating between both nostrils until the entire volume was given.

Blood Collection. Blood (200 ml) was collected by the retro-orbital route in isofluorane-anesthetized mice at the time of virus inoculation (Day 0). Blood was transferred to Eppendorf tubes and placed 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. All mice were bled at 0900 to eliminate the confounding variable of circadian rhythm on corticosterone levels. On Day 7 following virus inoculation, mice were anesthetized with sodium pentobarbital (80 mg/kg, intraperitoneal) and exsanguinated via cardiac puncture. At the time of euthanasia, nasal washes were collected to determine the extent of HSV-1 colonization of the nasal passages. The superficial cervical lymph nodes (CLN), mediastinal (MLN) lymph nodes, and brains were also collected for further analysis.

Quantitation 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.

Determination 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. 200 µL PBS/1%FBS were pipetted in and out of the nasal cavity 10 times. This step was repeated four times resulting in a total of 1 ml of nasal wash which was then placed into Eppendorf tubes. Nasal wash samples were frozen/thawed three times in a 95% ethanol/dry ice slurry, sonicated, and centrifuged at 16,000 x g for ten minutes. Supernatants were then transferred to new Eppendorf tubes. The levels of HSV in these samples were determined using standard plaque assay. Briefly, Vero cells were grown to 90-100% confluency in 60 mm2 tissue culture plates. Various dilutions (prepared in PBS containing 1% [v/v] fetal bovine serum) of the supernatants were added to duplicate wells containing Vero cell monolayers and allowed to incubate at 37°C for one hour. At the end of this one-hour incubation, methylcellulose overlay media was added to each well. The cells were then allowed to incubate at 37°C for 4 days. The overlay media was removed and the cells were then stained with 0.5% (w/v) crystal violet/5% (w/v) formaldehyde to visualize the plaques for counting. The plaques were counted and the concentration of virus in the supernatants was calculated and expressed in terms of plaque forming units (pfu)/ml.

Isolation of Cells from Lymph Nodes. Superficial cervical and mediastinal lymph nodes were removed and placed in Iscove's-modified Dulbecco's media (IMDM; Invitrogen, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (FBS), 50 mM 2-mercaptoethanol, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin sulfate. Lymph nodes were mechanically dissociated by passage through a 70 mm 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.

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. DNA from the brain was purified 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 a 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.

Synthetic Peptides. Synthetic peptides corresponding to the HSV-1 CTL recognition epitope gB498–505 (SSIEFARL)12 and ovalbumin amino acid residues 257-264 (OVA257-264; 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.

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 Fca receptors on isolated mononuclear cells were blocked with antibody in a 2.4G2 hybridoma cell culture supernatant supplemented with 20% (v/v) mouse serum (Sigma)3. Cell surface expression of CD8 was detected using APC-labeled anti-CD8 antibody (clone 53-6.7; eBioscience). For the detection and quantification of gB498-505 epitope-specific T lymphocytes, cells were incubated with a PE-labeled tetramer that was prepared as described previously1 and was provided by the National Institute of Health tetramer facility. This tetramer detects the H-2Kb-restricted, gB498-505-specific T cell receptor complex[10], which has previously been described as the immunodominant epitope in C57BL/6 mice12,29. 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.

Intracellular Cytokine Staining for IFN-. The lymph node-derived lymphocytes were isolated as described above and incubated with 1 µM of gB498-505 peptide for 2 hours at 37ºC in supplemented IMDM. 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 Fc g 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% (w/v) paraformaldehyde and incubated at room temperature and in the dark for 20 minutes. Cells were then washed twice with FACS buffer and incubated with anti-IFN-g 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.

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].

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.

RESULTS

1. Experimental Design

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 to be a form of enrichment, we wanted to determine if single housing would result in higher stress levels (as measured by CORT) and a lower HSV-specific immune response as compared to group-housed mice. We also wanted to determine if the enrichment devices could lower stress levels as measured by a reduction in the levels of CORT.

