Hypothalamic brain-derived neurotrophic factor regulates lymphocyte immunity and cancer progression in environmental enrichment

Undergraduate Research Thesis

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By

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Project Title: Hypothalamic brain-derived neurotrophic factor regulates lymphocyte immunity and cancer progression in environmental enrichment

Abstract:

Cancer is a heavily studied, multifactorial disease with an incidence of nearly 1.7 million in the U.S. alone. In a clinical study, a supportive social environment was shown to improve outcomes for breast cancer patients. Yet, animal model experiments have mainly focused on the negative effects of distress or social isolation on cancer outcomes. Our model of social enrichment, an enriched environment (EE), is a model of eustress, and we uniquely study the positive effects of the social environment on cancer outcomes. We have previously shown that EE exerts an anticancer effect. Here, we show that CD8 T-cells are required for this effect. Furthermore, we show a shift to CD8 T-cells in secondary lymphoid tissue that is mediated by a molecule in the hypothalamus, brain-derived neurotrophic factor. We also show a shift in thymocyte development toward CD8 T-cells in EE that is regulated by the same molecule. We have further studied the complex effect of EE on the adipose tissue microenvironment, specifically on natural-killer cells. Here, we show that adipose tissue-specific IL-15 overexpression results in increased NK cells in adipose tissue as well as increased NK cells at a nearby tumor and decreased mass of the nearby tumor. Finally, we extend the current knowledge of EE's anticancer effect to a blood cancer, acute myeloid leukemia, in which we see a substantial increase in progression-free survival but a modest, yet still significant, increase in overall survival. These results together show the complex connection between the environment, the nervous system, the immune system, and the cancer microenvironment in mice. The continual regulation of EE's anticancer effect by brain-derived neurotrophic factor suggests the possibility of manipulating a single gene in the brain for beneficial outcomes in certain cancers. Our model shows the importance of studying the positive effects of the environment on cancer outcomes as a model for human disease.

Overview:

In our collaboration with the Cao lab, we have shown an anticancer effect in an enriched environment model of mice, an environment that is physically, cognitively, and socially stimulating. In studying this effect, we seek to find the pathways that connect the macroenvironment, namely an enriched environment, to the tumor microenvironment and by which an enriched environment confers its anticancer effect. In this collaboration, we have extensively studied this connection, identifying hypothalamic brain-derived neurotrophic factor as a key mediator of this anticancer effect.

Here, I report our findings on T-cells in the thymus and the periphery, changes that create a T-cell repertoire that is more readily poised to lyse cancer cells. Furthermore, I report our initial findings on the role of natural killer cells in nearby adipose tissue in monitoring neighboring tumors. Finally, I report our preliminary results on EE's beneficial effects on the progression of acute myeloid leukemia. Together, these results show a model for macroenvronmental stress, in the form of eustress, in the context of cancer. The pervasive control of hypothalamic brain-derived neurotrophic factor in controlling the immune changes associated with an enriched environment's anticancer effect suggest the possibility of manipulating a single gene in the brain for beneficial outcomes in certain cancers.

Our collaboration with the Cao lab has produced a large amount of data, only some of which is presented here. I have chosen to report the data that most clearly relate to cancer, as this is the research and specialty I have always found most interesting and I am most likely to pursue. With that being said, some of the experiments presented here were designed after substantial investigation into adipose tissue in the enriched environment, a topic that I do not include here. I have included a brief background of these experiments in order to explain the rationale for these experiments.

Finally, this work has been highly collaborative. This work is a result of thorough work on the part of my mentor, Stephen Bergin, our direct collaborator in the Cao lab, Run Xiao, and myself, as well as many others in both of our labs. While I have contributed to the entire scientific process of these experiments, this work is not my work alone. Specifically, I have been a part of the mouse sacrifice, tissue processing, preparation and sample running on flow cytometry, data analysis of flow cytometry, and figure creation. Molecular work, such as polymerase chain reaction (PCR) and vector creation, as well as vector injection into the hypothalamus was completed by our collaborators in the Cao lab.

Background:

Stress has long been known as a key mediator of disease onset and progression[1,2]. Distress has been studied frequently in the context of both obesity and cancer. With obesity, stress is a concomitant risk factor for hypertension and metabolic diseases[3]. Furthermore, obesity has been theorized as an adaptive response to chronic stress[4]. Specific to cancer, distress is hypothesized to affect the progression of cancer. A patient's subjective sense of stress concerning their cancer diagnosis is predictive of a weakened immune system[5]. In a clinical study of breast cancer, stress was correlated with incidence of cancer, and a more supportive social environment has been linked with improved outcomes[6].

Traditionally, there are two stress axes: the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis. The sympathetic nervous system (SNS) is the component of the autonomic nervous system that is typically referred to as the "fight-or-flight" response. Activation of SNS results in release of stress hormones from the adrenal medulla, preferential use of glycolysis for energy, and decreased immune function. The hypothalamic-pituitary-adrenal (HPA) axis, on the other hand, is an endocrine response to stress. The release of corticotropin releasing hormone (CRH) from the hypothalamus into the blood triggers release of adrenocorticotropic releasing hormone (ACTH) from the anterior pituitary. ACTH then travels through the blood and causes the adrenal cortex to release glucocorticoids, another classic stress hormone.

These stress pathways are classically studied in the context of distress. Eustress, as opposed to distress, is "positive stress" involving cognitive activation without the classic detrimental and exhaustive effects of distress[7]. The role of eustress is less understood on a molecular level[8], even though clinical studies suggest a link with cancer outcomes[6]. Our lab collaborates with the Lei Cao lab that has extensively studied the effect of an enriched environment (EE) in mice[9,10,11]. EE is physically, cognitively, and socially stimulating compared to standard environment (SE) housing, and as such can be used as an animal model of eustress. EE has previously been associated with an anti-obesity effect and positive metabolic changes, which are not extensively discussed here.[9]

The Cao lab previously showed that EE exerts an anticancer effect, through reduced tumor growth and increased remission, in models of melanoma and colon cancer. They also reported that hypothalamic brain-derived neurotrophic factor (BDNF) was upregulated in EE, independent of extra exercise. BDNF has previously been shown as an important, early molecule in brain development[12]. It is highly responsive to exercise and the environment and has been linked with satiation of appetite and energy homeostasis[13,14]. Furthermore, overexpression of BDNF in SE led to reduced tumor burden, and BDNF knockdown blocked the anticancer effect of EE[9], leading to the conclusion that BDNF was at least partially responsible for EE's anticancer phenotype.

The immune and molecular mechanisms by which EE exerts its anticancer phenotype are previously unknown. We propose a peripheral and developmental model for T-cell control of EE's anticancer phenotype. Additionally, we present a novel understanding of the relationship between the adipose immune microenvironment and local tumor proliferation. Finally, we show that EE has beneficial results in a blood

cancer, acute myeloid leukemia, which is phenotypically different from melanoma, a solid tumor.

Figure Outline:

(1) T-cell depletion experimental design
(2) Confirmation of T-cell depletion
(3) CD8 T-cells partially mediate EE's anticancer effect
(4) T-cell phenotype in SLT of EE
(5) BDNF overexpression and depletion
    a. Confirmation of overexpression in hypothalamus
    b. T-cell phenotype in SLT reproduced in overexpression
    c. Confirmation of knockdown in hypothalamus
    d. T-cell phenotype in SLT eliminated in knockdown
(6) SNS regulation of T-cell phenotype in SLT
(7) HPA regulation of T-cell phenotype in SLT
(8) Thymus development changes in EE
    a. Thymic involution
    b. Thymus phenotypes at one, four, and twelve weeks
    c. Representative flow images for thymus phenotype
(9) Thymocyte retention in EE
(10) BDNF regulation of thymus phenotype (overexpression)
(11) BDNF regulation of thymus phenotype (depletion)
(12) HPA regulation of thymus phenotype (adrenalectomy)
(13) Binding of GC's to developing T-cells (GR-LCK mice)
(14) IL-15 overexpression
    a. Tumor photograph
    b. Tumor mass
    c. Immune changes
(15) AML preliminary study
    a. Survival curve
    b. Immune changes at three weeks
    c. Immune changes at five weeks
(16) Photograph of EE

Flow cytometry gating strategy

Statistical considerations: p-values are represented by relative significance using stars. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$.

Results:

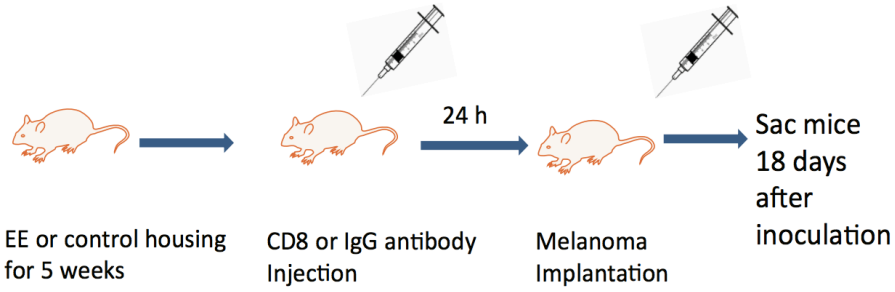
(1) T-cells in EE

Cytotoxic T lymphocytes (CTLs) recognize and kill cancer cells. CD8 is the defining marker of CTLs, in contrast to CD4 T cells. T-cells develop in the thymus but migrate to and are stored in secondary lymphoid tissue (SLT), such as the lymph node (LN) and spleen, before exportation to the periphery. Therefore, **we hypothesize that CD8 cytotoxic T lymphocytes mediate the anticancer effect of EE.**

<u>EE requires CD8 T-cells to mediate anticancer phenotype</u>

To test whether CTLs were required to mediate EE's anticancer phenotype, we administered CD8-depleting antibody or IgG antibody as control to mice in either EE or SE housing. Mice were housed in their respective cages for five weeks, which is the approximate amount of time when we have previously observed EE-associated metabolic changes. At this time, EE mice weighed less, positively confirming the metabolic effects of EE (data not shown). At that time, mice were injected with either CD8-depleting or IgG antibody. After 24 hours, mice were injected with a B16 melanoma cell line and were sacrificed 18 days later for tumor analysis. The experimental design is shown below (Figure 1).

