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Young Germ Cell Depleted Ovaries in Post-Reproductive Mice and Its Effects on Immune Function

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**YOUNG GERM CELL DEPLETED OVARIES IN POST-
REPRODUCTIVE MICE AND ITS EFFECT ON IMMUNE
FUNCTION**

by

McKenna Walters

**Capstone submitted in partial fulfillment of
the requirements for graduation with**

UNIVERSITY HONORS

with a major in

**ADVS: Bioveterinary Sciences
in the Department of Animal, Dairy, and Veterinary Sciences**

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Abstract

It has previously been shown that young, cycling ovarian transplantation in aged female mice increased the general health and life span in regard to their post-reproductive health. It has further been hypothesized that this enhancement of health is directly influenced by the ovarian somatic cells. To address this hypothesis, transplants of young germ cell depleted and germ cell containing ovaries were performed on female mice. The purpose of this study is to continue to discern the reproductive influence on aging health, specifically in the area of immunological well-being. Control group mice were separated by age and treatment mice were subsequently age matched to receive either germ cell depleted or germ cell containing ovary transplantations. All groups underwent various health span assays including immunoassays using flow cytometric analysis to determine T-cell subset alterations. Data collected from the immunoassays were analyzed with two-factor ANOVA and a Tukey-Kramer post-hoc test to determine any difference between groups. Ratio differences and trends were analyzed between the groups and between central naïve and central memory T-cells. Results showed that the group having received germ cell depleted ovaries displayed a shift in the central naïve to central memory ratio to an increased central naïve population, thus indicating an improvement of immunological function as compared to the other test groups. This may indicate that the ovarian somatic cell participates heavily in the regulation of age-associated health.

Keywords: germ cell depleted (GD); germ cell containing (GC); ovarian somatic cells

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Introduction

The concept of health and the onset of disease relies on a variety of factors, but the maintenance of good health in an individual relies heavily on the immunological systems of the body. Following the reproductive decline and end of cycling that occurs in aging women, also known as menopause, the risk of contracting or developing disease increases substantially as the effectiveness of the immunological systems also decline. The immune system is responsible for recognizing, regulating, and eliminating or deactivating factors that cause disease within the body such as infections, common illnesses, and many severe diseases including cancer. This diminishing of immunological health leads to a rapidly decreased quality of life for women everywhere.

The effect of aging on the immune system includes the reduced production of T-cells in the thymus. As a result, aging individuals do not respond to challenges to the immune system as efficiently as young individuals do. A well-known feature of aging is the increase in number of memory T-cells. Memory T-cells are generated following presentation of an antigen, (or foreign substance which induces the immune response), and remain within the body for long after the problem has been solved. These T-cells can then respond quickly to any re-exposure to said antigen and aid in clearing out the infection or begin the signaling of other disease-battling cells (Montecino-Rodriguez *et al.*, 2013). On the other hand, aging individuals also begin to experience decreased output numbers of naïve T-cells. Naïve T-cells are those which have never been primed by an antigen before. These T-cells are important for identifying and fighting new pathogens that may cause disease or infection (Salam *et al.*, 2013). Maintenance of both naïve and memory T-cells is critical for overall immune function and health.

There are several forms of T-cells with varying functions. CD8 T-cells, also called cytotoxic CD8 T-cells, play a critical role in the destruction of cells that may cause infection, including cells containing infectious viral or bacterial agents, as well as cancer cells. CD8 cells are very efficient at killing infected cells without killing surrounding, healthy cells and causing tissue damage (Janeway *et al.*, 2013). A second, extremely important type of T-cell is the CD4 T-cell. CD4 T cells are of singular importance in regard to the humoral response. CD4 T-cells may be activated or they may differentiate into other forms of effector cells that act in immune function, as well as lead to the production of antibodies by B cells. Another aspect of aging is a significant decrease in CD4 T-cell population. This diminishing of population can cause lead to susceptibility to a wide variety of infectious diseases and inability to respond effectively to vaccines (Lefebvre and Haynes, 2012). Therefore, a healthy population of naïve CD4 T-cells, along with balanced populations of other corresponding T-cell subsets, is a fairly reliable indicator of overall immunological health.

