The Influence of Macronutrients on Splanchnic and Hepatic Lymphocytes in Aging Mice

David G. Le Couteur,1,2,3 Szun S. Tay,4 Samantha Solon-Biet,1,2,3 Patrick Bertolino,4 Aisling C. McMahon,1,2,3 Victoria C. Cogger,1,2,3 Feyza Colakoglu,1 Alessandra Warren,1,2,3 Andrew J. Holmes,1 Nicolas Pichaud,5 Martin Horan,5 Carolina Correa,5 Richard G. Melvin,6 Nigel Turner,7 J. William O. Ballard,5 Kari Ruohonen,8 David Raubenheimer,1,9,10 and Stephen J. Simpson1

1Charles Perkins Centre, 2Ageing and Alzheimers Institute and the Centre for Education and Research on Ageing, Concord Hospital, and 3ANZAC Research Institute, Concord Hospital, University of Sydney, Sydney, Australia; 4Liver Immunology Group and AW Morrow Gastroenterology and Liver Centre, Centenary Institute, Royal Prince Alfred Hospital and University of Sydney, Sydney, Australia; 5School of Biotechnology and Biomolecular Sciences, University of New South Wales, Sydney, Australia; 6Institute of Biotechnology, University of Helsinki, Helsinki, Finland; 7University of New South Wales, Sydney, Australia; 8EWOS Innovation, Dirdal, Norway; 9Faculty of Veterinary Science and 10School of Biological Sciences, University of Sydney, Sydney, Australia.

Author for correspondence to David G. Le Couteur, PhD, AAAI and CERA, Concord Hospital, Sydney 2138, Australia. Email: david.lecouteur@sydney.edu.au

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Abstract

There is a strong association between aging, diet, and immunity. The effects of macronutrients and energy intake on splanchnic and hepatic lymphocytes were studied in 15 month old mice. The mice were ad-libitum fed 1 of 25 diets varying in the ratios and amounts of protein, carbohydrate, and fat over their lifetime. Lymphocytes in liver, spleen, Peyer’s patches, mesenteric lymph nodes, and inguinal lymph nodes were evaluated using flow cytometry. Low protein intake reversed aging changes in splenic CD4 and CD8 T cells, CD4:CD8 T cell ratio, memory/effector CD4 T cells and naïve CD4 T cells. A similar influence of total caloric intake in these ad-libitum fed mice was not apparent. Protein intake also influenced hepatic NK cells and B cells, while protein to carbohydrate ratio influenced hepatic NKT cells. Hepatosteatosis was associated with increased energy and fat intake and changes in hepatic Tregs, effector/memory T, and NK cells. Hepatic NK cells were also associated with body fat, glucose tolerance, and leptin levels while hepatic Tregs were associated with hydrogen peroxide production by hepatic mitochondria. Dietary macronutrients, particularly protein, influence splanchnic lymphocytes in old age, with downstream associations with mitochondrial function, liver pathology, and obesity-related phenotype.

Key Words: Liver—Lymphocytes—Macronutrients
mechanism underlying immunesenescence is thymic involution, leading to a reduction in T cell number and function, as well as changes in their subsets. Established T cell changes with aging include: increased proportion of memory T cells with a reduction in the proportion of naïve T cells; increased numbers of Tregs (4); and a reduction in the proportion of CD4 T cells with an increase in the proportion of CD8 T cells, leading to reduced CD4:CD8 T cell ratio (5–8). Moreover, these T cell changes have been shown to predict remaining life span in old age. For example, old mice with high levels of memory CD4 T cells and low levels of naïve T cells, and/or high levels of CD8 cells with low levels of CD4 T cells are shorter lived than those with opposite patterns (8–10). B cell numbers increase with aging and this is associated with dysregulated production of immunoglobulins (11,12). On the other hand, inflamming refers to the low grade activation of the immune system in old age, associated with increased circulating cytokines (especially interleukin-6 and tumor necrosis factor-α) and poorly regulated activation of innate immune cells (3,13). Inflamming is considered to be mechanistically linked with much of the aging phenotype including frailty and degenerative diseases (13).

