THE EFFECTS OF CALORIE RESTRICTION AND AGING ON NATURAL KILLER CELLS IN C57BL/6 MICE

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ABSTRACT

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Calorie restriction extends lifespan in a variety of species, even though it may be detrimental to the innate immune response. This research focuses on natural killer (NK) cells in aged (22 mo) calorie restricted (CR) C57Bl/6 mice. NK cells are innate immune cells capable of killing tumor or virus-infected cells. We used flow cytometry to analyze NK cell maturation in young *ad libitum* (AL) fed, young CR, aged AL, and aged CR mice. Aged CR mice had significantly reduced levels of terminally mature ($CD27^-$ $CD11b^+$) NK cells and had increased expression of the immature marker CD127 and decreased expression of the mature marker DX5. We also compared NK cells from 22 mo old aged mice that were calorie restricted from either 4 months of age (young-onset CR) or 20 months of age (old-onset CR). The total number of NK cells among live cells was significantly lower in the lung and spleen of old-onset CR mice compared to aged AL mice, while there was no significant difference between young-onset CR and aged AL mice. Old-onset CR mice also had significantly less early mature ($DX5^+$ and $CD27^+CD11b^+$) NK cells. Overall, we found that CR in aged C57Bl/6 mice is detrimental to the maturation of NK cells, which is exacerbated when CR is initiated in old-age.

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KEY TO ABBREVEATIONS

ACK	Ammonium chloride potassium
ADCC	Antibody dependent cellular cytotoxicity
ANOVA	Analysis of variance
AL	*Ad libitum*
BM	Bone marrow
CALERIE	Comprehensive assessment of long-term effects of reducing intake of energy study
CR	Calorie restriction
HSC	Hematopoietic stem cell
HAU	Hemagglutinating units
IFN-γ	Interferon gamma
IL	Interleukin
KIR	Inhibitory Ig-like receptors
KLR	Inhibitory killer-like lectin receptors
M6P	Mannose-6-Phosphate
ND	No data
NIA	National Institute of Aging
NK cell	Natural killer Cell
NKP	NK cell precursor
NOS	Nitric oxide synthase
NS	Not significant
SIRT	Sirtuin

Th1	T-helper 1
Th2	T-helper 2
TNF-α	Tumor necrosis factor alpha
TRAIL	Tumor necrosis factor-related apoptosis-inducing ligand

CHAPTER 1- LITERATURE REVIEW

1. Aging and influenza demographics

Currently, the aged (65+) population in the United States is growing at a faster rate than the rest of the U.S. population, this trend is expected to continue into the coming decades (Cherlin, 2010). This is important because aged populations are more susceptible to certain diseases including seasonal influenza. In fact, those over 65 years of age account for 60% of influenza related hospitalizations and 90% of influenza related deaths (CDC). This is in part due to immune senescence, which refers to the decline of the immune system with aging. Calorie restriction is a paradigm that has been shown to prevent immune senescence. Despite these findings, calorie restriction is detrimental when facing influenza infection (Gardner, 2005). Calorie restriction, influenza, aging and their relation to natural killer cells are discussed in the next sections.

2. Calorie restriction (CR)

2.1 History of CR benefits

Almost 100 years ago, Rous studied transplanted tumors in underfed mice. Following these experiments (Rous, 1914), scientists began researching calorie restriction, defined as reduced caloric intake without malnutrition. Frequently, at higher levels of calorie restriction, additional vitamins and minerals are provided to ensure proper nutrition. In the 1930's CR was shown to increase median and maximal life span in rats by McCay et al. (McCay et al., 1935). Since this report, longevity studies in yeast, flies, worms, fish, and rats have also proven life extension benefits with CR (Heilbronn and Ravussin, 2003). The question remains whether or not calorie restriction could extend human life.

Answers to this question are beginning to come to light; two research groups began using non-human primate models to determine whether CR results in life extension in a species evolutionarily close to humans. Many promising results have surfaced from these experiments in regard to overall health of non-human primates. A 20-year longitudinal study on rhesus monkeys conducted at the Wisconsin National Primate Research Center (WNPRC), declared that calorie restriction reduces the incidence of cancer, diabetes, cardiovascular disease, brain atrophy, and age-associated death in rhesus monkeys (Colman et al., 2009). The authors concluded that CR delays mortality and the onset of aging in a primate species. However, it is important to note analysis of these data only included monkeys who had died from age-related disease. Another figure in this report shows no significant difference in all-cause mortality when comparing CR monkeys to *ad libitum* (AL) fed monkeys.

A second study released in 2012 was conducted by the National Institute of Aging (NIA), and also examined CR in rhesus monkeys for over 20 years (Mattison et al., 2012). This group demonstrated many positive benefits of CR on health in a primate species, including decreased risk of cancer, controlled weight, and decreased serum triglycerides, cholesterol, and glucose. However, in contrast to the WNPRC study, the NIA-sponsored study did not show a decrease in age-associated mortality, cardiovascular disease, diabetes or the occurrence of age-associated morbidities. This research group concluded that both young-onset and old-onset CR in rhesus monkeys did not cause life extension. How could the results of two studies using very similar research conditions have such opposite results?

The scientists at the NIA conclude the differences lie in the variations between the two protocols. For example, the NIA restricted their control monkeys 10% in order to prevent the primates from becoming obese, whereas the WNPRC fed their control monkeys *ad libidum* (AL). This restriction implemented in NIA control animals may have led to an advantage over WNPRC monkeys in terms of survival. The diet compositions were also different; the WNPRC diet was derived from purified compounds while the NIA diet was derived from natural compounds which may contain trace minerals and phytochemicals. In addition, the diet varied in source of protein, fat, antioxidant, and carbohydrates. Finally, the NIA monkeys were more genetically diverse than the WNPRC monkeys (Mattison et al., 2012).

It is not feasible to do a long-term mortality study in humans due to cost and length of lifespan. However, certain conditions have allowed for researchers to study naturally occurring cases of CR in humans. On the island of Okinawa (Japan), there is

a high prevalence of centenarians. Kagawa et al. analyzed data from residents of Okinawa and determined that this group has a 31-41% lower death rate due to cerebral vascular disease, heart disease, or malignancy compared to the rest of Japan (Kagawa, 1978). While there may be some genetic differences to account for, adults on the island of Okinawa consume 20% fewer calories than the national average while the children consume only 62% of the national recommended intake.

A clinical trial called the CALERIE (Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy) study is underway, which has been designed to determine feasibility and health effects of CR in humans. In phase I, CR was determined to be feasible and cause weight loss, however the actual level of CR achieved (11.5%) was lower than the desired level (20%) (Racette et al., 2006). Phase I studies also showed that 6 months of CR in humans improved longevity biomarkers such as fasting blood insulin, core body temperature, and weight (Heilbronn et al., 2006). Other CR studies in humans from this time period showed improvements in atherosclerosis risk factors (Fontana et al., 2004), and improved diastolic function (Meyer et al., 2006). Phase II of the CALERIE study is a 2-year clinical trial which has a primary aim of determining if a 25% CR in humans will have the same health benefits observed in rodents and other species under similar CR regimens (Rochon et al., 2011). In addition, phase II secondary aims include determining CR safety, immune modulation, and potential mechanistic factors which have long been debated.

2.2 CR mechanism

Throughout the years many different theories have been derived to explain the life extension benefits of CR. Some scientists propose improvement in redox state to

be the cause of lifespan extension. While the redox state during CR does improve, it does not seem to be the primary cause of lifespan extension (Kowaltowski, 2011). In the early 2000's the popular potential mechanism proposed was that CR caused upregulation of Sirtuin (SIRT) proteins resulting in inhibition of PPARγ and decreased white adipose tissue (Wolf, 2006). There are seven types of SIRT proteins which regulate biological pathways in bacteria and other microorganisms. Decreased adipose tissue may result in changes in insulin signaling which has been proposed to increase longevity (Bluher et al., 2003). In addition during CR, SIRT1 has been shown to enhance cell adaptation to hypoxia (Kume et al., 2010) and insulin sensitivity (Schenk et al., 2011) while SIRT3 has been shown to prevent hearing loss (Someya et al., 2010). However, the life extension benefits of sirtuins have since been questioned; several studies have shown opposing results in lower organisms. For example, Smith et al. found that CR extends lifespan in *Saccharomyces cerevisiae*the in the absence of sirtuins (Smith et al., 2007). Guarente *et al.* conclude that while sirtuins may play some role in the regulation of lifespan extension, all seven need to be further studied in overexpression and mammalian knock out models (Guarente, 2012).

The CALERIE study hopes to shed light on lifespan extension mechanism by studying human triiodothryonine (T_3) levels and TNF-α in CR humans (Rochon et al., 2011). T_3 is a hormone secreted from the thyroid which declines with aging; it generally promotes metabolic activity by synthesizing proteins and enzymes (Kabadi and Rosman, 1988). TNF-α is a cytokine that can be released into the serum by various immune cells in order to cause inflammation, it also is related to decreased muscle mass in aging (Pedersen et al., 2003). Both T_3 (Heilbronn et al., 2006) and TNF-α

(Holloszy and Fontana, 2007) have already been shown to be significantly reduced from baseline in CR humans.

In addition to lifespan extension, CR can also cause prevention of age-associated diseases. In 2005, CR was shown to activate endothelial nitric oxide synthase (NOS) which increases mitochondrial biogenesis and oxygen consumption (Nisoli et al., 2005). CR increases NOS and mitochondrial proteins in the brain which enhanced respiratory capacity and survival of neurons (Cerqueira et al., 2012b). Enhanced mitochondria biogenesis increases NOS which has been proposed to be mediated through enhanced insulin sensitivity as a result of increased adiponectin in serum of CR rodents (Cerqueira et al., 2012a). Calorie restriction can also cause immune modulation which can either be detrimental or beneficial; these studies will be discussed in the next section.

2.3 CR and immunity

As previously mentioned, the increased risk of morbidity and mortality during aging are related to immune senescence, which refers to the decline in immune function with aging. Hematopoietic stem cells (HSC) in bone marrow are responsible for generating immune cells and red blood cells throughout the lifespan. The proliferative capacity of HSCs is improved in aged CR animals compared to aged AL animals (Chen et al., 2003), thus improving immune production in aging. Calorie restriction also delays T cell immune senescence, which occurs utilizing several different mechanisms. One mechanism is through maintenance of the naïve pool of CD4 and CD8 T cells (Chen et al., 1998) which tends to decline with aging as the T cell pool shifts from naïve to memory cells. The naïve T cell pool is important for immunological responses to new threats. Walford *et al.* was the first group to show an improvement in T cell antigen-

specific proliferative capacity, which usually declines with aging (Walford et al., 1973). This observation has since been confirmed by many other groups; however, the mechanism of action is unclear (Nikolich-Zugich and Messaoudi, 2005). Recent trials in rhesus monkeys have also shown reduced T cell senescence with CR (Messaoudi et al., 2006).

In addition to these immune cell benefits during CR, age-associated morbidities can also be improved or reduced. As previously mentioned, CR research began by studying transplanted tumors in underfed mice (Rous, 1914). Since this study almost 100 years ago, several different types of tumors and cancers have been examined in a number of species. Studies in CR mice and rats have shown a reduced incidence of three of the most prevalent types of cancers in these species including hepatomas, lymphomas, and lung tumors (Weindruch et al., 1982). While the longevity data between the two long-term rhesus monkey CR experiments was controversial, the cancer prevention data was not (Colman et al., 2009; Mattison et al., 2012). Both the WNPRC and the NIA study found reduced incidence of tumors in aged CR rhesus monkeys compared to aged AL rhesus monkeys. Another disease that is improved with CR is autoimmune disease including uveoretinitis, Sjogren syndrome, and auto-immune renal disease (Jolly, 2004). Most autoimmunity and calorie restriction work has been performed using mouse models. The potential mechanisms behind the improvements to autoimmune diseases are likely related to: a reduction in inflammatory cytokine production (IFN-γ, lL-12, IL-6 and TNF-α), an increase in immunosuppressive cytokine production (Nikolich-Zugich and Messaoudi, 2005), and restored production of the cytokine IL-2, which is essential for T cell maturation (Kubo et al., 1992).

CR has clearly been shown to have beneficial effects on immunosenesence; however animals on a CR diet may be at increased risk of infection. A pertinent study on the susceptibility of C57Bl/6 CR mice to polymicrobial sepsis by cecal ligation and puncture showed CR mice died earlier after sepsis compared to AL mice (Sun et al., 2001). In addition, these researchers observed a decrease in macrophage activation in response to LPS (a component of bacteria which is recognized by macrophages for phagocytosis), which may have been due to an observed decrease in LPS receptors (CD14) and TLRs on the macrophage surfaces (Sun et al., 2001). Another study reported increased susceptibility of CR mice to primary parasite infection. *Heligmosomoides bakeri* infection in CR mice resulted in increased worms, which produced more eggs compared to worms in AL mice (Kristan, 2007). In 2005, Gardner showed decreased survival of CR mice when infected with primary influenza. This research also revealed a decrease in NK cell cytotoxicity in the lung during infection (Gardner, 2005).

3. Natural killer (NK) cells

3.1 NK cell development

NK cells, or large granular lymphocytes, are innate immune cells capable of killing tumor or virus-infected cells. Before the discovery of NK cells, scientists began to observe background or ‘natural’ cytotoxicity against tumor cells in the spleen, bone marrow, and blood of mice (Herberman et al., 1975; Kiessling et al., 1975). Several strains and target cell lines were studied before Keissling *et al.* termed the cells responsible, natural killer cells (Kiessling et al., 1975). NK cell development and maturation were then studied extensively.

In the bone marrow, hematopoietic stem cells give rise to common lymphoid progenitors, followed by NK cell precursors (NKPs). S1P1, which is expressed on the surface of immature progenitors, induces secretion of the chemokine SDF-1 by macrophages into circulation which promotes the egress of progenitors form the bone marrow into circulation (Golan et al., 2012). Gordon *et al*. recently showed that increased production of the transcription factor Eomes, causes increased expression of the integrin and NK cell marker DX5, which correlates to S1P1 (Gordon et al., 2012). This would imply that Eomes and DX5 are at least somewhat responsible for the egress of NKPs to circulation. NKPs can be found in the bone marrow, thymus, lymph nodes, liver, and spleen (Huntington et al., 2007). It is unclear whether NKPs are generated by stem cells that populate these peripheral tissues (i.e. in the thymus) or if they enter circulation via the bone marrow and migrate (Sitnicka, 2011). Vosshenrich *et al.* proposed an alternate NK maturation pathway which occurs in the thymus and is dependent on the transcription factor GATA-3 and cytokine interleukin 7 (recognized by

IL-7Rα or CD127) (Vosshenrich et al., 2006). These $CD127^+$ thymic NK cells are functionally different than bone marrow derived NK cells, exhibiting reduced cytotoxicity and enhanced cytokine production (Vosshenrich et al., 2006).