Figure 1. T-cell Depletion Experimental Design



To confirm depletion of CD8 T-cells in the depletion group, we ran flow cytometry of blood. All mice in both depleting groups showed sufficient depletion of CD8 T-cells that did not deplete CD4 T-cells. A representative flow image is show in Figure 2.

Figure 2. CD8 T-cell depletion confirmation

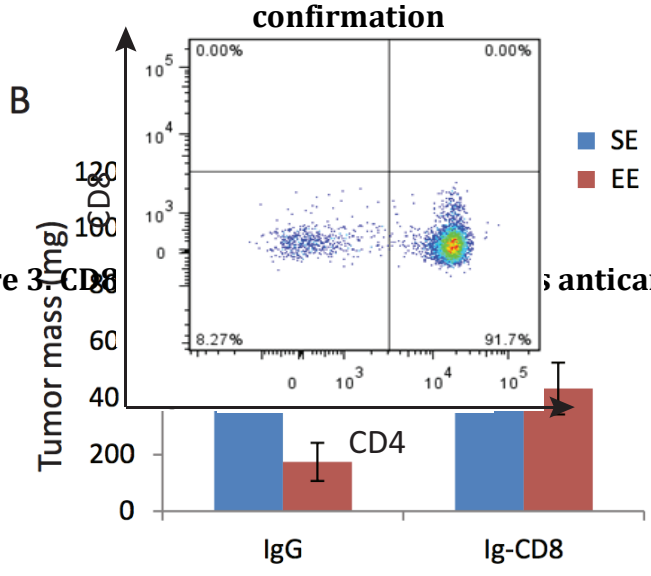


Figure 3. CD8 [illegible] anticancer effect

Within the IgG control group, EE tumors weighed significantly less ($p<0.001$) than SE, confirming our previous studies. Within the CD8-depleting antibody groups, there was no significant difference between EE and SE ($p>0.07$). Importantly between the EE groups, mice administered CD8-depleting antibody had significantly larger tumors than those administered IgG control, suggesting that CTLs play at least some role in mediating EE's anticancer effect (Figure 3).

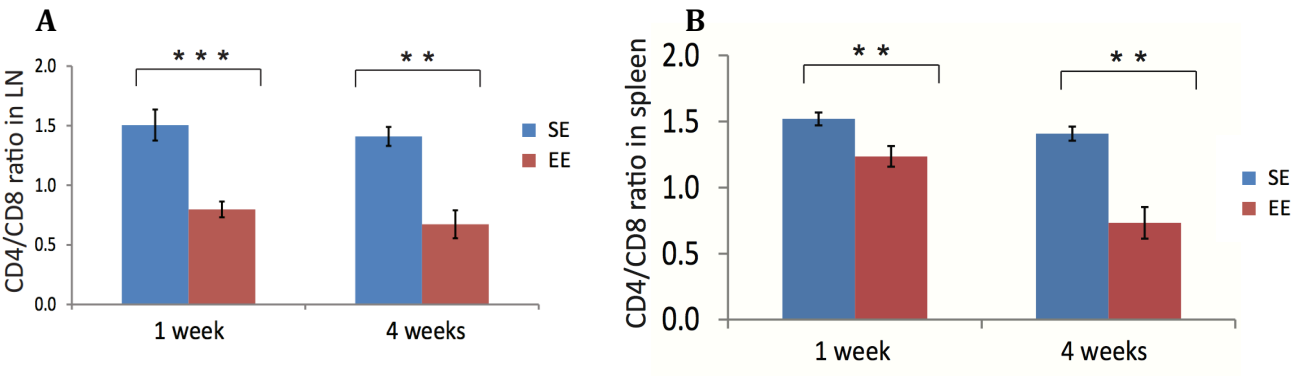
EE shifts CD8 T cell populations

We next wanted to test whether EE affects T-cell populations in the periphery, such as in SLT, to support this anticancer effect. To do this, we measured the CD4:CD8 ratio. A lower ratio, with a higher percentage of CD8 T-cells, represents a T-cell repertoire more readily poised to lyse cancer cells by detecting the down-regulation of MHC-I. Furthermore, we wanted to see whether this potential T-cell change preceded other immunological changes we had previously observed in macrophages and NK cells.

We housed mice in either EE or SE for one or four weeks. We sacrificed mice and analyzed SLT, specifically lymph node (LN) and spleen for T-cell populations using flow cytometry. (Gating strategies are described in methods.)

At both one and four weeks, both LN and spleen showed a decreased CD4:CD8 ratio (Figure 4A and 4B). Exposure to EE for only one week produced a distinct change in T-cell populations in SLT. The CD4:CD8 ratio does not continue to decrease with extended exposure to EE, indicating that EE induces a distinct T-cell balance that is consistently induced at an early time point.

Figure 4. T-cell phenotype in SLT

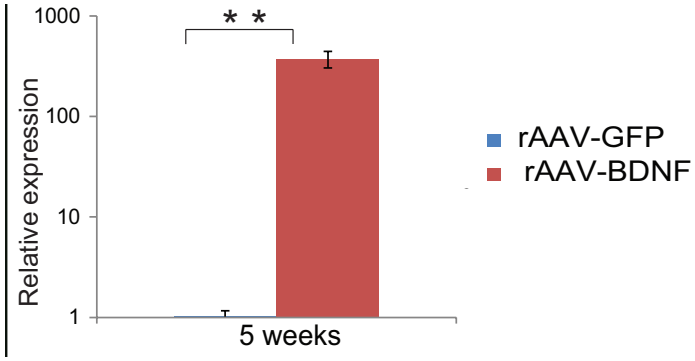


BDNF controls T-cell populations in EE

We have previously seen BDNF as a key regulator of EE's metabolic effects on weight and obesity as well as tumor resistance. Therefore, we hypothesized that BDNF regulated the T-cell changes we had observed. To test this hypothesis, we carried out both an overexpression experiment and a depletion experiment.

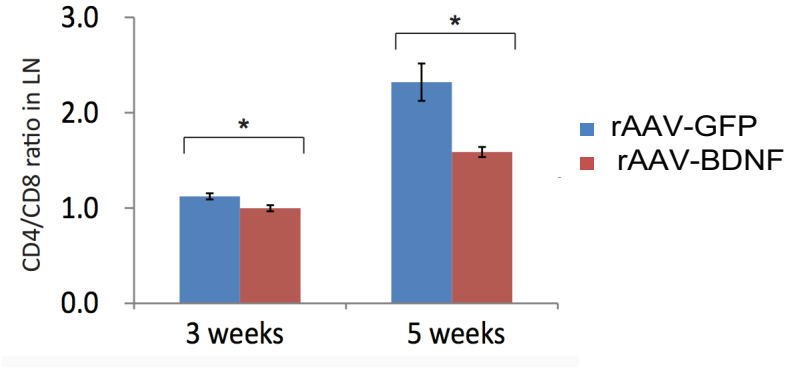
To test whether overexpression of BDNF was sufficient to induce our observed T-cell phenotype, we injected a recombinant adeno-associated virus (rAAV) vector containing BDNF or GFP as a control directly into the hypothalamus of mice that were maintained in SE housing. (rAAV-GFP is represented by the blue bar, and rAAV-BDNF is represented by the red bar.) Vector localization to the hypothalamus was confirmed by GFP fluorescence (data not shown). BDNF overexpression was confirmed by quantitative RT-PCR (Figure 5A).

Figure 5A. BDNF Overexpression Confirmation



Mice administered rAAV-BDNF had a significantly lower CD4:CD8 ratio compared to rAAV-GFP (Figure 5B). This suggests that BDNF overexpression is sufficient to induce T-cell changes in SE and that BDNF controls this anticancer T-cell change, at least to some extent.

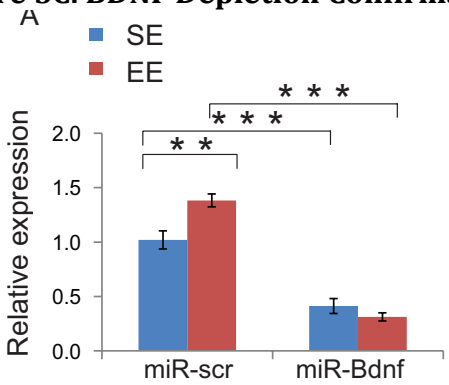
Figure 5B. BDNF Overexpression



Furthermore, we then tested whether knockout of BDNF eliminated the T-cell changes in EE. To do so, we injected either microRNA targeting BDNF or a microRNA

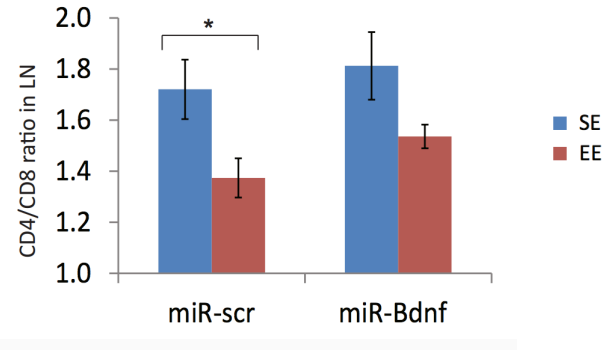
targeting a scrambled sequence that does not target any known genes directly into the hypothalamus of mice living in either EE or SE. We confirmed the knockdown of BDNF expression by quantitative RT-PCR (Figure 5C).