Reproductive health has previously been shown to be influential upon general health. The components of reproductive health within women is very complex. For our studies, we focused upon the ovarian function as it is essential for reproductive cycling. The ovary consists of two main cells: the germ cells and the somatic cells. The germ cells are that which we often refer to as “the eggs” of an ovary. Germ cells function in fusing with sperm to generate a zygote. The somatic cells of the ovary are the “body cells” which make up the ovarian pouch and support the germ cells. It has previously been shown that in primitive organisms, longevity was increased when their ovarian germ cells were depleted (Martins *et al.*, 2016). This presumably indicates that the somatic cell is an influential factor on longevity. In our recent studies, post-reproductive mice that had received transplantations of young ovaries were shown to have extended longevity

and several improved aspects of health including increased populations of naïve CD4 T-cells, indicating an improvement in immunological health. For this study we brought together these two ideas and transplanted young, germ cell depleted and germ cell containing ovaries into post-reproductive female mice. As compared to mice with germ cell containing ovaries, the longevity of mice with germ cell depleted ovaries experienced a further longevity (Peterson *et al.*, 2017). The reason for this influence of germ cell depletion and young ovarian somatic cells on post-reproductive longevity and health is not clearly understood as of yet, but the purpose of this study is to further derive its influence on immunological function, specifically. We have hypothesized that in a form of trade-off, the lack of ovarian germ cells stimulates the ovarian somatic cells to support the overall organismal health rather than the germ cells while not reproducing, resulting in improved immune function.

Materials and Methods

Animals

CBA/J strain female mice were chosen for this study as a result of their early ovarian follicle loss which occurs around three hundred days of age (Thung *et al.*, 1956; Jones and Krohn, 1961; Faddy *et al.*, 1987). This quality made CBA/J strain mice a good model organism for our study of menopause and age-related conditions, as loss of ovarian follicles is associated with menopause in humans.

Female CBA/J mice of ages seventy-five days, two-hundred days, five-hundred days, and four-hundred and fifty days were obtained from Jackson Laboratory (Bar Harbor, ME). The mice were individually housed in ventilated cages (Green Line IVC Sealsafe Plus, Tecniplast, West

Chester, PA). Each cage included corncob bedding (7097 Corncob, Harlan Teklad, Bartonville, IL), deionized water, laboratory rodent diet ad libitum (2018 Teklad Global 18% Protein Rodent Diet, Harlan Teklad, Bartonville, IL), and other added enrichments. The laboratory environment consisted of fresh filtered air (15 changes per hour), temperature of $21 \pm 2^{\circ}\text{C}$, humidity of $50 \pm 20\%$, and a light-dark cycle (12:12 h) in a specific pathogen free colony. The mice were placed in an American Association for Accreditation of Laboratory Animal Care (AAALAC)-approved facility using the National Institute of Health's animal-use guidelines. All mice were cared for under the Utah State University Institutional Animal Care and Use Committee (IACUC-2277) guidelines.

Anesthetics were utilized during the surgeries of both ovary donor and ovary recipient mice. Analgesia was also provided to ovary recipient mice for at least forty-eight hours post operation or longer when necessary. Euthanasia was administered via cervical dislocation and a thoracotomy was performed immediately after euthanasia, followed by rapid exsanguination via cardiocentesis in the donor mice. Mice that experienced acute weight loss were treated with subcutaneous fluids and moistened food. Mice with rectal/vaginal prolapse or acute urine staining were manually cleaned and treated with Desitin®. The mice were monitored twice daily or more and weights were recorded at least monthly, with more frequent recordings where concerns occurred. Aged, moribund mice that exerted overt clinical signs (catatonia) were promptly euthanized. The criteria for euthanasia for aged mice was determined with the help of the attending veterinarian and included, but was not limited to, mice found in poor condition with or without crusting around the perineum, diarrhea, urine staining, persistent vaginal prolapse, chronic vulva/rectal swelling, kyphosis, respiratory distress, anorexia, poor coat condition with noticeable lack of grooming, moribund mentation, hind-limb paresis, wounds not

healing, neoplastic growths, and unusual weight loss or gain. It is understood that aged CBA/J female mice experience an average weight loss from peak weight to death of 12% per month (Mason *et al.*, 2010). An increased rate of weight loss, (not total weight loss), was the most critical factor for determining a moribund state. Unexpected deaths occurred uncommonly and included neoplastic growths, decubitus ulcers, and uncontrolled cataleptic seizures.

