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ORIGINAL PAPER

# Dietary effect on immunological energetics in mice

**Sebastián I. Martel · Sebastián A. Riquelme · Alexis M. Kalergis · Francisco Bozinovic**

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**Abstract** Defense against natural aggressors, such as bacterial infections, requires both energy and an immune-cellular response. However, the question as to how these two components are interconnected in small endotherms by means of the host diet remains only poorly understood. Here, we tested in laboratory mice whether dietary proteins and carbohydrates can modulate the interplay between energy expenditure, food intake and the innate and adaptive immune response when confronting a bacterial challenge (Bacillus Calmette-Guérin, BCG). We observed that mice fed with a high protein diet (HP) developed a better immune response associated to increased numbers of circulating monocytes. In addition, HP diet directly influenced the peripheral blood proportions of both T and B lymphocytes even before the BCG challenge. Interestingly, animals that developed this type of immune response after BCG challenge showed an increased rate of metabolism and food consumption before being challenged. Thus, HP diet induced in non-challenged animals a similar energy expenditure and food intake described by BCG-treated mice. These data suggest that a high amount of proteins in diet can modify the energetic and nutrient dynamic in the host causing a better immune reaction against a microbial challenge.

**Keywords** Ecoimmunology · Food intake · Energy expenditure · Immune-cellular response · Small mammals

## Introduction

What an animal eats defines its biology (Bozinovic and del Río 1996). Food habits and diet selection are associated with specific traits ranging from molecular to physiological, as well as from behavioral to ecological and evolutionary traits (Bozinovic 1993; Bozinovic and Muñoz-Pedreros

S. I. Martel, S. A. Riquelme authors contributed equally to this work.

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S. I. Martel · F. Bozinovic (✉)
Departamento de Ecología and Center of Applied Ecology and Sustainability (CAPES), Facultad de Ciencias Biológicas, P. Universidad Católica de Chile, Santiago 6513677, Chile
e-mail: fbozinovic@bio.puc.cl

S. A. Riquelme · A. M. Kalergis
Departamento de Genética Molecular y Microbiología, Facultad de Ciencias Biológicas, Millennium Institute on Immunology and Immunotherapy, Santiago, Chile

S. A. Riquelme · A. M. Kalergis (✉)
Departamento de Inmunología Clínica y Reumatología, Facultad de Medicina, Pontificia Universidad Católica de Chile, Santiago 6513677, Chile
e-mail: akalergis@bio.puc.cl

S. A. Riquelme · A. M. Kalergis
INSERM, UMR 1064, Nantes, France

S. A. Riquelme · A. M. Kalergis
CHU Nantes, ITUN, Nantes, France

S. A. Riquelme · A. M. Kalergis
Faculté de Médecine, Université de Nantes, Nantes, France

1995; Cruz-Neto and Bozinovic 2004; Karasov and Diamond 1988). Recent studies (Catalán et al. 2011; Schulenburg et al. 2009) have suggested that nutrients (i.e. dietary chemical composition) could affect immune function. When given a choice between different diets, pathogen-infected consumers select those diets that improve their immune system and hence increase survival or fitness. This notion suggests that the nutritional state may play a role in energetics of the host and influence pathogen challenge-dependent metabolic rates (Ardia et al. 2012; Książek and Konarzewski, 2012). Thus, since triggering the immune system is costly (Armitage et al. 2003; Moret and Schmid-Hempel 2000; Rolff and Reynolds 2009), feeding on low-quality diets and/or deficient nutrition may harm immune function (Chandra 1996). Because substantial costs are associated with immune activation and the maintenance of the immune system, a trade-off between the physiological demands associated with diet, maintenance (energy metabolism) and immune function (defense) would be expected (Lochmiller and Deerenberg 2000; Read and Allen 2000) (sensu Garland 2014). Amongst endothermic vertebrates, it was shown in white mice that an increment of immunoglobulin G due to an immune challenge augmented metabolic rate (Demas et al. 1997). In birds, a nearly 30 % increase in resting metabolism was reported for in house sparrows after an immune challenge (Martin et al. 2003). Furthermore, in collared doves it was observed that antibody production against sheep red blood cells also increased basal metabolic rate (Eraud et al. 2005). Interestingly, Scott et al. (2005) demonstrated that in a mouse-nematode interaction model, infected mice fed with a low energy diet increased food intake. Altogether, these results suggest that high immune activity in endothermic vertebrates increases energy expenditure. Thus, this notion would imply that animals must to reallocate resources from other physiological function to sustain an efficient immune response, which could affect the Darwinian fitness of the individual.