CD122, NK1.1, NKp46, and DX5 are used to define committed NKPs and NK cells. NKPs are $CD122^+$ (Rosmaraki et al., 2001), however $CD122^+NK1.1^-DX5^-$ cells have been shown to be capable of producing T cells in vivo (Nozad Charoudeh et al., 2010). Therefore, NK cells can be defined as $CD122^+CD3^-NK1.1^+$ in certain strains of mice (Ballas and Rasmussen, 1990). In mice which do not express NK1.1 (Nogusa, 2010), NK cells can be defined using DX5, which is considered the NK pan-marker (Arase et al., 2001). NKp46 was more recently discovered and is used to identify murine immature NK cells and some immature human NK cells (Sitnicka, 2011). When immature NK cells differentiate into functionally mature NK cells they begin to express other surface markers such as CD11b, CD43, KLRG1, and Ly49s (Kim et al., 2002).

Several stages of NK cell maturation have been defined in mice and humans. Hayakawa *et al.* discovered that $CD11b^+$ mature NK cells could be split into two sub-groups based on their expression of CD27. $CD11b^{high}CD27^{high}$ NK cells have greater cytotoxicity against tumor cells and a heightened response to activating cytokines (Hayakawa and Smyth, 2006). Expanding on this discovery, Chiossone *et al.* defined a 4-stage developmental program of NK cells based on expressed on CD11b and CD27 (Chiossone et al., 2009). In this model early immature NK cells are defined as $CD11b^-CD27^-$, followed by $CD11b^-CD27^+$ immature NK cells, $CD11b^+CD27^+$ mature NK cells,

and finally $CD11b^{+}CD27^{-}$ terminally mature NK cells (Chiossone et al., 2009). This model was again expanded upon by Narni-Mancinelli *et al*., in which they propose six stages of NK cell maturation defined by: expression of CD122 (stage 1), NK1.1 (stage 2), NKp46 (stage 3), CD16 (stage 4), CD11b (stage 5), and the downregulation of CD27 (stage 6) (Narni-Mancinelli et al., 2011). In addition, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been identified as a maturation marker after NK cells have acquired NKp46 and before they acquire DX5 (Gordon et al., 2012). NK cell maturation stages are summarized in figure 1.

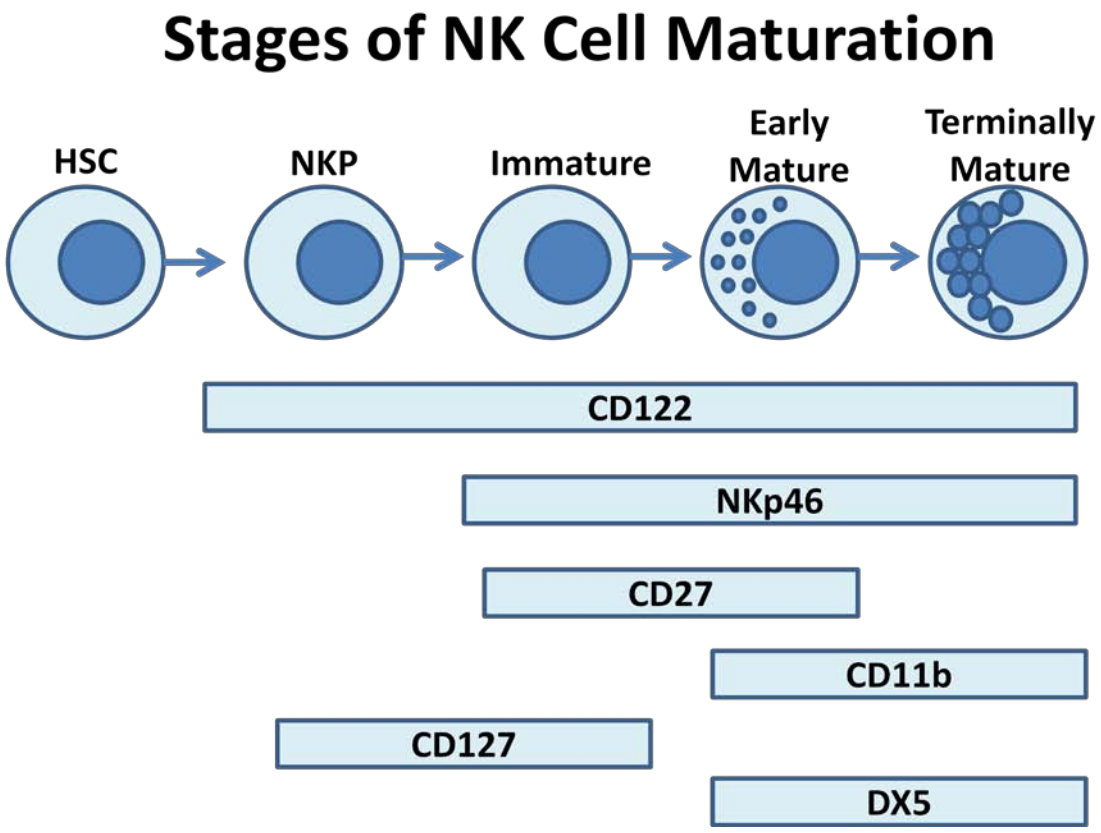

Figure 1.1 Representation of NK cell maturational stages and distinct marker expression during each stage. HSC= hematopoietic stem cell, NKP= NK cell precursor. For interpretation of the references to color in this and other figures, the reader is referred to the electronic version of this thesis.

3.2 The role of NK cells in immunity

The immune response can be sub-divided into two categories, innate and adaptive. Innate immune responses act almost immediately and without specificity, while adaptive immune responses take six to ten days or more to expand the proper lymphocyte populations in order to mount protection (Murphy et al., 2008). NK cells are considered an innate immune cell; however, they also help bridge the gap between the innate and adaptive systems. During immune responses to extracellular bacteria and pathogens in tissues, pathogen associated molecular patterns on bacteria are recognized by macrophages. The bacteria are then ingested by macrophages which are stimulated to release cytokines and chemokines to recruit neutrophils and monocytes from the blood. Some of the cytokines released by macrophages include: IL-12, IL-15, and IL-18; which prime NK cells to secrete additional cytokines, primarily tumor necrosis factor alpha (TNF-α) and interferon gamma (IFN-γ; (Vivier et al., 2008). Together, TNF-α and IFN-γ stimulate adaptive immune cells to undergo further maturation and differentiation in order to mount an adaptive response. NK cells also have innate properties (non-specific) to kill tumor and virus-infected cells. Viral particles replicate by invading healthy host cells and are therefore classified as intracellular pathogens. NK cells kill viral-infected cells by releasing cytotoxic granules (Murphy et al., 2008), controlling the infection until an adaptive immune response can be mounted to clear the virus (Vivier et al., 2008).

MHC Class I is expressed on the surface of nearly every nucleated cell in a mouse model. Immature NK cells do not exhibit inhibitory receptors, however they are also not fully functional, which makes them functionally incompetent of attacking the self

cell (Yokoyama and Kim, 2006). Examples of NK cell inhibitory receptors are inhibitory Ig-like receptors (KIRs) in humans, and inhibitory killer-like lectin receptors (KLRs) or Ly49s in mice (Jost and Altfeld, 2013; Murphy et al., 2008). When a virus enters a cell, it can inhibit the host-cells protein synthesis, rendering it incapable of producing MHC Class I (Murphy et al., 2008). Decreased expression of MHC Class I on the virus-infected cell results in increased susceptibility to NK cell mediated killing. The virus infected cell is also capable of displaying viral antigen on its MHC Class I receptor. Upon recognition of the altered MHC Class I molecules, the inhibitory receptor does not recognize MHC Class I as self, and therefore will not inhibit the NK cell from activating. The activating receptors, such as activating KIRs and natural cytotoxicity receptors in humans and activating KLRs and Ly49s in mice activate NK cell-mediated lysis of virus-infected cells (Jost and Altfeld, 2013). A well classified example of an activating receptor on NK cells is NKG2Ds receptor, an MHC-like molecule expressed during cellular stress (Sun and Lanier, 2009).

Natural killer cells utilize several mechanisms in order to kill virus-infected or tumor cells: release of toxic granules, antibody dependent cellular cytotoxicity (ADCC), reverse ADCC, and FAS ligand or TRAIL mediated apoptosis (Murphy et al., 2008). As previously mentioned, NK cells are not activated when they receive signals from both activating and inhibitory receptors. However, NK cells receiving activating signals only results in killing of the target cell, thus inhibitory signals are critical in regulating NK cell responsiveness. The most prominent mechanism of NK cell killing is release of its toxic granules, perforin and granzyme. Granzyme forms an extracellular complex with perforin, which is then recognized by a tumor or virus-infected cells mannose-6-

phosphate (M6P) receptor, targeting the complex for destruction in an endosome (Trapani and Smyth, 2002). Perforin pores are formed on the membrane of the endosome, which allows for release of granzyme B into the target cell cytosol. Granzyme B activates BIM and BAX proteins inducing caspase mediated apoptosis of the cell (Thiery et al., 2011). Some tumors avoid granzyme/perforin mediation killing by inhibiting expression of M6P receptors, and as such, NK cells have evolved alternative mechanisms for killing target cells (Trapani and Smyth, 2002). ADCC occurs when Fc receptors on NK cells recognize antibody bound to target cells, which causes NK cell degranulation (Murphy et al., 2008). Reverse ADCC is a commonly used assay in which the antibody first binds the NK cells Fc receptor, and then the bacteria for NK cell degranulation (Perussia and Loza, 2000). The final mechanism of NK-cell mediated cell death occurs when TRAIL or FAS-LIGAND are expressed on the NK cell surface which is recognized by the TRAIL or FAS death receptors, respectively, on the target cell. Both of these pathways initiate the caspase cascade and cause apoptosis of the target cell (Alderson et al., 1995; Zamai et al., 1998). The TRAIL pathway is usually utilized to kill self-reactive lymphocytes.

3.3 Diet and NK cells

There are several dietary factors capable of influencing NK cells. One example is the increase in NK cell activity observed with probiotic consumption. In an aged population, *Lactobacillus casei Shirota* enhanced NK cell activity (Dong et al., 2013). Similarly, Lactococcus lactis subsp. cremoris FC enhances IL-12 and IL-18 cytokine production by macrophages and dendritic cells, resulting in IFN-γ production by NK cells (Kosaka et al., 2012). In addition, several strains of bifidobacterium have also been

shown to significantly increase IFN-γ production by NK cells, stronger responses are seen in young participants (You and Yaqoob, 2012). Other dietary factors that positively impact NK cell function or maturation include: black and brown rice bran (Choi et al., 2012), breast vs. formula feeding (Ortiz-Andrellucchi 2008), fucoidan administration (Azuma et al., 2012), and active hexose correlated compound supplementation (Ritz et al., 2006).

Leptin is an influential adipokine on NK cell development and activation is leptin, a hormone secreted by adipose tissue to control food intake. Leptin controls food intake by counteracting neuropeptide Y, which is a hormone released by the hypothalamus and gut cells to stimulate feeding (Stanley et al., 1985). NK cells express leptin receptors, and are stimulated when leptin concentration is high in circulation. Mice lacking the leptin receptor (*db/db)* have lower NK cell activation and lower percentage of NK cells in the liver, spleen, lung and blood (Tian et al., 2002), suggesting leptin receptors play a role in NK cell activation. Diet-induced obese mice have higher circulating leptin and expression of leptin receptors on NK cells than normal weight mice. Because NK cells are stimulated by leptin, one may assume that obese mice have higher NK cell activity. However, diet-induced obese mice have skewed Janus kinase-2 downstream signaling from the leptin receptor, leaving their NK cells less responsive to leptin than lean mice, resulting in leptin resistance (Nave et al., 2008). While calorie restriction improves leptin signaling in diet-induced obese models (Wilsey and Scarpace, 2004), non-obese calorically restricted animals have significantly lower levels of circulating leptin than normal weight animals (Clinthorne et al., 2013). CR mice

also have lower number, percent, maturation, and cytotoxicity of NK cells compared to normal weight controls (Clinthorne et al., 2013; Gardner, 2005; Ritz et al., 2008).

4. CR, aging, and NK cells

Our laboratory has extensively studied the effects of CR on NK cells. In 2005, Gardner reported a study in which aged AL and aged CR mice were infected with varying hemagglutinating units (HAU) of influenza virus. NK cell cytotoxicity was reduced in both groups 24 hours after infection compared to baseline. At 48 hours after infection, aged AL mice demonstrated normal NK cell cytotoxicity, however aged CR mice did not. While this observation was not significant, increased death of CR mice infected with influenza may be attributed to altered weight and NK cell response (Gardner, 2005). In 2008, Ritz *et al.* infected young CR mice with influenza to compare survival and NK cells. They showed a decrease in survival, and an increase in lung viral titers, lung pathology, and weight loss in young CR compared to young AL mice. In addition, CR mice also had lower percentages and numbers of NK cells, the differences in NK cell number were significant at baseline and at 1 and 3 days post-infection, while the differences in NK cell percent were significant at days 1, 2, and 3 post-infection. NK cell cytotoxicity was also tested, while there was a decrease in cytotoxicity, it was not significant (Ritz et al., 2008).

In 2010, Clinthorne *et al.* switched young CR mice to an AL diet, hypothesizing an improvement in NK cell activity (Clinthorne et al., 2010). Upon receipt of the AL diet, the previously CR mice restored their body weight in 2 days, and their body fat in 6 days. During influenza infection, the percent and number of NK cells in re-fed mice was attenuated from CR levels, but not fully restored to AL levels. However, the number and percent of $CD69^{+}$ (an activation marker) NK cells was restored to AL levels (Clinthorne et al., 2010). A more recent publication from our laboratory represents the first detailed

report of functional and maturational changes of NK cells in CR. This study showed a significant decrease in NK cell percent in the lung, spleen, and blood along with a significant increase of NK cells in the bone marrow, perhaps due to an inability of NK cells to properly migrate from the bone marrow. NK cells in CR mice were more immature based on increased expression of CD127, and a decreased percent of terminally mature ($CD27^{-}CD11b^{+}$) NK cells (Clinthorne et al., 2013). In addition, NK cells stimulated with IL-2 and IL-12 were less capable of producing IFN-γ in CR mice compared to AL mice (Clinthorne et al., 2013).

