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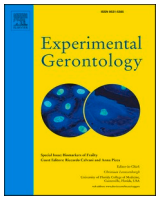

The interaction between aging and protein malnutrition modulates peritoneal macrophage function: An experimental study in male mice

Gabriela Kodja Vivian [a], Renaira Oliveira da Silva [a], Andressa Cristina Antunes Santos [a], Araceli Aparecida Hastreiter [a], Carolina Carvalho Dias [a], Edson Naoto Makiyama [a], Primavera Borelli [a], Claudia de Oliveira Rodrigues [b], Ricardo Ambrósio Fock [a,*]

[a] Department of Clinical and Toxicological Analysis, School of Pharmaceutical Sciences, University of São Paulo, São Paulo, Brazil
[b] Department of Biomedical Science, Charles E. Schmidt College of Medicine, Florida Atlantic University, Florida, USA

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ABSTRACT

Malnutrition is considered one of the most common problems in the elderly population worldwide and can significantly interfere in health evolution in these individuals, predisposing them to increased infection susceptibility. The immune response triggered by infections comprises several mechanisms, and macrophages play important roles in this response. This study aimed to evaluate mechanisms related to macrophage function in a model of protein malnutrition in the elderly. Two age groups (young: 3–5 months and elderly: 18–19 months) male C57BL/6NTac mice were subjected to protein malnutrition with a low-protein diet (2 %). The nutritional status, hemogram and number of peritoneal cells were affected by both age and nutritional status. Additionally, the spreading capacity as well as the phagocytic and fungicidal activity of peritoneal macrophages were affected by the nutritional status and age of the animal. Interestingly, the percentages of $F4/80^{+}/CD11b^{+}$ and $CD86^{+}$ cells were reduced mostly in elderly animals, while the $TLR\text{-}4^{+}$ population was more affected by nutritional status than by age. The production of pro-inflammatory cytokines such as TNF-α, IL-1α, and IL-6 was also influenced by nutritional status and/or by age, and malnourished animals of advanced age produced higher amounts of the anti-inflammatory cytokine IL-10. Furthermore, the phosphorylation ratio of the transcription factor NFκB (pNFκB/NFκB) was directly affected by the nutritional status, independently of age. Thus, these results allow us to conclude that aging and protein malnutrition compromise macrophage function, likely affecting their immune function, and in aged protein-malnourished animals, this impairment tends to be more pronounced.

1. Introduction

The elderly population increases in size every year and, in the next decades, will represent a quarter of the world's population (United Nations Department of Economic and Social Affairs, 2020; Engelheart and Brummer, 2018). This global scenario presents a different perspective on quality of life, since nutrition helps to maintain a healthy balance, and several diseases that affect elder people result from dietary imbalances (Siddique et al., 2017; Abd Aziz et al., 2017). Under these circumstances, the incidence of malnutrition is relatively high, affecting >60 % of the elderly population, especially in hospitalized or institutionalized elderly. Notably, malnutrition, mainly related to inadequate protein intake, predisposes the elder population to increased infection susceptibility (Abd Aziz et al., 2017; Rush, 1997; Volkert et al., 2019; Corcoran et al., 2019).

The immune response triggered by infections comprises several cellular and humoral mechanisms that have the ability to protect the organism (Medzhitov and Janeway, 1997). Among them, macrophages are effector cells of the innate immune system involved in the processes of phagocytosis, antigen presentation, secretion of several cytokines, regulation of lymphocyte activation, proliferation, and others (Bonnardel and Guilliams, 2018; Locati et al., 2013). As such, these cells play a key role in host cellular defense against infection. The initial sensing of infection and strengthening of innate immunity are mediated by pattern recognition receptors (PRRs) (Bonnardel and Guilliams, 2018; Locati et al., 2013; Vijay, 2018). Toll-like receptors (TLRs) are important PRRs

* Corresponding author at: Laboratory of Experimental Haematology, Department of Clinical and Toxicological Analysis, School of Pharmaceutical Sciences, University of São Paulo, Avenida Lineu Prestes, 580 - Bloco 17, São Paulo, SP, Brazil.
E-mail address: hemato@usp.br (R.A. Fock).

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found in macrophages that have important roles in pathogen recognition, modulating several cellular mechanisms and pathways to mount an adequate response against pathogens (Locati et al., 2013; Vijay, 2018). Among the TLRs, TLR-4 acts as a receptor for lipopolysaccharide (LPS) (Vijay, 2018).

Lipopolysaccharide (LPS) is an important constituent of the membranes of gram-negative bacteria and takes part in stimulation of the host immune response. Macrophages are one of the cell types involved in such responses (Bonnardel and Guilliams, 2018; Vijay, 2018). The recognition of LPS by the complex toll-like receptor 4 (TLR-4) triggers the activation of several cellular pathways in macrophages, one of which activates the transcription factor nuclear factor kappa b (NFκB) (Vijay, 2018; Mitchell et al., 2016). This transcription factor plays a key role in regulating the innate immune system and, once activated, triggers the production of several pro-inflammatory cytokines (Mitchell et al., 2016; Duque and Descoteaux, 2014), such as interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), which are essential for a normal inflammatory response (Duque and Descoteaux, 2014). Another transcription factor important for an adequate cellular immune response is the signal transducer and activator of transcription 3 (STAT-3), an acute-phase response element that is also activated by several pro-inflammatory cytokines (Levy and Lee, 2002; Fernando et al., 2014).