Several studies have suggested the occurrence of significant energetic costs associated to immunity through a trade-off between immunocompetence and life history traits (Martin et al. 2001; Norris and Evans 2000; Ricklefs and Wikelski 2002). However, a direct cost of the immune response on host energetics has been highly difficult to demonstrate (Malvin and Kluger 1979; Mendes et al. 2006). Thus, considering that the activation of an efficient immune response is a costly process (Martin et al. 2003; Eraud et al. 2005), a deficient nutrition may impair proper immune function and increase the susceptibility of animals to infectious diseases. As part of the present study, we have hypothesized that since the cellular immune defense is a final line of protection against parasites, an adequate provision of dietary macromolecules would be required to maintain the energetic cost-based immunocompetence (Amat et al. 2007; Zuk and Stoehr 2002). To evaluate this postulate, laboratory mice were fed with different diets and then challenged with Bacillus Calmette-Guérin (BCG), a live attenuated bacterium (*Mycobacterium bovis*). We measured food consumption, resting metabolic rate and cellular immune response in peripheral blood of dietary acclimated mice before and after the bacterial challenge.

## Materials and methods

### Mice and maintenance

Six to 8 week old C57BL/6 mice were obtained from The Jackson Laboratory (http://www.jax.org/) and maintained at the Pontificia Universidad Católica animal facility. Each individual was kept separately in individual cages provided with food and water ad libitum, *a* LD = 12:12 photoperiod and a controlled ambient temperature of 23 ± 1 °C. Cages were cleaned and water and food was renewed every week or as required. All mice were maintained at the pathogen-free animal facility at the Pontificia Universidad Católica de Chile (Santiago, Chile). All animal work was performed according to institutional guidelines and supervised by a veterinarian.

### Treatment of mice

At the beginning of the experiment, 14 days before the immunological challenge, individuals were randomly separated into three groups of seven animals. Each group was fed with one of three food types: high protein or HP diet and low protein or LP diet (MP Biomedicals, Santa Ana, CA. http://www.mpbio.com/). As a control (C) diet we used a standard commercial mice food pellet (LabDiet, St. Louis, MO; http://www.labdiet.com/), Nutritional details are described in detail in Table 1. After 2 weeks of acclimation (day 0), individuals were challenged by inoculation with Bacillus Calmette-Guérin or BCG (see below), an attenuated strain of *Mycobacterium bovis*.

### Challenge with bacillus calmette-guérin (BCG)

The immunological challenge consisted of *Mycobacterium bovis* or Bacillus Calmette-Guérin (BCG), which is a worldwide used live attenuated vaccine against tuberculosis. It has been reported that the innate immune response against BCG involves inflammatory peripheral blood polymorphonuclear cells, such as neutrophils (Mantovani et al. 2011).

BCG was grown in Middlebrook 7H9 medium (Difco) supplemented with 10 % AOCD (oleic acid, albumin, dextrose, catalase, Becton–Dickinson) and 0.05 % Tween 80

**Table 1** Nutritional value and chemical composition of the experimental diets used in this study. Values are averages of dry-weight and % respectively

| Ingredients | Control | | High-protein | | Low-protein | |
|---|---|---|---|---|---|---|
| | Grams | % | Grams | % | Grams | % |
| Protein | 115 | 23 | 285 | 57 | 60 | 12 |
| Carbohydrates | 295 | 59 | 160 | 32 | 360 | 72 |
| Insoluble fibre | 25 | 5 | 5 | 1 | 25 | 5 |
| Vegetable oil | 25 | 5 | 25 | 5 | 35 | 7 |
| Vitamin mix | 40 | 8 | 25 | 5 | 20 | 4 |
| Total | 500 | 100 | 500 | 100 | 500 | 100 |

The protein composition of all diets contains the essential aminoacids. The low-protein diet was supplemented with methionine (0.3 %) as suggested by www.mpbio.com. The carbohydrate fraction in all diets was composed fundamentally by starch and sucrose and traces of glucose and fructose (<0.2 %). In addition diets were supplemented with a vitamin and mineral mix

(Bueno et al. 2008; Cautivo et al. 2010; Espinoza et al. 2013; Palavecino et al. 2014). Cultures were incubated at 37 °C without agitation in medium without antibiotics to reach an optical density (600 nm) equal to 0.6. Each mouse was vaccinated subcutaneously on the back with $1 \times 10^8$ CFU of BCG suspended in 100 µl of PBS, 14 days after acclimation to the diets (day 0).