Figure 5C. BDNF Depletion Confirmation



As expected, mice administered microRNA targeting a scrambled sequence living in EE showed a decrease compared to their SE counterparts, confirming the control. Mice administered microRNA targeting BDNF in EE did not show a statistically significant difference from SE counterparts (Figure 5D). These results show that depletion of BDNF eliminates EE's anticancer effect. Together with the overexpression experiment, BDNF is a key regulator of EE's anticancer effect.

Figure 5D. BDNF Depletion



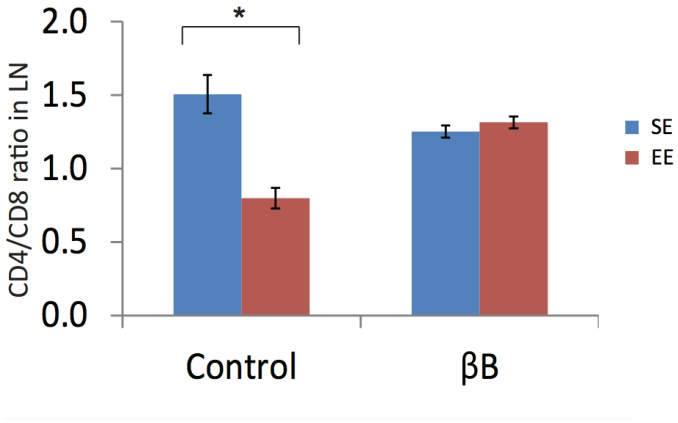
<u>SNS and HPA axes both control EE downstream of BDNF</u>

In our previous study, we showed that sympathetic nervous system (SNS) signaling was required for EE's anticancer effect. Furthermore, we showed that hypothalamic-pituitary-adrenal (HPA) signaling is upregulated in EE. As SNS and HPA are two classic stress control systems, we investigated whether they play a role in regulating our T-cell phenotype downstream of BDNF in the hypothalamus.

We used a non-specific beta-blocker (βB), propranolol, to investigate the role of SNS in the EE-induced changes in T-cell homeostasis in EE. Propanolol prevents the binding of epinephrine to beta-receptors on effector cells.

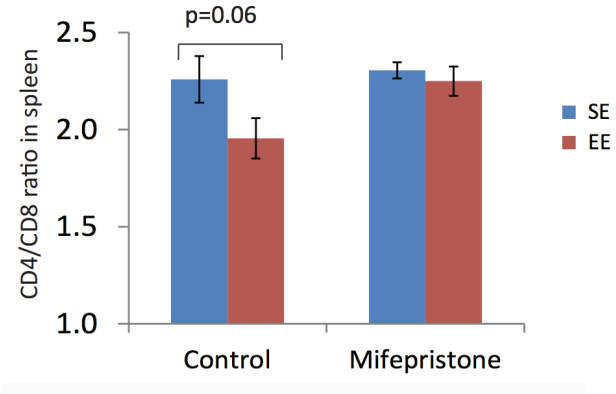
We housed mice in either EE or SE and administered propranolol in the drinking water of half of the mice and regular drinking water as a control. Mice administered propranolol had no significant CD4:CD8 ratio change between EE and SE (Figure 6), indicating that βB had blocked the T-cell effect.

Figure 6. SNS Regulation



To investigate the role of HPA signaling, we used an antagonist of cortisol receptors, mifepristone. Mifepristone prevents the binding of cortisol to its receptor on effector cells. Similar to the previous experiment, mice were housed in EE or SE, and half of the mice in each group were administered mifepristone by oral injection, while the other half received an oral injection of vehicle control. Administration of mifepristone completely blocked the T-cell CD4:CD8 ratio change observed in EE (Figure 7).

Figure 7. HPA Regulation



Intermediate Conclusion #1: Environmental and genetic activation of hypothalamic BDNF modulates T-cell immunity to exert an anticancer phenotype.

(2) EE induces changes to the thymus and T-cell development

The experiments outlined in (1) show the peripheral changes in T-cells that mediate an anticancer effect in EE, as well as the regulation of this immune change. But, the immune development that leads to this change was not investigated. The thymus is the primary organ of T-cell development, and it serves a vital role in maintaining immune homeostasis through this function.

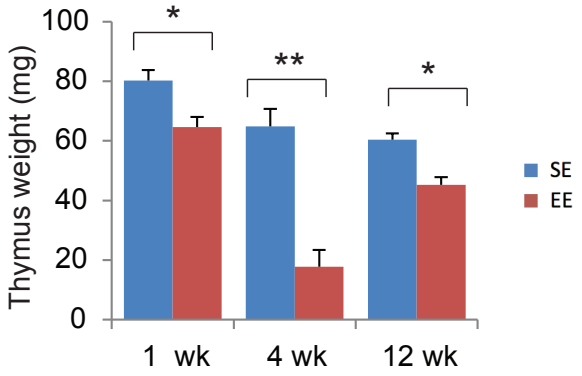
The thymus undergoes significant changes in response to stress and age, typically decreasing in size through a process called thymic involution. We have previously observed thymic involution in EE but have not studied the specific effects of involution on T-cell development or its regulation. T-cells undergo a standard developmental process, defined by the presence or absence of CD4 and CD8. T-cells develop from CD4-CD8- double negative (DN) cells to double positive (DP) and to single positive (SP) CD4 T-helper or CD8 CTLs.

Therefore, **we hypothesize that EE shifts thymocyte development in the thymus toward CD8 CTLs and that this development is regulated similarly by BDNF and the stress axes.**

<u>EE alters thymus development and retains single-positive T-cells</u>

To test the effect of EE on T-cell development in the thymus, we housed mice in either EE or SE for 6 days, 4 weeks, or 12 weeks. A six-day exposure to EE was sufficient to induce a significant decrease in thymus mass in EE compared to SE, and this difference in thymic mass persisted at both 4 and 12 weeks (Figure 8A). A decrease in thymus mass is termed thymic involution and is a consistent finding in EE. Thymic involution is typically studied in the context of distress (psychological stress, infection, and cancer), but EE shows that thymic involution is associated with eustress as well.

Figure 8A. Thymic Involution



Within the stages of T-cell development, at each time point, we saw a higher proportion of DN and SP CD8 T-cells with a decrease in DP cells (Figure 8B and 8C).