Our laboratory has also studied NK cell changes observed in aged AL fed mice. In 2008, Nogusa *et al.* infected young and aged AL mice with influenza virus. Aged mice had significantly decreased NK cell cytotoxicity 2 days post-infection compared to young mice. Aged mice also had reduced NK cell numbers (day 2) and percentages (day 1, 2, and 3) post-influenza infection. Beli *et al.* confirmed a reduction of NK cells in the lungs of aged mice, in addition to a decrease in IFN-γ production 2 days post-infection when stimulated with either YAC-1 cells or IL-2 + IL-12 cytokines. In addition, aged mice also have more immature ($CD27^{+}CD11b^{-}$) and less terminally mature ($CD27^{-}CD11b^{+}$) NK cells (Beli et al., 2011).

As evidenced by the six papers reviewed here from our laboratory, both CR and aging cause detrimental changes to NK cells in the presence and absence of influenza infection. Often, elderly populations do not consume appropriate calories (Wakimoto and Block, 2001) which may cause changes in NK maturation and function in a CR and aging population. Therefore, we found it necessary to combine the two paradigms of CR and aging, and study changes in NK cells. In addition, previous research by Messaoudi

et al. explored the possibility of an optimal age window for onset of CR (Messaoudi et al., 2008). This study showed that old-onset CR is detrimental to T cells, compared to juvenile or young-adult onset CR (Messaoudi et al., 2008). Therefore, an additional set of experiments was conducted to determine whether old-onset CR is more detrimental than young-onset CR in aged CR mice. The results of both the aged CR experiments are discussed in the next chapter.

CHAPTER 2- CALORIE RESTRICTION EXACERBATES AGE-RELATED NATURAL KILLER CELL DEFECTS IN C57BL/6 MICE

1. Introduction

Calorie restriction (CR) has been studied since the early 20th century (Rous, 1914), and has become accepted as a mechanism of increasing life span in yeast, worms, flies, and rodents. Existing data on the effects of CR on lifespan extension in non-human primates are controversial, as evidenced by two recent longitudinal 20-year CR studies on rhesus monkeys. In the Wisconsin National Primate Research Center study (Colman et al., 2009), CR increased non-human primates lifespan. However, using a similar restriction protocol, but different exclusion criteria, Mattison et al. (Mattison et al., 2012) showed no significant effect on lifespan. The effect of CR on the immune system is also debatable. The benefits of CR include, but are not limited to, prevention of: tumors in rhesus monkeys (Mattison et al., 2012), autoimmune disease in mice (Piccio et al., 2008), and T cell senescence in rhesus monkeys (Messaoudi et al., 2006). On the other hand, CR in mice may cause natural killer (NK) cell defects (Ritz et al., 2008) and increased susceptibility to influenza (Gardner, 2005) and bacteria-induced peritonitis (Sun et al., 2001).

NK cells are innate immune cells that have the ability to kill virus-infected and tumor cells. During NK cell maturation, common lymphoid progenitors in the bone marrow develop into NK1.1^{+}CD122^{+} NK cells (Kim et al., 2002). Subsequently, in both bone marrow and peripheral tissues (i.e. spleen and liver), immature NK cells acquire expression of markers such as TNF-related apoptosis-inducing ligand (TRAIL) and

integrin α_v (CD51) (Kim et al., 2002). NK cell maturation then progresses in a four-stage developmental program during which NK cells differentially express CD11b and CD27. Peripheral immature NK cells express CD27, but lack C11b, while mature NK cells upregulate C11b and progressively lose expression of CD27 (Chiossone et al., 2009). Maturation can also be defined by the downregulation of the immature marker TRAIL and the upregulation of the early mature marker DX5, as immature NK cells advance from $TRAIL^{+}DX5^{-}$ to $TRAIL^{-}DX5^{+}$ (Gordon et al., 2012).

Once NK cells have matured they migrate from the bone marrow to peripheral tissues (i.e. spleen, lymph nodes), which is at least partially mediated by the chemokine CXCL12 (SDF1-α) (Bernardini et al., 2008). Leptin is a hormone directly proportional to levels of adipose tissue; it is released by the gut cells and hypothalamus to stimulate feeding (Stanley et al., 1985). NK cells express leptin receptors and are stimulated in increased leptin environments (Tian et al., 2002). Additionally, NK cells in peripheral tissues may express IL-7Rα (CD127), and have been hypothesized to represent cells that were either derived from the thymus (Vosshenrich et al., 2006) or are immature bone marrow-derived NK cells (Di Santo, 2006).

Our laboratory has previously shown that shown that CR mice have decreased inflammation and circulating leptin (Clinthorne et al., 2012). We have also shown that CR decreases survival of influenza-infected mice which is accompanied by decreased NK cell numbers and cytotoxicity (Ritz et al., 2008). Similarly, compared to young controls, aged *ad libitum* (AL) fed mice infected with influenza have decreased NK cell survival and functional responses (Beli et al., 2011; Gardner, 2005). Interestingly, other immune cells may benefit from CR. For example, Messaoudi *et al.* (Messaoudi et al.,

2008) show that initiating CR in young adult (post-puberty) primates prevents the T cell senescence that occurs with aging, while juvenile-onset (pre-puberty) CR and old-onset CR may have a detrimental effect on T cells. However, we have shown, when CR is initiated in young adult mice, NK cells do not mature completely nor develop their full function (Ritz et al., 2008).

While we have described NK cell changes in young CR mice, we have not done so in aged CR mice. Therefore, the first aim of these studies was to determine NK cell changes in aged (22 mo) C57BL/6 mice consuming a CR diet. Based on our previous observations regarding aging and CR, we hypothesized there would be more significant NK cell defects in aged CR mice compared to controls. In addition, as seen with T cell senescence, CR may affect NK cells differently depending on the age of the animals at the time of CR initiation. Therefore, the second aim of our study was to determine whether young adult-onset CR affects NK cells differently than old-onset CR. We hypothesized the age of onset of CR would have detrimental effects on NK cell maturation. Our results indicate that CR exacerbates the detrimental effects of aging on NK cell numbers and phenotype which could contribute to increased risk of viral infection.

2. Materials and methods

2.1 Mice

C57BL/6 male mice were purchased from the National Institute of Aging (NIA) Charles River Laboratories (Wilmington, MA, USA). Mice were individually housed in micro-isolator cages at the Michigan State Research Containment Facility, a facility certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice were acclimated for at least 1 wk in a biosafety level 2 room prior to euthanasia and fed diets purchased from the NIA. Control animals received NIH-31 diet AL, and calorie restricted (CR) animals received 3 g/d of NIH-31/NIA fortified diet, resulting in 40% CR as previously described (Ritz et al., 2008). Mouse body weight and composition were measured weekly using an EchoMRI-500 (Echo Medical Systems, Houston, TX, USA) as previously described (Clinthorne et al., 2013). Mice were euthanized by CO_2 asphyxiation followed by cardiac puncture for blood collection. All animal procedures were in accordance with the National Research Council guidelines and approved by the Michigan State Institutional Animal Care and Use Committee.

2.2 Groups and feeding protocol

For the aged CR protocol, 4 groups of mice were purchased from the NIA: 10 control young AL (Y-AL), 10 young CR (Y-CR), 10 aged AL (A-AL), and 10 aged CR (A-CR). The restriction protocol implemented by the NIA consisted of feeding mice AL until 14 wk of age. Diets were switched at weekly intervals by the NIA to increasing levels of calorie restriction: 10%, 25% and 40% (Turturro et al., 1999). The mice were maintained at a 40% CR until purchased at 6 mo (2 mo of 40% CR) and 22 mo (18 mo

of 40% CR) of age. Age-matched AL fed animals were also purchased at this time. After mice were received they were acclimated for at least 1 wk and kept on an AL or a 40% CR diet until euthanasia.

For the young vs. old onset of CR onset protocol, 3 groups of 22-mo-old mice were analyzed: 10 aged AL (A-AL), 10 aged old-onset CR (OO-CR) and 10 aged young-adult onset CR (YO-CR). A-AL and YO-CR were purchased from the NIA at 20 mo. The YO-CR mice had been calorie restricted by the NIA as described above. Mice were fed their respective diets until euthanasia at 22 mo, resulting in an 18 mo restriction for the YO-CR group. OO-CR mice had been fed AL by the NIA, until they were purchased at 19 mo. We then calorie restricted the animals in-house beginning at 19.5 mo, using the same protocol as the NIA (described above), switching diets at weekly intervals to increasing levels of CR: 10%, 25%, and 40% (Supplemental Figure 1B). Forty percent CR was reached by 20 mo. OO-CR animals were held on this restriction until euthanasia at 22 mo, for a total of a 2-mo restriction.

2.3 Tissue processing

Blood was centrifuged at 2000xg for 20 min. Serum was stored at -80°C. Liver samples were dissociated in RPMI using the gentleMACS dissociator. The dissociated liver cells were resuspended in 10 mL of 40% isotonic Percoll solution and layered over 60% isotonic Percoll solution. Cell layers were separated by centrifugation and the intermediate lymphocyte band was harvested and washed. Lungs and spleens were digested with 1 mg/mL collagenase D (Roche, Indianapolis, IN, USA) and 40 units/mL DNAase I (Roche), they were then dissociated twice using a gentleMACS dissociator (Miltenyi Biotec, Cologne, Germany) with a 30 min 37°C incubation between

dissociations. In the age of CR onset protocol, spleens were processed as previously described (Ritz et al., 2006). Bone marrow processing was performed as previously described (Clinthorne et al., 2012). Red blood cells from liver, lung, spleen and bone marrow were lysed with ammonium chloride-potassium (ACK) lysis buffer (Lonza, Walkersville, MD, USA).

2.4 Flow cytometry

Non-specific binding was blocked by incubating cells for 10 min with anti-CD32/CD16 antibody (2.4G2) (BD Bioscience, San Jose, CA, USA). Cells were incubated with optimal concentrations of fluorochrome-conjugated antibodies for 30 minutes on ice, in the dark. The following fluorochrome conjugated antibodies were purchased from either eBioscience (San Diego, CA, USA) or BD Bioscience (San Jose, CA, USA): NK1.1 (APC), CD127 (PercpCy5.5), CD3 (AlexaFluor 700), CD11b (PECy7), CD27 (PE), DX5 (FITC), CD51 (biotinylated APC Cy7), IFN-γ (FITC) and TRAIL (biotinylated PE CF594). Data was acquired on a FACS Canto II flow cytometer (BD Bioscience, San Jose, CA, USA) and analyzed using Flow Jo software (Treestar, Ashland, OR, USA). Lymphocytes were gated using FSC vs. SSC, and NK cells were identified as $CD3^{-}NK1.1^{+}$.

2.5 Serum analytes

Mouse serum proteins were analyzed by ELISA, per manufacturer instructions: Corticosterone (Promega, Madison, WI, USA), Leptin (R&D Systems, Minneapolis, MN, USA), TNF-α (eBioscience, San Diego, CA, USA), IL-7 (Ray BioTech, Norcross, GA, USA), and SDF1-α (Ray BioTech, Norcross, GA, USA).

2.6 Statistics

Data were analyzed with GraphPad Prism 4 (Graph Pad Software, Inc, La Jolla, CA, USA) using either a one-way ANOVA followed by Tukey's *post hoc* test, or two-tailed t-tests. Figure and table data are expressed as mean ± SEM. Significance was set at $P \leq 0.05$.

3. Results

3.1 CR mice have altered body composition compared to AL mice

Specific ages and characteristics of Y-AL, Y-CR, A-AL, and A-CR mice are listed in Table 1. Following the NIA protocol, all CR mice consumed 3 g/d of NIH-31/NIA fortified diet, which resulted in 40% restriction in young mice and 39% restriction in aged mice. Both groups of CR mice had significantly lower body weight than aged-matched AL controls. As determined by MRI, the percent body fat of CR mice was significantly lower than age-matched controls. No significant difference was observed in percent lean body mass between groups. Finally, percent water weight was significantly higher in A-CR mice compared to A-AL mice, while no significant difference was observed between their young counterparts.

Table 1. Characteristics of young CR, young AL, aged CR, and aged AL C57Bl/6 mice.

	Y-AL[1]	Y-CR	A-AL	A-CR
Age (mo)	6	6	22	22
Weight (g)	28.5±1.0[a,2]	21.0±0.5[b]	34.4±2.0[c]	22.4±0.8[b]
Fat (% of weight)	16.0±0.9[a]	9.8±0.4[b]	18.4±1.8[a]	10.8±0.15[b]
Lean Body Mass (% of weight)	75.7±0.8[a]	76.2±0.5[a]	75.1±2.0[a]	78.3±0.2[a]
Water (% of weight)	0.33±0.04[a]	0.50±0.06[ab]	0.27±0.05[a]	0.63±0.08[b]

[1]Y-AL= young *ad libitum*, Y-CR= young calorie restricted, A-AL= aged *ad libitum,* A-CR= aged calorie restricted

[2]Values (means ± SEM) with different letters within the same row are statistically different (P≤0.05).

3.2 Age and CR create changes in systemic environment

In agreement with previous reports (Clinthorne et al., 2013), CR mice had a significantly higher concentration of corticosterone than AL mice in serum, while age had no effect (Figure 2.1A). As expected, leptin levels were significantly lower in CR mice than AL mice (Clinthorne et al., 2013), while age had no significant effect on leptin levels (Figure 2.1B). SDF1-α levels were significantly increased in aged mice compared to young, but SDF1-α was not changed with CR (Figure 2.1C). CR reduced TNF-α, a pro-inflammatory cytokine, compared to age-matched controls, while aged mice had significantly higher TNF-α levels than young mice in the AL group (Figure 2.1D). IL-7 could not be detected in the serum (data not shown).