Experimental Design

Each of the four-hundred day old female CBA/J mice were randomly selected for germ cell depleted transplant recipients, germ cell containing transplant recipients, and control groups.

The control group mice consisted of the following individuals:

- One-hundred-day-old reproductively cycling mice (n = 19)
- Two-hundred-day-old mice whom were both cyclic and acyclic throughout the course of the study (n = 10)
- Four-hundred-day-old acyclic mice (n = 5)
- Six-hundred-day-old acyclic mice (n = 3)

The ovarian transplant mice consisted of the following individuals:

- Four-hundred-day-old mice whom were acyclic at the time of surgery and received sixty-day-old ovaries from cycling donor mice (n = 9)
- Four-hundred-day-old mice whom were acyclic at the time of surgery and received sixty-day-old germ cell depleted ovaries from cycling donor mice (n = 11)

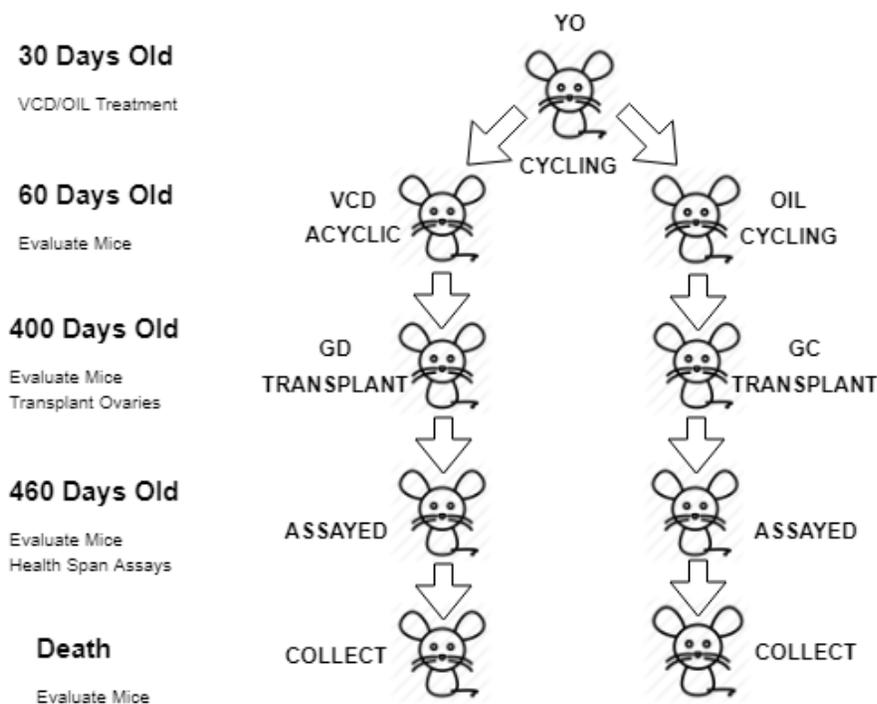


Figure 1. Experimental design. Thirty-day old mice (YO) were treated with VCD or sesame oil for fifteen days. At 60 days of age, germ cell depleted (GD) and germ cell containing (GC) ovaries were transplanted into four-hundred-day-old mice. After a month of recovery time, all groups were evaluated and underwent health span assays, including the immunoassay. Following assays, mice were collected at death for further evaluation.

Age at Ovarian Manipulation

The donor mice were of twenty-eight days of age and were randomly chosen to receive placebo injections of sesame oil or treatment with 4-vinylcyclohexene diepoxide (VCD) to germ cell deplete the ovaries. The germ cell depleted donor mice received intraperitoneal injections of 160 mg/kg VCD daily for fifteen days, as a fifteen-day time frame was previously shown to stop reproductive cycling and deplete ovarian follicles (Mason *et al.*, 2018).