Figure 8B. EE Thymus Phenotype

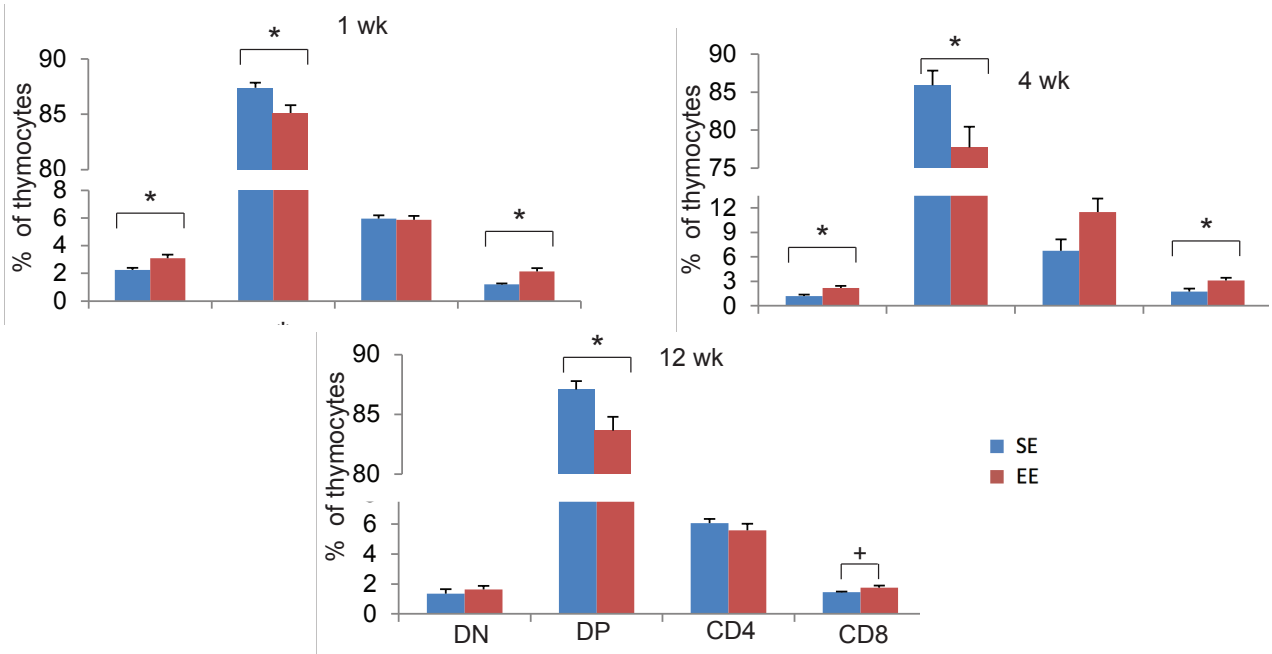
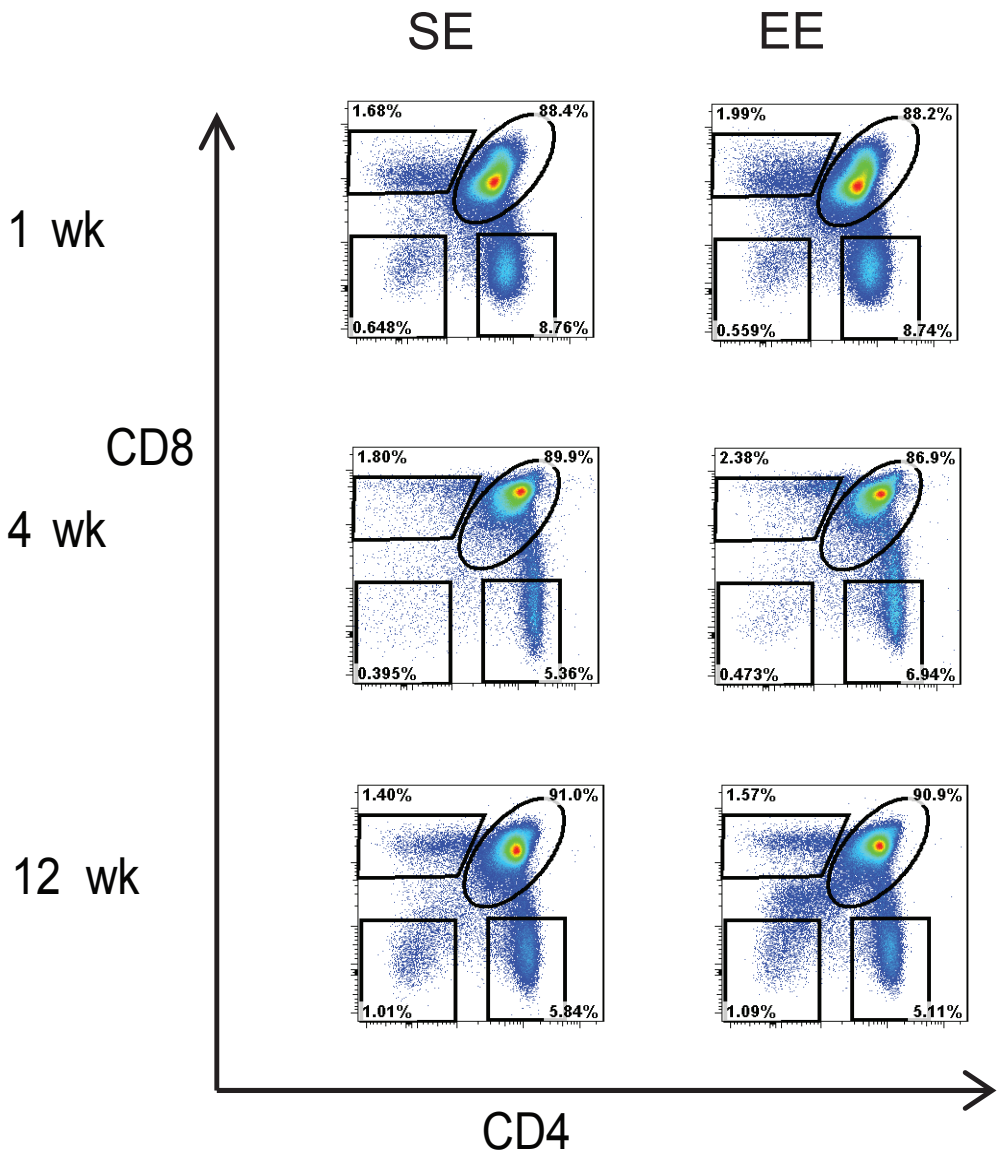


Figure 8C. EE Thymus Phenotype Flow Cytometry



We further hypothesized that changes in thymus retention of SP T-cells may contribute to the SLT phenotypes reported in (1). To test this hypothesis, we obtained RAG2-GFP mice in which GFP is expressed in T-cells that have recently undergone T-cell receptor (TCR) rearrangement. TCR rearrangement occurs in the DN T-cell stage and results in unique T-cell receptors on developing T-cells. RAG2 is a gene expressed during TCR rearrangement. RAG2-GFP mice have GFP inserted into the open reading frame of the RAG2 gene such that whenever RAG2 is expressed, GFP is expressed. Thus, T-cells that have recently left the thymus can be detected as expressing GFP.

RAG2/GFP mice were placed in SE or EE for 2 weeks. In SLT, we observed a significantly reduced CD4/CD8 ratio of GFP+ T-cells but no change when gated on GFP- T-cells (Figure 9A). Consistent with our hypothesis that EE housing preferentially retains CD4 T-cells, GFP expression was reduced in CD4 but not CD8 T-cells (Figure 8B). Overall, the data demonstrate that EE housing rapidly alters thymocyte development and retains SP T-cells within the thymus.

Figure 9A. Thymocyte Retention

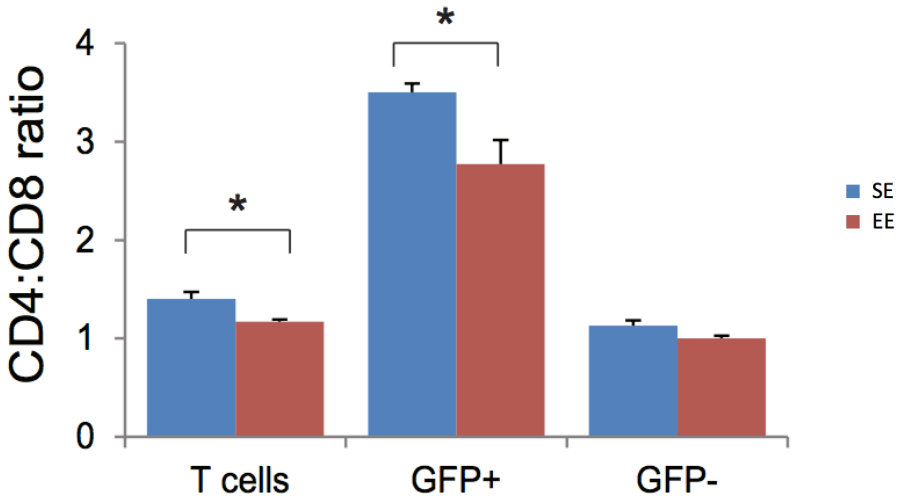
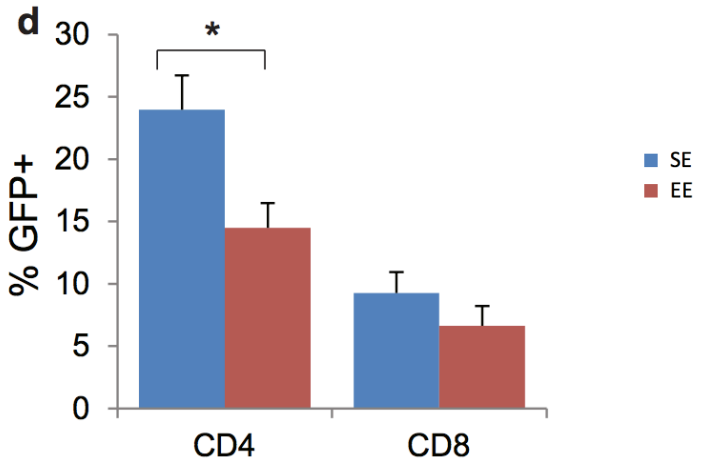


Figure 9B.



BDNF controls EE's thymus developmental changes

The experiments in (1) show BDNF control of T-cells in SLT, so we hypothesized that BDNF controls EE's change in T-cell development in the thymus. Similar to (1), we used both an overexpression and a depletion model of BDNF.

To test whether overexpression of BDNF was sufficient to induce our observed thymic development change, we injected a recombinant adeno-associated virus (rAAV) vector containing BDNF or GFP as a control directly into the hypothalamus of mice that were maintained in SE housing. (rAAV-GFP is represented by the blue bar, and rAAV-BDNF is represented by the red bar.) Overexpression was similarly confirmed using quantitative RT-PCR (data not shown).

Mice administered rAAV-BDNF significantly smaller thymuses than control (Figure 10A). Furthermore, within thymocyte populations, there was a reduction in DP cells and an increase in SP CD8 cells, as was observed in EE at several timepoints (Figure 10B).

