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Age, Calorie Restriction, and Age of Calorie Restriction Onset Reduce Maturation of Natural Killer Cells in C57Bl/6 Mice

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Abstract

Calorie restriction (CR), also known as energy restriction, has been shown to have a deleterious impact on both adult and aged mouse survival during influenza virus infection. Natural killer (NK) cell phenotypic differences contribute to increased susceptibility of adult CR mice. We hypothesized NK cell phenotype from adult and aged C57Bl/6 mice fed NIH-31 diet *ad libitum* (AL) would be different than NK cell phenotype from adult and aged mice fed NIH-31/NIA fortified diet at 40% CR. We hypothesized NK cell phenotype from mice consuming 40% CR initiated at 20 months of age would not be different than 40% CR initiated at 3 months of age. We initiated the 40% restriction either at the standard 12 weeks of age or at 78 weeks of age. NK cells were isolated and quantified from various tissues using flow cytometry. Aged CR mice had significantly reduced levels of terminally mature (CD27⁺CD11b⁺) NK cells, increased expression of the immature marker CD127, and decreased expression of the mature marker DX5. Total number of NK cells among cells was significantly lower in the lung and spleen of old-onset aged CR mice compared to aged AL mice, while there was no significant difference between young-onset aged CR and aged AL mice. Old-onset aged CR mice had significantly less early mature (DX5⁺ and CD27⁺CD11b⁺) NK cells compared to young-onset aged CR and aged AL fed mice. Overall, we found that CR in aged mice is detrimental to maturation of NK cells, which is exacerbated when CR is initiated in old-age.

Keywords

Natural killer cell; Calorie restriction; Aging; Mice; Cell maturation

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1. Introduction

Calorie restriction (CR) has been studied since the early 20th century [1], and has been generally accepted as a mechanism of increasing life span in yeast, worms, flies, and rodents. However, more recent data on the effects of CR on lifespan extension in non-human primates are controversial, evidenced by two recent longitudinal 20-year CR studies on rhesus monkeys. In the Wisconsin National Primate Research Center study [2], CR increased lifespan in non-human primates. However, using a similar CR protocol, but different exclusion criteria and different diets, Mattison et al. showed no significant effect on lifespan [3]. The degree of CR and age of CR onset are important considerations for both increased lifespan and immune benefits. While CR may not extend lifespan in all cases, CR can prevent tumor progression in rhesus monkeys [3], autoimmune disease in mice [4], reduce T cell senescence in rhesus monkeys [5], and reduce other immune-related disease. In contrast, we and others have shown differences in natural killer (NK) cell phenotype [6], increased susceptibility to primary influenza infection [7], and bacteria-induced peritonitis [8] in CR mice.

NK cells are innate immune cells that kill virus-infected cells and cancer cells. During NK cell maturation, common lymphoid progenitors in the bone marrow develop into NK1.1⁺CD122⁺ NK cells [9]. 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) [9]. 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 CD11b, while mature NK cells upregulate CD11b and progressively lose expression of CD27 [10]. Even though terminally mature NK cells lack expression of CD27, upon activation CD27 expression increases as a co-stimulatory marker. 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⁺ [11].

Migration of NK cells from the bone marrow to peripheral tissues is, at least partially, mediated by the chemokine CXCL12, also known as SDF1- α [12]. Our previous observations have shown reduced mature NK cells in peripheral tissues of CR mice, which correlated with reduced serum CXCL12 [13]. Peripheral tissue NK cells that express IL-7R α (CD127) represent cells that were either derived from the thymus [14] or immature bone marrow-derived NK cells [15]. Several signals induce NK cell maturation including IL-7, IL-15, and leptin. Leptin is a hormone directly proportional to levels of adipose tissue; it is released by the gut cells, adipose tissue, and hypothalamus to stimulate satiety [16]. NK cells express leptin receptors and were activated in increased leptin environments [17]. Furthermore, leptin is a pro-survival signal for several immune cells. Therefore, the balance of inducing and inhibiting signals in the environment impacts NK cell maturation and development.

Survival of influenza-infected adult CR mice was reduced within the first 3–5 days of infection. This increased susceptibility of adult CR mice correlated with decreased NK cell numbers and cytotoxicity in adult mice [6]. Similarly, *ad libitum* (AL) fed aged mice

infected with influenza had decreased NK cell survival [18] and functional responses [7] compared with adult mice. The phenotype of NK cells from aged CR mice has yet to be characterized. Age of CR onset is important for development of immune benefits associated with CR. Messaoudi *et al.* showed that initiating CR in young adult (postpuberty) primates prevents T cell senescence associated with aging, while young-onset (pre-puberty) CR and old-onset CR had a detrimental effect on T cell development and function [19]. However, when CR was initiated in young adult mice, NK cells did not mature completely nor develop their full function [6]. Due to different effects of CR on NK cells compared to T cells when initiated in young adult animals, it is possible that age of onset affects NK cells differently than T cells.

While we have described NK cell changes in adult CR mice and observed increased susceptibility to primary influenza infection in aged CR mice, we have yet to determine if this increased susceptibility of aged CR mice to influenza was related to alterations in NK cell maturation or function. In the present studies, we evaluated NK cell number and phenotype in aged (22 month) C57Bl/6 mice consuming a CR diet. Based on our previous observations regarding aging and CR, we hypothesized there would be more NK cell phenotypic differences in aged CR mice compared with controls. In addition, CR may affect NK cells differentially depending on the age of the animals at the time of CR initiation. Therefore, the second aim of our study was to determine whether adult-onset CR affects NK cells differently than old-onset CR in aged mice. We hypothesized the age of onset of CR would not have different 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. Methods and Materials

2.1 Animals and housing

C57Bl/6 male mice were purchased from the National Institute of Aging (NIA) maintained at Charles River Laboratories (Wilmington, MA, USA). Mice were individually housed in micro-isolator cages at the Michigan State University Research Containment Facility, a facility certified by the Association for Assessment and Accreditation of Laboratory Animal Care International. Mice were fed diets purchased from the NIA. Control animals received NIH-31 diet AL, and calorie restricted (CR) animals received 3 g/day of NIH-31/NIA fortified diet, resulting in 40% CR (Table 1). These diet compositions have been described previously [6]. Body composition was measured weekly using an EchoMRI-500 (Echo Medical Systems, Houston, TX, USA) as previously described [13]. Mice were euthanized by CO₂ asphyxiation followed by cardiac puncture. 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

To characterize the effect of age and CR on NK cell phenotype, 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 CR feeding protocol used by the NIA consists of

weaning all mice in each group onto AL diet. At 12 weeks of age, 10% CR is induced by feeding NIH-31/NIA fortified diet (about 4g per day) and maintained for one week, followed by one week at 25% CR by feeding NIH-31/NIA fortified diet (about 3.5g per day), and finally 40% CR at 14 weeks of age by feeding NIH-31/NIA fortified diet (about 3g per day) [20]. The CR mice were maintained at 40% CR until purchased at 6 months (2 month duration of 40% CR) and 22 months (18 month duration of 40% CR) of age. Age-matched AL fed animals were also purchased at this time. After mice were received they acclimated for at least 1 week and were maintained on their respective diet until euthanasia.

For the age of onset of CR feeding protocol, 3 groups of 22 month old mice were assessed: 10 aged AL (A-AL), 10 old-onset aged CR (O-A-CR) and 10 young onset aged CR (Y-A-CR). A-AL and Y-A-CR were purchased from the NIA at 20 months of age. The Y-A-CR mice had been calorie restricted by the NIA as described above [20]. Mice were fed their respective diets until euthanasia at 22 months of age, resulting in an 18 months of restriction for the Y-A-CR group. O-A-CR mice had been fed AL by the NIA, until they were purchased at 19 months of age. We then calorie restricted the animals in-house beginning at 19.5 months, using the same protocol as the NIA (described above), switching diets at weekly intervals to increasing levels of CR: 10% (approximately 4g of NIH-31/NIA fortified diet per day), 25% (approximately 3.5g of NIH-31/NIA fortified diet per day), and 40% (3g of NIH-31/NIA fortified diet per day). Forty percent CR was reached by 20 months of age and O-A-CR animals were held on this restriction until euthanasia at 22 months of age, for a total of a 2 months of calorie restriction (Supplemental Figure S1).