Mice do not experience menopause as humans do, rather they undergo an estropause-like decrease in reproductive function. This reproductive decline usually begins in CBA/J strain mice at around two-hundred and forty days of age and most are reproductively incompetent by three-hundred and thirty days of age (Cargill *et al.*, 2003). Female CBA/J mice generally become

reproductively active between forty-five and sixty days of age. We manipulated the ovaries at twenty-eight days of age to avoid any dramatic up-regulation of the reproductive system as is found at the onset of puberty, and to eliminate alternative influences the ovaries may have in addition to direct hormonal effects.

Ovarian Transplantation

The germ cell containing and germ cell depleted donor mice were anesthetized and the ovaries were extracted when they reached sixty days of age and were actively cycling. Microscopic analysis of vaginal lavage were used to determine cycles. After ovarian removal, the donor mice were euthanized using the previously mentioned methods.

The germ cell containing and germ cell depleted ovary recipient mice were anesthetized and had the ovarian transplant surgery using donor ovaries as previously described (Mason *et al.*, 2018; Cargill *et al.*, 2003). In brief, a vertical incision was made in the lateral aspect of the abdomen between the ribs and coxa. The ovarian fat pad was identified and removed into a field of view outside of the body cavity. A small opening was made in the ovarian bursa and the ovary extracted. The donor ovary was then placed into the emptied bursa. The bursa and abdominal cavity were then sutured closed and the skin stapled closed. This procedure was performed on both left and right ovaries. All recipient mice were allowed one month of recovery before beginning assays.

Immunoassay

Change in immune function was evaluated by measuring the alterations in T-cell subsets present in the blood from the mice in each group (Figure 2). About 200 μL of whole blood was collected in heparin-treated blood tubes from the superficial temporal vein after a 4-5 hour fast, as older mice do not fare well following longer or overnight fasting. The blood cells were processed and immunostained using a Mouse Naïve/Memory T-Cell Panel (BD PharMingen) including anti-mouse CD4, anti-mouse CD3, anti-mouse CD62 L, and anti-mouse CD44, as well as stained with a Fixable Live/Dead viability dye (Life Technologies). Flow cytometric analysis of the samples was subsequently performed by Dr. Chris Davies using standard settings on a SORP FACSAria II Flow Cytometer (BD Biosciences). The samples were then analyzed using FACSDiva Version 6.1.3 (BD Biosciences) and reported as CD4 and CD8 central naïve, central memory, peripheral naïve, and peripheral memory T cells, as well as the ratio of CD4 central naïve to central memory cells (Figure 3).

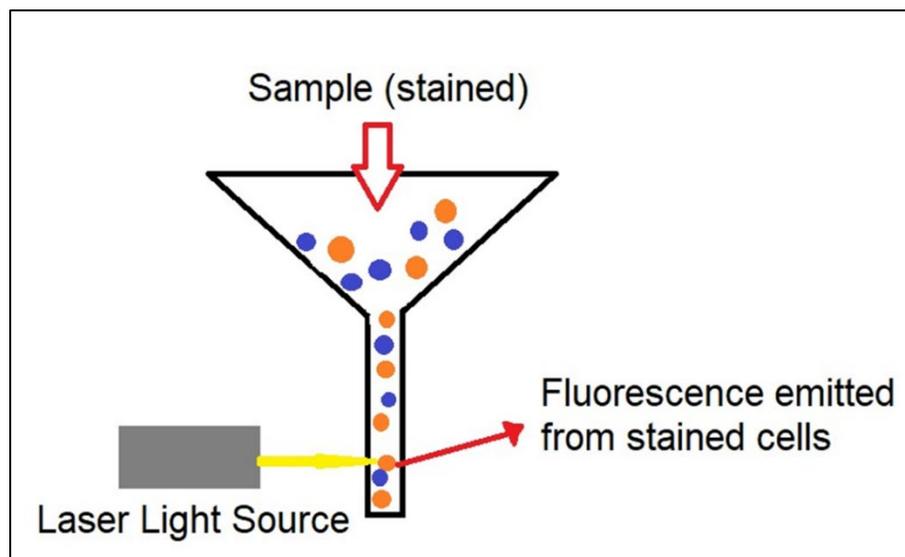


Figure 2. Simplified schematic of the process of flow cytometric analysis. Whole blood samples containing immunostained blood cells were used for the analysis. Example of results found in Figure 2