### Resting metabolic rate (RMR)

RMR was measured during 1.5 h once a week through $O_2$ consumption by open-flow respirometry, both before and after the BCG challenge. Prior to the measurements, mice were fasting for at least 3 h and then weighed. Measurements were made during the period of inactivity (between 09:00 and 16:00 h) and each individual was always measured at the same time to prevent circadian effects. Custom made metabolic chambers of 400 mL were used (www.sablesys.com). Chambers received dried air at a rate of 800 mL/min from mass flow controllers (MFS2, Sable systems), which is enough to ensure adequate mixing inside the chamber. Air passed through $CO_2$-absorbent granules of Baralyme and $H_2O$-absorbent granules of Drierite both before and after passing through the chamber. The metabolic chamber was located inside an incubator and the ambient temperature (Ta) was set to 30.0 ± 0.5 °C, which is within the thermoneutral zone of this species. In each trial, oxygen consumption was sampled every 1 s. Oxygen consumption values were measured and calculated with a FC-10a sensor and Expedata© data program, respectively (http://www.sablesys.com/). The resting metabolic rate was estimated as the lowest steady state period of 5 min, recorded during $VO_2$ measurements. Also, every 2 days and before and after the challenge, individuals and their food were weighed to determine body mass changes and food intake over time.

### Flow cytometry analyses of immune blood cells

To measure the percentage of different immune cell types in the blood of mice, every week and after RMR measurements, flow cytometry assays were performed. Between 200 and 300 µl of blood were drawn from each chloroform-anesthetized mouse by the method of cheek bleeding by using a sterile 25G syringe. Blood from each individual mouse was collected in 1.5 mL eppendorff tubes containing 0.1 ml of anticoagulant (heparin or EDTA) and kept in ice to avoid coagulation and/or cell lysis. Each tube was centrifuged at 1,800 rpm for 6 min at 4 °C. The serum was extracted and ACK buffer (150 mM $NH_4Cl$, 10 mM $KHCO_3$, 0.15 mM EDTA) was added to each tube to lyse the erythrocyte fraction in blood samples. ACK-treated tubes were left for 5 min at room temperature and then centrifuged as described above. This procedure was repeated twice to ensure the complete absence of red blood cells. Finally, each sample was washed, resuspended and homogenized in 600 µl of PBS, divided in two tubes and stained with anti CD11b-FITC (cat. 553310, BD Pharmingen™), anti Gr1-APC (cat. 553129), anti CD11c-PE (cat. 553802), anti 2.4G2-PerCP-Cy™5.5 (cat. 560540), anti CD4-FITC (cat. 553651), anti CD8a-APC (cat. 553035) and anti B220-PE (cat 553090). Data acquisition was performed on a FACSCalibur cytometer (BD Biosciences) and analyzed using FCS Express v3 software.

### Statistical analyses

Data were analyzed through a one-way ANOVA (StatSoft 2001) at $\alpha = 0.05$. After ANOVA we used the a posteriori Fisher`s LSD (least significant difference) test for multiple comparisons.