One of the most widely reported interventions for delaying aging is caloric restriction (14–16). Caloric restriction is achieved experimentally by limiting the amount of food available to animals, by about 30%–50% compared with the intake of ad-libitum fed controls. This is usually, but not always, associated with increased median and/or maximum life span and a delay in many age-related changes and pathologies. There has been extensive research on the effects of caloric restriction on immunological aging. Overall, it has been shown that caloric restriction maintains naïve T cells, prevents the rise of memory CD4 T cells and B cells, and normalizes the CD4 to CD8 ratio (6,7,11,17–19). Such studies suggest that many of the beneficial effects of caloric restriction on aging are mediated by delaying age-related changes in the immune system.

Recently, the assumption that it is the reduction in calories per se that delays aging has been challenged by studies that have used nutritional geometry to evaluate the interacting effects of macronutrients and energy intake on aging in ad-libitum fed animals (20–23). In caloric restriction experiments, animals are provided with reduced food, therefore the influence of compensatory feeding and the role of endogenous appetite targets cannot be assessed, nor can the interacting and/or contributory influences of each of the macronutrients (24). In mice and humans, food intake is driven most strongly by the protein content of the diet, with compensatory increases in food intake occurring when animals are provided with low protein diets and vice versa (23). When these compensatory changes outweigh appetite systems for other nutrients, resulting in these being over-or under-ingested, this is a phenomena termed “protein leverage (25,26)”. Studies in several ad-libitum fed invertebrate models have shown that diets low in protein and high in nonprotein energy (ie, carbohydrates) are associated with longer life spans, even though there is increased caloric intake (22,27,28). Such experiments are increasingly analyzed using an approach called the Geometric Framework which allows the interactions between macronutrients and energy on outcomes such as longevity to be visualized (29). We recently completed a study in 858 mice provided with 1 of 25 diets varying in protein, carbohydrate, fat, and energy content and utilized this Geometric Framework approach (23). Life span was optimized in animals on the low protein, high carbohydrate diets, as were late-life phenotypic features including lipoproteins, blood pressure, insulin levels, and glucose tolerance. Paradoxically, animals on the optimal low protein/high carbohydrate diets tended to be overweight with increased body fat and hepatosteatosis because of their increased energy intake. The life-extending mechanism appeared to be related to the effects of dietary protein and carbohydrates on circulating branched chain amino acids and glucose, with subsequent downstream effects on hepatic mTOR activation and mitochondrial function.

In this study we also collected tissue from liver, spleen, Peyer’s patches, and mesenteric and inguinal lymph nodes in a cohort of mice aged 15 months for analysis of lymphocyte populations. Here we used the Geometric Framework to test our major hypotheses, which are (1): that lymphocyte populations in old age are influenced more by macronutrients than energy intake in ad-libitum fed animals, and (2): that low protein/high carbohydrate diets, which are associated with delayed aging, will be associated with favorable T lymphocyte responses (higher CD4:CD8 T cell ratio, higher naïve T cell: memory T cell ratio; lower number of Tregs). Furthermore, the relationship between these lymphocyte responses and downstream nutritional outcomes including obesity-related parameters, mitochondrial function, and fatty liver were also evaluated.

Methods

Animals and Nutritional Intervention

The primary methods are described elsewhere (23). In summary, C57BL6-weanling mice (n = 858, male and female) were ad-libitum fed over their lifetime 1 of 25 diets varying in content of protein, carbohydrate, and fat (Supplementary Table 2). Energy content was altered by varying the nondigestible fiber concentration. Food intake was measured by dry weight of food consumed taking into account spillage which was also weighed. Mice were maintained in specific pathogen-free conditions until death and then median life spans were calculated. One cohort of mice was killed at 15 months for determination of circulating amino acids, liver pathology, lymphocyte populations, and hepatic mitochondrial function (mitochondrial oxygen consumption, maximal hydrogen peroxide production, and citrate synthase activity). All protocols were approved by Sydney Local Health District Animal Welfare Committee (Protocol No. 2009/003).