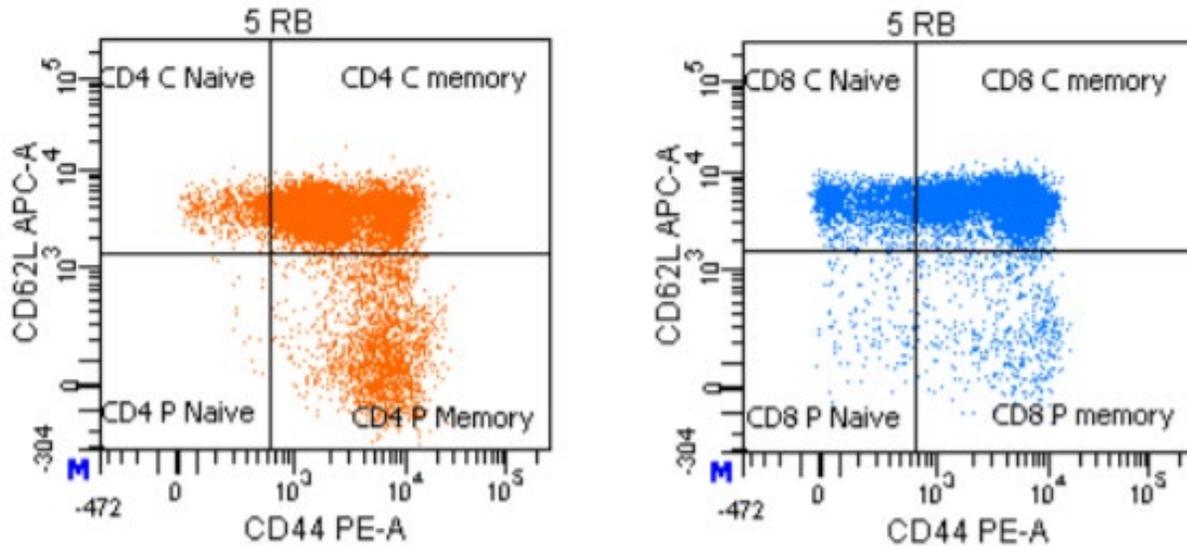


Figure 3. Results of T-cell subset assay: following flow cytometric analysis, ratios of central naïve and central memory cells of both CD4+ and CD8+ T-cells were determined. Central naïve cells were selected with high CD62 L and low CD44, central memory cells with high CD62 L and high CD44. Peripheral naïve cells were selected with low CD26 L and low CD44 and peripheral memory cells with low CD62 L and high CD44.

Statistical Analysis

Statistical analysis was performed with GraphPad Prism 7.04 (GraphPad Software, Inc., La Jolla, CA). Normality was determined using a D'Agostino-Person omnibus test. The data was also analyzed with a two-factor ANOVA and a Tukey-Kramer post-hoc test to determine any difference between groups. Student's two-tailed t-test was performed on individual treatments, assuming unequal distribution of variance. Any test result with $P < 0.05$ was considered significant.

Results

The ratio of central naïve cells to central memory cells were used as a measure of immune function in this experiment (Figure 3). These ratios are expected to decrease with aging, and it was found that our 575 day old females had significantly decreased ratios in both CD4 and CD8 T-cells (Figures 4 and 5). Following analysis, we determined that regardless of germ cells status within the ovary, transplantation of new ovaries improved the central naïve to central memory T-cell ratio significantly, though the germ cell depleted ovary recipient ratios experienced a more noticeable effect. Also notable is the fact that this experiment increased the general population of CD4 T-cells in the aged test mice, which is an indicator of improved immune function (Figure 6). These results indicate that the somatic cells of the ovary may play an important role in age-associated regulation of health.