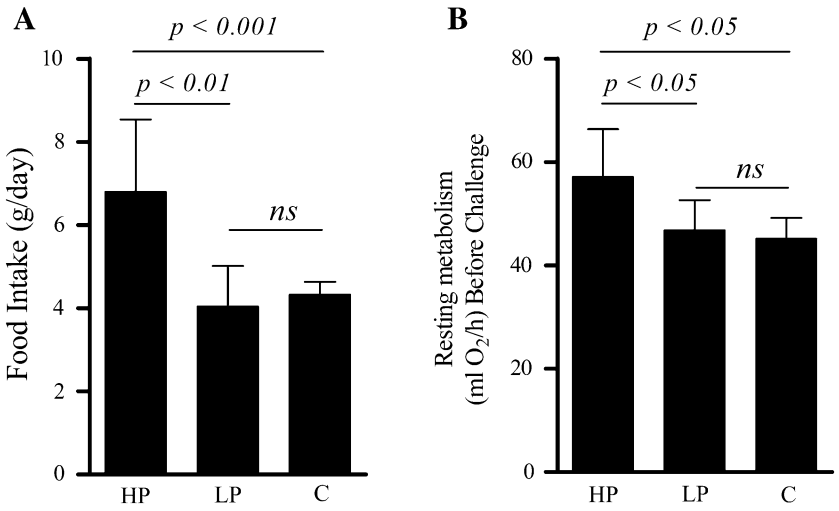


**Fig. 1** **a** Relationship between diet quality and food intake before the bacterial challenge. **b** Relationship between diet quality and resting metabolic rate before the bacterial challenge. P-levels after a one-way ANOVA are indicated. Significant differences at $P < 0.05$ as well as non-significant differences (ns) are indicated after a one-way ANOVA. *HP* high protein, *LP* low protein and *C* control diets

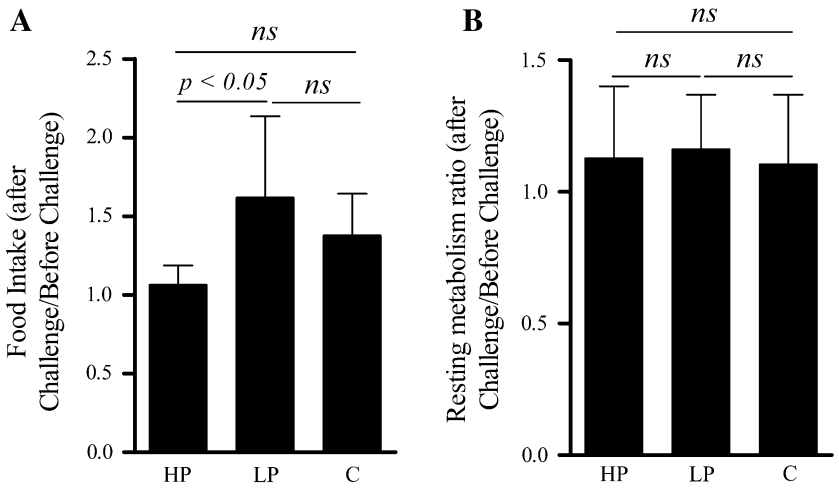


**Fig. 2** **a** Relationship between diet quality and food intake ratio before and after the bacterial challenge in dietary acclimated laboratory mice. **b** Relationship between diet quality and resting metabolic rate ratio before and after the bacterial challenge in dietary acclimated laboratory mice. Significant differences at $P = 0.05$ as well as non-significant differences (ns) are indicated after a one-way ANOVA. *HP* high protein, *LP* low protein and *C* control diets

## Results

### Mice fed with High Protein diet consumed more food and showed increased metabolic rate before the immune challenge

We observed that mice fed with a high protein content diet (HP) consumed increased amounts of food before the immune challenge (one-way ANOVA, $F_{2,\ 18} = 11.835$, $P = 0.0005$; Fig. 1a). However, these animals did not increase their body mass (data not shown). In addition, animals fed with the HP diet increased RMR before the immune challenge (one-way ANOVA, $F_{2,18} = 6.451$, $P = 0.007$; Fig. 1b).

### The amount of proteins in diet modulates food intake but not metabolic rate after a challenge with BCG

As shown in Fig. 2a, only animals fed with LP diet exhibited a significant increase in food consumption after BCG challenge (~60 % for LP, one-way ANOVA, $F_{2,18} = 4.596$, $P = 0.024$b). In addition, no significant differences were found for RMR either in time (pre- versus post-challenge) or between experimental groups (one-way ANOVA, $F_{2,18} = 0.093$, $P = 0.911$; Fig. 2b).