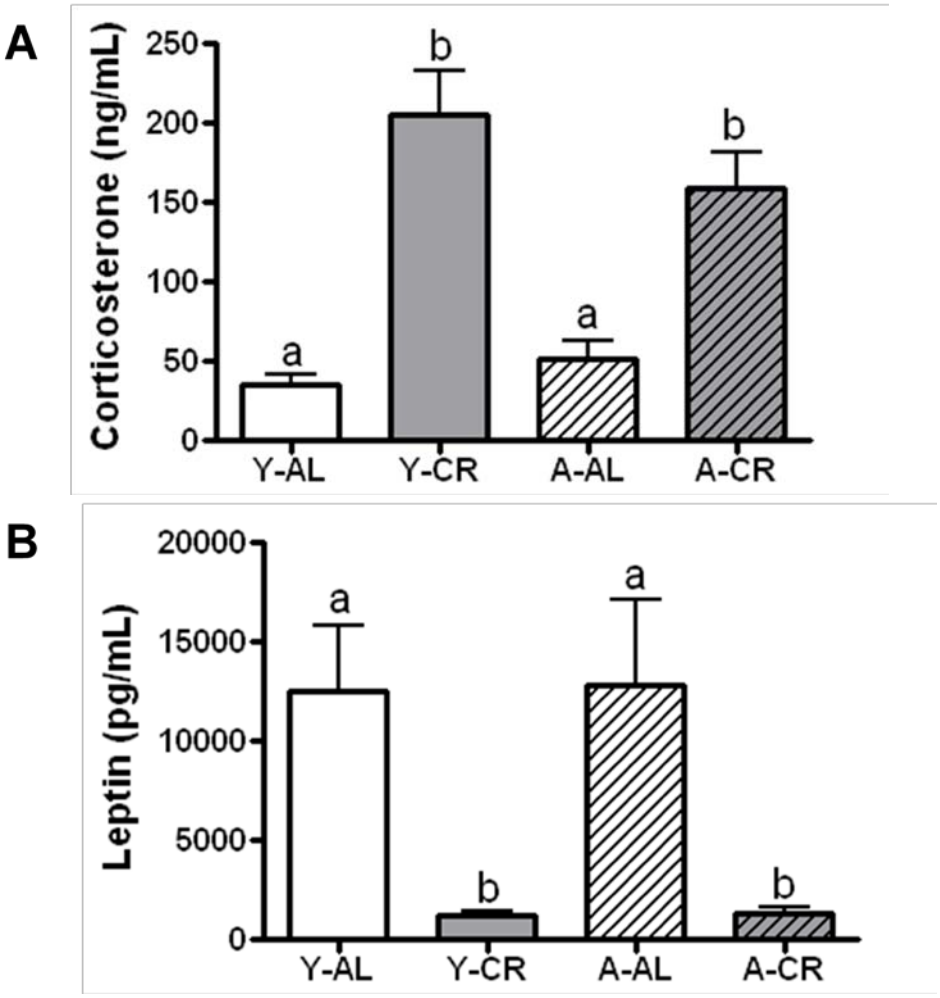

Figure 2.1. Analysis of corticosterone, leptin, SDF1 alpha, and TNF alpha in the serum of young AL, young CR, aged AL, and aged CR mice. Mouse serum was analyzed by ELISA for corticosterone (A), leptin (B), SDF1 alpha (C), and TNF alpha (D). Bars with different letters are significantly different ($P \leq 0.05$) as determined by one-way ANOVA with Tukey's *post hoc* test. ND=not detectable, Y-AL=young *ad libitum*, Y-CR=young calorie restricted, A-AL=aged *ad libitum*, and A-CR=aged calorie restricted.

Figure 2.1 (cont'd).

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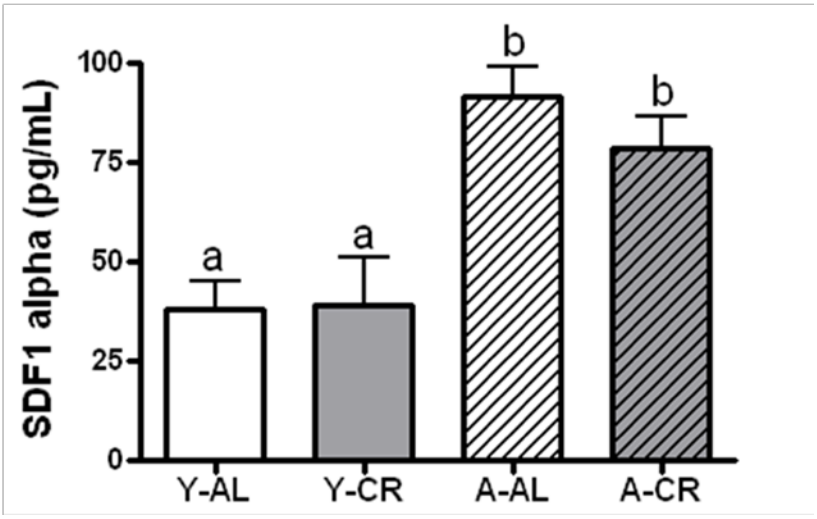

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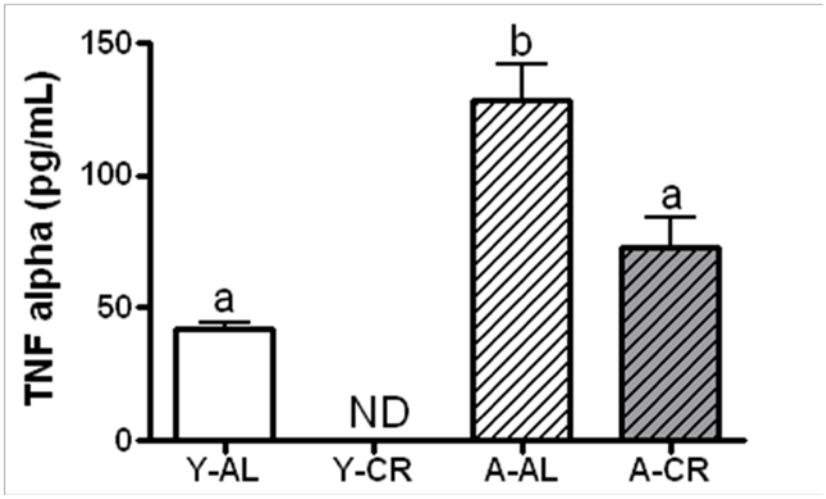


3.3 NK cells are reduced in A-CR mice

The bone marrow and liver are sites of early NK cell development and maturation, while the spleen is thought to facilitate later stages of NK cell maturation (Huntington et al., 2007). Using flow cytometry, we assessed the effects of CR and aging on NK cell percent (Figure 2.2A) and number (Figure 2.2B) in these tissues. In all tissues, A-CR mice had the lowest number and percent of NK cells among lymphocytes and live cells, although this was not always significant. In bone marrow, the number of NK cells was significantly decreased in A-CR mice compared to A-AL mice. Because the lung contains terminally differentiated NK cells that have homed in, we investigated whether the changes observed in the bone marrow and spleen were reflected in a site independent from NK cell development. In the lung, the percent of NK cells among live cells was significantly lower in the A-CR group when compared to all other groups, indicating a combined effect of aging and calorie restriction on NK cell percent.

3.4 Detrimental effects on NK cell maturation markers in the spleen of A-CR mice

We then determined if maturation markers were altered in A-CR mice. Characterization of the spleen (Figure 2.2C), revealed a significant increase in percent of NK cells expressing CD127 (immature marker) and a significant decrease in DX5 (early mature marker) in the A-CR group compared to all other groups of mice. Liver NK cells also expressed higher levels of CD127 and lower levels of DX5 (data not shown). A-CR bone marrow NK cells expressed significantly higher levels of both CD127 and DX5 (data not shown). However, the percent of $DX5^{+}$ NK cells among live cells was not increased in the bone marrow (data not shown), indicating the increase may be a reflection of alterations in other markers within the subset, and not a true increase in

$DX5^+$ NK cells. In all tissues, most significantly in the spleen, we observed changes in expression of CD27 and CD11b, which led us to analyze NK cell maturation based on 4-stage development of CD27 and CD11b.

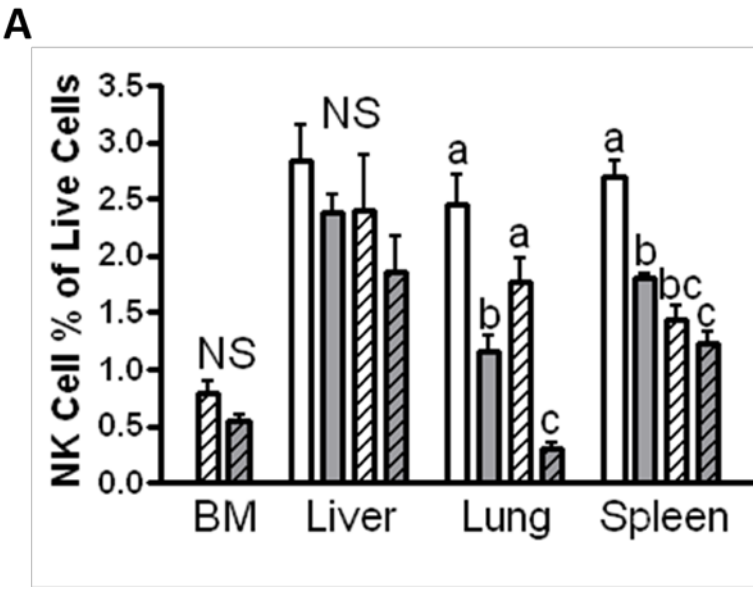
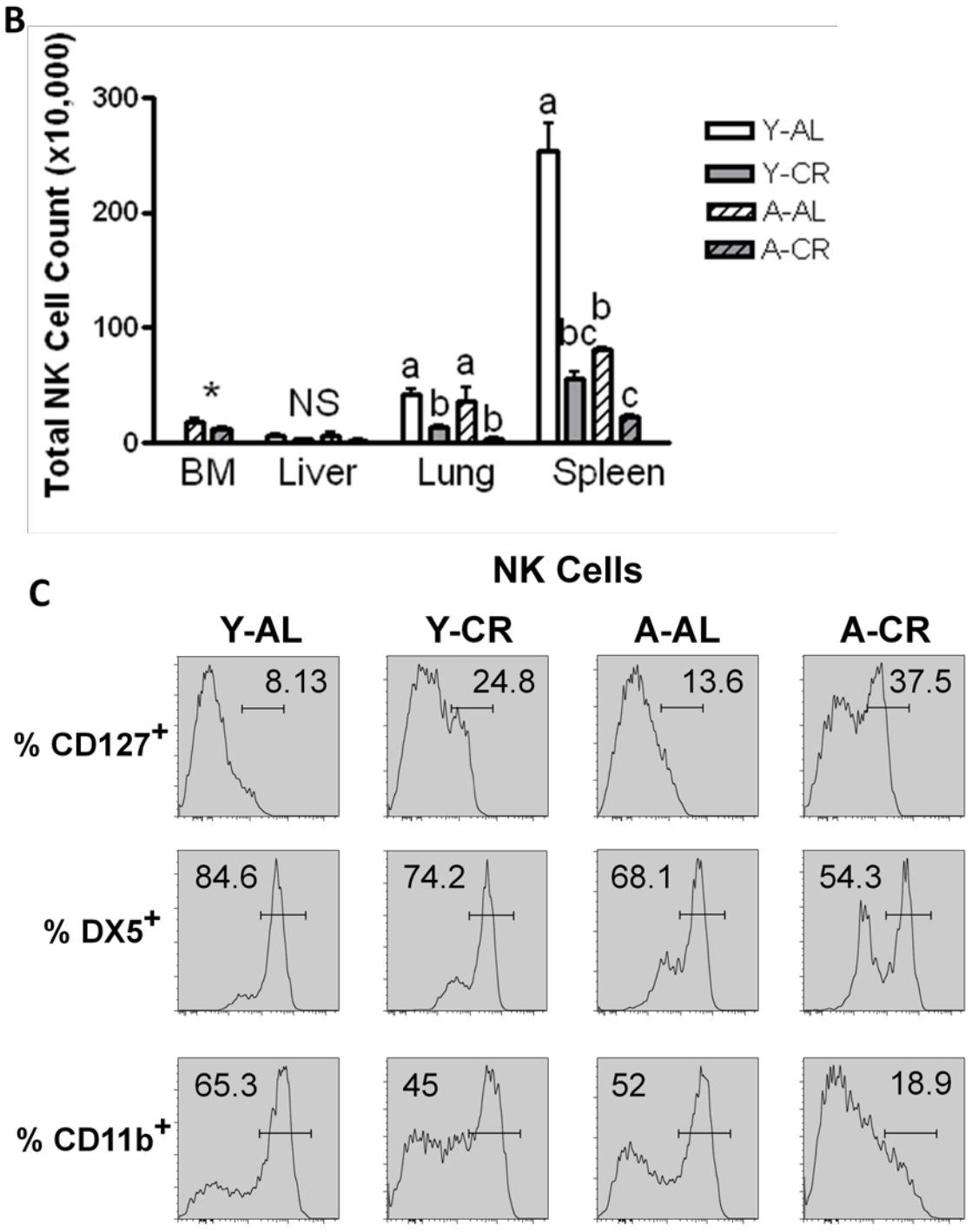


Figure 2.2. NK cell percent, number, and basic maturation markers (spleen only) in bone marrow, liver, lung, and spleen of young AL, young CR, aged AL, and aged CR mice. Natural killer (NK) cell percent among live cells (A) and total NK cell number (B) in bone marrow (BM), liver, lung, and spleen (n=10 per group) were analyzed using flow cytometry. Bars with different letters within the same tissue are statistically different (P≤0.05) as determined by one-way ANOVA with Tukey's *post hoc* test. Asterisks represent a significant difference (P≤0.05) as determined by t-test. NS=not significant, Y-AL=young *ad libitum*, Y-CR=young calorie restricted, A-AL=aged *ad libitum*, and A-CR=aged calorie restricted. (C) Splenocytes were analyzed for changes in immature (CD127) and mature (DX5 & CD11b) markers. Each histogram is a representative example of the group. The number in each box represents the percent of NK cells positive for the identified marker.

Figure 2.2 (cont'd).



3.5 A-CR NK cells are less mature based on CD27 and CD11b expression

As previously mentioned, murine NK cell maturation can be described by a 4-stage developmental program defined by CD27 and CD11b as they progress from immature to mature NK cells (Chiossone et al., 2009). Using this step-wise program, we determined the maturation status of NK cells in tissues of aged CR mice compared to controls was altered. As shown in Figure 2.3B, aging and CR had detrimental effects on NK cell maturation independently which was exacerbated in the A-CR group. The most notable effects were observed in terminally mature NK cells ($CD11b^{+}CD27^{-}$). In all tissues analyzed, A-CR mice had significantly reduced frequency of terminally mature NK cells compared to all other groups (Figure 2.3C).