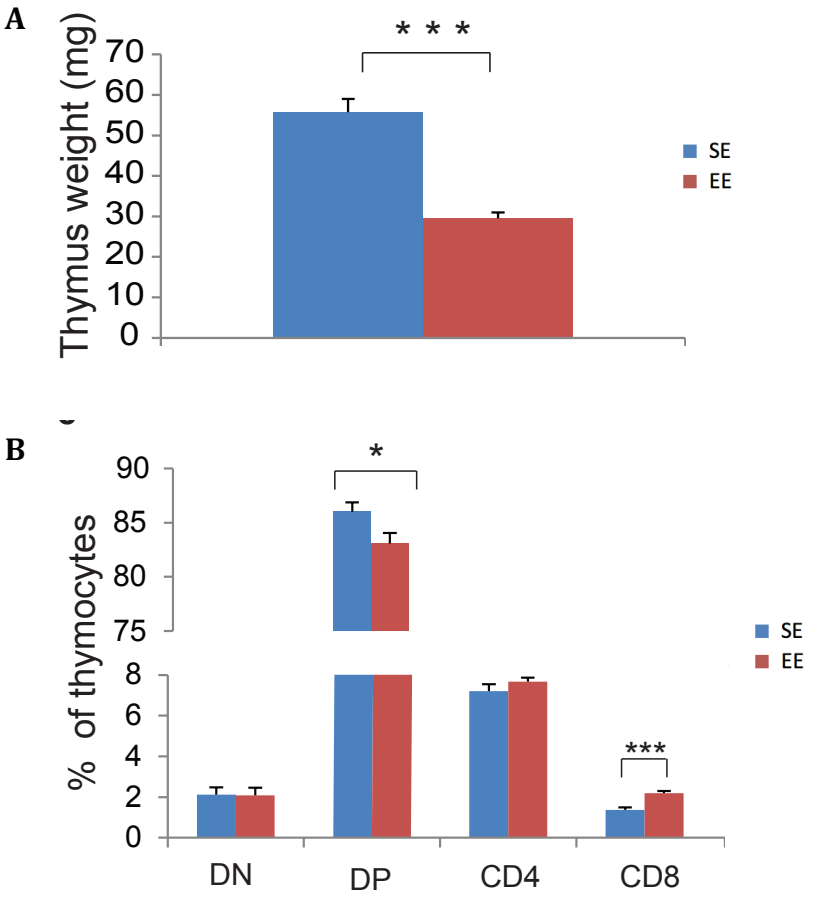


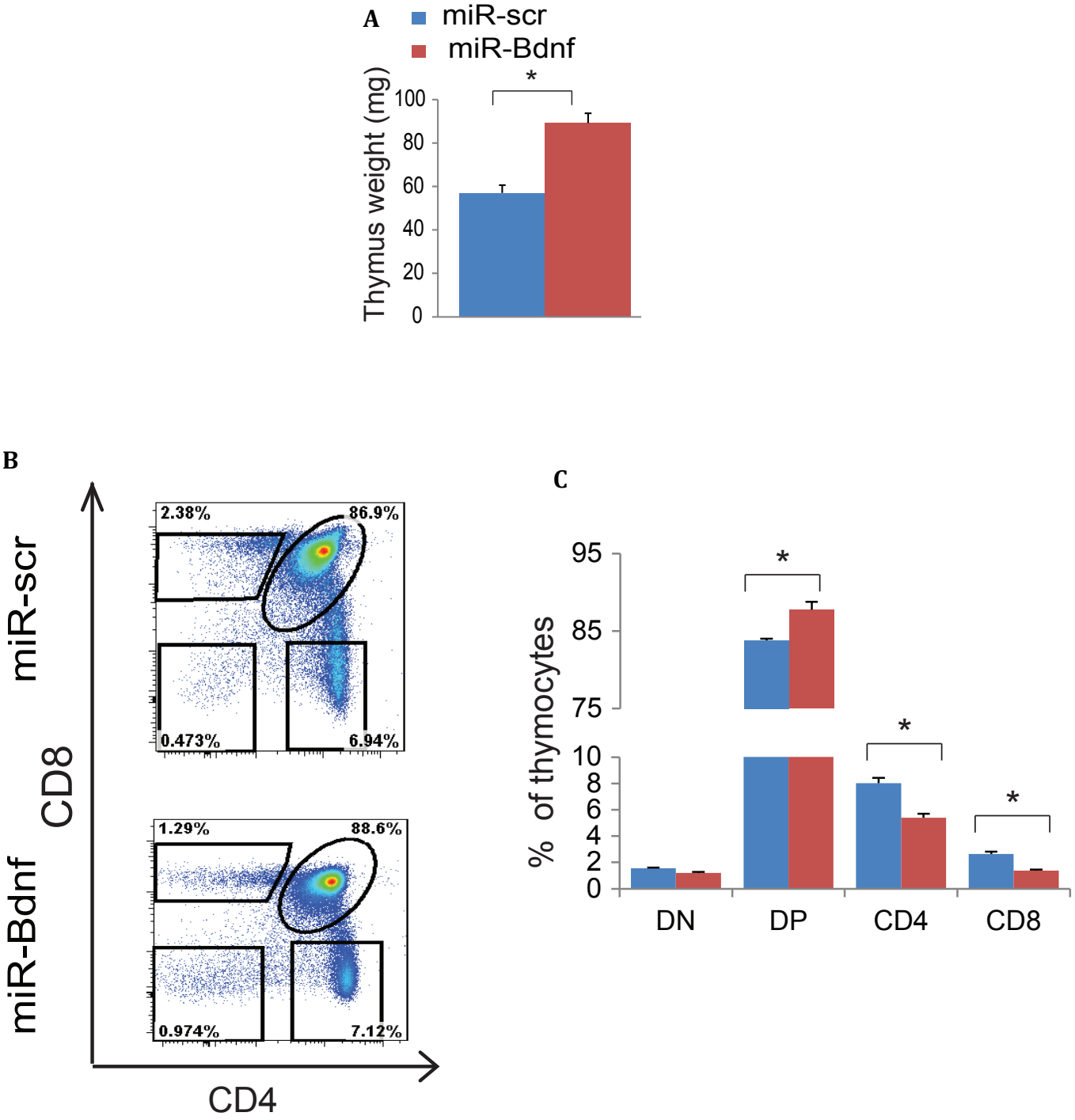
Figure 10. BDNF Regulation of Thymus Phenotype (Overexpression)

Furthermore, we tested whether knockout of BDNF eliminated the changes in thymocyte development. To do this, we injected either microRNA targeting BDNF or a microRNA targeting a scrambled sequence directly into the hypothalamus of mice living

in either EE or SE. BDNF knockdown was similarly confirmed using quantitative RT-PCR (data now shown).

Comparing the two groups injected with microRNA targeting BDNF, we observed an *increase* in thymus weight in EE (Figure 11A). We also observed an increase in DP cells and a decrease in SP CD8 cells (Figure 11B and 11C). These results in the knockdown experiment actually represent a reversal of the results seen in EE in Figure 8B, consistent with our hypothesis that BDNF regulates thymocyte development.

Figure 11. BDNF Regulation of Thymus Phenotype (Depletion)



HPA signaling mediates EE immune phenotypes in SLT

As shown in (1), blockade of the HPA axis disrupted the T-cell changes in SLT. So we hypothesized that HPA may affect thymocyte development leading to these changes. In the HPA axis, corticotropin-releasing hormone (CRH) stimulates secretion of aderenocorticotrophin hormone (ACTH) in the anterior pituitary, which stimulates corticosteroid production in the adrenal glands.

Glucocorticoids (GC), such as cortisol, have extensively been studied in the context of distress and have previously been studied in EE. Distress is associated with a substantial increase in GC beyond the normal physiological range. EE on the other hand is associated with a mild increase in GC that is significant but within the physiological range. This finding supports the possibility that eustress, represented by EE, is an intermediate and healthy stressor state between lack of stimulation and distress.

Furthermore, the large increase in GC in distress is associated with an immunosuppressive state, whereas the mild increase in GC in EE is actually associated with an increase in CD8 T-cells and natural killer (NK) cells. With regard to T-cell development, GC has previously been shown to promote positive selection of T-cells.

To test the hypothesis that activation of the HPA axis mediates the EE thymic phenotypes, we obtained mice that had either undergone an adrenalectomy surgery or sham control surgery, and randomly assigned them to SE or EE housing for five weeks. After five weeks, we observed that sham EE-housed mice had an elevated serum corticosteroid level but no change in serum ACTH was observed, confirming that the adrenalectomy had prevented corticosteroid production and that the sham surgery had not prevented corticosteroid production (confirmatory data not shown).

Adrenalectomy (ADX) eliminated the EE-induced reduction in thymus weight (Figure 12A). When we investigated the thymocyte populations, we observed that the EE-induced increase in CD8 T-cells observed in sham mice was eliminated in adrenalectomized-EE mice (Figure 12B and 12C). Overall, the data supports the hypothesis that EE acts through adrenal gland-derived corticosteroids to regulate thymocyte development.