We developed a model to determine the impact of EE on both the level of stress and the immune response to HSV infection in both group-housed (4 mice/cage) and single-housed mice. Upon their arrival, mice were placed into either group- or single-housing conditions and allowed to acclimate to these conditions for five days. Following this acclimation, EE, consisting of both an igloo and a suspended tunnel, was added to one-half of the cages in each of the housing conditions. Following 7 days in either the enriched or non-enriched environment, mice were bled retro-orbitally and infected intranasally with HSV-1. At 7 days post-infection, mice were bled once again and euthanized via an overdose of sodium pentobarbital. The brains and nasal washes were collected and assessed for virus titers; the CLN and MLN were collected and assessed for HSV-specific immune function, and the serum collected on both this day and on the day of infection was assessed for levels of corticosterone as described in the Materials and Methods.

2. The impact of single/group housing, environmental enrichment, and/or HSV infection on serum corticosterone levels.

Given that CORT is the principle indicator of stress in mice[61], we measured serum CORT levels in an attempt to determine the impact of EE on the degree of stress exhibited by mice in both group-housed and single-housed conditions, both pre- and post-HSV-1 infection. We hypothesized that EE would decrease levels of stress in both group-housed and single-housed mice.

As is illustrated in Figure 1A, providing EE enrichment to the group-housed mice either prior to HSV infection or after HSV infection did not alter the CORT levels. Interestingly, the level of CORT in EE group-housed mice after HSV infection were significantly lower ($p = 0.002$) than EE group-housed mice prior to HSV infection. Although not statistically significant, there was a trend ($p = 0.130$) for NE group-housed mice to have lower levels of CORT than NE group-housed mice prior to HSV infection.

As was stated earlier, group housing alone may be considered to be a form of enrichment for mice. Therefore, it was important to determine if single housing would in higher levels of serum CORT and, if so, determine if EE could reduce these CORT levels. As is illustrated in Figure 1B, there were no significant differences in CORT levels between the single-housed EE and NE mice either pre- or post-HSV infection. There were also no difference in the level of CORT between single-house and group-housed mice under any conditions.

3. The impact of single/group housing and environmental enrichment on the generation of CD8+ HSV-1 gB498-505+

cells in the CLN of 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 CLN drain the upper respiratory tract and nasal cavity, and the MLN drain the lower respiratory tract. Both sets of lymph nodes have previously been shown to contain HSV-specific CTL in response to intranasal HSV-1 infection. The vast majority of these CTL in C57BL/6 mice recognize the HSV-1 gB498-505 immunodominant peptide in the context of the H-2Kb MHC class I molecule[12].

Using the experimental approach described above, both single-housed and group-housed mice living in either EE or NE conditions were infected intranasally with HSV-1. Seven days later, lymphoid cells obtained from the CLN were stained with an APC-labeled anti-CD8 antibody and a PE-conjugated gB498-505 tetramer and analyzed using flow cytometry. All comparisons were made to the NE group (control group) which was arbitrarily designed a value of 100%. Expressing this data as the percent of control was required as the data represents pooled data from three independent experiments (n = 4 mice per group per experiment). As is illustrated in Figure 2, neither group-housed mice (Figure 2A) nor single-housed mice (Figure 2B) living in EE conditions exhibited any differences in the percentage of CD8+ gB498-505+ cells as compared to NE controls.

4. The impact of single/group housing and environmental enrichment on the generation of CD8+ HSV-1 gB498-505+ cells in the MLN of HSV-1 infected mice.