2.3 Tissue processing

Blood was collected by cardiac puncture and centrifuged at 2000×g for 20 min. Isolated serum was stored at −80°C until analysis. Lungs and spleens were digested with 1 mg/mL collagenase D (Roche, Indianapolis, IN, USA) and 40 units/mL DNase 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 into single cell suspensions by dounce homogenization as previously described [21]. Bone marrow cell processing was performed, as previously described [13], by flushing bones with sterile cell culture media to create a single cell suspension. Red blood cells from lung, spleen and bone marrow were lysed with ammonium chloride-potassium (ACK) lysis buffer (Lonza, Walkersville, MD, USA). Hemacytometer cell counts with trypan blue exclusion dye were used to obtain total cell numbers from each tissue.

2.4 Flow cytometry

Non-specific binding was blocked by incubating cells for 10 min with anti-CD32/CD16 antibody (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): anti-NK1.1-APC, anti-CD127-PerCPy5.5, anti-CD3-AlexaFluor 700, anti-CD11b-PECy7, anti-CD27-PE, anti-DX5-FITC. 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, according to manufacturer instructions; Corticosterone (Promega, Madison, WI, USA), Leptin (R&D Systems, Minneapolis, MN, USA), TNF- α (eBioscience, San Diego, CA, USA), and IL-7 (Ray BioTech, Norcross, GA, USA).

2.6 Statistical Analyses

Data were analyzed with GraphPad Prism 7 (GraphPad Software, Inc, La Jolla, CA, USA). Data comparing Y-AL, A-AL, Y-CR, and A-CR was analyzed using two way ANOVA followed by Tukey's *post hoc* test to determine the effects of age, diet, and agediet interaction on NK cell phenotype. Data comparing age of onset (A-AL, Y-A-CR, and O-A-CR) was analyzed using one way ANOVA followed by Tukey's *post hoc* test to determine the effects of CR onset on NK cell phenotype. All data are expressed as means $\pm$ SEM and significance was set at $p < 0.05$. Post-hoc power analyses were performed with G*Power 3.1.9.2 on total NK cell percent for each tissue. For one-way ANOVA, at 0.05 alpha, 10 samples in each of 3 groups (30 total), total spleen NK cell percent had an effect size of 0.196 and 13.6% power, total lung NK cell percent had an effect size of 0.855 and 98.3% power, and total bone marrow NK cell percent had an effect size of 0.516 and 66.5% power.

3. Results

3.1 Age and calorie restriction create changes in the systemic environment

In agreement with previous reports [13], CR mice had a significantly higher serum concentration of corticosterone than age-matched AL mice (6 month AL; 36.64 ± 5.52 ng/mL vs 6 month CR; 205.58 ± 27.31 ng/mL, Tukey's $p < 0.001$, 22 month AL; 51.04 ± 11.36 ng/mL vs 22 month CR; 158.82 ± 22.92 ng/mL, Tukey's $p = 0.0021$) (Figure 1A). Age was not a significant factor for serum corticosterone concentration. Corticosterone from 6 month AL serum was significantly different than 22 month CR serum (Tukey's $p = 0.0005$) and corticosterone from 6 month CR serum was significantly different than 22 month AL (Tukey's $p < 0.0001$). Diet was the only significant term of the two way ANOVA for corticosterone ($p < 0.001$). Age had no effect on serum leptin concentration (Figure 1B). As expected based on body weight and composition (Table 2), serum leptin concentration was significantly different by diet ($p = 0.0003$) but not age or the interaction of age and diet [13]. Serum leptin concentrations were significantly decreased in 6 month CR (1233.7 ± 222.6 pg/mL, Tukey's $p = 0.0255$) and 22 month CR (1340.5 ± 228.1 pg/mL, Tukey's $p = 0.0435$), compared to 6 month AL (12512.2 ± 3282.0 pg/mL) mice; as well as significantly decreased in 6 month CR (Tukey's $p = 0.0311$) and 22 month CR (Tukey's $p = 0.0496$), compared to 22 month AL (12825.1 ± 4262.7 pg/mL). Diet was the only significant term of the two way ANOVA for leptin ($p = 0.0003$). Serum concentration of a pro-inflammatory cytokine, TNF- α (Figure 1C), was reduced in 22 month CR (72.97 ± 11.19 pg/mL) mice compared to age-matched controls (22 month AL; 128.59 ± 13.41 pg/mL, Tukey's $p = 0.0049$), while 22 month AL mice had significantly higher TNF- α levels

(Tukey's $p=0.0006$) than 6 month AL mice (42.48 ± 2.20 pg/mL). Serum concentrations of TNF- α were not detected in 6 month CR mice (0.00 ± 0.00 pg/mL), which was significantly different than both 22 month AL (Tukey's $p<0.0001$) and 22 month CR mice (Tukey's $p=0.0006$). Diet ($p=0.0004$) and age ($p<0.0001$), independently, were significant terms of the two way ANOVA for TNF- α . Serum IL-7 could not be detected in any samples (data not shown). Body weight and composition was different between diet and age groups of mice (Table 2). Age ($p=0.0075$) and diet ($p<0.0001$) independently were significant terms for body weight. Body weight was significantly increased in 22 month AL mice (34.4 ± 2.0 g) compared to 6 month AL mice (28.5 compared to 6 month AL mice 1.0 g, Tukey's $p=0.0143$), 6 month CR mice (21.0 ± 0.5 g, Tukey's $p<0.0001$), and 22 month CR mice (22.4 ± 0.8 g, Tukey's $p<0.0001$).

Body weight was increased in 6 month AL mice compared to 6 month CR (Tukey's $p=0.0022$) and 22 month CR (Tukey's $p=0.0121$) mice. Diet was a significant factor ($p<0.0001$) for percent body fat. Percent body fat was decreased in 6 month CR mice ($9.8 \pm 0.4\%$) compared to both 6 month AL ($16.0 \pm 0.9\%$, Tukey's $p=0.0036$) and 22 month AL ($18.4 \pm 1.8\%$, Tukey's $p=0.0001$) mice. Percent body fat was decreased in 22 month CR ($10.8 \pm 0.15\%$) compared to 6 month AL (Tukey's $p=0.0139$) and 22 month AL (Tukey's $p=0.0005$) mice. There were no significant differences in lean body mass between any diet or age group (6 month AL; $75.7 \pm 0.8\%$, 6 month CR; $76.2 \pm 0.5\%$, 22 month AL; $75.1 \pm 2.0\%$, 22 month CR; $78.3 \pm 0.2\%$). Diet was a significant factor ($p=0.0006$) for percent water weight. Percent water weight was significantly decreased in both 6 month AL ($0.33 \pm 0.04\%$, Tukey's $p=0.0152$) and 22 month AL ($0.27 \pm 0.05\%$, Tukey's $p=0.0042$) compared to 22 month CR mice ($0.63 \pm 0.008\%$). Percent water weight of 6 month CR mice ($0.50 \pm 0.06\%$) was not significantly different than 6 month AL, 22 month AL, or 22 month CR mice.