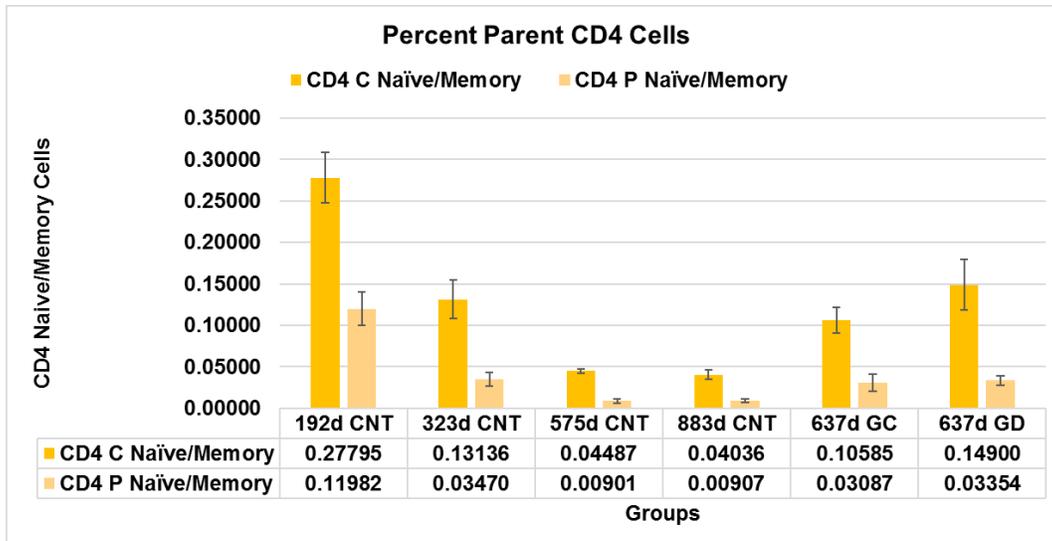


Figure 4. CD4 T-cell subset changes with age. CD4 naïve cells decreased with age as is demonstrated by decreased central naïve cells and decreased naïve to memory cell ratio in CD4 T-cell populations. Ratios were determined in control (CNT), germ cell containing (GC), and germ cell depleted (GD) mice of varying ages. GC and GD mice both experienced increased ratios, especially in central naïve to memory ratios.

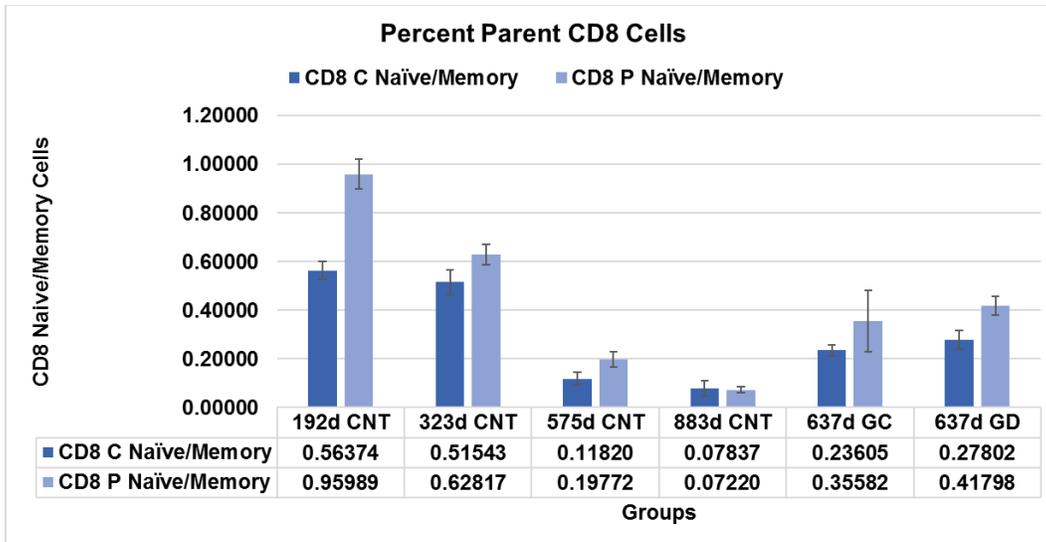


Figure 5. CD8 T-cell subset changes with age. CD8 naïve cells also decreased with age as demonstrated by decreased central naïve cells and decreased naïve to memory cell ratios in CD8 T-cell populations. GC and GD mice experienced increases in both central and peripheral memory to naïve ratios.