Although HSV was delivered into the upper respiratory tract of the mice used in this study, HSV is able to spread to and infect the lower respiratory tract where it stimulates an immune response in the mediastinal lymph nodes. Therefore, it was important for us to also determine the impact of EE on the frequency of lymph-node-derived CD8+ gB498-505+ cells in the MLN. The MLN-derived lymphoid cells were stained with anti-CD8 APC antibody and PE-conjugated gB498-505 tetramer and analyzed using flow cytometry as for the CLN-based studies described above. Mice exposed to EE demonstrated significantly fewer ($p = 0.016$) CD8+ gB498-505+-specific cells than did the mice in the NE environment. Although not statistically significant, there was a trend ($p = 0.170$) for EE single-housed mice to have higher levels of CD8+ gB498-505+-specific cells than NE single-housed mice (Figure 3B).

5. The impact of single/group housing and environmental enrichment on the generation of CD8+ IFN-γ+ cells in the CLN of HSV-1 infected mice.

To further delineate the effects of EE on the HSV-specific cellular immune response in the draining lymph nodes, we determined the percentage of CD8+ IFN-γ+ cells in the lymphocytes derived from the CLNs. Determining the percentage of functional CD8+ T cells as measured by their ability to produce IFN-γ is important given the number of roles that IFN-γ plays in the overall immune response and, in particular, the defense against HSV infection. As is illustrated in Figure 4A, EE administered to group-housed mice had no effect on the number of CD8+IFN-γ+ cells in the CLNs as compared to the NE group-housed mice. Similarly, EE administered to single-housed mice had no effect on the number of CD8+IFN-γ+ cells in the CLNs as compared to the NE single-housed mice (Figure 4B).

6. The impact of single/group housing and environmental enrichment on the generation of CD8+ IFN-γ+ cells in the MLN of HSV-1 infected mice.

As is illustrated in Figure 5A, there were no difference in the percentages of CD8+IFN-γ+ cells in the MLN of EE group-housed mice as compared to NE group-housed mice. Although not statistically significant, there was a trend ($p = 0.194$) for EE single-housed mice to have an increased percentage of CD8+IFN-γ+ cells in the MLNs relative to the NE control (Figure 5B).

DISCUSSION

To date, the use of EE has been based on trial-and-error, general observations, anecdotes, and/or recently published practices. Thus, recommendations as to whether or not environmental enrichment should be provided to research animals are often conflicting. The studies described herein have attempted to objectively evaluate the effect of EE on stress levels, as measured by serum corticosterone, and the immune response to a clinically relevant pathogen (HSV-1), as measured by HSV-specific CD8+ T lymphocyte number and function. These studies were performed in both group- and single-housed mice under carefully controlled experimental conditions. The findings presented are particularly important for the field of laboratory animal medicine in that they add to our understanding of the influence of environment on the susceptibility to and pathogenesis of a variety of rodent pathogens that are

immunologically resisted.

We were initially concerned that the addition of EE to group-housing conditions would result in the mice struggling to determine who would control the use of the EE devices, and thus leading to aggression, stress, and increased CORT levels. However, as is illustrated in Figure 1A, this did not appear to be the case either pre- or post-HSV infection. These findings concur with those of other studies which have demonstrated that EE does not increase levels of psychological stress[15,31,61]. In fact, there have been some reports that EE actually decreases CORT levels in group-housed mice[7,30,34]. This decrease in CORT may be attributed to reduced stress levels, alteration in dominance hierarchy, and/or decrease in aggression. Our contrasting finding that EE did not alter CORT levels in group-housed mice may be a function of either the type of enrichment provided or the extent of enrichment provided per cage. Perhaps if only one of the enrichment devices was added per cage and/or the number of mice per cage had been greater than four, the added enrichment per cage would have resulted in differing levels of corticosterone between the EE and NE groups. We had also hypothesized that single-housed NE mice would experience higher levels of CORT than their single-housed EE counterparts. However, this finding was not observed (Figure 1B). It is possible that the components of the single-housing conditions alone provided an enriched environment. For example, the corncob bedding as well as areas under the food bin and water bottle may have provided some level of enrichment that could not be further enhanced by the presence of the igloo and tunnel in each cage.