3.2 Natural killer cells are reduced in aged calorie restricted mice

The effects of CR and aging on NK cell percentages (Figure 2A) and numbers (Figure 2B) were assessed in bone marrow, lung, and spleen. In all tissues, 22 month- CR mice had reduced percent NK cells among lymphocytes (determined by forward and side scatter properties) and total NK cell number, although this was not always statistically significant. In the bone marrow, there was a significant reduction in the percentage of NK cells in terms of age ($p<0.0001$) and interaction of diet and age ($p=0.0002$). However, total number of bone marrow NK cells were only statistically significant in the interaction of diet and age (two way ANOVA; $p=0.0309$). Based on Tukey's post-hoc analysis there were no significant differences in total bone marrow NK cell number between any diet or age groups (6 month AL; $17.00 \pm 0.38 \times 10^4$ cell, 6 month CR; $18.67 \pm 2.28 \times 10^4$ cell, 22 month AL; $18.34 \pm 11.36 \times 10^4$ cell, 22 month CR; $12.64 \pm 1.05 \times 10^4$ cell). Bone marrow NK cell percentage was significantly increased in 6 month CR mice (6 month CR; $1.28 \pm 0.02\%$) compared to 6 month AL mice (6 month AL; $1.00 \pm 0.04\%$, Tukey's $p=0.0122$), 22 month AL ($0.78 \pm 0.11\%$, Tukey's $p<0.0001$), and 22 month CR mice ($0.55 \pm 0.04\%$, Tukey's $p<0.0001$). In addition, percent of bone marrow NK cells from 6 month AL mice was significantly increased compared to bone marrow NK cells from 22 month CR mice (Tukey's $p<0.0001$). The interaction of diet and age was not significant, but independently diet ($p<0.0001$) and

age ($p=0.0002$) were significant two way ANOVA terms for the percentage of lung NK cells. Total lung NK cell number was significant between diet groups ($p<0.0001$) in the two way ANOVA. Percent and total number of 6 month AL lung NK cells (percent; $2.45 \pm 0.26\%$, total number; $42.23 \pm 4.46 \times 10^4$ cell) were significantly increased compared to lung NK cell of 6 month CR (percent; $1.17 \pm 0.14\%$, Tukey's $p<0.0001$, total number; $13.76 \pm 1.42 \times 10^4$ cell, Tukey's $p=0.0102$) and 22 month CR mice (percent; $0.30 \pm 0.05\%$, Tukey's $p<0.0001$, total number; $3.65 \pm 0.75 \times 10^4$ cell, Tukey's $p=0.0004$). Percent and total number of lung NK cells from 6 month CR mice were significantly increased compared to lung NK cell percent from 22 month CR mice in terms of percentage (Tukey's $p=0.0104$) but not total cell number. Lung NK cell percentage and total number from 22 month AL mice (percent; $1.78 \pm 0.21\%$, total number; $36.46 \pm 11.09 \times 10^4$ cell) were significantly increased compared to 22 month CR mice (percent; Tukey's $p<0.0001$, number; Tukey's $p=0.0025$). In the spleen, age (percent; $p<0.0001$, total number; $p<0.0001$), diet (percent; $p<0.0001$, total number; $p<0.0001$), and the interaction of diet and age (percent; $p=0.0044$, total number; $p<0.0001$) were all statistically significant for both the percentage and total number of NK cells. Percent and total number of spleen NK cells from 6 month AL mice (percent; $2.69 \pm 0.15\%$, total number; $253.91 \pm 24.14 \times 10^4$ cell) were significantly increased compared to 6 month CR mice (percent; $1.80 \pm 0.05\%$, Tukey's $p<0.0001$, total number; $56.20 \pm 5.40 \times 10^4$ cell, Tukey's $p<0.0001$), 22 month AL mice (percent; $1.44 \pm 0.13\%$, Tukey's $p<0.0001$, total number; $81.16 \pm 1.64 \times 10^4$ cell, Tukey's $p<0.0001$), and 22 month CR mice (percent; $1.23 \pm 0.10\%$, Tukey's $p<0.0001$, total number; $23.27 \pm 1.36 \times 10^4$ cell, Tukey's $p<0.0001$). Percent of spleen NK cells from 22 month CR mice were significantly reduced compared to 6 month CR mice (Tukey's $p=0.0038$), but not 22 month AL mice (Tukey's $p=0.1241$) while total spleen NK cells from 22 month CR mice were not significantly different compared to 6 month CR mice (Tukey's $p=0.2679$), but significantly reduced compared to 22 month AL mice (Tukey's $p=0.0161$).

3.3 Aged calorie restricted natural killer cells are less mature

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 [10]. Using this step-wise program, the maturation status of NK cells in tissues of aged CR mice compared to controls was determined (Figure 3). The percent of immature ($CD27^+/CD11b^-$) NK cells (Figure 3A) were not significantly different in bone marrow between diet and age groups of mice (6 month AL; $50.07 \pm 1.9\%$, 6 month CR; $50.33 \pm 2.08\%$, 22 month AL; $45.30 \pm 5.64\%$, 22 month CR; $48.78 \pm 5.60\%$). However, age ($p<0.0001$), diet ($p<0.0001$), and the interaction ($p=0.001$) were significant factors for percentage of immature NK cells present in the lung. Immature NK cells were increased in the lung of 22 month CR mice ($27.03 \pm 2.93\%$) compared to other diet and age groups of mice (6 month AL; $6.59 \pm 0.48\%$, Tukey's $p<0.0001$, 6 month CR; $11.54 \pm 1.05\%$, Tukey's $p<0.0001$, 22 month AL; $9.64 \pm 1.01\%$, Tukey's $p<0.0001$). Age ($p<0.0001$) and diet ($p<0.0001$) were significant factors for spleen immature NK cell percentage. Immature NK cells were increased in spleen of 22 month CR mice ($50.19 \pm 1.90\%$) compared to other diet and age groups of mice (6 month AL; $23.47 \pm 1.25\%$, Tukey's $p<0.0001$, 6 month CR; $41.06 \pm 1.35\%$, Tukey's $p=0.0021$, 22 month AL; $38.19 \pm 2.11\%$, Tukey's $p<0.0001$). In addition, 6 month CR (Tukey's $p<0.0001$) and 22 month AL (Tukey's $p<0.0001$) mice had significantly increased percentage of spleen

immature NK cells compared to 6 month AL mice. Age was a significant factor for bone marrow double positive (CD27⁺/CD11b⁺) NK cells (Figure 3B) ($p=0.0035$), but diet and age groups were not significantly different using Tukey's post-hoc analysis (6 month AL; $33.40 \pm 1.64\%$, 6 month CR; $34.69 \pm 2.0\%$, 22 month AL; $44.04 \pm 4.82\%$, 22 month CR; $45.34 \pm 4.70\%$). Diet was a significant factor for lung double positive NK cell percent ($p=0.027$) but diet and age groups were not significantly different using Tukey's posthoc analysis (6 month AL; $11.34 \pm 1.84\%$, 6 month CR; $16.14 \pm 3.77\%$, 22 month AL; $12.18 \pm 1.91\%$, 22 month CR; $18.65 \pm 2.03\%$). The interaction of diet and age ($p=0.0112$) was a significant term for double positive spleen NK cell percentage. Double positive spleen NK cell percent was significantly increased in 6 month CR mice ($21.12 \pm 1.28\%$) compared to 6 month AL ($15.35 \pm 0.94\%$, Tukey's $p=0.0189$) and 22 month CR ($15.43 \pm 1.40\%$, Tukey's $p=0.021$), but not 22 month AL (16.80 ± 1.67) mice. In the bone marrow, terminally mature (CD27⁻/CD11b⁺) NK cells (Figure 3C) were significantly different based on diet ($p=0.0002$). Terminally mature bone marrow NK cell percent was significantly increased in 6 month AL ($9.87 \pm 1.07\%$) compared to 6 month CR ($5.93 \pm 1.02\%$, Tukey's $p=0.0497$) and 22 month CR ($3.71 \pm 0.87\%$, Tukey's $p=0.0012$), but not 22 month AL mice ($8.50 \pm 1.34\%$). In addition, 22 month AL terminally mature bone marrow NK cell percent was significantly increased compared to 22 month CR mice (Tukey's $p=0.0219$). Diet ($p<0.0001$), age ($p<0.0001$), and the interaction ($p=0.0175$) were all significant factors for terminally mature lung NK cell percentage. All diet and age groups had significantly increased percent of terminally mature lung NK cells (6 month AL; $74.86 \pm 2.14\%$, Tukey's $p<0.0001$, 6 month CR; $63.79 \pm 4.31\%$, Tukey's $p<0.0001$, 22 month AL; $65.93 \pm 3.49\%$, Tukey's $p<0.0001$) compared to 22 month CR mice ($35.07 \pm 5.27\%$). Diet ($p<0.0001$) and age ($p<0.0001$) were significant factors for terminally mature spleen NK cell percentage. All diet and age groups were significantly different with Tukey's post-hoc analysis. Terminally mature spleen NK cell percent was highest in 6 month AL ($52.30 \pm 1.78\%$) and significantly increased compared to other diet and age groups (6 month CR; $33.40 \pm 2.21\%$, Tukey's $p<0.0001$, 22 month AL; $24.92 \pm 1.47\%$, Tukey's $p<0.0001$, 22 month CR; $11.61 \pm 1.00\%$, Tukey's $p<0.0001$).