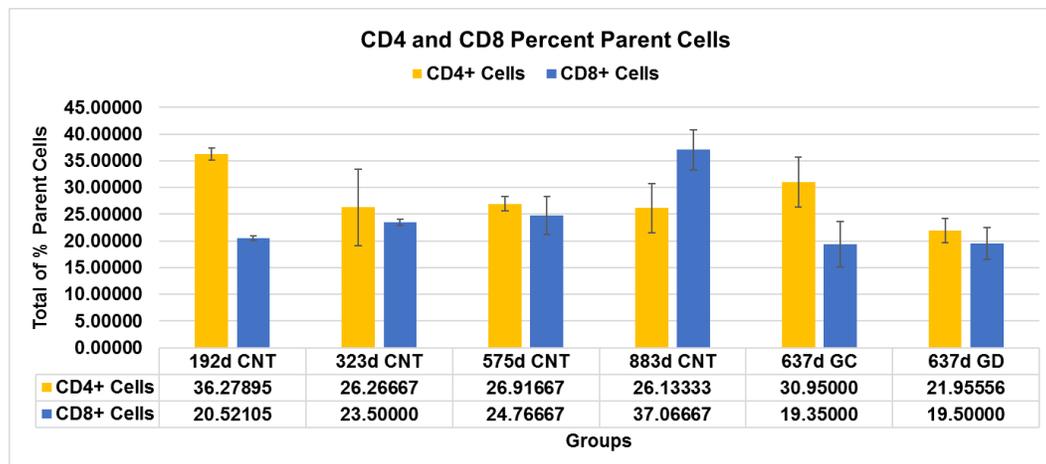


Figure 6. Percentages of parent cells for CD4 and CD8 cells. Aging mice experienced a general decrease in CD4 T-cells as compared to total parent cells. GC and GD mice showed an increase in percentage of CD4 cells in comparison to parent cells.

Discussion

As mentioned earlier, by six months of age, female CBA/J mice are considered reproductively competent and are reproductively cycling. At twelve months of age, ovaries in the same mice will have become senescent and will have stopped reproductively cycling. In this experiment, a group of twelve-month-old mice received young ovaries from two-month-old donors. As a result, six-month-old control mice and sixteen-month-old recipient mice were both in possession of six-month-old ovaries and it was found that both groups generally benefitted from the effects of young ovarian function.

The immune system is reliant upon many factors, but the predominant cells of function are the T-cells. The T-cells are responsible for recognizing, attacking, and otherwise regulating infection-causing pathogens and other various diseases. Maintenance of the CD4 T-cell population is especially important for the upkeep of adaptive immunity, which remembers pathogens it has been previously introduced to and prevents the body from becoming sick from them again. The CD8 T-cells have a significant job in that they destroy many diseased cells including cancer cells. Aging is known to cause a general reduction in protective immunity which can lead to inadequate responses to vaccines, increased susceptibility to disease, and inability to prevent or fight infection. There is evidence suggesting an age-associated decline in the naïve subset of T-cells and a subsequent increase in memory T-cells that have gone through at least one cycle of antigen-stimulated activation and proliferation (Montecino-Rodriguez, *et al.*, 2013). Maintenance of naïve T-cell numbers is critical for response to new or introduced disease pathogens, as well as for general immune function upkeep. Re-establishment of healthy naïve T-cell numbers in aging individuals could aid in the continued defense against pathogens and result in overall improvement of health and quality of life.

In this study, it was noted that there was a significant age-associated reduction in central and peripheral naïve T-cell subsets from six to sixteen months of age. This decline was notably improved by ovarian transplantation within the sixteen-month-old females. Unfortunately, the study was limited to a small number of animals per treatment group, which is not unusual for aging studies, but it should be noted that even the small number of animals resulted in large, significant differences between groups.

Conclusions

To summarize, our study has shown that immune function, which is crucial for maintaining health and battling disease, is affected strongly by aging in a negative manner but can be positively influenced by the transplantation of young, germ cell depleted ovaries in post-reproductive mice. The findings of this experiment suggest that this positive influence may be due to the somatic cells of the ovary. Though the effect of the ovary on health and longevity is not well known and is being continuously debated, we believe that these findings provide a basis for further studies involving the effect of young ovaries on restoration of health in post-reproductive females.