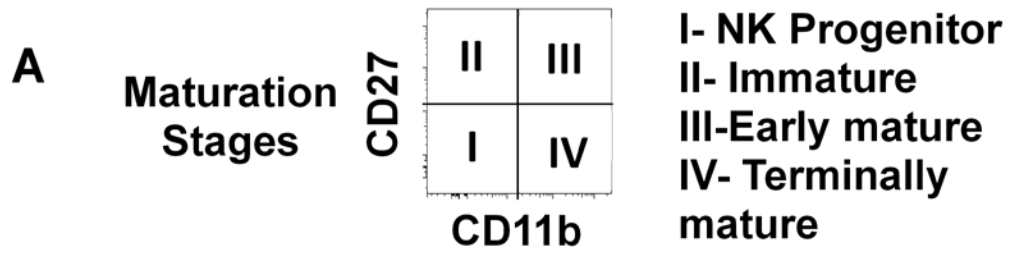
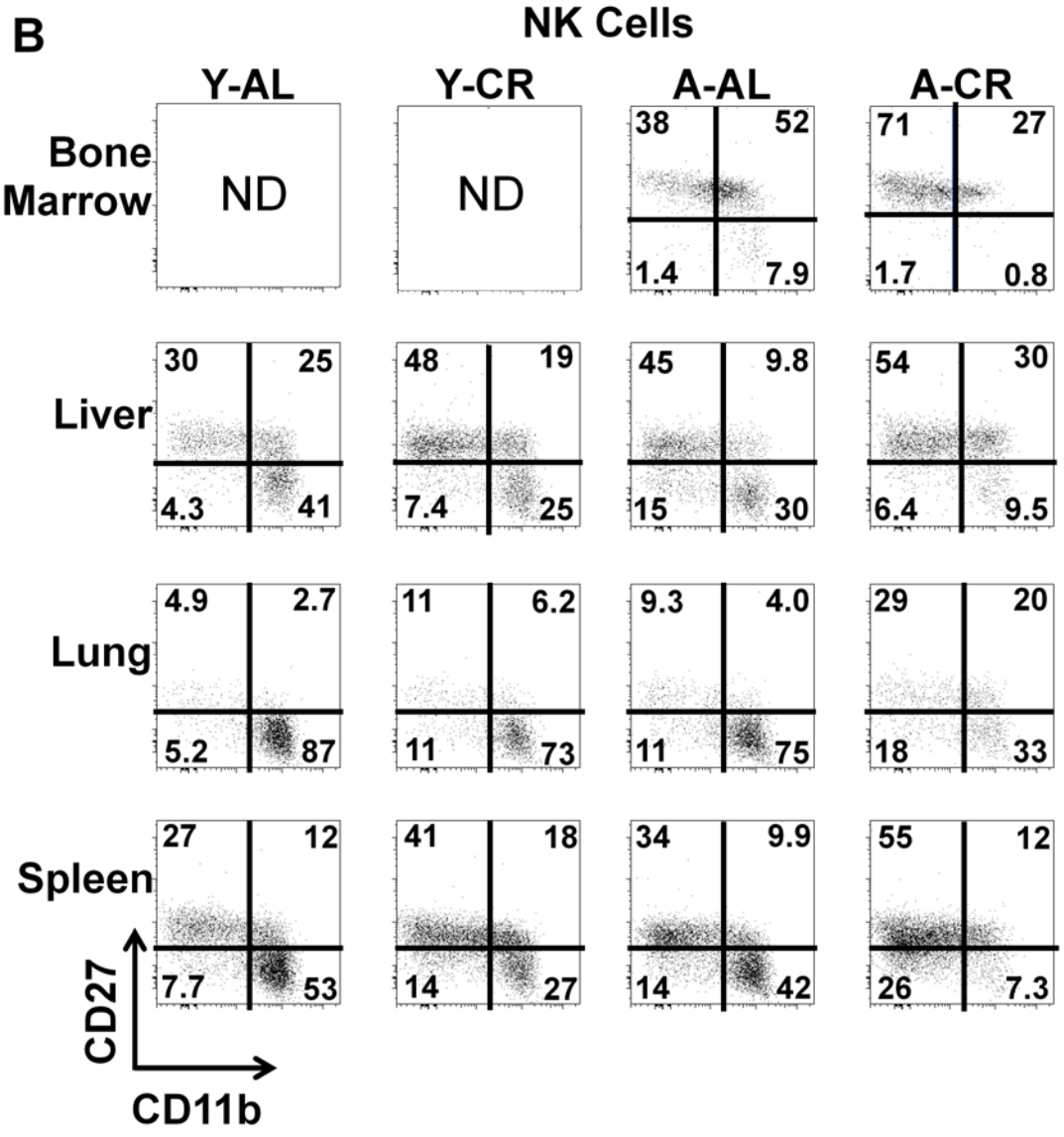


Figure 2.3. NK cell maturation based on expression of CD27 and CD11b in the spleen, lung, liver, and bone marrow of young AL, young CR, aged AL, and aged CR mice. (A) Diagram describes NK cell maturation stages based on expression of CD27 and CD11b which correlate to each of the four quadrants in the representative flow plots using flow cytometry. (B) Representative flow plots describe the changes in NK cell maturation seen in the bone marrow (BM), liver, lung, and spleen. Each flow plot is a representative example of the group. The number in each box represents the percent of NK cells positive for the identified markers. ND= no data, Y-AL=young *ad libitum*, Y-CR=young calorie restricted, A-AL=aged *ad libitum*, and A-CR=aged calorie restricted. (C) Means ± SEM of quadrant IV ($CD27^{-}CD11b^{+}$) are represented as bar graphs for each tissue (n=10 per group). Bars with different letters within the same tissue are significantly different (P≤0.05) as determined by one-way ANOVA with Tukey's *post hoc* test. Asterisks represent a significant difference (P≤0.05) as determined by t-test.

Figure 2.3 (cont'd).



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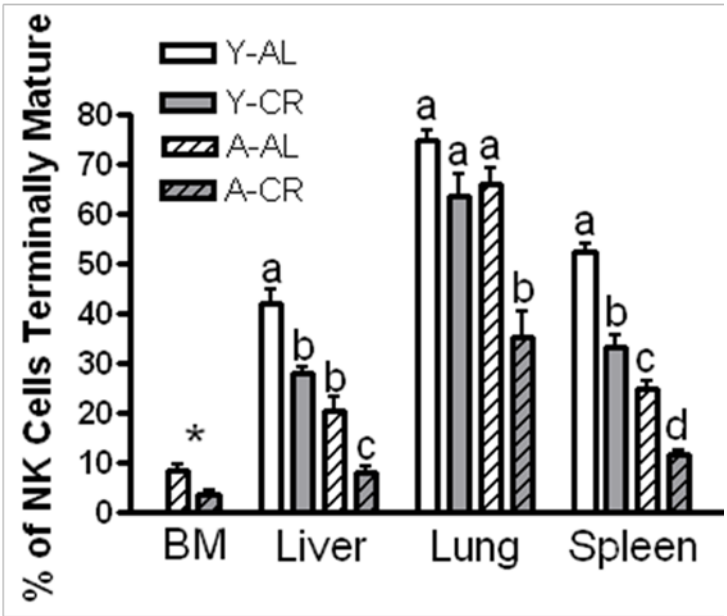


3.6 CD127 expression is increased and DX5 expression is decreased on A-CR terminally mature NK cells

Decreased percent of terminally mature NK cells was observed across all tissues, leading us to investigate the expression of other markers within this subset which might be related to a delay in maturation. After gating terminally mature NK cells, we compared expression of CD127 (immature marker) and DX5 (mature marker) between groups. As shown in Figure 2.4, terminally mature NK cells in A-CR mice had increased expression of CD127 in the bone marrow and spleen. Terminally mature NK cells from A-CR mice also had decreased expression of DX5 in the spleen, lung, and liver. DX5 was expressed on 98.5 ± 0.3% (mean ± SEM) of Y-AL terminally mature NK cells in the spleen and 60.0 ± 8.4% (mean ± SEM) of A-CR terminally mature NK cells, a 1.6x decrease. Also in the spleen, CD127 was expressed on 2.6 ± 0.2 % of Y-AL terminally mature NK cells, while it was expressed on 45.6 ± 5.6% of A-CR terminally mature NK cells, a 17.5x increase.

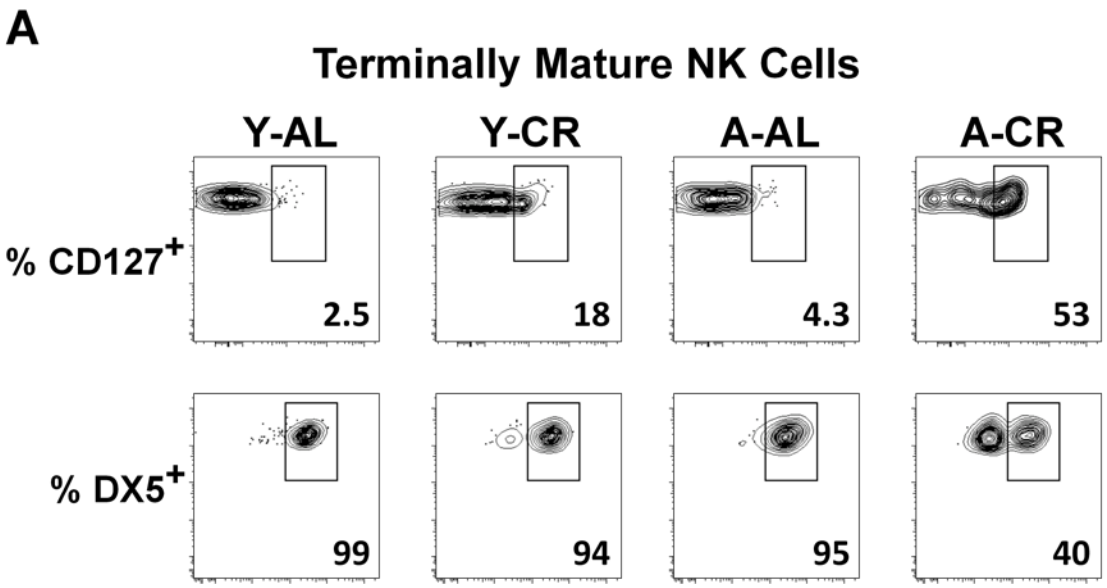
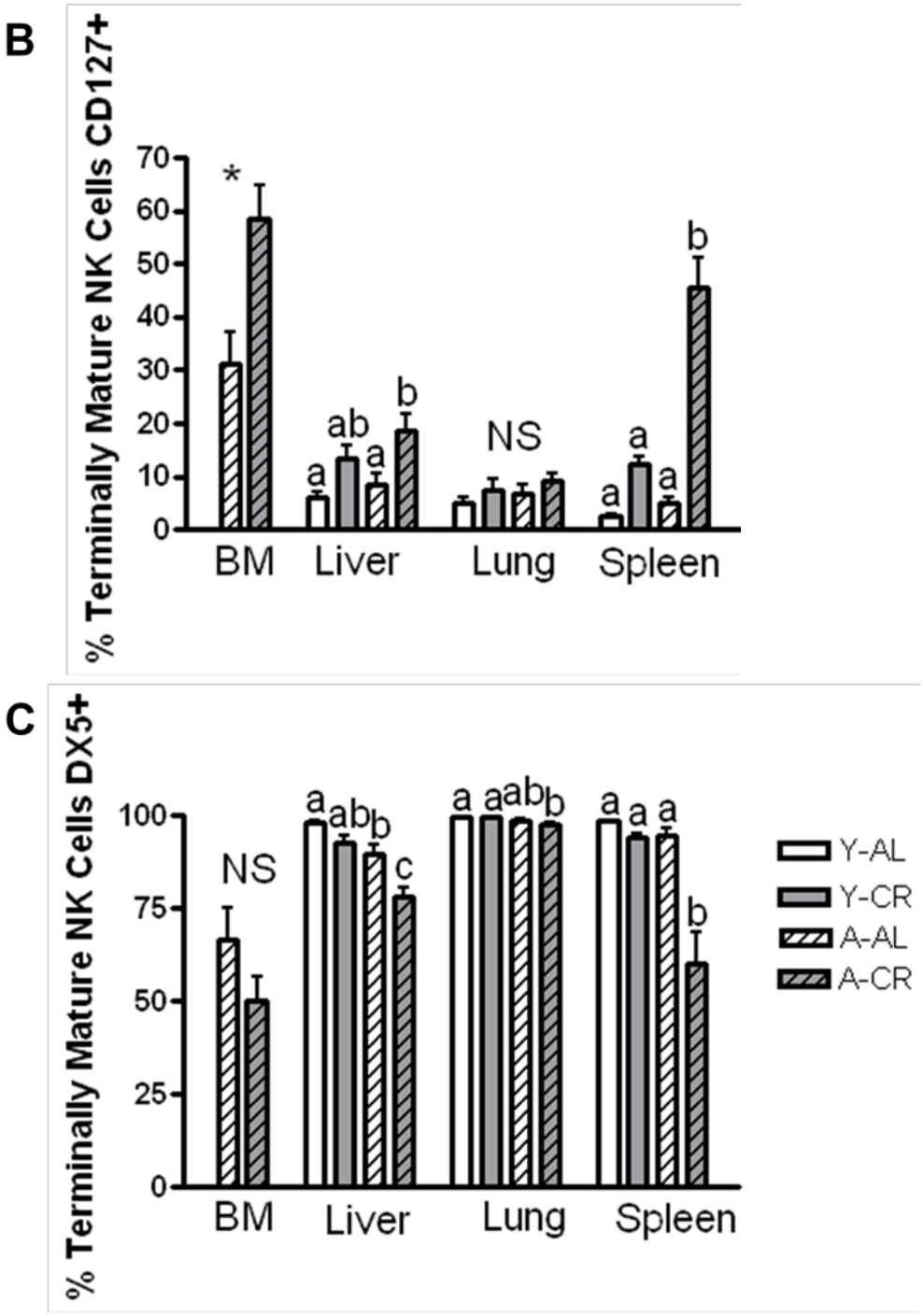


Figure 2.4. Expression of CD127 and DX5 on terminally mature bone marrow, liver, and spleen NK cells of young AL, young CR, aged AL, and aged CR mice. (A) Representative flow plot diagrams gated on $CD127^+$ (immature marker) and $DX5^+$ (mature marker) spleen NK cells within the $CD27^-CD11b^+$ (terminally mature) NK cell subset by flow cytometry. Each flow plot is a representative example of the group. The number in each box represents the percent of terminally mature NK cells positive for the identified marker. Y-AL= young *ad libitum*, Y-CR= young calorie restricted, A-AL= aged *ad libitum*, A-CR= aged calorie restricted. Means ± SEM of terminally mature ($CD27^-$ $CD11b^+$) NK cells expressing either $CD127^+$ (B) or $DX5^+$ (C) are represented as bar graphs for bone marrow (BM), liver, lung, and spleen (n=10 per group). Bars with different letters within the same tissue are statistically different ($P \leq 0.05$) as determined by one-way ANOVA with Tukey's *post hoc* test. Asterisks represent a significant difference ($P \leq 0.05$) as determined by t-test. NS= not significant.

Figure 2.4 (cont'd).