Terminally mature spleen NK cell percent in 6 month CR was significantly increased compared to 22 month AL (Tukey's $p=0.0052$) and 22 month CR mice (Tukey's $p<0.0001$) and 22 month AL terminally mature spleen NK cells were significantly increased compared to 22 month CR mice (Tukey's $p<0.0001$). Terminally mature (CD27⁻/CD11b⁺) NK cells were further characterized based on expression of CD49b (DX5) as a mature marker and CD127 (IL-7 receptor) as an immature marker (Figure 3D). Diet was a significant factor ($p=0.0307$) in the analysis of percent terminally mature bone marrow NK cells expressing DX5 (Figure 3E), but no diet or age groups were significantly different based on Tukey's post-hoc analysis (6 month AL; $78.35 \pm 3.06\%$, 6 month CR; $54.82 \pm 7.45\%$, 22 month AL; $66.39 \pm 8.84\%$, 22 month CR; $50.07 \pm 6.74\%$). Age was a significant factor ($p=0.0010$) in the analysis of percent terminally mature lung NK cells expressing DX5. Terminally mature lung NK cells expressing DX5 were significantly decreased in 22 month CR ($97.40 \pm 0.74\%$) mice compared to 6 month AL ($99.38 \pm 0.13\%$, Tukey's $p=0.0129$) and 6 month CR ($99.70 \pm 0.08\%$, Tukey's $p=0.0031$) mice, but not compared to 22 month AL ($98.58 \pm 0.41\%$) mice. Diet ($p<0.0001$), age ($p=0.002$), and the interaction ($p=0.0017$) were all

significant factors in the analysis of percent terminally mature spleen NK cells expressing DX5. The percent of terminally mature NK cells expressing DX5 was significantly reduced in 22 month CR ($60.05 \pm 8.39\%$) mice compared to other diet and age groups (6 month AL; $98.52 \pm 0.30\%$, Tukey's $p < 0.0001$, 6 month CR; $93.97 \pm 1.23\%$, Tukey's $p < 0.0001$, 22 month AL; $94.78 \pm 1.71\%$, Tukey's $p < 0.0001$). Percent of terminally mature bone marrow NK cells expressing CD127 (Figure 3F) was significantly affected by diet ($p = 0.0003$). In the bone marrow, percent terminally mature NK cells expressing CD127 was significantly increased in 22 month CR mice ($58.55 \pm 6.17\%$) compared to 6 month AL ($25.43 \pm 5.05\%$, Tukey's $p = 0.0126$) and 22 month AL ($31.26 \pm 5.97\%$, Tukey's $p = 0.0077$), but not 6 month CR mice (55.32 ± 4.01). Percent of terminally mature lung NK cells expressing CD127 was not significantly different (6 month AL; $4.90 \pm 1.28\%$, 6 month CR; $7.38 \pm 1.99\%$, 22 month AL; $6.69 \pm 1.80\%$, 22 month CR; $8.99 \pm 1.52\%$). Diet ($p < 0.0001$), age ($p < 0.0001$), and the interaction ($p < 0.0001$) were all significant factors for percent of terminally mature spleen NK cells expressing CD127. In the spleen, percent terminally mature NK cells expressing CD127 were significantly increased in 22 month CR mice ($45.61 \pm 5.64\%$) compared to other diet and age groups (6 month AL; $2.58 \pm 0.15\%$, Tukey's $p < 0.0001$, 6 month CR; $12.28 \pm 1.38\%$, Tukey's $p < 0.0001$, 22 month AL; $5.05 \pm 0.89\%$, Tukey's $p < 0.0001$).

3.4 Young onset – aged – calorie restricted and old onset – aged – calorie restricted mice have similar environments

Following the NIA protocol, CR mice received 3 g/d of NIH-31/NIA fortified diet, which resulted in an approximately 40% restriction (Table 3). The A-AL mice had significantly higher body weight ($34.4 \pm 2.0\text{g}$) and percent body fat ($18.4 \pm 1.8\%$) than O-A-CR ($25.2 \pm 0.3\text{g}$ body weight, $8.8 \pm 0.6\%$ body fat) and Y-A-CR ($22.4 \pm 0.76\text{g}$ body weight, $10.8 \pm 0.15\%$ body fat). There were no significant differences in percent lean body mass between any age of onset groups (A-AL; $75.1 \pm 2.0\%$, Y-A-CR; $78.3 \pm 0.2\%$, O-A-CR; $78.5 \pm 0.5\%$). A-AL mice had significantly lower percent water weight ($0.27 \pm 0.05\%$) than Y-A-CR ($0.63 \pm 0.09\%$) and O-A-CR (0.72 ± 0.11) mice.

As described above, CR mice (Y-A-CR; $158.57 \pm 18.19\text{ng/mL}$ Tukey's $p = 0.0045$, O-A-CR; $167.13 \pm 41.65\text{ng/mL}$ Tukey's $p = 0.0025$) had increased serum corticosterone concentrations (Figure 4A) compared to A-AL mice ($32.67 \pm 4.62\text{ng/mL}$). There was no significant difference in serum corticosterone concentrations between mice fed CR for 2 months (O-A-CR) or for 18 months (Y-A-CR). Based on the body composition data, serum leptin concentration (Figure 4B) was significantly reduced in CR mice (Y-A-CR; $1912.44 \pm 133.73\text{pg/mL}$ Tukey's $p < 0.0001$, O-A-CR; $1736.05 \pm 243.08\text{pg/mL}$ Tukey's $p < 0.0001$) compared to A-AL mice ($13080 \pm 2016.97\text{pg/mL}$). There was no significant difference in serum leptin concentrations between O-A-CR and Y-A-CR mice. There were no significant differences in serum TNF- α concentrations (A-AL; $88.41 \pm 17.73\text{pg/mL}$, Y-A-CR; $74.27 \pm 18.61\text{pg/mL}$, O-A-CR; $81.85 \pm 25.49\text{pg/mL}$).