Interestingly, HSV-infection of the group housed mice, regardless of enrichment status, resulted in lower levels of CORT (Figure 1A). This finding may be due to the body's homeostatic response to the infection by down-regulating HPA axis activity in an attempt to mount for a stronger immune response in hope of clearing the infection. Although not statistically significant, there appeared to also be a trend toward lower levels of CORT in the HSV-infected single-housed mice (Figure 1B). Although glucocorticoids such as corticosterone are generally thought to be immunosuppressive under conditions of stress, it is important to realize that they also play a role in role in regulating immune function under non-stress conditions.

It is important to note that at no time were any of the corticosterone levels above the baseline levels (< 100 ng/ml) that are typically seen in non-stressed, C57BL/6 male mice (Figure 1A and 1B). This finding confirms that the environmental conditions in which the mice were housed were well-controlled, thus minimizing the extent of housing-related stress and, in turn, minimizing the levels of CORT. Thus, it is possible that under non-ideal conditions (e.g. constant and/or intermittent animal room noise, investigator traffic, cage-changing at or near the times of blood collection) the use of enrichment devices would have resulted in lower levels of CORT. Given that the levels of CORT in the present studies were already low may have made it physiologically impossible for EE to achieve corticosterone levels that were any lower than what was observed.

The magnitude of the immune response that occurs in response to any infectious pathogen is critical for mounting a successful defense against the development of disease. Our use of a well-established intranasal model of HSV infection in these studies provides insight into the impact of EE and stress on not only the immune response to HSV but also the immune response to other pathogens that are cleared by mucosally-based immune defense mechanisms. Our finding that EE did not affect the number (Figures 2A and 2B) and function (Figures 4A and 4B) of HSV-1 gB498-505-specific CD8+ T cells generated in the CLN was not surprising given that the CLN were removed 7 days post-infection, a time when the immune response in these lymph nodes may have already stabilized or even began to wane. It is possible that an assessment of this response at an earlier time following infection would have resulted in different findings.

Many of the commonly acquired, respiratory-based infections are able to readily spread from the upper respiratory tract to the lower respiratory tract. As a result, the draining lymphoid tissues in which a local immune response occurs can vary both spatially and kinetically. Previous studies have shown that an experimentally-induced intranasal HSV infection in mice results in CD8+ T cell-based immune response in the MLN. Taking advantage of this knowledge, we also assessed the immune response in the MLN. Our finding that EE group-housed mice exhibited significantly fewer HSV-1 gB498-505-specific CD8+ T cells (Figure 3A) and a trend toward fewer HSV-1 gB498-505-specific CD8+ T cells with IFN-g-producing function (Figure 5A) lends itself to a variety of interpretations. For example, one may initially assume that this finding is due solely to a diminished immune response. However, this finding may be due to a lower level of HSV-1 spread into the lower respiratory tract and thus a lower antigenic load to stimulate a MLN-based immune response. It is possible that

the lower level of CORT exhibited by these group-housed EE mice (Figure 1A) resulted in a stronger immune response in the upper and/or lower upper respiratory tract — an immune response that was sufficient to decrease the extent of viral replication, resulting in an infection of a lesser magnitude in the lower respiratory tract. Despite our finding that group-housed EE mice did not exhibit an increased CD8+ T cell-based immune response in the cervical lymph nodes, it is important to realize that there are many other components of both the innate and adaptive immunity that may play a role in the clearance of an HSV infection in the upper respiratory tract. Although we were not able to detect any virus in either the nasal washes via plaque assay (data not shown), it is possible that either the infection was cleared by day 7 post-infection or that the efficiency of the plaque assay technique was too low to detect the relatively small amounts of virus that may have been present. We were also unable to detect any virus in the brain as determined by real-time (RT-PCR). These latter findings provide support for the hypothesis that the mice mounted an adequate local (intranasal) immune response to viral infection.