### High Protein diet increased the inflammatory response to a BCG challenge in mice

Because the innate immune response against BCG involves inflammatory peripheral blood cells, such as neutrophils (Muñoz-Durango et al. 2013; Riquelme et al. 2011), we evaluated whether each diet could modify the immunological response induced by BCG. We observed that only the HP diet conferred to mice, the ability to develop an increased immune response based on peripheral blood $Gr1^{+}CD11b^{+}$ neutrophils at day 7 post challenge (repeated measures ANOVA, significant association between time and diet $F_{4,36} = 3.611$, $P = 0.014$; Fig. 3). LP and C diets also developed this type

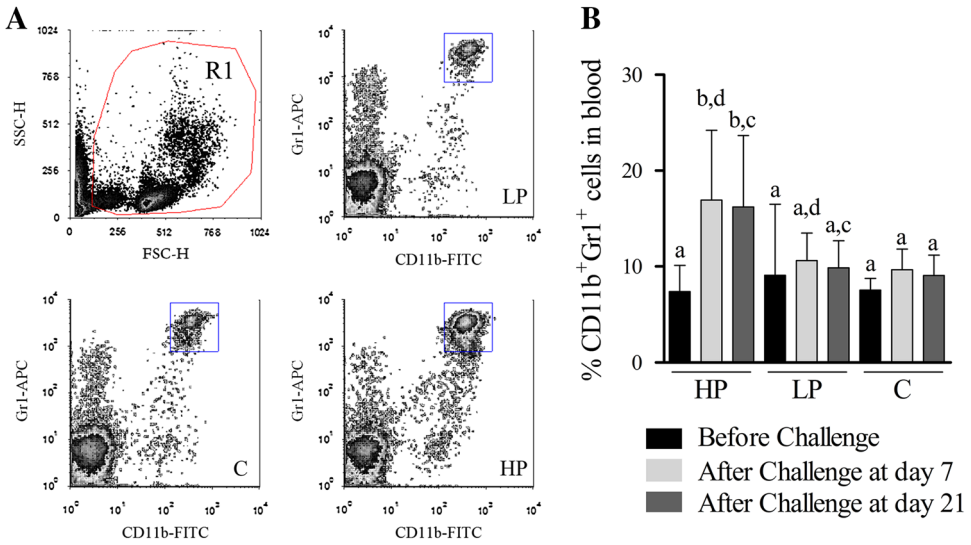


**Fig. 3** Relationship between diet quality and frequency of $Gr1^+CD11b^+$ neutrophils in blood in dietary acclimated C57BL/6 mice before and after the bacterial challenge (7 and 21 days). **a** Blood samples from mice were analyzed by flow cytometry. *Upper left* image shows a representative forward (cell size, FSC-H)-side (cell surface complexity, SSC-H) scatter plot of cells in blood. The region described as R1 excludes cellular debris from the analyses (low for FSC-H). From these cells, neutrophils expressing both Gr1 and CD11b surface markers ($Gr1^+CD11b^+$) were quantified (squared gate). Representative dot plots for each treatment at 7 days post-BCG challenge are shown. **b** Quantifications of neutrophil population expressing Gr1 and CD11b ($Gr1^+CD11b^+$) before and after BCG challenge are shown. Similar letters indicate non-significant differences after a repeated measure ANOVA and an a posteriori Fisher test. *HP* high protein, *LP* low protein and *C* control diets

of response but at reduced levels when compared to HP diet. Interestingly, even at 21 days post challenge the $Gr1^+CD11b^+$ immune response in HP mice remained higher than mice fed with the other two diets (Fig. 3).

### Lymphocyte maintenance and response is regulated by protein content in diet before and after a BCG challenge

T cells are important for the clearance of pathogenic bacteria (Muñoz-Durango et al. 2013; Riquelme et al. 2011). In addition, BCG vaccination induces a T cell response, which can modulate both B and T cell immune memory (Cautivo et al. 2010; Palavecino et al. 2014). We observed that animals that were fed with LP diet displayed lower percentages of peripheral blood $CD4^+$ and $CD8^+$ T cells before the BCG challenge (repeated measures ANOVA, significant association between time and diet, $F_{4,36} = 3.706$ $P = 0.012$ for $CD4^+$ T cells, $F_{4,36} = 2.943$ $P = 0.033$ for $CD8^+$ T cells; Fig. 4a, b, respectively). After BCG challenge (7 days post challenge), only those animals fed with LP diet showed a significant increment in both $CD4^+$ and $CD8^+$ T cell populations (Fig. 4a, b). Furthermore, after the BCG challenge mice fed with LP diet displayed a significant increase in the frequency of peripheral blood B cells ($B220^+2.4G2^+$ cells), as compared to control and HP diets (repeated measures ANOVA, significant interaction between time and diet $F_{4,36} = 7.914$ $P < 0.001$ Fig. 4c).