3.7 Natural killer cells are reduced in old onset – aged – calorie restricted bone marrow, lung, and spleen

The effects of the initiation of CR during the aging process on NK cell percent and number were determined using flow cytometry (Figure 5). Percent of bone marrow NK cell from A-

AL mice ($0.76 \pm 0.06\%$) was significantly increased compared to percent of bone marrow NK cell from O-A-CR mice ($0.54 \pm 0.05\%$, Tukey's $p=0.0273$). Bone marrow NK cell percent was not different between A-AL and Y-A-CR ($0.69 \pm 0.06\%$) or between Y-A-CR and O-A-CR mice. Total number of bone marrow NK cells were not significantly different between any age of onset groups (A-AL; $19.49 \pm 2.05 \times 10^4$ cell, Y-A-CR; $15.57 \pm 2.06 \times 10^4$ cell, O-A-CR; $13.89 \pm 1.73 \times 10^4$ cell). Percent and number of lung NK cell from A-AL mice (percent; $0.86 \pm 0.05\%$, total number; $12.33 \pm 0.74 \times 10^4$ cell) was significantly increased compared to O-A-CR mice (percent; $0.53 \pm 0.04\%$ Tukey's $p=0.0003$, total number; $6.98 \pm 0.92 \times 10^4$ cell Tukey's $p=0.0013$), but not Y-A-CR mice (percent; $0.71 \pm 0.06\%$, total number; $9.59 \pm 1.58 \times 10^4$ cell). Percent of spleen NK cells were not significantly different between any age of onset groups (A-AL; $1.38 \pm 0.15\%$, Y-A-CR; $1.15 \pm 0.10\%$, O-A-CR; $1.29 \pm 0.21\%$). Total spleen NK cells from A-AL mice ($49.37 \pm 8.50 \times 10^4$ cell) were significantly increased compared to O-A-CR mice ($22.28 \pm 6.73 \times 10^4$ cell, Tukey's $P=0.0181$) and Y-A-CR mice ($19.48 \pm 3.26 \times 10^4$ cell, Tukey's $P=0.0088$).

3.8 old onset – aged – calorie restriction impairs natural killer cell maturation

To determine if O-A-CR was beneficial or detrimental, we assessed NK cell maturation between the Y-A-CR and O-A-CR groups (Figure 6) using the 4-step developmental program based on expression of CD27 and CD11b [10]. Both aged CR groups (Y-A-CR; $40.89 \pm 6.34\%$, O-A-CR; $40.77 \pm 7.79\%$) had a higher percent of immature ($CD27^+CD11b^-$) NK cells in the spleen than the A-AL group ($22.67 \pm 4.61\%$), although not significant. In the lungs (A-AL; $12.29 \pm 1.67\%$, Y-A-CR; $21.71 \pm 3.40\%$, O-A-CR; $14.51 \pm 2.84\%$) and bone marrow (A-AL; $50.33 \pm 1.56\%$, Y-A-CR; $48.35 \pm 2.73\%$, O-A-CR; $53.18 \pm 2.72\%$), there were no significant differences in the percent of immature NK cells. Double positive $CD27^+CD11b^+$ NK cells were significantly reduced in O-A-CR ($17.02 \pm 2.30\%$) spleens compared to A-AL mice ($30.9 \pm 2.19\%$, Tukey's $P=0.0083$) and Y-A-CR mice ($26.62 \pm 3.46\%$, Tukey's $P=0.0486$). In the bone marrow, the double positive NK cells were significantly reduced in O-A-CR mice ($36.97 \pm 2.05\%$) compared to Y-A-CR ($43.59 \pm 2.13\%$, Tukey's $P=0.0485$), but not A-AL mice ($38.58 \pm 1.36\%$). There were no significant differences in percent of double positive NK cells in the lung (A-AL; $17.98 \pm 2.71\%$, Y-A-CR; $25.70 \pm 2.73\%$, O-A-CR; $17.77 \pm 2.88\%$). Terminally mature NK cells ($CD27^-CD11b^+$) in the spleen were significantly reduced in Y-A-CR ($16.99 \pm 5.05\%$, Tukey's $p=0.0105$) and O-A-CR ($19.46 \pm 4.84\%$, Tukey's $p=0.0242$) mice compared to A-AL mice ($39.19 \pm 5.02\%$). In the lung, the terminally mature NK cells were significantly reduced in Y-A-CR mice ($40.53 \pm 5.10\%$, Tukey's $p=0.0052$) compared to A-AL ($62.73 \pm 3.72\%$) and reduced, but not significantly, compared to O-A-CR ($55.27 \pm 4.76\%$). No significant differences of terminally mature NK cells were observed in the bone marrow (A-AL; $6.32 \pm 0.76\%$, Y-A-CR; $3.76 \pm 1.39\%$, O-A-CR; $3.85 \pm 0.85\%$). Terminally mature ($CD27^-CD11b^+$) NK cells were assessed for expression of DX5 and CD127 in bone marrow, spleen, and lung (Figure 6D–E). No differences were observed in $DX5^+$ terminally mature NK cells between the age of onset groups in bone marrow (A-AL; $88.86 \pm 1.46\%$, Y-A-CR; $90.30 \pm 1.57\%$, O-A-CR; $84.44 \pm 2.44\%$) or lung (A-AL; $97.43 \pm 1.01\%$, Y-A-CR; $97.39 \pm 0.33\%$, O-A-CR; $97.43 \pm 0.38\%$). Percent of terminally mature NK cells expressing DX5 in the spleen was significantly reduced in O-A-CR mice ($94.45 \pm 0.69\%$) compared to A-AL ($98.33 \pm 0.26\%$, $p=0.0041$), but not Y-A-CR ($96.07 \pm 1.11\%$). Percent of terminally

mature NK cells expressing CD127 were not significantly different in the bone marrow (A-AL; $18.37 \pm 7.65\%$, Y-A-CR; $34.03 \pm 12.86\%$, O-A-CR; $26.01 \pm 10.21\%$) or the lung (A-AL; $4.32 \pm 1.78\%$, Y-A-CR; $8.16 \pm 1.52\%$, O-A-CR; $5.21 \pm 0.84\%$). Terminally mature NK cells expressing CD127 in the spleen were significantly increased in Y-A-CR ($19.77 \pm 6.97\%$) compared to A-AL ($4.02 \pm 0.85\%$, Tukey's $p=0.0299$) and O-A-CR ($5.29 \pm 0.76\%$, Tukey's $p=0.0485$).

4. Discussion

We have previously observed changes in NK cell frequency and number, distribution, and maturation in adult (6 month) CR mice [13], as well as increased susceptibility to influenza virus infection in both adult (6 month) and aged (22 month) CR mice compared to adult (6 month) AL mice [7]. We observed, in agreement with our hypothesis, that CR exacerbates age-related changes in NK cell maturation phenotype. Contradictory to our hypothesis, old-onset CR was more detrimental to NK cell maturation than young-onset CR, as evidenced by reduced percent of early mature NK cells from O-A-CR mice co-expressing CD27 and CD11b or terminally mature NK cells expressing DX5. This research has implications not only for an aging US population, which may voluntarily calorie restrict, but also for aged individuals who may involuntarily undergo calorie restriction.

Environment, including nutrient status, growth factors, cytokines, and hormones, contribute to NK cell development and maturation. Corticosterone was measured to determine stress levels of CR mice. Calorie restriction, but not aging, significantly increased serum corticosterone. Calorie restriction [22], as well as aging [23], 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 [24], which is a more severe stress that also results in increased corticosterone. Corticosterone is immune-suppressive and may have contributed to reduced NK cell maturation observed in CR mice. TNF- α is a pro-inflammatory cytokine released by macrophages and NK cells [25]. As expected, CR reduced serum TNF- α when CR was initiated at a young age (14wks), which has been previously described by Spaulding et al. [26]. Aging is associated with a pro-inflammatory environment (i.e. increased TNF- α). In this research, aging increased circulating TNF- α , as previously described [27]. In the age of onset experiment, we did not observe differences in A-AL and Y-A-CR mice because A-AL serum TNF- α levels were unexpectedly lower than in our other experiments. Reduced TNF- α in CR mice may have contributed to reduced NK cell maturation observed. As expected, leptin was significantly reduced in CR mice. NK cells express leptin receptors and were activated in increased leptin environments [17]. Reduced leptin environments may result in reduced maturation and activation of NK cells or affect the half-life of NK cell subsets. A limitation of our work was the lack of complete characterization of biochemical mechanisms on NK cell maturation such as other cytokines, growth factors, and metabolic parameters. The complex interaction of environmental factors created by aging and CR influenced NK cell development and maturation.