Figure 12A. HPA Regulation of Thymus Phenotype

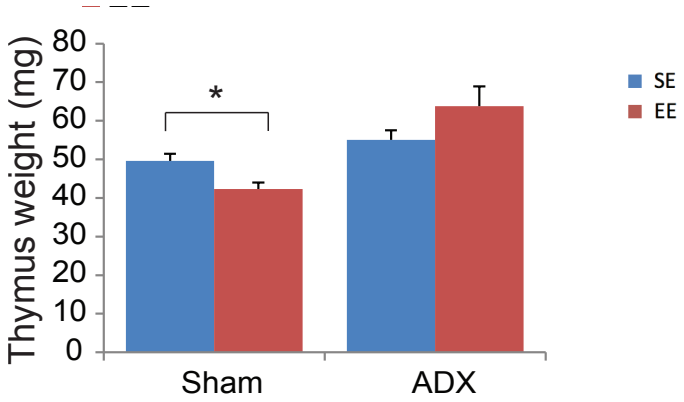


Figure 12B. Thymus Phenotypes in ADX

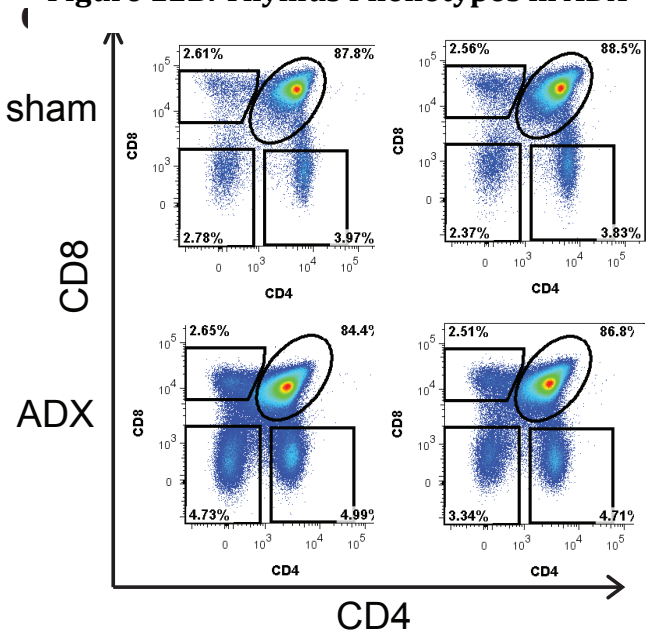
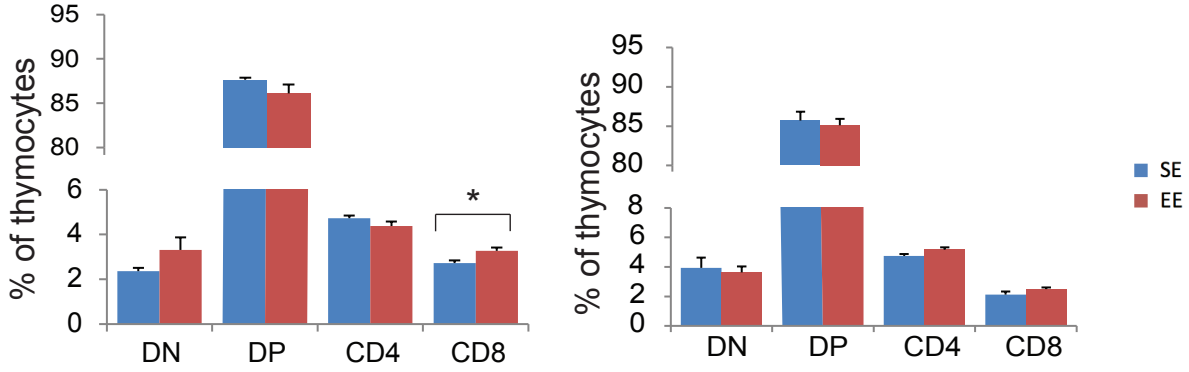
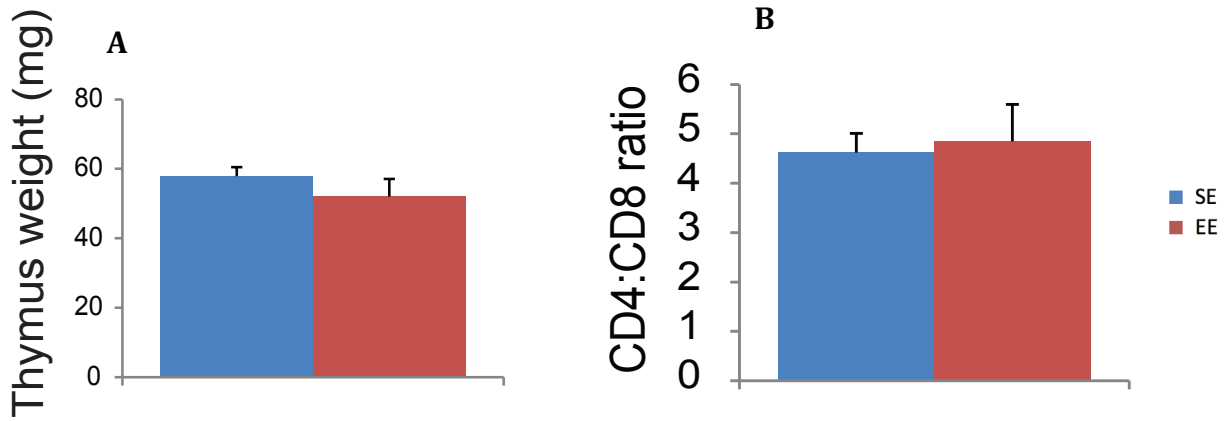


Figure 12C.



To test whether the effects of corticosteroids directly regulated thymocyte development, we used a conditional knockout mouse train $LCK^{Cre}GR^{lox}$ ($GR^{lck\text{-}Cre}$), in which the glucocorticoid receptor (GR) gene is deleted in the thymus in the DN4 stage (when LCK is expressed), such that DP, SP CD4, and SP CD8 cells lack responsiveness to glucocorticoids. When $GR^{lck\text{-}Cre}$ mice were housed in EE for five weeks, we observed no changes in thymus weight (Figure 13A) or CD4/CD8 ratio (Figure 13B) on mature SP cells.

Figure 13. GR-LCK Mice



As may be expected from (1), we also tested the effect of ablation of SNS in thymocyte development. Upon ablation of SNS, all EE-associated thymocyte changes persisted (data not shown). This could indicate either that SNS has its effects on T-cells after export from the thymus or downstream of HPA axis.

Intermediate Conclusion #2: Hypothalamic BDNF regulates thymocyte development in enriched environment mice leading to CD8 T-cell development

(3) IL-15 overexpression leads to decreased tumor mass

The Cao lab recently created an adeno-associated viral vector that travels specifically to adipose tissue. They have published its use in delivering a Cre recombinase to delete insulin receptor from mice with floxed insulin receptor.

Other work between our groups has focused on the immune microenvironment in EE within adipose tissue of several depots. We have studied extensively cells of the innate immune system, specifically innate lymphoid cells. Innate lymphoid cells (ILCs) are a newly-classified cell in the innate immune system that includes natural killer cells as well as three distinct classes of ILCs that are involved in functions ranging from metabolism to helminth expulsion in the gut. (Our unpublished studies on ILCs and other immune cells in adipose tissue are not included here, as they do not directly relate to EE's anticancer phenotype.)

Our preliminary results in these studies have shown that EE increases NK cells in visceral adipose tissue. With the discovery of this adipose-specific vector, we wondered whether delivery of interleukin-15 (IL-15), which recruits NK cells, specifically to adipose tissue would increase NK cells in adipose tissue. Furthermore, we wanted to test whether this presumed increase in NK cells would lead to decreased tumor mass in the local area.

Therefore, **we hypothesized that vector-mediated, adipose-specific IL-15 overexpression would lead to increased NK cells in adipose tissue and decreased local tumor growth**.

To test this hypothesis, we injected mice with AAV containing IL-15 overexpression or scrambled sequence as control and subsequently injected with melanoma tumor on the flank. Three weeks later, mice were sacrificed and tumors were separated from surrounding tissue, measured for volume, weighed, and photographed. Tumors and adipose tissue were then processed for flow cytometry analysis.

Tumor masses were significantly decreased in mice administered AAV containing IL-15 (Figure 14A and 14B). (One outlier in the IL-15 group increased p value.) NK cells were increased in the adipose tissue (data now shown), as expected in the IL-15 group. NK cells were also increased in the tumor (Figure 14C), suggesting some level of recruitment or infiltration from the adipose tissue immune environment.

Figure 14A. IL-15 Overexpression

CT

IL15

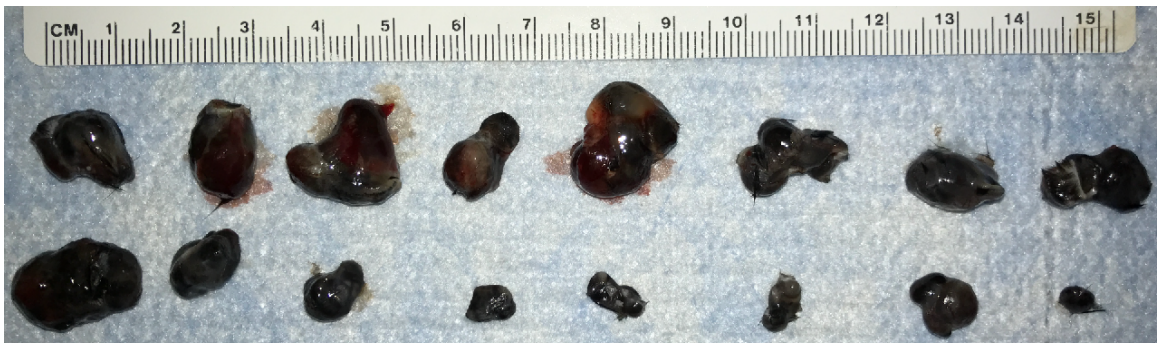


Figure 14B.

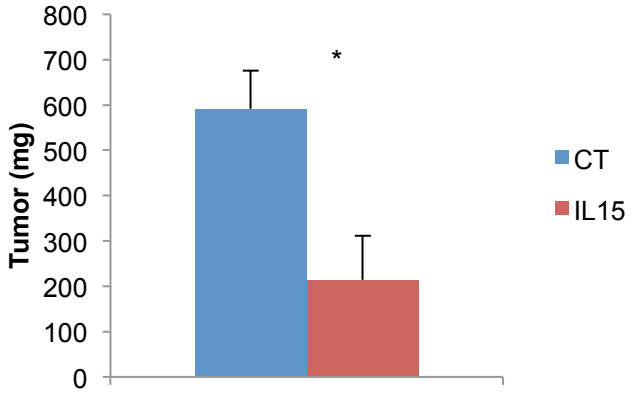
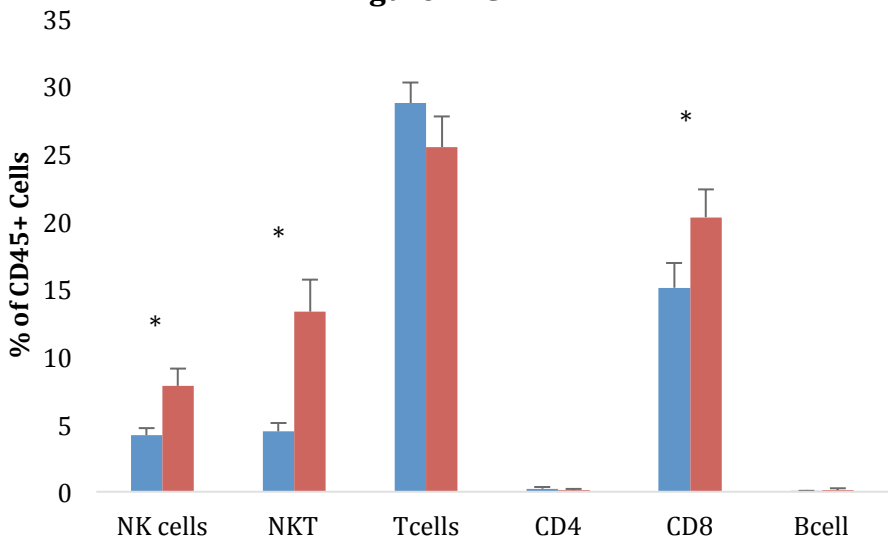


Figure 14C.