## Discussion

On an evolutionary timescale, it has been hypothesized that diet is a powerful selective agent shaping rates of energy expenditure in birds and mammals (Bozinovic and del Río 1996, Cork 1994). In addition, energy metabolism is associated with the rate in which animals acquire and process energy to fuel their existence and survival. Because metabolic rates set the pace of life, measurements and analyses of their variability have been of paramount importance to several contemporary evolutionary and ecological theories (Brown et al. 2004; Cruz-Neto and Bozinovic 2004; Kooijman 2000) and recently to ecological immunology (Shulenburg et al. 2009; Sheldon and Verhulst 1996). Consistently with this notion, it has been shown that activation of an efficient immune system involves several energy costs (Klasing 2004). Although energy has been the typical currency examined in the study of the immune function (Sheldon and Verhulst 1996), scarceness of other resources also may affect the immunocompetence of the host and the proper function of the immune system (Amat et al. 2007; Zuk and Stoehr 2002, Martin et al. 2003). Indeed, dietary macronutrient composition may cause a reallocation of resources and consequently a trade-off between different physiological processes (Ricklefs and Wikelski 2002, Eraud et al. 2005). Here, we have evaluated whether diet composition can modulate the function of the immune

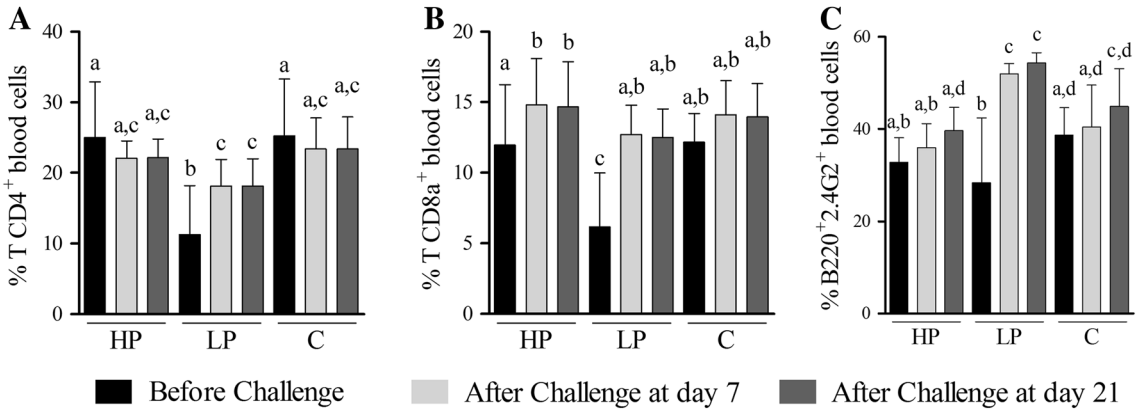


**Fig. 4** Relationship between diet quality and **(a)** percentage of $CD4^+$ T cells in blood; **(b)** percentage of $CD8^+$ T cells in blood and **(c)** percentage of $B220^+2.4G2^+$ B cells in blood in dietary acclimated lab mice before and after the bacterial challenge (7 and 21 days post challenge). Similar letters indicate non-significant differences after a repeated measure ANOVA and an a posteriori Fisher test. *HP* high protein, *LP* low protein and *C* control diets

system, both in terms of maintenance and use, as well as the metabolic costs associated to the immune response.