Measurement of Lymphocyte Populations

Leukocytes from the spleen, mesenteric, and inguinal lymph nodes were isolated by passing through a stainless steel mesh (100 μm) and centrifuged at 400g for 5 minutes before suspension in staining buffer (phosphate-buffered saline without Ca2+/Mg2+ supplemented with 2% fetal calf serum, 5 mM sodium azide, and 2 mM ethylenediaminetetraacetic acid). The liver and the Peyer’s patches were passed through the steel mesh, pelleted (400g for 5 minutes) and resuspended in 15 mL phosphate-buffered saline plus 9 mL of isotonic Percoll solution (GE Healthcare, UK). After centrifugation at 800g for 8 minutes, a large cell fraction was removed by passing through 250 μm nylon mesh. The cell suspensions were washed and resuspended in staining buffer before further analysis. Lymphocyte subsets were identified by staining with CD3-PerCPCy5.5, CD4-AlexaFluor700, CD8-Pacific Blue, CD25-PE-Cy7, CD44-APC-Cy7, NK1.1-APC, CD11b-FITC, and CD19-PE for 30 minutes at 4°C. Dead cells were excluded by 4,6-diamidino-2-phenylindole staining. Cells were acquired on a BD LSR-II flow cytometer (BD Biosciences) and analyzed with FlowJo v9 (TreeStar Inc., Ashland, OR). CD3+ T cells, CD3+ NK1.1+ NK cells and NK1.1+ NK cells were first identified before gating of CD19+ B cells and CD11b+ macrophages within the CD3+NK1.1+ and CD3+ NK1.1+ CD19+ populations, respectively. T cells were further divided into CD4+ or CD8+ subsets, naïve (CD44hiCD25lo), memory/effector CD44hi, or CD25hi CD4+ Treg. Fluorescence minus-one controls for CD25 and CD44
staining were performed. Positively identified subsets were summed and the percentages of each subset calculated.

Liver Histology
Steatosis and perisinusoidal fibrosis were assessed using light microscopic interpretation of liver tissue stained with hematoxylin and eosin by four-blinded investigators (DLC, AW, VC, AM) using a 0, +, ++, +++ semiquantitative scales.

Statistics
As described previously (23) generalized additive models (GAM) with thin-plate splines as implemented in the package mgcv of the R language were used to categorize the response surfaces of macronutrient intake versus lymphocyte populations. Correlograms were performed using the corrplot package in R to visualize patterns in the response of lymphocytes with macronutrients and mitochondrial function. Linear regression was used

Figure 1. Geometric framework analysis showing relationship between macronutrients and splenic T lymphocytes. There were significant relationships between (a) CD4 T cells and protein intake (p < .05), (b) CD8 T cells and protein (p < .001) and carbohydrate (p < .05) intake, (c) CD4:CD8 T cell ratio and protein intake (p < .001), (d) naive CD4 T cells and protein intake (p < .001), and (e) memory CD4 T cells and protein intake (p < .005). In each surface, the blue represents the lowest value while the red represents the highest value. Each graph represents a slice through the median value of the third macronutrient (value provided in parenthesis below the x-axis label).
to evaluate the relationship between branched chain amino acids and lymphocytes (SigmaPlot v 11.2.05, Systat Software Inc.). Non-parametric analysis of variance with post hoc Dunns method was used to evaluate the relationship between the four grades of hepatic steatosis with dietary intakes, mitochondrial activity, and lymphocytes (SigmaPlot v 11.2.05, Systat Software Inc.) with \( p < .05 \) considered significant.