3.7 OO-CR and YO-CR mice have similar body composition

We first validated our in-house CR protocol by comparing weight, body composition (Supplemental Table 1), and NK cell percent, number, and maturation (Supplemental Figure 2A-F) between groups of young mice calorie restricted in-house and young mice calorie restricted by the NIA. Our young in-house CR mice had significantly higher body fat percent than young CR mice purchased from the NIA. However, the body fat percent of young in-house CR mice was not significantly different compared to the Y-CR mice from Table 1. No other significant differences in any of these parameters were found. We concluded our protocol was sound and comparable to the NIA protocol. Specific ages and characteristics of A-AL, YO-CR, and OO-CR mice are listed in Table 2. Following the NIA protocol, CR mice received 3 g/d of NIH-31/NIA fortified diet, which resulted in a 39% restriction. The AL mice had significantly higher body weight and percent of weight as fat than OO-CR and YO-CR. There were no significant differences in percent lean body mass between the groups. A-AL mice had significantly lower percent water weight than YO-CR and OO-CR mice. Time course data of OO-CR mice from AL (19.5 months) to euthanasia (22 months) are displayed in Supplemental Figure 3.

Table 2. Characteristics of aged AL, young-onset CR, and old-onset CR C57Bl/6 mice.

	A-AL[1]	YO-CR	OO-CR
Length of CR (mo)	N/A	18	2
Age (mo)	22	22	22
Weight (g)	34.4±2.0 [a,2]	22.4±.76[b]	25.2±0.3[b]
Fat (% of weight)	18.4±1.8[a]	10.8±0.15[b]	8.8±0.6[b]
Lean Body Mass (% of weight)	75.1±2.0[a]	78.3±0.2[a]	78.5±0.5[a]
Water (% of weight)	0.27±0.05[a]	0.63±0.09[b]	0.72±0.11[b]

[1]A-AL= aged *ad libitum*, YO-CR= young-onset calorie restricted, OO-CR= old-onset calorie restricted

[2]Values (means ± SEM) with different letters within the same row are statistically different (P≤0.05).

3.8 NK cells are reduced in OO-CR bone marrow, lung, and spleen

The overall effects of the initiation of CR during the aging process on NK cell percent and number were determined using flow cytometry. A-AL mice had a significantly higher percent (BM and lung, Figure 2.5A) and number (lung and spleen, Figure 2.5B) of NK cells than OO-CR mice. There were no significant differences of NK cell percent of live or NK cell number between the OO-CR and YO-CR groups.

3.9 OO-CR has detrimental effects on NK cell maturation

In order to determine if OO-CR was beneficial or detrimental, we assessed NK cell maturation between the YO-CR and OO-CR groups using the 4-step developmental program based on expression of CD27 and CD11b (Chiossone et al., 2009). Both aged CR groups had a higher percent of immature NK cells than the A-AL group. However, the early mature $CD27^{+}CD11b^{+}$ NK cells were significantly reduced in OO-CR compared to controls in the spleen (Figure 2.5E). Because we found terminally mature ($CD27^{-}CD11b^{+}$) NK cells to be sensitive to CR and aging (Figure 2.2 and 2.3) we compared expression of CD127 and DX5 on terminally mature NK cells form YO-CR and OO-CR. No differences were observed between the YO-CR and OO-CR groups.

Maturation of NK cells can be described as the loss of immature marker TRAIL, and upregulation of the integrin and mature marker, DX5 (Gordon et al., 2012). Therefore, we analyzed NK cells for expression of TRAIL (data not shown) and DX5 (Figure 2.5D). No significant differences in TRAIL were observed. However, the percent of $DX5^{+}$ NK cells was significantly reduced in OO-CR mice compared to YO-CR and A-

AL ($P \leq 0.05$). The reduction in splenic $DX5^+$ and $CD27^+CD11b^+$ NK cells suggests a more immature phenotype of OO-CR NK cells.

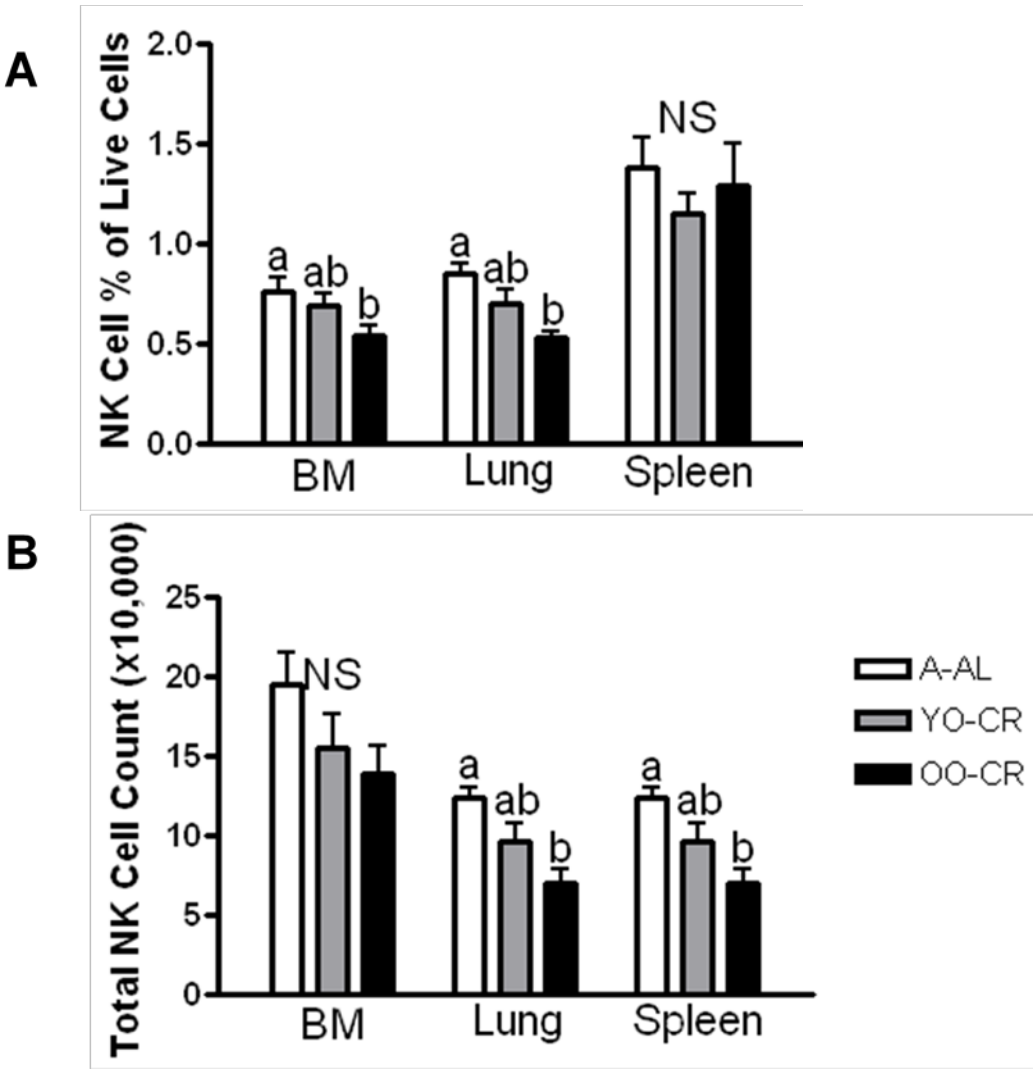


Figure 2.5. Analysis of NK cell percent, number, and maturation in the bone marrow, lung, and spleen of aged AL, young-onset CR, and old-onset CR mice. NK cell percent among live cells (A) and total NK cell number (B) in bone marrow (BM), lung, and spleen (n=10 per group) were analyzed by flow cytometry. Bars with different letters within the same tissue are statistically different (P≤0.05) as determined by one-way ANOVA with Tukey's *post hoc* test. NS= not significant, A-AL=aged *ad libitum*, YO-CR= young-onset calorie restricted, OO-CR=old-onset calorie restricted. (C) Representative flow plot diagrams describe the changes in maturation seen in the bone marrow (BM), lung, and spleen according to CD27 and CD11b, as described in Figure 2.3A. Each histogram is a representative example of the group. The number in each box represents the percent of NK cells positive for the identified marker. Percent of spleen NK cells $DX5^+$ (D) and $CD27^+CD11b^+$ (early mature) (E) (means ± SEM) are represented as bar graphs (n=10 per group). Bars with different letters within the same tissue are statistically different (P≤0.05) as determined by one-way ANOVA with Tukey's *post hoc* test.

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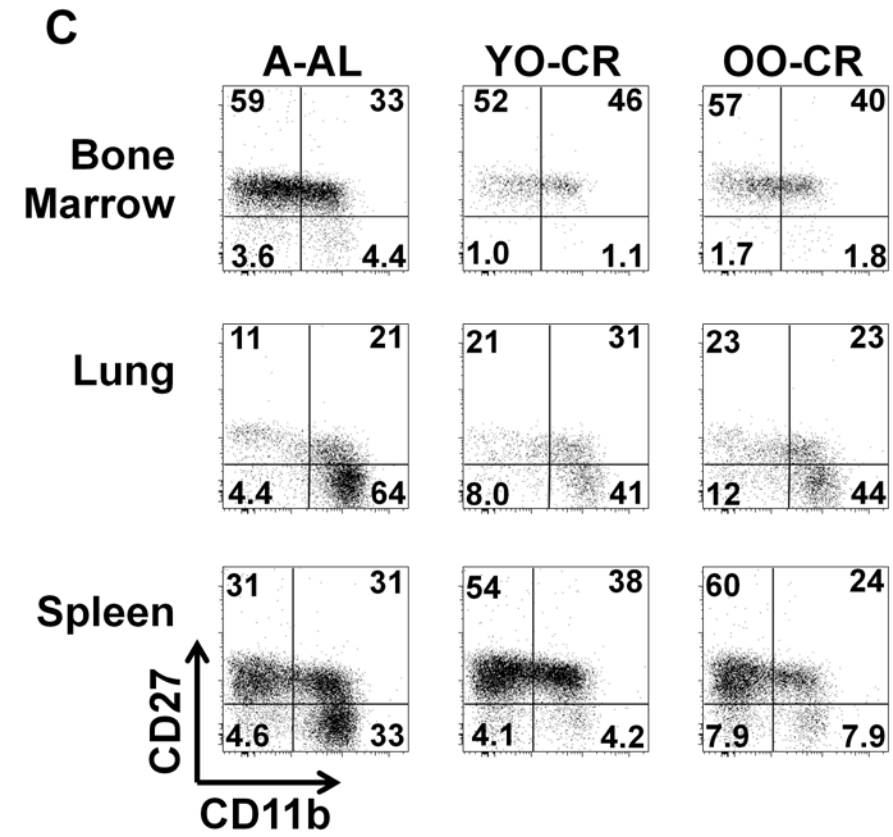


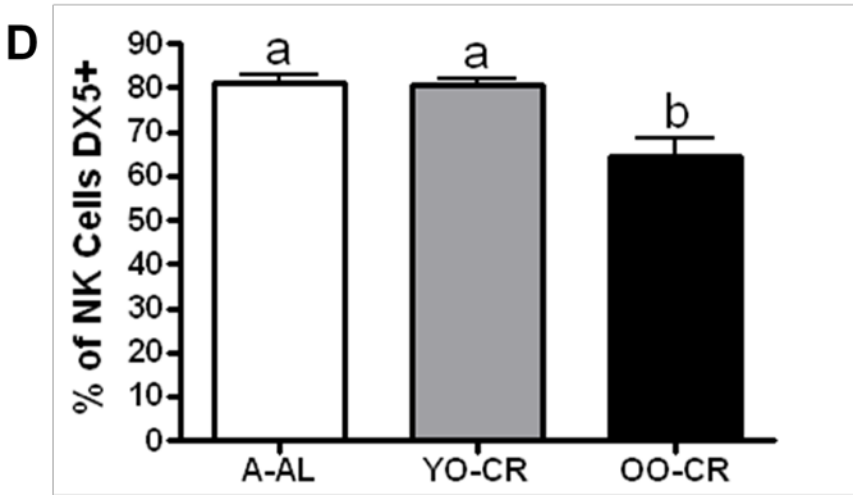
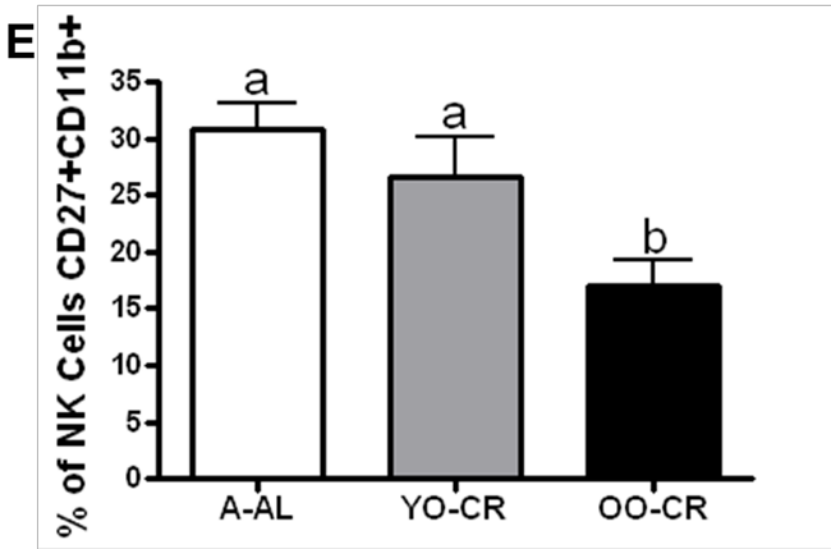


Figure 2.5 (cont'd)



4. Discussion

This research investigated the combined effects of CR and aging on NK cells. We found that CR exacerbates age-related defects to NK cells as evidenced by a more immature NK cell phenotype in A-CR mice. In addition, old-onset CR is more detrimental to NK cell maturation than young-onset CR as evidenced by the reduced percent of early mature NK cells from OO-CR mice expressing CD27 and CD11b or DX5. This research has implications not only for an aging population which may choose to calorie restrict, but also for aged individuals who may involuntarily undergo calorie restriction.

Independently, CR and aging affected circulating serum levels of corticosterone, TNF-α, leptin, and SDF1-α. However, there were no additive effects of combining the two paradigms. Corticosterone is a glucocorticoid that was measured to determine stress levels. Calorie restriction, but not aging, significantly increased serum corticosterone as expected. Calorie restriction (Harris et al., 1994) as well as aging (DeKosky et al., 1984) have previously been shown to raise corticosterone levels. Though CR is a stress resulting in elevated corticosterone, it is important to note CR is not protein-energy malnutrition (Ritz and Gardner, 2006), which is a more severe stress that also results in increased corticosterone. TNF-α is a pro-inflammatory cytokine released by macrophages and NK cells (Jewett et al., 1997). As expected, CR reduced TNF-α in circulating serum, which has been previously described by Spaulding et al. (Spaulding et al., 1997). Aging is associated with a pro-inflammatory environment (i.e. increased TNF-α). In this research, aging increased circulating TNF-α, as previously described (Bruunsgaard et al., 2000).