In contrast to the EE group-housed mice, EE single-housed mice showed a trend toward greater numbers of both HSV-1 gB498-505-specific CD8+ T cells (Figure 3B) and HSV-1 gB498-505-specific CD8+ T cells with IFN-g-producing function (Figure 5B). It is difficult to attribute these greater numbers strictly to either an inherent increase in overall immunocompetence or to a greater antigenic load in the lower respiratory tract. However, an intriguing explanation for this finding is that the lack of social contact in singly-housed mice, which itself is considered a form of enrichment, may result in a less than optimal immune response that can be enhanced under EE conditions.

Each set of data presented herein is based on a sample size of twelve mice per group pooled from three independent experiments. The degree of variability within some of the data was somewhat higher than expected and may be responsible for some of the comparisons between groups not being statistically significant ($p < 0.05$) but rather only a trend toward significance ($0.20 > p > 0.05$). Our expected variability was a function of using an inbred strain of mouse (C57BL/6) in which each mouse was genetically identical. However, it has been well established that there are individual differences in a variety of physiological and behavioral traits even within inbred strains of mice. Thus such differences could account for the variability that was observed. It is also important to point out that there are many immune-related steps that must function in a well organized fashion to result in efficient antigen-specific T cell activation, expansion, and function. As is noted above, the degree of antigenic load is also a critical factor in the magnitude of an immune response. Thus, an alteration in even just one of these immune- or virus-related factors can have significant effects on the magnitude of the T cell response.

Although our studies provided what we thought was an increased enriched environment in the form of an igloo and a tunnel, it is possible that the housing of these mice in standard cage environment is sufficiently enriched. Rather than providing EE, it would be interesting to determine the impact of removing all probable forms of enrichment on CORT levels and immune function. For example, by shifting group- and single-housed mice from a standard cage environment to one with no bedding, no lid on which to climb, no areas in which to "hide" (e.g. a cage with no corners, food bin, or water bottle), we would expect that CORT levels to rise and immune function to be less than robust. It would also be interesting to determine if cage-based enrichment can counter the effects of others stressors (e.g. noise) that are inherent to many laboratory animal facilities. Although the studies described herein focused exclusively on the primary T cell response, it is important to determine 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. 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.

The studies described herein illustrate the difficulty in assessing the true impact of EE on immune function. Even under the best of controlled conditions, there are still a number of variables for which one is unable to account. Although our studies were designed to evaluate a pathogen-specific immune response using state-of-the-art flow cytometry-based techniques, we have addressed the relationship between EE and immune function using only one strain of mice (C57BL/6), two housing conditions (four per cage; single-house), one route of infection (intranasal), one pathogen (HSV-1), two sets of lymph nodes (CLN and MLN), and two measures of immunity (number and function of CD8+ T cells). Thus, one has to be cautious in attempting to generalize the results from one study to all relationships among EE and immune function or other physiological

parameters. Additional data is necessary to determine how EE broadly and specifically influences immune functions in other ways. One should carefully consider the use of adding any type of enrichment device as it may function as additional variable with unknown consequences on the measures that are being made. However, an attempt to provide an environment for mice that allows for species-specific behavior such as gnawing, nesting and hiding, and foraging should be made whenever possible. Further studies need to be conducted to determine EE effects on all aspects of the overall welfare of laboratory mice.