**Results**

**The Influence of Macronutrients and Energy Intake on Lymphocyte Populations in Ad-libitum Fed Mice**

The Geometric Framework was used to evaluate the effects of macronutrients and total energy intake on the splanchnic and hepatic lymphocyte populations in 125 mice that were killed at 15 months of age. We investigated the effect of diet in three different compartments (liver, spleen, lymph nodes) which normally contain different lymphocyte subsets (Supplementary Figure 1). Figures 1 and 2 show the surfaces generated by the Geometric Framework method where there were statistically significant relationships determined by GAM, and that are of particular interest for aging. In the spleen (Figure 1), protein intake was negatively associated with CD4 T cells and positively associated with the CD8 T cell subset. Subsequently, the CD4:CD8 ratio decreased as protein intake increased. Likewise CD4 memory T cells were positively associated while naïve CD4 T cells were negatively associated with protein intake.

In the liver (Figure 2), protein intake was positively associated with B cells and negatively associated with NK cells. The population of NKT cells was influenced by the interaction between protein and carbohydrate intake, with low protein to carbohydrate intake ratios associated with higher proportions of NKT cells. All the statistically significant associations are shown in Supplementary Table 1 where it can be seen that dietary intake mostly influences lymphocytes in the spleen and liver, while the main macronutrient that influences lymphocytes is protein.

In addition to the Geometric Framework approach, simple correlations between individual intakes of macronutrients and energy versus each of the cell subsets were evaluated. Figure 3 shows the correllograms from this analysis, performed in order to illustrate the patterns of the relationships between dietary intake and lymphocyte subsets. Overall, the patterns of change with dietary intake tended to be similar between liver, spleen, and lymph nodes. The strong impact of protein intake on values for splenic CD4, CD8, CD4:CD8 ratio, naïve CD4 T cells and memory CD4 T cells and for hepatic B, NK and NKT cells are apparent. These are similar to changes seen using the Geometric Framework. Neither the Geometric Framework nor the correllogram analyses showed any major impact of total energy intake on these particular cell subsets.

In our previous study of macronutrients and aging (23), we found that protein intake was strongly correlated with circulating branched chain amino acids with downstream associations with hepatic mTOR phosphorylation and mitochondrial activity. To determine whether there was any relationship with lymphocytes,

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**Figure 2.** Geometric framework analysis showing relationship between macronutrients and B, NK and NKT hepatic lymphocytes. There were significant relationships between (a) B cells and protein intake \( (p = .05) \), (b) NK cells and protein intake \( (p < .05) \), and (c) NKT cells and the interaction between protein and carbohydrate intake \( (p < .05) \). In each surface, the blue represents the lowest value while the red represents the highest value. Each graph represents a slice through the median value of the third macronutrient (value provided in parenthesis below the x-axis label).
the associations between circulating branched chain amino acids and frequencies of hepatic CD4 T cells, CD8 T cells, naive T cells, memory T cells, and Tregs and CD4:CD8 T cell ratio were evaluated. There were statistically significant relationships with all but memory CD4 T cells, with higher branched chain amino acids associated with changes seen in aging, that is, ↓CD4:CD8 ratio, ↓naive T cells and ↑Treg (Figure 3).

The Relationship Between Hepatic Lymphocytes and Hepatic Mitochondrial Function, Hepatosteatosis, and Fibrosis
Mitochondrial function was assessed using mitochondria isolated from liver and included oxygen consumption rates (primarily state III and state IV with different combinations of substrates) and maximal hydrogen peroxide production (measured in the presence of