Intermediate Conclusion #3: IL-15 overexpression in adipose tissue leads to increased NK cells in adipose tissue and decreased tumor size in local tissue.

(4) EE confers survival benefit in AML

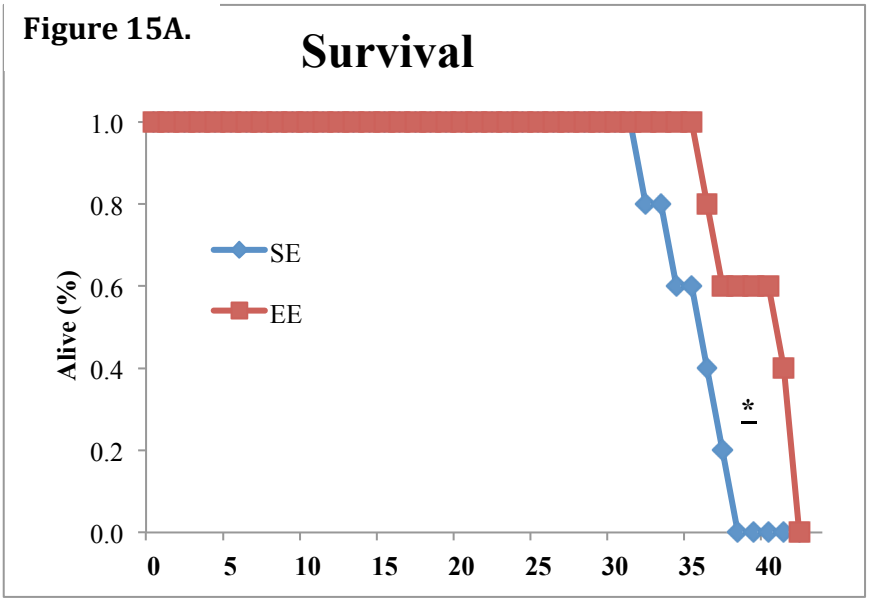
Our lab has had a long-term focus on blood cancers, with much recent effort into acute myeloid leukemia (AML). While our previous studies of EE (and all others in the literature) have shown an anticancer effect in EE for solid tumor models, no studies have focused on blood cancers, such as AML, in EE.

Therefore, we wanted to test whether EE improves disease outcomes for AML in mice, and **we tentatively hypothesized that in an AML survival study mice living in EE would have a survival benefit over SE.**

To test EE's effect on AML, mice with CD45.1(+) immune cells were sublethally irradiated (400 Cgy) to partially destroy immune cells in the bone marrow. Six hours later, we transplanted *Mll*$^{PTD/WT}$:*Flt3*$^{ITD/WT}$ leukemia cells into these mice via tail injection at a dose of two million cells per mouse. (Two million cells from this mouse donor has previously shown a survival of around 30 days.) Mice were randomly assigned to either EE or SE housing following injection.

EE mice had a median survival that was 14% longer than SE mice (Figure 15A). EE mice also had an improved hazard rate, a measure of the rate of death at a given age.

Figure 15A.



We also monitored AML progression through white blood cell (WBC) counts. At both 3 and 5 weeks, EE had significantly lower WBC counts (Figure 15B and 15C). Through video surveillance of mice, EE mice were drastically more active. Overall, these data suggest that EE substantially improves progression-free survival (the length of time in which the disease does not get worse), as evidenced by the differences in WBC counts and mouse activity. But EE gives a modest, but significant improvement in survival.

Figure 15B.

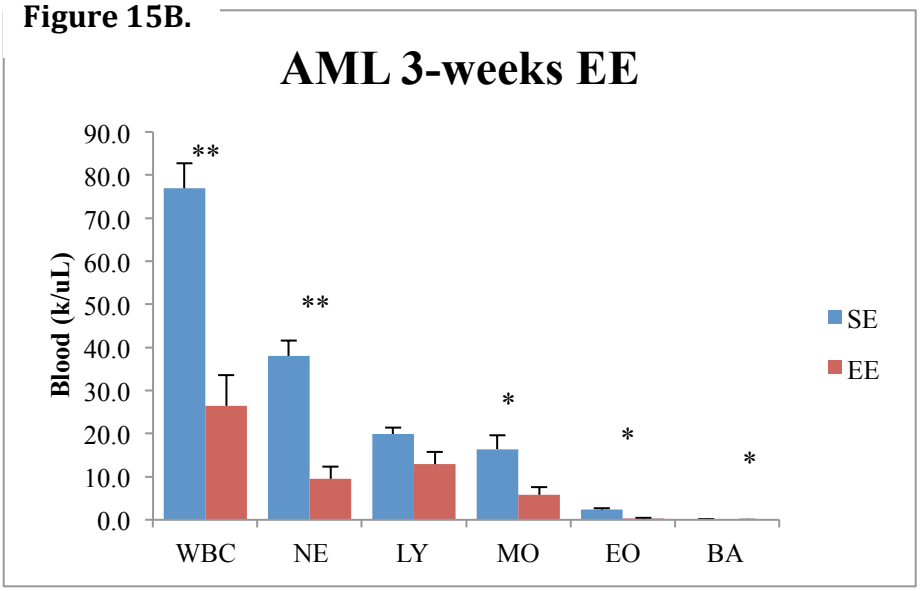
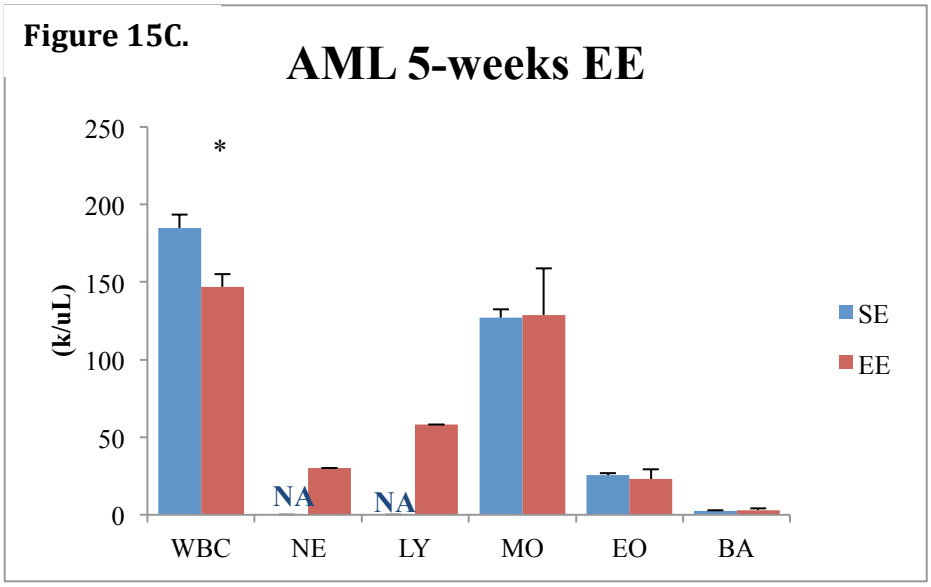


Figure 15C.



Intermediate Conclusion #4: Enriched environment confers a modest, yet significant, survival benefit in acute myeloid leukemia along with a substantial increase in progression-free survival.

Methods:

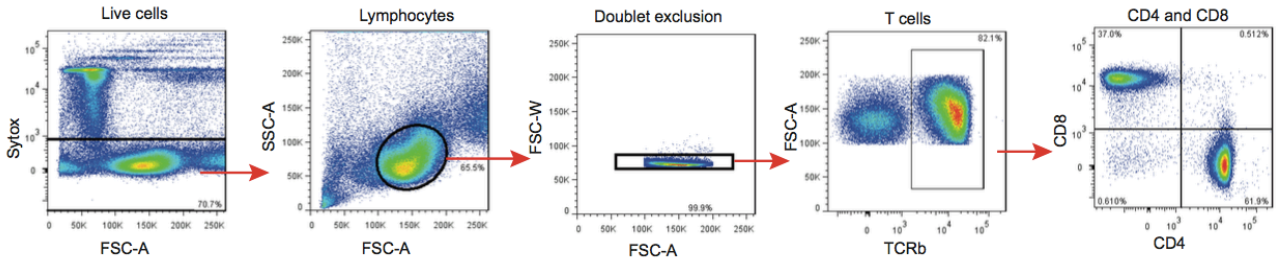
For mouse experiments, male 3-week-old C57/BL6 mice were purchased from Charles River (Spencerville, OH) unless otherwise noted. We carried out all mouse experiments in compliance with the regulations of The Ohio State University Institutional Animal Ethics Committee.

EE protocol

For enriched environment housing, mice are housed in cages 1.5 m x 1.5 m x 1.0 m with 10-20 mice per cage. Cages are supplemented with wheels, tunnels, igloos, huts, retreats, wood toys, a maze, and nesting material in addition to standard lab chow and water. An EE cage can be seen in Figure 16. Both EE and SE mice were housed in temperature- (22-23 °C) and humidity-controlled environments.