Through extensive analysis of NK cell maturation stages, we observed less mature NK cells in both aged compared to adult mice and CR compared to AL mice. In addition, we observed a combined effect of these two factors in that 22 month CR had reduced mature

NK cells than both 22 month AL and 6 month CR mice. The most notable observation, was the significant decrease of bone marrow, spleen, and lung terminally mature (CD27⁻CD11b⁺) NK cells in mice that were both aged and calorie restricted (22 month CR) compared to all other age and diet groups of mice. Further analysis showed terminally mature NK cells from 22 month CR mice had decreased expression of DX5 (a mature marker) and increased expression of CD127 (IL-7 Receptor; an immature marker). Thymic-derived NK cells express CD127 (IL-7R) and depend upon IL-7 signaling for development [14]. We did not detect IL-7 in the serum of any group of mice. Age-associated thymic involution was reduced in CR mice [28]. Another limitation of our work was the lack of thymus measurements. Increased CD127⁺ NK cells may be due to reduced thymic involution and/or undetectable levels of serum IL-7 to support maturation of CD127⁺ immature NK cells. Immature (CD27⁺CD11b⁻) and early mature (CD27⁺CD11b⁺) NK cells were associated with higher, but less controlled, cytotoxicity and higher production of cytokines such as interferon-gamma. The increased cytotoxicity of murine early mature (CD27⁺CD11b⁺) NK cells was due to reduced expression of inhibitory Ly49 receptors compared to terminally mature (CD27⁻CD11b⁺) NK cells [29]. Increased immature and early mature NK cells in CR may result in increased collateral tissue damage upon primary virus infection increasing susceptibility. However, if CR populations can survive the early innate infection the increased interferon-gamma production by early mature NK cells may facilitate improved adaptive and memory immune responses.

Previous studies have shown that young-onset, but not mid-adult or old-onset CR extends lifespan in rodents [30], [31]. Additionally, old-onset calorie restriction did not extend the lifespan of rhesus monkeys [3]. Messaoudi *et al.* determined that old-onset calorie restriction had a negative effect on immune function, specifically T-cells [19]. Here, we provide evidence that old-onset calorie restriction may also be detrimental to NK cell maturation compared to young-onset calorie restriction. O-A-CR mice had significantly lower numbers of NK cells in the lung and spleen compared to A-AL mice, while Y-A-CR numbers were not significantly different from A-AL numbers. There was also a significantly lower proportion of spleen DX5⁺ and CD27⁺CD11b⁺ (early mature markers) NK cells in the O-A-CR group compared to Y-A-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.

The Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy (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 [32], [33]. In addition, 6 month CR humans possessed markers of longevity, including reduced body temperature and reduced fasting blood insulin [34]. 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 [35]. Effros *et al.* observed a declining antibody response to influenza vaccination in aging, which was improved with 40% CR in mice [36]. In addition, Messaoudi *et al.* has shown an improvement in T cell responses, important in naïve B cell maturation into antibody producing plasma cells, in mid-adult onset CR in rhesus monkeys [19]. Therefore, calorie restriction may improve the antibody response to vaccines in the

CALERIE study, yet expose participants to primary virus infection due to impaired NK cell maturation and innate immunity.

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 reduced in all tissues studied with increased expression of CD127 and decreased expression of DX5. In addition, a lower percentage of early mature (CD27⁺CD11b⁺ or DX5⁺) NK cells were present in spleen of old-onset CR mice NK cells compared to young-onset CR mice. Early mature (CD27⁺CD11b⁺) NK cells are the most effective at releasing cytokines in the proper environment [37]. Our laboratory has previously shown CR impairs NK cell maturation and function [13, 6] and aged CR mice infected with influenza have decreased survival and higher lung viral titers [7]. These findings suggest that age-associated decline of natural killer cell maturation is a mechanism of increased susceptibility to viral infections that is worsened by CR.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ACK	ammonium chloride potassium
AL	<i>ad libitum</i>
A-AL	aged <i>ad libitum</i>
A-CR	aged calorie restricted
BM	bone marrow
CALERIE	Comprehensive Assessment of Long-Term Effects of Reducing Intake of Energy
CR	calorie restriction
ND	no data
NK	natural killer
NIA	National Institute of Aging
O-A-CR	old onset aged calorie restricted
TNF-α	tumor necrosis factor alpha

TRAIL	tumor necrosis factor-related apoptosis-inducing ligand
Y-A-CR	young onset aged calorie restricted
Y-CR	young calorie restricted

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Figure 1.

Analysis of corticosterone, leptin, and TNF- α in the serum of 6 month AL, 6 month CR, 22 month AL, and 22 month CR mice. Diet ($p = 0.0001$) was a significant term for serum corticosterone (A). 6 month AL mice were significantly different than 6 month CR (Tukey's $p < 0.0001$) and 22 month CR (Tukey's $p = 0.0005$) mice. 22 month AL mice were significantly different than 6 month CR (Tukey's $p < 0.0001$) and 22 month CR (Tukey's $p = 0.0021$) mice. Diet was a significant term ($p = 0.0003$) for serum leptin (B). 6 month AL mice were significantly different than 6 month CR (Tukey's $p = 0.0255$) and 22 month CR (Tukey's $p = 0.0435$) mice. 22 month AL mice were significantly different than 6 month CR (Tukey's $p = 0.0311$) and 22 month CR (Tukey's $p = 0.0496$) mice. Diet ($p = 0.0004$) and age ($p = 0.0001$) independently were significant terms for serum TNF alpha (C). 22 month AL mice were significantly different than 6 month AL (Tukey's $p = 0.0006$), 6 month CR (Tukey's $p < 0.0001$), and 22 month CR (Tukey's $p = 0.0049$) mice. 22 month CR mice were significantly different than 6 month CR (Tukey's $p = 0.0006$) mice. Bars with different letters are significantly different ($p = 0.05$) as determined by two way ANOVA with Tukey's *post hoc* test. ND=not detectable.

Figure 2.

NK cell percent and number in bone marrow, lung, and spleen of 6 month AL, 6 month CR, 22 month AL, and 22 month CR mice. Natural killer cell percent among cells (A) and total NK cell number (B) in bone marrow, lung, and spleen (n=10 per group) were analyzed using flow cytometry as NK1.1⁺/CD3⁻. Percent of bone marrow NK cells had significant interaction (p=0.0002) and age (p=0.0001) terms (A). 6 month AL mice bone marrow NK cell percent was significantly different between 6 month CR mice (Tukey's p=0.0122) and 22 month CR mice (Tukey's p<0.0001). 6 month CR mice were significantly different than 22 month AL mice (Tukey's p<0.0001) and 22 month CR mice (Tukey's p=0.0001). The interaction term (p=0.0309) was significant for total bone marrow NK cell number (B). No groups were significantly different for bone marrow NK cell number using Tukey's post-hoc analysis. Percent lung NK cells had significant age (p=0.0002) and diet (p<0.0001) terms (A). 6 month AL mice lung NK cell percent was significantly different than 6 month CR mice (Tukey's p<0.0001) and 22 month CR mice (Tukey's p<0.0001). 22 month CR mice lung NK cell percent was significantly different than 6 month CR mice (Tukey's p=0.0104) and 22 month AL mice (Tukey's p<0.0001). Diet (p=0.0001) was significant for total lung NK cell number (B). 6 month AL mice total lung NK cells were significantly different than 6 month CR mice (Tukey's p=0.0102) and 22 month CR mice (Tukey's p=0.0004). 22 month AL mice total lung NK cells were significantly different than 22 month CR mice (Tukey's p=0.0025). Percent (A) and total number (B) of spleen NK cell were significant for age (p=0.0001), diet (p=0.0001), and the interaction of diet and age (percent; p=0.0044, number; p<0.0001). 6 month AL mice spleen NK cell percent was significantly different than 6 month CR mice (Tukey's p<0.0001), 22 month AL mice (Tukey's p<0.0001), and 22 month CR mice (Tukey's p<0.0001). 6 month CR mice spleen NK cell percent was