Figure 3. (a) Correlograms showing the patterns of the relationships between protein, carbohydrate, fat, and energy intakes with lymphocytes in liver, spleen, Peyer’s patches, inguinal, and mesenteric nodes. The strength and direction of the correlation coefficient is shown by the color (blue = positive association; red = negative correlation), intensity and size of the ellipses (demonstrated in the scale bar). (b–e) Relationship between circulating branched chain amino acids and lymphocyte changes within the liver. There was a negative relationship with (b) CD4:CD8 T cell ratio ($p < .001$) and (e) naive CD4 T cells ($p < .05$), (c) a positive relationship with Tregs ($p < .01$), but (d) no relationship with memory CD4 T cells.
rotenone and the same substrates used for the mitochondrial respiration), as well as citrate synthase activity [a marker of mitochondrial density (30)]. There were mixed relationships between lymphocyte populations and citrate synthase activity, most notably the positive relationship between CD4:CD8 ratio. There were no statistically significant relationships with Respiratory Control Ratio (RCR = state III/state IV as a marker of mitochondrial coupling) except for Tregs when palmitate–malate was used as the substrate. Indeed the most striking observation is the positive association between the frequency of hepatic Tregs among liver leucocytes and many measures of mitochondrial activity, including maximal hydrogen peroxide production by hepatic mitochondria ($p < .001$ with pyruvate–malate as substrates, $p < .001$ with succinate–rotenone, $p < .001$ with glutamate–malate, $p = .001$ with palmitate–malate, Figure 4).

Aging is associated with the development of hepatosteatosis (31) therefore we studied its relationship with hepatic lymphocytes and mitochondrial function ($n = 112$ mice where technically satisfactory specimens were available). Hepatosteatosis was graded as 0 (55% of mice), + (17%), ++ (12%), or +++ (16%). Dietary macronutrients had significant relationships with the grade of hepatosteatosis (Supplementary Figure 2). There were no statistically significant associations between mitochondrial assays and the four grades of hepatosteatosis (data not shown), consistent with the study of Franko and colleagues (32) where diet-induced hepatosteatosis was not associated with altered mitochondrial function in young mice.

There were some associations between hepatic lymphocytes and the degree of hepatosteatosis (Figure 5). Overall, hepatosteatosis was associated with increased frequency of Tregs, memory CD8 T cells, and a trend towards increased frequency of NK cells, while the percentage of memory CD4 T cells was reduced. The relationship with NK cells was particularly interesting because NK cells were also strongly correlated with body fat, leptin, and the glucose tolerance test (Figure 6).

**Discussion**

Old age is associated with impairment of the immune system which contributes to age-related susceptibility to frailty, morbidity, and mortality. Age-related changes in the immune system are complicated but generally lead to a reduction in response to antigenic challenges called immunosenescence while there is a dysregulated increase in basal activity called inflammaging. Age-related changes in total number of lymphocytes are less physiologically important than the changes in the subsets of lymphocytes. As a result of thymic involution and lifelong antigenic stresses, old age is associated with an increase in the proportion of memory T cells to the detriment of...
naive T cells. There is also a reduction in the frequency of CD4 T cells with increases in the percentage of CD8 T cells, Tregs, and B cells (1–12).

There has been great interest in developing therapies that delay age-related changes in the immune system in order to delay age-related morbidities, and to date these have focused primarily on nutritional interventions such as caloric restriction. Studies on caloric restriction and aging immune system were pioneered in the early 1970s (17,33,34) and although the methodologies for evaluating the immune system have evolved enormously since those times, the overall conclusion has been consistent—caloric restriction delayed most of age-related immunological changes that were able to be measured (19). One of the earliest studies of the effect of caloric restriction on T cell subsets was published by Grossman and colleagues (35) who found that there was a reduction of CD4 numbers and proliferation in the spleen, and that caloric restriction increased CD4 proliferation. Subsequently Fernandes’ group performed a number of studies showing that caloric restriction reversed the age-related shift from naive T cells to memory T cells in rats (6); the decrease in CD4 and increase in CD8 splenic cells in mice (7); and the increase in B cells and reduction of naive T cells in mice (11).