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Reflection

This project has been a large part of undergraduate career. I joined Dr. Mason's lab when I was a freshman student and I have enjoyed the work so much that I've stuck with the lab for all four years of my education at USU. We have been through multiple phases of the project including determination of lifespan and the effects on health of ovarian transplant recipients, all leading up to this project and the experiments that are now being conducted to continue the work and derive the causes of this positive influence. It is our hope that eventually this work will be applied to women's health, increasing the quality of life of post-menopausal women everywhere. I'm incredibly proud of the progress that has been made so far. My dream is to one day hear about some progression of this current project being applied and aiding women in regaining health, and being able to say that I was a part of that project in its early stages!

There are so many different aspects of health and several of these aspects were being tested in the lab, so we decided I should focus on just one topic for this capstone project. This is how I ended up studying the effects of the germ cell depleted ovaries on immunological health. I knew when I was assigned this topic that I didn't have a very broad knowledge of the immune system, only the basics that I had picked up in some of my other biology courses. I decided that it would be beneficial for me to take a course on immunology both for this project as well as to prepare me for future veterinary immunology courses. I took the course in the fall, along with the PhD candidate student that I work under in the laboratory, and it was absolutely beneficial to my education, my capstone project, and my own understanding of health and immunity. During this time we were already in the process of the experiment and performing immunological assays, but it wasn't until I completed the course and did some extra research that I fully understood what the assay results were showing us.

I joined Dr. Mason's lab in hopes that I would receive some research experience specifically in the area of veterinary medicine, as Dr. Mason is a professor of the WIMU veterinary program at USU. What I found is that I was able to gain both veterinary experience through doing research with the mice that we worked with, as well as human health experience as our animal research always relates back to women's health. Before I came to USU, I actually didn't know that research was such a big part of veterinary science. I mainly had dreams of working in a clinic at on my family's ranch back home. I quickly fell in love with the research process and I'm now hopeful that I will be able to continue research in my future career, even while I'm working in a clinic. Working in a research lab is a very different experience from working within a biology or chemistry lab for courses. However, it's also very similar because we use many of the techniques that are gained from course labs. I couldn't be more grateful for my experience in the lab because it taught me how to apply my skills from my courses to "real life" situations. I find that sometimes, even if I am excelling in a course, I might not see the whole picture of the importance of the knowledge I'm receiving. Working within Dr. Mason's lab was highly instrumental in aiding me to make connections between gaining an education and utilizing my education.

One of the best things that I gained from working in the lab were close relationships with both Dr. Mason and the other members of the lab. Dr. Mason has been a wonderful mentor who has provided a million opportunities to each of his students along with never-ending support. He often leaves the members of the lab with a lot of freedom to work how they choose, so long as the job gets done, and that worked well for me. He was always patient in trying to guide me through lab projects, and his graduate students and laboratory technicians were extremely capable and knowledgeable – Dr. Mason seems to just attract excellence. His friendly lab

atmosphere also facilitated many friendships. In fact, a few of my best friends and favorite college experiences have come as a result of working in the lab. I have been accepted to the WIMU program and begin in the fall. My lab experience contributed highly to my acceptance, I know, and I'm grateful for that! I'm also glad because Dr. Mason will be one of my professors within the program, meaning that I will be able to continue to benefit from his mentorship and support throughout the next two years. I will also be allowed to participate in summer research within Dr. Mason's lab, which means that I can continue to be a part of his exciting projects and aid him in this project that means so much to me.

All of the time spent on the various research projects in Dr. Mason's lab was well worth it to me. I got everything that I hoped to out of the experience and so much more. I was provided an incredible amount of opportunities for hands-on veterinary experiences and I have participated in things that not many people can say that they have. I received a wonderful education through the ADVS program at USU, but I think that my time in the lab contributed the most to my future career because it put me in the position of really applying what I know and forced me to find my own answers to problems. This project was very rewarding to me as a student because I was able to follow an idea from beginning to end and see some results. It was also rewarding to me as a woman because I feel like I may be making a difference in the future lives of women of society. I know many women personally who would benefit from research like this if we are able to continue the process and bring it to humans. I am extremely grateful that I had the opportunity to be a part of such an interesting and potentially beneficial project for so long, and I hope that it continues to progress and eventually do wonderful things for society. My experience as an undergraduate worker has done nothing but good things for me, and I will cherish it for as long as I live.