Leptin is a satiety hormone that NK cells depend on for proper maturation (Tian et al., 2002). Calorie restriction, but not aging, significantly reduced serum leptin. SDF1-α is suggested to regulate NK cell retention in bone marrow when SDF1-α concentration is higher in the bone marrow than in the circulation (Bernardini et al., 2008). In this study, SDF1-α was increased in aged mice, with no changes caused by calorie restriction. Even though we do not provide direct evidence, it is likely these environmental changes occurring with CR and aging contribute to the observed phenotype of NK cells.

Through extensive analysis of NK cell maturation stages, we confirmed NK cells are less mature in both aged and CR mice independently. In addition, we have clearly shown that there is a combined effect of joining these two paradigms in C57Bl/6 mice. The most notable observation, is the significant decrease of terminally mature ($CD27^-$ $CD11b^+$) NK cells in mice that were both aged and calorie restricted in the bone marrow, spleen, lung, and liver, compared to all control mice. Further analysis showed terminally mature NK cells had decreased expression of DX5 (a mature marker) and increased expression of CD127 (IL-7 Receptor; an immature marker). The downregulation of CD127 in $CD27^-CD11b^+$ NK cells in A-CR mice was expected because $CD127^+$ NK cells are generally thymic derived (Vosshenrich et al., 2006) and aging is associated with thymic involution (Aspinall and Andrew, 2000). IL-7 knockout mice are unable to produce thymic derived $CD127^+$ NK cells (Vosshenrich et al., 2006). We could not detect IL-7 in the serum, giving no further indication of a mechanism for improper CD127 expression in A-CR terminally mature NK cells.

Previous studies have shown that juvenile-onset, but not mid-adult or old-onset CR extends lifespan in rodents (Taylor et al., 1995; Weindruch et al., 1982). Additionally, old-onset calorie restriction does not extend the lifespan of rhesus monkeys (Mattison et al., 2012). Messaoudi *et al.* determined that old-onset calorie restriction has a negative effect on immune function, specifically T-cells (Messaoudi et al., 2008). Here, we provide evidence that old-onset calorie restriction may also be detrimental to NK cell maturation compared to young-onset calorie restriction. OO-CR mice had significantly lower numbers of NK cells in the lung and spleen compared to A-AL mice, while YO-CR numbers were not significantly different from A-AL numbers. There was also a significantly lower proportion of $DX5^+$ and $CD27^+CD11b^+$ (early mature markers) NK cells in the OO-CR group compared to YO-CR and A-AL controls. Therefore, initiating CR at advanced age has a negative impact on NK cell number and maturation in comparison to initiating CR at a young age.

During a T_H1 immune response, dendritic cells and macrophages produce IL-12 and IL-18 among other cytokines, which work synergistically to stimulate NK cells to produce IFNy (Lauwerys et al., 1999). IFNy is then released from NK cells eliciting a slew of functions, including activation of cytotoxic T cells, in order to kill virus infected cells. In this study IFN-γ production was measured following stimulation of NK cells *in vitro* with cytokines IL-12 and IL-18. No significant differences in IFN-γ production were seen (n=5; data not shown).

The CALERIE project has found some benefits to CR in humans, two separate studies showed that humans on a 25% CR diet for an average of 6 years had decreased atherosclerosis risk factors and improved diastolic function in aging (Fontana

et al., 2004; Meyer et al., 2006). In addition, 6 month CR reduced markers of longevity including body temperature and fasting blood insulin (Heilbronn et al., 2006). No data on immune function has yet been reported from the CALERIE project, however phase 2 will look at delayed-type hypersensitivity and antibody response to vaccines (Rochon et al., 2011). Effros *et al.* observed a declining antibody response to influenza vaccination in aging, which was improved with 40% CR in mice (Effros et al., 1991). In addition, Messaoudi *et al.* has shown an improvement in T cell response (important in naïve B cell maturation into antibody producing plasma cell) in mid-adult onset CR in rhesus monkeys (Messaoudi et al., 2008). Therefore, calorie restriction may improve the antibody response to vaccines in the CALERIE study.

NK cells are important during the innate immune response to primary influenza, because they have the capability of killing virus-infected cells via the recognition of altered MHC Class I. Therefore, depleting NK cells leads to increased susceptibility to primary influenza infection. Our laboratory has shown CR impairs NK cells (Clinthorne et al., 2012; Ritz et al., 2008) and aged CR mice infected with influenza have decreased survival and higher lung viral titers (Gardner, 2005). Therefore, additional research within the CALERIE project or other human research should measure innate immune parameters to determine the effects of CR on the immune response.

In conclusion, we have shown that CR and aging have a combined detrimental effect on the maturation of NK cells. Aged CR terminally mature ($CD27^{-}CD11b^{+}$) NK cells were significantly reduced in all tissues studied with increased expression of CD127 and decreased expression of DX5. In addition, a lower percentage of old-onset CR mice NK cells are in an early mature stage ($CD27^{+}CD11b^{+}$ or $DX5^{+}$) compared to

young-onset CR mice. Early mature ($CD27^{+}CD11b^{+}$) NK cells are the most effective at releasing cytokines in the proper environment (Fu et al., 2011). These findings suggest that CR in aging is a mechanism of declining natural killer cell maturation, which could result in increased susceptibility to viral infections.

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CHAPTER 3- CONCLUSION AND FUTURE DIRECTIONS

Aging and calorie restriction are independently detrimental to NK cell number and maturation status in C57Bl/6 mice. Combining the two paradigms resulted in an exacerbated detrimental effect on NK cell number, percent, and maturation. The most distinctive observation was the decrease in percent of terminally mature ($CD27^-$ $CD11b^+$) NK cells in all tissues studied (spleen, lung, bone marrow, and liver) in aged CR mice. These terminally mature NK cells in spleens of aged CR mice had high expression of the IL-7 receptor CD127 (a marker of immaturity) and low expression of the integrin DX5 (a marker of maturity). Aged CR mice were also subjected to two different times of CR onset. CR initiated during old age (20 months) resulted in a more immature NK cell phenotype as evidenced by decreased early mature NK cells ($DX5^+$ or $CD27^+CD11b^+$), compared to CR initiated during young age (4 months).

Combining the aging and CR paradigms to study NK cells in the absence of infection is new research adding to the current knowledge base. However, previous research from our laboratory supports these conclusions as well. In 2005, Gardner showed aged CR mice are more susceptible to death from influenza infection compared to aged AL mice (Gardner, 2005), which is attributed to decreased NK cell cytotoxicity and lower body weight in CR mice. Ritz *et al.* showed young CR mice infected with influenza have higher mortality which may be related to their decreased number and percent of NK cells (Ritz et al., 2008). Clinthorne *et al.* gave a detailed report of declined NK cell maturation in young CR mice (Clinthorne et al., 2013). And finally, Beli *et al.*

observed detrimental changes in NK cell maturation in aged influenza-infected mice (Beli et al., 2011).

This research provides insight into many different fields. Some researchers define NK cells based on expression of DX5, which has been considered an NK cell pan marker (Arase et al., 2001). However, approximately 35% of old-onset aged CR mice do not express DX5 (Figure 2.5); implying DX5 may not be an ideal marker to identify NK cells. Here, we show that terminally mature NK cells may not be developing due to decreased expression of IL-7 receptor (CD127) and increased expression of the integrin DX5; which may be relevant in other paradigms. Finally, it is well known that elderly people do not always consume the appropriate amount of calories (Wakimoto and Block, 2001). In the absence of malnutrition this mimics the CR diet. When determining susceptibility to certain diseases in the elderly, particularly viruses, NK cell status should be studied as a potential cause for a decrease in innate immune response.

Future studies should verify the results observed here by studying NK cells in aged calorie restricted humans. We may then determine dietary modulations which may be capable of improving the NK cell response. Previous research has shown benefits of active hexose correlated compound (Ritz et al., 2006) and probiotics (Dong et al., 2013; Kosaka et al., 2012; You and Yaqoob, 2012) on NK cell status; these modulations may prove beneficial in the aging CR paradigm. NK cells are crucial in controlling viral infections until an adaptive immune response can be mounted. Because there are lower numbers and maturation of NK cells in aged CR mice, future studies should also examine the effectiveness of the NK cell response to influenza or other viruses in aged CR mice. Terminally mature NK cells in aged CR mice have altered

expression of CD127 and DX5 which could not be explained mechanistically in the scope of this research. Understanding the mechanisms behind this observation will prove beneficial in the field of NK cell biology. Finally, only two ages of onset of CR were tested in this research, young-onset (4 months) and old-onset (20 months). Messaoudi et al. showed an improved T cell response in rhesus monkeys that were calorie restricted at a mid-age onset (Messaoudi et al., 2008). It would be interesting to determine if perhaps a mid-age onset of CR would be less detrimental to NK cells than old-onset.

In conclusion, combining aging and CR is detrimental to NK cell number, percent, and maturation; this is exacerbated when CR-onset occurs late in life. This conclusion is supported by previous research from our laboratory characterizing NK cell status in the absence and presence of influenza infection in either aged or CR mice. Future research needs to explore the implications and potential dietary modulations to improve NK cell status in aged CR individuals.

APPENDIX

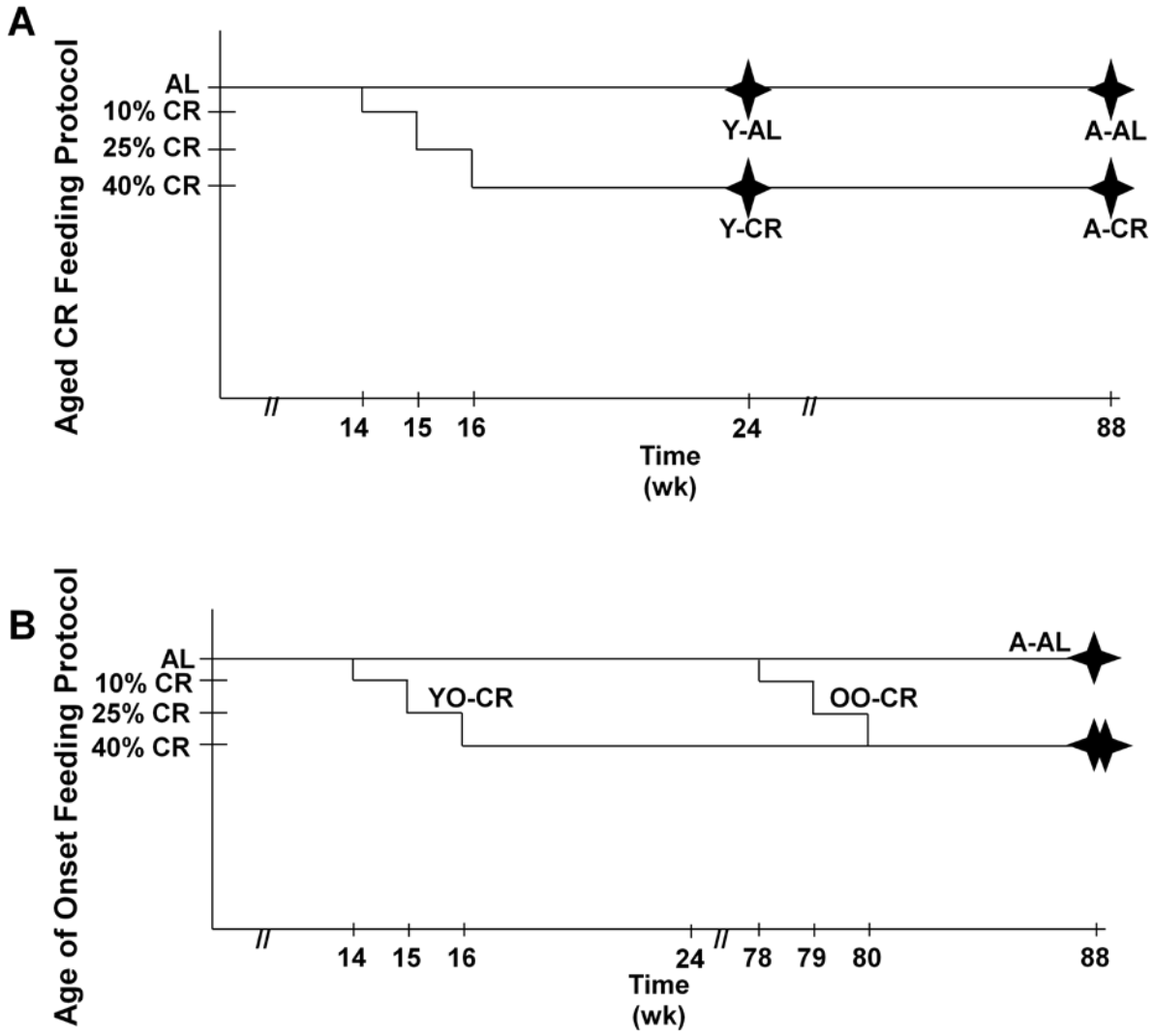
Supplemental Table 1. Characteristics of young purchased CR and young in-house CR C57Bl/6 mice.