In our study, similar changes in T lymphocyte subsets and B cells that have been seen previously with caloric restriction were achieved in ad-libitum fed animals by manipulating the balance of the dietary macronutrients. Protein intake was strongly associated with CD4 and CD8 T cells, CD4:CD8 ratio, naive T cells, memory CD4 T cells in the spleen, and B cells in the liver. Low protein intake was associated with changes seen with caloric restriction (↑CD4 and ↓CD8 leading to ↑CD4:CD8 T cell ratio, ↑ naive T cells, ↑ memory T cells, ↓ B cells), while high protein intake was associated with changes that have been linked to increased mortality in old age (↑CD4 and ↑CD8, ↓naive T, ↓memory T). Moreover, total energy intake did not influence any of these parameters, noting that these are ad-libitum fed mice. The results are consistent with our life span study that showed reduced protein intake associated with a low protein, high carbohydrate diet is linked with longer life span, while total energy intake had no impact on health or life span. It is important to note that low protein intake could only be achieved with very low protein,

Figure 5. The relationship between hepatic lymphocyte subsets, hepatosteatosis and perisinusoidal fibrosis. Hepatosteatosis was associated with (a) Treg (p < .001), (b) memory CD4 T cells (p < .05), (c) memory CD8 T cells (p < .05) and (d) a borderline association with NK cells (p = .055). Hepatosteatosis was graded from 0 to +++ (e).
in mice with the lowest circulating levels of branched chain amino acids were more consistent with those seen in younger animals. We propose that some of the well-established beneficial effects of caloric restriction on age-related immune function may be related to the fact that caloric restriction protocols limit protein intake, and that reduced calorie intake per se does not have beneficial effects on the immune system—at least in animals with ad libitum access to food. It is noteworthy that Fernandes and colleagues (17) showed that a low protein diet (6% vs 22% in mice) was associated with reversal of splenic enlargement and thymic involution, with normalization of circulating immunoglobulins and functional cell mediated immunity, consistent with our findings.

We also studied the relationship between nutrition, lymphocytes, and the liver. The liver is the master regulator of systemic responses to nutrition and caloric restriction and moreover, it has an increasingly recognized impact on aging and age-related diseases (36,37). Old age is associated with impaired mitochondrial function and increased generation of oxygen-derived free radicals (38) which could influence hepatic lymphocyte populations. Tregs are more resistant to oxidative stress than other lymphocytes. This difference is thought to explain why Tregs are found in higher proportions in neoplasia and infections in which oxidative stress is increased (39,40). We found that hepatic Tregs were positively correlated with hepatic mitochondrial hydrogen peroxide production and in fact this was the only lymphocyte subset strongly linked with any mitochondrial function. The increase in Tregs with old age might be related to increased oxidative stress and infections that characterize aging.

Tregs were also increased in livers with the highest grade of hepatosteatosis, consistent with their role in suppressing inflammation. On the other hand, hepatosteatosis caused by a high fat diet has been reported to be associated with decreased Tregs (41), although this study was short-term in young mice, which might explain the difference. Hepatosteatosis was also associated with reduced memory CD4 T cells, increased memory CD8 T cells and a trend towards increased NK cells. We found no effect of the diet in NKT cell frequencies despite other reports of increased NKT cells in mice following short term high fat diets (42). Although the relationship between NK cells and hepatosteatosis was of borderline statistical significance (p = .055) there were strong relationships between hepatic NK cells and body fat, glucose tolerance and leptin, suggesting a role for NK cells in the relationship between obesity and hepatosteatosis.

In conclusion, the key finding from this study is that macronutrients influence splanchnic lymphocyte populations in late life. Mice with low intake of protein compared with those with higher protein intakes had patterns of T lymphocyte subsets that would be considered to be more favorable for aging and similar to those seen with caloric restriction (↑CD4 T cells, ↓CD8 T cells, ↑CD4:CD8 ratio, ↑naive T cells, ↓memory CD4 T cells). In addition we found an association between Tregs and mitochondrial hydrogen peroxide production, which raises the possibility that the increase in Tregs in old age is related to oxidative stress. We also observed an association between macronutrient and caloric intake with hepatic NK cells, hepatosteatosis, leptin, and body fat. The results underscore the importance of distinguishing the contributions of specific nutrients to relationships between diet and ageing.

**Supplementary Material**

Supplementary material can be found at: [http://biomedgerontology.oxfordjournals.org/](http://biomedgerontology.oxfordjournals.org/)
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References