significantly different than 22 month CR mice (Tukey's $p=0.0038$). 6 month AL mice total spleen NK cells were significantly different than 6 month CR mice (Tukey's $p<0.0001$), 22 month AL mice (Tukey's $p<0.0001$), and 22 month CR mice (Tukey's $p<0.0001$). 22 month AL mice total spleen NK cells were significantly different than 22 month CR mice (Tukey's $p=0.0161$). Bars with different letters within the same tissue are statistically different ($p \leq 0.05$) as determined by two way ANOVA with Tukey's *post hoc* test.

Figure 3.

NK cell maturation based on expression of CD27, CD11b, CD127, and DX5 in the bone marrow, lung, and spleen of 6 month AL, 6 month CR, 22 month AL, and 22 month CR mice. Flow cytometry was used to identify NK cells as in Figure 2 (CD3⁻/NK1.1⁺), and subsequently gated for expression of CD27 and CD11b. Percentage of mature NK cells (CD11b⁺/CD27⁻) expressing CD127 or DX5 were measured. (A) Immature NK cell (NK1.1⁺/CD3⁻/CD27⁺/CD11b⁻) percentage of total NK cells. No significant differences were observed in the bone marrow. Diet (p = 0.0001), age (p = 0.0001), and the interaction of diet and age (p=0.0010) were significant in the lung. 22 month CR mice were significantly different than 6 month AL mice (Tukey's p<0.0001), 6 month CR mice (Tukey's p<0.0001), and 22 month CR mice (Tukey's p<0.0001). Diet (p = 0.0001) and age (p = 0.0001) were significant in the spleen. 6 month AL mice were significantly different than 6 month CR mice (Tukey's p<0.0001), 22 month AL mice (Tukey's p<0.0001), and 22 month CR mice (Tukey's p<0.0001). 22 month CR mice were significantly different than 6 month CR mice (Tukey's p=0.0021) and 22 month AL mice (Tukey's p<0.0001). (B) Mature, activated NK cell (NK1.1⁺/CD3⁻/CD27⁺/CD11b⁺) percentage of total NK cells. Age (p=0.0035) was significant in the bone marrow. No significant differences were observed with Tukey's post-

hoc analysis. Diet ($p=0.0270$) was significant in the lung. No significant differences were observed with Tukey's post-hoc analysis. The interaction of diet and age ($p=0.0112$) was significant in the spleen. 6 month CR mice were significantly different than 6 month AL mice (Tukey's $p=0.0189$) and 22 month CR mice (Tukey's $p=0.0210$). (C) Mature NK cell ($NK1.1^+/CD3^-/CD27^-/CD11b^+$) percentage of total NK cells. Diet ($p=0.0002$) was significant in the bone marrow. 6 month AL mice were significantly different than 6 month CR mice (Tukey's $p=0.0497$) and 22 month CR mice (Tukey's $p=0.0012$). 22 month AL mice were significantly different than 22 month CR mice (Tukey's $p=0.0219$). Diet ($p=0.0001$), age ($p=0.0001$), and the interaction of diet and age ($p=0.0175$) were significant in the lung. 22 month CR mice were significantly different than 6 month AL mice (Tukey's $p<0.0001$), 6 month CR mice (Tukey's $p<0.0001$), and 22 month AL mice (Tukey's $p<0.0001$). Age ($p=0.0001$) and diet ($p=0.0001$) were significant in the spleen. 6 month AL mice were significantly different than 6 month CR mice (Tukey's $p<0.0001$), 22 month AL mice (Tukey's $p<0.0001$), and 22 month CR mice (Tukey's $p<0.0001$). 6 month CR mice were significantly different than 22 month AL mice (Tukey's $p=0.0052$) and 22 month CR mice (Tukey's $p<0.0001$). 22 month AL mice were significantly different (Tukey's $p<0.0001$) than 22 month CR mice. (D) Representative flow cytometry gating of CD127 and DX5 from mature ($CD27^-/CD11b^+$) NK cells of the spleen. (E) Percent of mature NK cells ($NK1.1^+/CD3^-/CD27^-/CD11b^+$) that express DX5 in bone marrow, lung, and spleen. Diet ($p=0.0307$) was significant in the bone marrow. No significant differences were observed with Tukey's post-hoc analysis. Age ($p=0.0010$) was significant in the lung. 22 month CR mice were significantly different than 6 month AL mice (Tukey's $p=0.0129$) and 22 month AL mice (Tukey's $p=0.0031$). Diet ($p=0.0001$), age ($p=0.0002$), and the interaction of diet and age ($p=0.0017$) were significant in the spleen. 22 month CR mice were significantly different than 6 month AL mice (Tukey's $p<0.0001$), 22 month AL mice (Tukey's $p<0.0001$), and 6 month CR mice (Tukey's $p<0.0001$). (F) Percent of mature NK cells ($NK1.1^+/CD3^-/CD27^-/CD11b^+$) that express CD127 in bone marrow, lung, and spleen. Diet ($p=0.0003$) was significant in the bone marrow. 22 month CR mice were significantly different than 6 month AL mice (Tukey's $p=0.0126$) and 22 month AL mice (Tukey's $p=0.0077$). No significant differences were observed in the lung. Diet ($p=0.0001$), age ($p=0.0001$), and the interaction of diet and age ($p=0.0001$) were significant in the spleen. 22 month CR mice were significantly different than 6 month AL mice (Tukey's $p<0.0001$), 6 month CR mice (Tukey's $p<0.0001$), and 22 month AL mice (Tukey's $p<0.0001$) mice. Bars with different letters within the same tissue are significantly different ($p=0.05$) as determined by two way ANOVA with Tukey's *post hoc* test.

Figure 4.

Analysis of corticosterone, leptin, and TNF alpha in the serum of aged mice initiated on 40% CR at different ages. Serum corticosterone concentrations (A) were significantly increased in Y-A-CR mice (Tukey's $p=0.0045$) and O-A-CR mice (Tukey's $p=0.0025$) compared to A-AL mice. Serum leptin concentrations (B) were significantly decreased in Y-A-CR mice (Tukey's $p<0.0001$) and O-A-CR mice (Tukey's $p<0.0001$) compared to A-AL mice. There were no significant differences in serum TNF alpha (C). Bars with different letters are significantly different ($p < 0.05$) as determined by one way ANOVA with Tukey's *post hoc* test.

Figure 5.