	Y-P-CR[1]	Y-H-CR
Age (mo)	6	6
Weight (g)	23.8±0.5[a,2]	23.8±0.2[a]
Fat (% of weight)	12.3±0.5[a]	10.2±0.5[b]
Lean Body Mass (% of weight)	77.5±1.5[a]	77.9±0.7[a]
Water (% of weight)	0.49±0.03[a]	0.68±0.10[a]

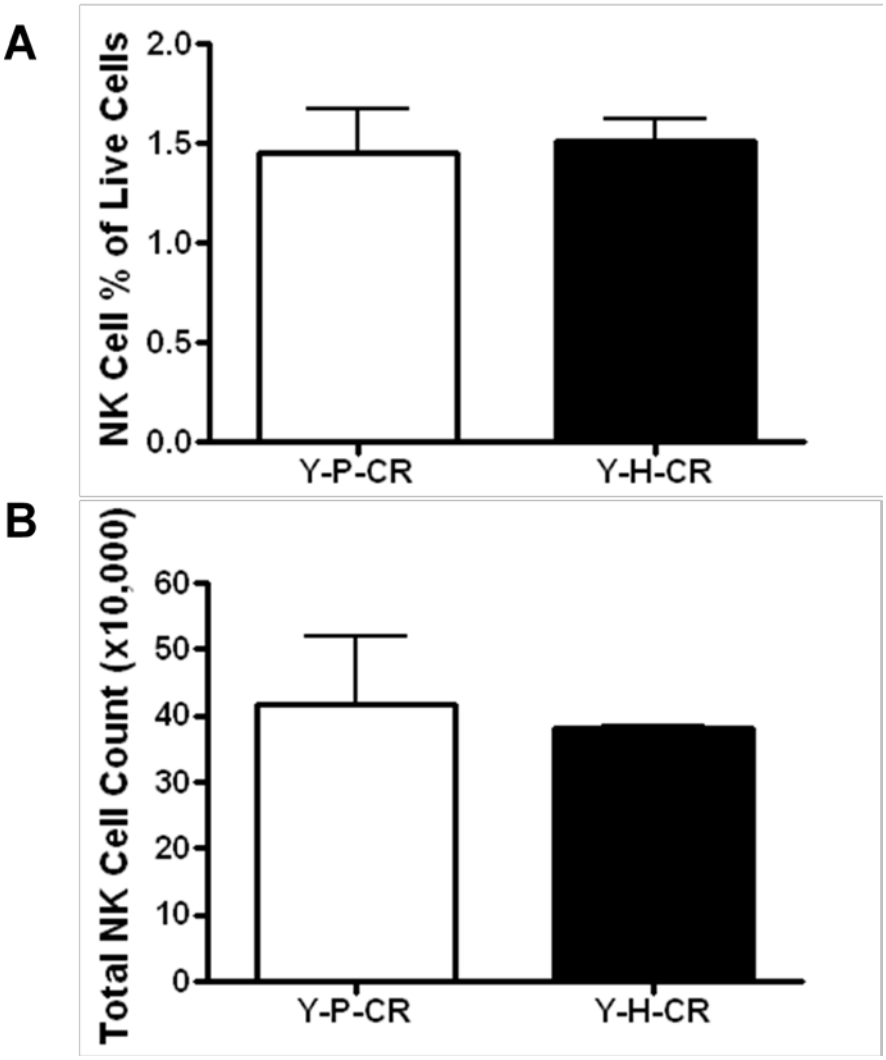
[1]Y-P-CR= young purchased calorie restricted,

Y-H-CR= young in-house calorie restricted

[2]Values (means ± SEM) with different letters within the same row are statistically different ($P \leq 0.05$).

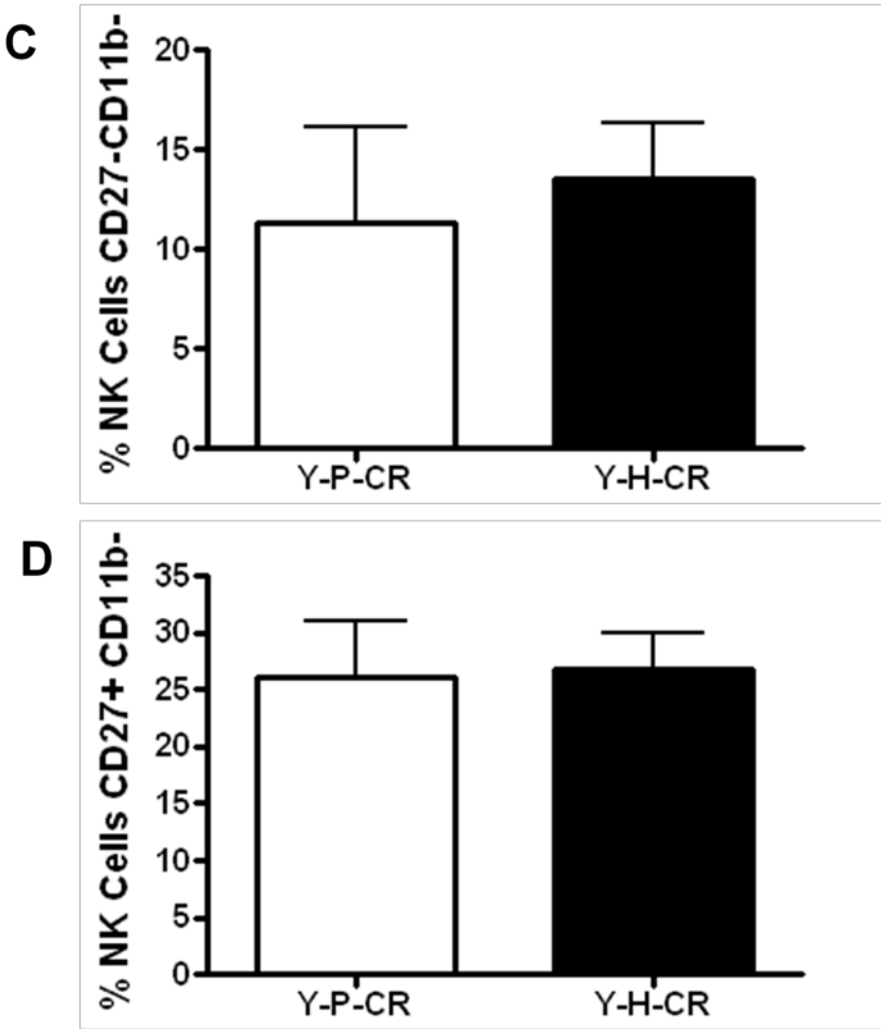


Supplemental Figure 1. Feeding protocols of all mice involved in this study. (A) Aged CR feeding protocol. Y-AL= young *ad libitum,* A-AL= aged *ad libitum,* Y-CR= young calorie restricted, A-CR= aged calorie restricted. Symbols represent time of CO_2 asphyxiation. (B) Age of onset feeding protocol. YO-CR= young-onset (CR at 4 mo) calorie restricted, OO-CR= old-onset (CR at 20 mo) calorie restricted.



Supplemental Figure 2. Validating in-house CR protocol by comparing young NIA-purchased CR mice to young in-house CR mice and comparing NK cell percent, number and maturation in the spleen. Young mice purchased and calorie restricted by the NIA (Y-P-CR, n=6) were compared to young mice calorie restricted in-house (Y-H-CR, n=10 per group). No significant differences were seen in NK cell percent (A), NK cell number (B), percent of $CD27^{-}CD11b^{-}$ (immature) NK cells (C), percent of $CD27^{+}CD11b^{-}$ (early mature) NK cells (D), percent of $CD27^{+}CD11b^{+}$ (late mature) NK cells (E), or percent of $CD27^{-}CD11b^{+}$ (terminally mature) NK cells (F). Data represented as means ± SEM, means were compared using an unpaired t-test ($P \leq 0.05$).

Supplemental Figure 2 (cont'd).



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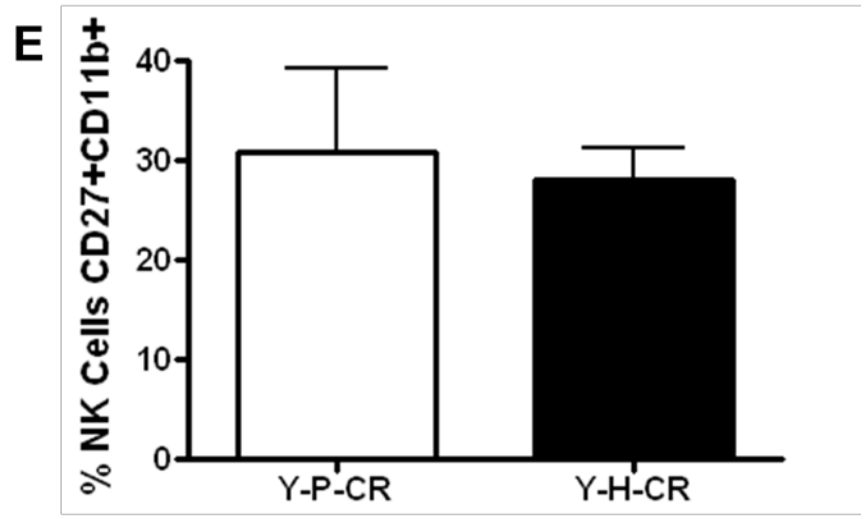
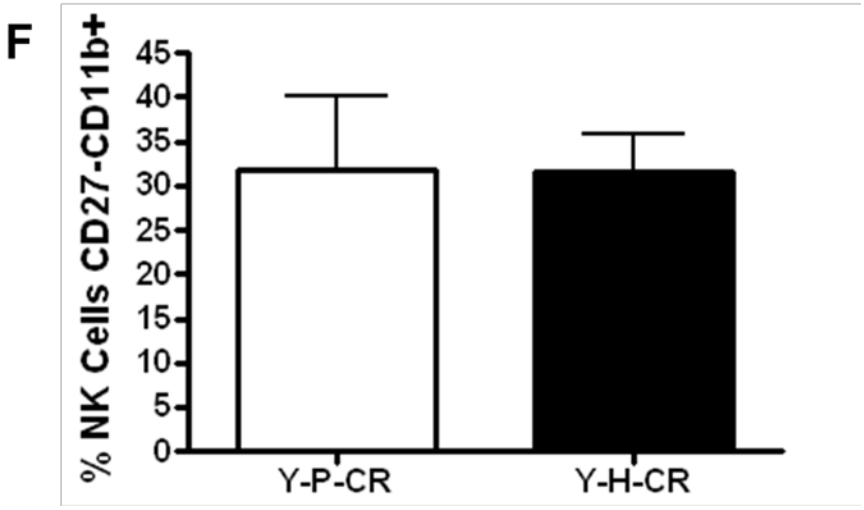
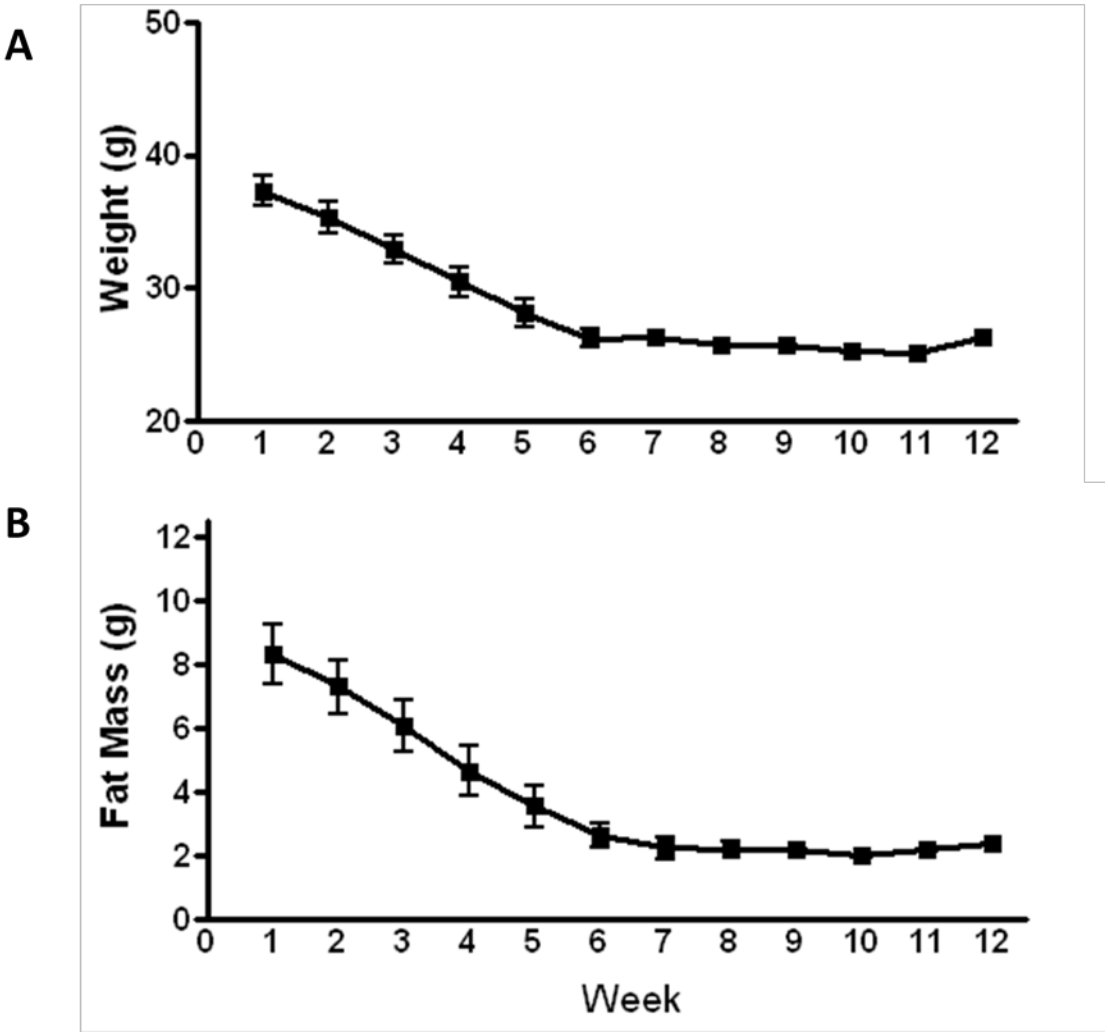


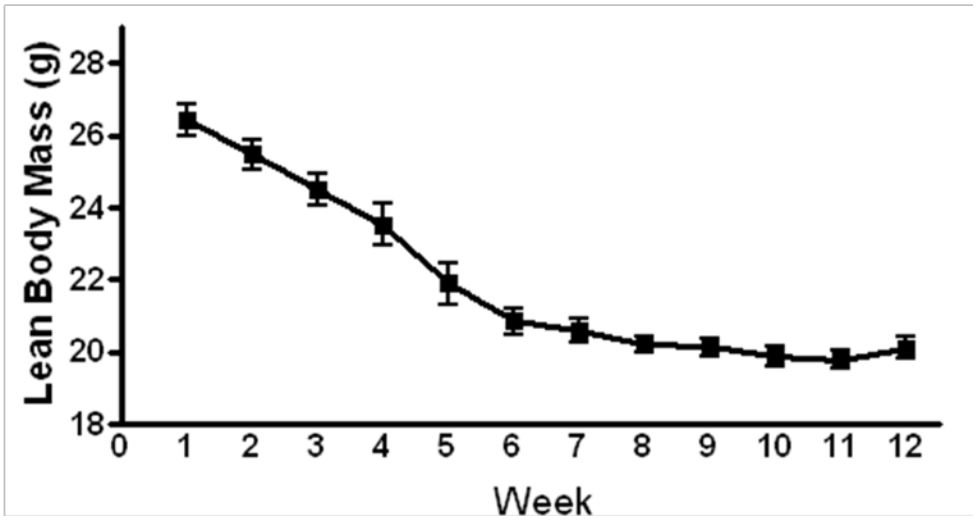




Supplemental Figure 3. Body composition data of aged old-onset CR mice from AL (19.5 months) to 2 months after 40% CR initiation (22 months). Week 1= AL diet, week 2= 3 days after 10% CR initiation, week 3= 3 days after 25% CR initiation, week 4= 3 days after 40% CR initiation, week 5-12= mice kept at 40% CR diet to stabilize. Weight (A), fat mass (B), and lean body mass (C) are all represented.

Supplemental Figure 3 (cont'd).

C



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