Figure 1

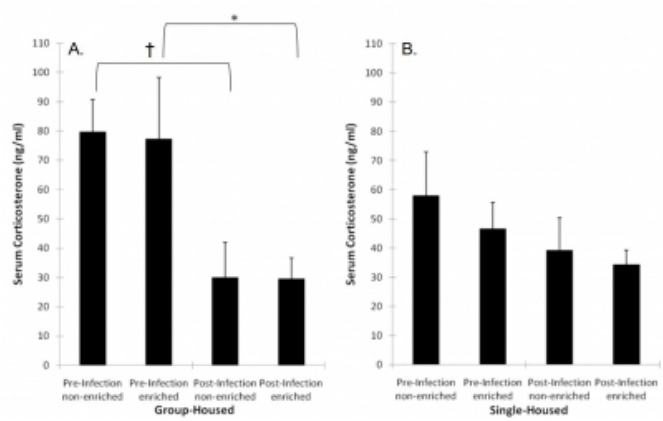


Figure 2

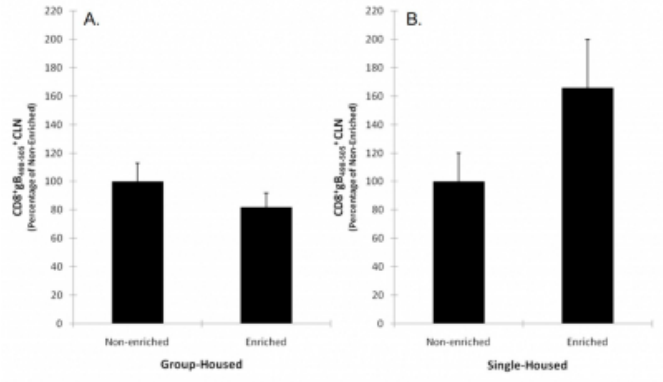


Figure 3

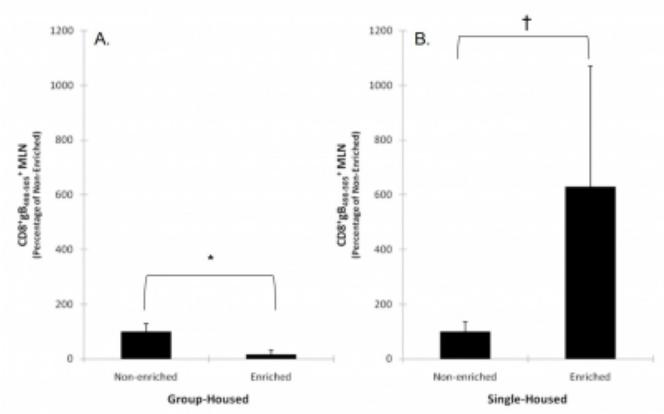


Figure 4

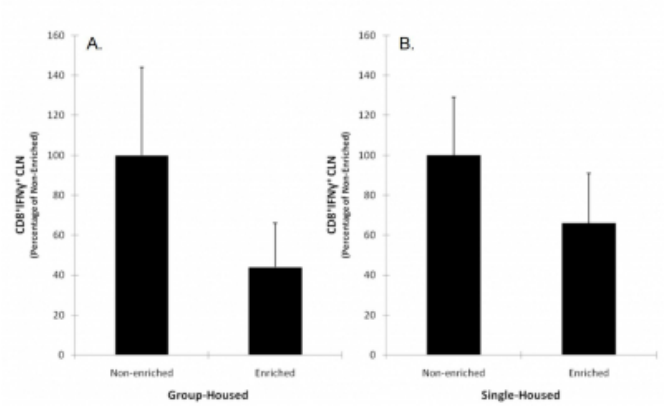
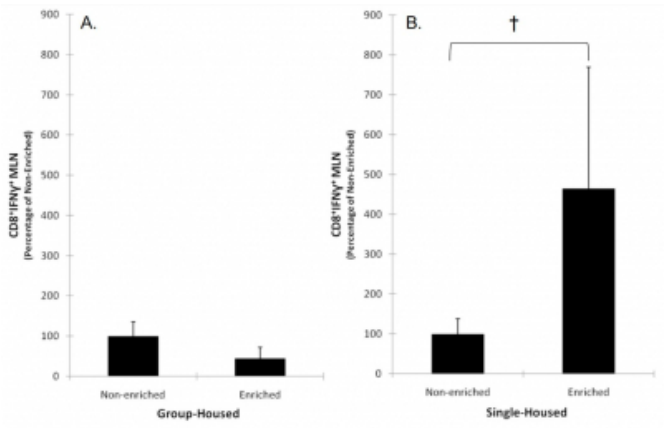


Figure 5



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