Our results suggest that higher amount of dietary protein, associated with low dietary carbohydrate, can promote an increase in food consumption as well as in RMR. As demonstrated by Veloso and Bozinovic (1993), long-time acclimatization with a high dietary protein increases the rate of metabolism. Nevertheless, the observation of elevated RMR in a short time scale might be also explained by the specific dynamic effects of a high dietary protein meal digestion. Thus, future studies are required to test explicitly this hypothesis. On the other hand, consumption of a high protein diet increased the inflammatory response mediated by $Gr1^+$ $CD11b^+$ neutrophils after a bacterial challenge. As a result, an enhanced short-term immune response against a bacterial pathogen was developed in animals receiving the high protein diet. Recruitment of neutrophils to the blood upon bacterial challenge suggests that these cells are stimulated by components of the infectious agent. BCG contains several immune stimulating molecules that trigger the mobilization of activated cells, such as neutrophils, to the blood (Abadie et al. 2005). As a consequence of high protein diet, the frequency of these cells, relative to other cell types, increased after bacterial challenge. Along these lines, our data showed a nearly 3-fold increase of pro-inflammatory blood monocytes within 7 days after bacterial exposure in mice fed with the high protein diet, as compared to control mice (Fig. 3). These observations suggest that the composition of the food consumed can be an significant factor in the performance that an organism will display when exposed to a bacterial pathogen. In addition, associated to the energetic costs derived from the immune system, mice fed with the high protein diet showed no relevant differences in resting metabolic rate before and after bacterial challenge.

Both RMR (Fig. 2b) and body mass (data not shown) exhibited no significant differences in any of the groups treated between 7 days before and after the BCG challenge. Nevertheless, there was a non-significant tendency to increase in the level of food intake in the C group (40 %) and a clear significant increment for LP group, nearly to 60 % higher than the levels prior to the BCG challenge. The HP group showed no significant changes in the level of food intake (Fig. 2a). When the energetic costs of mounting an immune response are intermediate, animals can cope with it by increasing food intake or reducing the energy allocated to other physiological functions (Garland 2014). In this case, increasing intake levels appear to be sufficient to deal with an attenuated bacterial pathogen. Along these lines, it has been suggested that when the cost of mounting an immune response is low, animals may handle it by increasing food intake or through a change in time budget (Amat et al. 2007), Thus, there is not a high energetic cost caused by infection, since the high proportion of protein in the diet helps to keep a well "armed" immune system. However, it is important to point out that the performance of others physiological functions was not evaluated in this study.

Finally, the strategy used by these individuals is the storage of cells and molecules involved in defense functions rather than produce new ones if the pathogens attack again. This is mainly because the cost of this reserve should be very low, considering the high protein intake of individuals subjected to the HP regime. In addition, if we focus on the state of the immune system before a pathogenic attack, we see that the quality of the diet in terms of macronutrients influences the preparation against a possible microbial aggression. Figure 4 shows a clear difference between the blood levels of $CD4^+$ and $CD8^+$ T lymphocytes between

animals fed LP diet as compared to those fed C and HP diet. These data suggest that there is a threshold in the diet for protein composition (~20 %), below which there will be no more expansion of peripheral blood T cells, but conservation of the lymphocyte proportions to ensure an accurate defense in response to a bacterial challenge.

In summary, (i) although there is an energetic cost associated, the consumption of a high percentage of protein in the diet is beneficial in both the maintenance and the response of the innate immune system. Once a host has been infected by a bacterial pathogen, the innate immune response is significantly faster and efficient at controlling microbial dissemination. Furthermore, the immune system is better prepared for an infection when the host has been fed with a protein enriched diet. However, there is a large cost associated with the energy allocated to immune system maintenance at rest. (ii) There is a threshold percentage of protein in the diet above which normal levels of lymphocytes, mainly T lymphocytes, remain within an optimal range. In other words, the maintenance of normal levels of major defense cells is subject to the dietary protein/carbohydrate (P/C) ratios ingested by an organism. We can conclude that the proportion of macronutrients in food can lead to important consequences relative to organism performance, ranging from the state of the defense system (immunocompetence) and the performance of the organism against immunological challenges. Apparently, the energy cost associated with immune function can be paid before or during a pathogenic challenge, depending on the proportion of dietary macronutrients. Thus, a rich protein diet confers significant advantages relative to immunocompetence and the immune response. The cost of storing reserves of proteins and defensive cells in comparison to a new production may explain this observation. Nevertheless, the energetic cost seems to be paid before, mediated by a high metabolic rate and food intake. Finally, we have provided evidence suggesting that diet interacts with the immune system providing the basic nutrients to build up the host defense. The availability of nutrients in food also seems to modulate the immune response but at an energetic cost that also depends of food quality.

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