Analysis of NK cell percent and number in the bone marrow, lung, and spleen of aged mice initiated on 40% CR at different ages. NK cell percent among cells (A) and total NK cell number (B) in bone marrow (BM), lung, and spleen (n=10 per group) were analyzed by flow cytometry as described in Figure 2 (CD3⁻/NK1.1⁺). O-A-CR mice NK cell percentage was significantly reduced compared to A-AL mice (Tukey's $p=0.0273$) in the bone marrow. O-A-CR mice NK cell percentage was significantly reduced compared to A-AL mice (Tukey's $p=0.0003$) in the lung. No significant differences observed in spleen NK cell percentage. No significant differences were observed in total bone marrow NK cell numbers. O-A-CR mice total lung NK cell number was significantly reduced compared to A-AL mice (Tukey's $p=0.0013$). Y-A-CR mice (Tukey's $p=0.0088$) and O-A-CR mice (Tukey's $p=0.0181$) total spleen NK cell numbers were significantly reduced compared to A-AL mice. 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.

Figure 6.

NK cell maturation based on expression of CD27, CD11b, CD127, and DX5 in the bone marrow, lung, and spleen of aged mice initiated on 40% CR at different ages. Flow cytometry was used to identify NK cells as in Figure 2 (CD3⁻/NK1.1⁺), and subsequently gated for expression of CD27 and CD11b. Percentage of mature NK cells (CD11b⁺/CD27⁻) expressing CD127 or DX5 were measured. (A) Immature NK cell (NK1.1⁺/CD3⁻/CD27⁺/CD11b⁻) percentage of total NK cells in bone marrow, lung, and spleen. No significant differences were observed. (B) Mature, activated NK cell (NK1.1⁺/CD3⁻/CD27⁺/CD11b⁺) percentage of total NK cells in bone marrow, lung, and spleen. O-A-CR mice were significantly reduced compared to Y-A-CR mice (Tukey's $p=0.0485$) in the bone marrow. No significant differences were observed in the lung. OA- CR mice percent mature, activated NK cells were significantly reduced compared to A-AL mice (Tukey's $p=0.0083$) and Y-A-CR mice (Tukey's $p=0.0486$) in the spleen. (C) Mature NK cell (NK1.1⁺/CD3⁻/CD27⁻/CD11b⁺) percentage of total NK cells in bone marrow, lung and spleen. No significant differences were observed in the bone marrow. Y-A-CR mice mature NK cell percent were significantly reduced compared to A-AL mice (Tukey's $p=0.0052$) in the lung. Y-A-CR mice (Tukey's $p=0.0105$) and O-A-CR mice (Tukey's $p=0.0242$) mature NK cell percent were significantly reduced compared to AAL mice in the spleen. (D) Percent mature NK cells (NK1.1⁺/CD3⁻/CD27⁻/CD11b⁺) that express DX5 in bone marrow, lung, and spleen. No significant differences were observed in bone marrow and lung. Spleen, O-A-CR

mice mature NK cells expressing DX5 were significantly reduced compared to A-AL mice (Tukey's $p=0.0041$). (E) Percent mature NK cells (NK1.1⁺/CD3⁻/CD27⁻/CD11b⁺) that express CD127 in bone marrow, lung, and spleen. No significant differences were observed in bone marrow and lung. Spleen, Y-A-CR mice mature NK cell percent expressing CD127 were significantly increase compared to A-AL mice (Tukey's $p=0.0485$) and O-A-CR mice (Tukey's $p=0.0299$). Bars with different letters within the same tissue are significantly different ($p \leq 0.05$) as determined by one way ANOVA with Tukey's *post hoc* test.

Figure 7.

Table 1

Macronutrient and nutrient content of the diets fed to mice

	NIH-31 ^{1,2}	NIH-31/NIA fortified ^{1,2}
Protein, g/kg	184.20	187.40
Fat, g/kg	44.70	44.10
Fiber, g/kg	40.50	45.80
Gross energy, kJ/g	16.82	16.53
Ash, g/kg	66.40	65.10
Nitrogen-free extract, g/kg	559.10	550.40
Amino acids, g/kg		
Arginine	10.60	11.00
Methionine	3.90	3.60
Histidine	4.10	4.20
Leucine	16.10	15.00
Lysine	9.50	9.60
Tryptophan	2.40	2.20
Valine	9.60	8.80
Cysteine	2.80	2.60
Isoleucine	9.00	7.60
Threonine	7.10	7.10
Phenylalanine	9.20	-
Tyrosine	7.00	-
Phenylalanine + Tyrosine	-	15.30
Vitamins		
Retinyl acetate, mg/kg	-	13.93
3-hydroxy retinol, mg/kg	10.57	-
Cholecalciferol, µg/kg	10.48	17.50
dl-α-Tocopheryl acetate, mg/kg	38.30	52.15
Choline, mg/g	1.96	2.60
Niacin, mg/kg	92.20	116.16
Pantothenic acid, mg/kg	39.50	55.07
Pyridoxine, mg/kg	10.20	13.16
Riboflavin, mg/kg	7.80	11.04
Thiamine, mg/kg	77.30	123.44
Menadione, mg/kg	22.00	111.01
Folic acid, mg/kg	1.70	2.13
Biotin, mg/kg	0.13	0.38
Cobalamin, µg/kg	53.00	93.80
Minerals		
Calcium, g/kg	10.60	10.30
Phosphorus, g/kg	9.20	9.30

	NIH-31 ^{1,2}	NIH-31/NIA fortified ^{1,2}
Sodium, g/kg	2.60	3.00
Chlorine, g/kg	4.20	4.80
Potassium, g/kg	5.90	5.90
Magnesium, g/kg	2.00	2.00
Iron, mg/kg	300.20	336.41
Manganese, mg/kg	152.80	156.01
Zinc, mg/kg	50.40	48.41
Copper, mg/kg	13.20	13.28
Iodine, mg/kg	1.94	2.01
Cobalt, mg/kg	0.53	0.53
Selenium, mg/kg	-	0.30

¹ Provided by the National Institute on Aging and Harlan Teklad. Ingredients: ground wheat, ground corn, ground oats, wheat middlings, fish meal, soybean meal, corn gluten meal, dehydrated alfalfa meal, soybean oil, dicalcium phosphate, brewers dried yeast, salt, calcium carbonate, choline chloride, menadione sodium bisulfite complex, thiamine mononitrate, calcium pantothenate, tocopheryl acetate, retinyl acetate, riboflavin, Cobalamin supplement, niacin, cholecalciferol supplement, pyridoxine HCl, folic acid, biotin, magnesium oxide, ferrous sulfate, manganous oxide, copper sulfate, zinc oxide, calcium iodate, and cobalt carbonate.

² Adapted from [6].

Table 2

Body composition of young and aged CR mice.

	6mon AL	6mon CR	22mon AL	22mon CR
Weight (g)	28.5 ± 1.0 ^{1,2,a}	21.0 ± 0.5 ^b	34.4 ± 2.0 ^c	22.4 ± 0.8 ^b
Fat (%)	16.0 ± 0.9 ^a	9.8 ± 0.4 ^b	18.4 ± 1.8 ^a	10.8 ± 0.15 ^b
Lean Body Mass (%)	75.7 ± 0.8 ^a	76.2 ± 0.5 ^a	75.1 ± 2.0 ^a	78.3 ± 0.2 ^a
Water (%)	0.33 ± 0.04 ^a	0.50 ± 0.06 ^{a,b}	0.27 ± 0.05 ^a	0.63 ± 0.08 ^b

¹Values (means ± SEM)

²Diet (p<0.0001) and age (p=0.0075) were significant factors for body weight. Diet (p<0.0001) was a significant factor for percent body fat. No factors were significant for percent lean body mass. Diet (p=0.0006) was a significant factor for percent water weight. Different letters within the same row are statistically different (Tukey's p 0.05).

Table 3

Body composition of young and aged onset of CR.

	A-AL	Y-A-CR	O-A-CR
Age (mon)	22	22	22
Length of CR (mon)	N/A	18	2
Weight (g)	34.4 ± 2.0 ^{Ia}	22.4 ± 0.76 ^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

^I Values (means ± SEM) with different letters within the same row are statistically different (Tukey's p 0.05).