Flow Cytometry

Flow cytometry was used to identify populations of immune cells in a mixture via selective antibody staining. Shown below (Figure 16) is the antibody staining used to identify populations of T-cells, specifically CD4 and CD8 T-cells. In all panels, sytox was used to identify dead from live cells. Within the live cell fraction (sytox-negatice), the lymphocyte population was gated based on relative size and granularity standards. Doublets were excluded using a second size gating. For T-cells, all T-cells were defined as TCR-β positive, then further categorized as either CD4 or CD8 positive (with minor populations as either the double-negative or double positive in SLT). For thymocyte classification in the thymus, DN cells are in the lower left-hand quadrant of the CD4 versus CD8 plot, while the DP cells are in the upper right-hand quadrant.



Melanoma Implantation and CD8 T-cell depletion

The mouse B16-F10 melanoma cell line was purchased from ATCC (ATCC CRL-6475^{TM}) in 2014, and culture conditions followed ATCC's instruction. The B16-F10 cell line was expanded in three passages, and the frozen stock was thawed and used for implantation in several experiments. For the CD8-depletion experiment, we housed mice

in their respective environments for 5 weeks and then intraperitoneally injected 0.2 mg of anti-CD8 (BioXcell, clone YTS 169.4.2) or IgG (BioXcell) in 200 μL PBS. The day after antibody injection B16-F10 melanoma cells were subcutaneously implanted on the flank (1×10^5 cells/per mouse, $n = 10$ per group). The antibodies were injected once per week until sacrifice 18 days after melanoma implantation. The tumors were dissected away from neighboring tissues, measured for approximate volume, and weighed.

rAAV Vector Construction and Packaging

The recombinant adeno-associated virus (rAAV) plasmid contains a vector expression cassette consisting of the CMV enhancer and chicken β-actin (CBA) promoter, WPRE (posttranscriptional regulatory element of woodchuck hepatitis virus) sequence, and bovine growth hormone poly-A region flanked by AAV inverted terminal repeats. Human BDNF cDNA was inserted into the multiple cloning sites between the CBA promoter and WPRE sequence. EGFP (enhanced green fluorescent protein) was cloned into the rAAV plasmid as a control. rAAV serotype 1 vectors were packaged, purified and the vectors were adjusted to 2×10^{13} vg/ml in PBS for injection.

rAAV-mediated BDNF overexpression in hypothalamus.
C57BL/6 mice, male, 5 weeks of age, were randomly assigned to receive AAV-BDNF ($n = 5–7$) or AAV-GFP ($n = 5$). Mice were anaesthetized with ketamine/xylazine and secured via ear bars and incisor bar on a Kopf stereotaxic frame. A mid-line incision was made through the scalp to reveal the skull and two small holes were drilled into the skull with a dental drill above the injection sites (−1.2 AP, ± 0.5 ML, −6.2 DV, mm from bregma). rAAV vectors were injected bilaterally into the hypothalamus (0.5 μl per site) at a rate of 0.1 μL per minute using a 10 μL Hamilton syringe attached to Micro4 Micro Syringe Pump Controller (World Precision Instruments, Sarasota, FL). At the end of infusion, the syringe was slowly raised from the brain and the scalp was sutured. Animals were placed back into a clean cage and carefully monitored post-surgery until recovery from anesthesia. After three or five weeks, those mice were sacrificed.

AAV-microRNA Experiment

AAV vectors containing microRNA targeting BDNF (miR-Bdnf) and the scramble control (miR-scr) were previously used in the Cao lab's study of EE's anticancer effect. We randomly assigned 6-week-old male C57BL/6 mice to receive AAV-miR-Bdnf ($n = 15$) or AAV-miR-scr ($n = 15$). We injected 0.7 μL of AAV vectors (1.4×10^{10} per site) bilaterally into the hypothalamus at the stereotaxic coordinates described above. Seven days after surgery, each vector-injected group was split to live in enriched ($n = 8$ miR-Bdnf, $n = 8$ miR-scr) or control housing ($n = 7$ miR-Bdnf, $n = 7$ miR-scr). Mice were sacrificed after five weeks in the EE.

Statistical analysis

All data were compared using Student *t*-test and plotted as mean ± SEM in column charts.

Discussion:

Overall, our results have shown a substantial change in T-cell development, migration, and function in EE. Our preliminary data showed that CD8 T-cells are required for EE's anticancer effect. Furthermore, we described a shift in T-cell populations within SLT toward CTLs. This shift in T-cell populations is early and consistent and is therefore likely to lead EE's T-cell-mediated anticancer effect. Furthermore, through depletion and overexpression experiments, we showed that BDNF is a mediator of this T-cell change. Through blockade of both SNS and HPA axis, we further showed that both of these stress axes mediate EE's T-cell change.

Since T-cells develop in the thymus, we hypothesized that EE exerts this T-cell phenotypic change in SLT through developmental shifts in the thymus. EE thymuses undergo thymic involution. Within the T-cell developmental pathway, there was a higher percentage of thymocytes in DN and CD8 stages and fewer in the DP population. This shift indicates pushing cells out of the intermediate double-positive stage toward CD8 single positive cells, which could contribute to the SLT phenotype of a decreased CD4:CD8 ratio. Furthermore, we sought to connect the thymic developmental changes in EE with BDNF and potential SNS and HPA control. Through similar overexpression and depletion experiments of BDNF, we showed that BDNF mediates EE's changes in T-cell populations in the developing thymus as well. Furthermore, we showed that HPA axis, and not SNS, controls the thymocyte shift. To more specifically test this connection, we used a mouse which deletes glucocorticoid receptor in all cells after the DN stage. There was no difference in EE thymocyte populations compared with SE, further confirming our hypothesis that EE works through HPA axis to mediate thymus changes.

Yet, CD8 T-cells are just one portion of the story. Our research into adipose tissue has shown us that EE confers a complex change in the immune microenvironment that is fat depot-specific. We have seen significant changes in innate immunity, notably an increase in NK cells. We hypothesized that recruitment of NK cells to adipose tissue could result in increased killing of tumor cells of a nearby tumor. To test this hypothesis we utilized the Cao lab's recently developed adipose tissue-specific AAV vector. We inserted IL-15 into the vector, which resulted in recruitment of NK cells specifically to the tumor and decreased tumor mass, compared to control.

With these experiments on solid tumors, there is still much to do. Our preliminary results studying AML showed a slight increase in survival for mice housed in EE with a significant increase in progression-free survival. It is still unknown what causes this increase in progression-free survival but then a steady decrease in survival of EE mice. Furthermore, we do not yet know whether T-cells, NK cells, or, most likely, some combination of several immune cells coordinates the increase in progression-free survival.

This work, and this entire collaboration, shows the importance of a model of eustress in the study of cancer. While clinical studies have begun to study the positive effects of a supportive environment, laboratory and animal-model studies tend to focus on distress, such as social isolation, to see how these environments make cancer worse. Our model uniquely studies how cancer outcomes can be improved. EE is applicable to phenotypically diverse cancers, including both solid-tumor and blood cancers.

Furthermore, our mechanistic studies create an opportunity for the manipulation of a single gene, BDNF, in the brain to better cancer outcomes.

Conclusion:

Cancer is a heavily studied, multifactorial disease with an incidence of nearly 1.7 million in the U.S. alone. Recent clinical evidence suggests a positive effect of the social environment on cancer outcomes.Yet, animal model experiments have mainly focused on the negative effects of distress or social isolation on cancer outcomes. Our model of social enrichment, an enriched environment, is a model of eustress, and we uniquely study the positive effects of the social environment on cancer outcomes. We have previously shown that EE exerts an anticancer effect. Here, we show that CD8 T-cells at least partially mediate this response, though they are not the entire story of EE's anticancer phenotype (Figure 3). Furthermore, we show a shift to CD8 T-cells in secondary lymphoid tissue that is mediated by a molecule in the hypothalamus, brain-derived neurotrophic factor, which we had previously been shown to mediate EE's anticancer effect in general.

We then sought to understand the T-cell developmental changes that lead to the change in the CD4:CD8 ratio in SLT. We showed a shift in thymocyte development toward single-positive CD8 thymocytes in EE that is also regulated by BDNF. We show through a genetic knockout mouse that EE's thymocyte development changes are mediated by the hypothalamic-pituitary-adrenal axis, specifically through binding of glucocorticoids to glucocorticoid receptor on developing thymocytes.

We have further studied the complex effect of EE on the adipose tissue microenvironment, specifically on natural-killer cells. Here, we show that adipose tissue-specific IL-15 overexpression results in increased NK cells in adipose tissue as well as increased NK cells at a nearby tumor and decreased mass of the nearby tumor.

Finally, we extend the current knowledge of EE's anticancer effect to a blood cancer, acute myeloid leukemia, in which we see a substantial increase in progression-free survival but a modest, yet still significant, increase in overall survival.

These results together show the complex connection between the environment, the nervous system, the immune system, and the cancer microenvironment in mice. The continual regulation of EE's anticancer effect by BDNF suggests the possibility of manipulating a single gene in the brain for beneficial outcomes in certain cancers. Our model shows the importance of studying the positive effects of the environment on cancer outcomes as a model for human disease.

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The experiments outlined in this thesis are the result of an extensive collaboration between the labs of Michael Caligiuri, MD and Lei Cao, PhD. Having them both as experienced mentors has helped me gain experience as a scientist. Stephen Bergin has been my direct mentor in the Caligiuri lab, and his expertise and mentorship have also been invaluable. As he went through his PhD and I went through my undergraduate years, he and I have learned together.

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I am very excited for the future of my medical career at the University of Chicago Pritzker School of Medicine, where I will undoubtedly continue the scientific pursuits I have begun in this lab. Thank you to everyone who has helped me along the way.