Although malnutrition has been shown to affect the immune response (De Bandt, 2015; Santos et al., 2017), there is a lack of studies of the mechanisms involved in the elderly. Thus, the objective of the current study was to investigate whether, and how, aging and protein malnutrition affect the immune response and the function of macrophages.

2. Materials and methods

2.1. Diets

The murine diets were prepared in house. Casein was the protein source (>85 % protein) for both normoproteic and hypoproteic diets, and the protein contents were 12 % and 2 %, respectively. The final protein concentration in the diets was confirmed by the standard micro-Kjeldahl method (Ward, 1963). Both diets contained 100 g kg^{-1} sucrose, 80 g kg^{-1} soybean oil, 10 g kg^{-1} fiber, 2.5 g kg^{-1} choline bitartrate, 1.5 g kg^{-1} L-methionine, 40 g kg^{-1} of mineral mixture, and 10 g kg^{-1} of vitamin mixture. The control diet contained 120 g kg^{-1} casein and 636 g kg^{-1} cornstarch, while the malnourishment diet contained 20 g kg^{-1} casein and 736 g kg^{-1} cornstarch. The diets were isocaloric and provided 1716.3 kJ/100 g. The mineral and vitamin mixture were prepared according to the recommendations of the America Institute of Nutrition for adult mice (Reeves et al., 1993).

2.2. Mice

This study was approved by the Ethics Committee on the Use of Animals of the Faculty of Pharmaceutical Sciences of the University of São Paulo (CEUA Protocol n°544). Male C57BL/6NTac (Taconic Biosciences, USA) mice, 3–5 months old, (young group), and 18–19 months old (elderly group), were obtained from the Animal Care Facility of Faculty of Pharmaceutical Sciences at University of São Paulo. They were maintained in individual cages at 20–24 °C, at relative humidity of 55 % ± 10 %, and on a regular 12-hour light/dark cycle. The animals received the control diet for an adaptation period of 7 days to stabilize their body weight and were subsequently divided into groups receiving either the control or the hypoproteic diet and water *ad libitum*. Four groups named as Control Young, Malnourished Young, Control Elderly and Malnourished Elderly were used in the study, according to age and the diet received.

Mice were subjected to experimental assays after 30 days of eating their respective diet, during which time the malnourished group lost 15 % or more of their original body weight. After induction of the malnutrition experimental protocol, mice were euthanized by anesthetic overdose with xylazine hydrochloride (Rompum®, Bayer, Brazil, 10 mg/kg) and ketamine hydrochloride (Ketamina®, Cristália, Brazil, 100 mg/kg), followed by exsanguination.

2.3. Nutrition assessment

The nutritional evaluation was performed by measuring body weight, diet and protein consumption, as well as serum protein and albumin concentrations. Every 48 h, body weight and diet consumption of all mice were monitored. The body weight variation was calculated considering the initial (after the adaptation period) and final weights (on the day of euthanasia) of the animals in all the groups.

Body composition was analyzed by X-ray tomography, using a current of 400 μA and a voltage of (35 kVp) to acquire 600 projections. After data collection, an algorithm (FBP, filtered back projection) was used to reconstruct the tomographic image, resulting in an image with 0.125-mm isotropic voxels. All steps were performed using PMOD software, version 3.706 (PMOD Technologies, Switzerland). Subsequently, the images obtained after each segmentation step were digitally reconstructed using ImageJ software (version 1.53, National Institutes of Health, USA).

In addition, after euthanasia, the gastrocnemius muscle from the left hind limb and the visceral (epididymal) fat pads were carefully removed, manually dissected, and weighed.

2.4. Hemograms and biochemical analyses

Blood samples were collected from the axillary plexus into conical tubes with or without EDTA (1 mg/mL; Sigma-Aldrich, USA). The blood samples collected with EDTA were used for hemograms, which were performed in an automated analyzer ABX Micros ABC Vet® equipment (Horiba ABX, France). Leucocyte differential and morphological analyses were performed on blood smears stained by employing the May-Grünwald-Giemsa (Merck, Germany) technique. The serum from blood samples collected without anticoagulant was separated by centrifugation, and serum total protein, albumin, urea, creatinine, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were assayed by using an automated dosing system (Roche, Cobas® 6000 analyzer, Switzerland).

2.5. Peritoneal cell count

After euthanasia, cells from the peritoneal cavity were harvested by injecting 10 mL of sterile RPMI-1640 medium (Vitrocell, Brazil) supplemented with 10 % fetal bovine serum (Vitrocell, Brazil), penicillin (100 units/mL), and streptomycin in an aseptic environment. Cell suspensions were centrifuged for 10 min at 400 ×*g* and 4 °C and resuspended in 1 mL of sterile RPMI-1640 medium. The total number of peritoneal cells was determined using a Neubauer chamber, and the different types of cells were quantified in cytocentrifuge smears stained by the May-Grünwald-Giemsa (Merck, Germany) technique. Cell viability was determined by trypan blue exclusion. Peritoneal macrophages were obtained by incubating 1×10^6 cells per dish in 24-well polystyrene culture plates for 1 h at 37 °C, and non-adherent cells were removed by three vigorous washes with RPMI-1640 medium. The entire procedure was executed under aseptic conditions, and all the materials used were previously sterilized and pyrogen-free.

2.6. Peritoneal cell flow cytometry

Peritoneal macrophages obtained as described above were characterized by flow cytometry analysis and defined as double-positive cells for F4/80 and CD11b. Peritoneal cells were centrifuged at 400 ×*g* for 7 min, and the supernatant was separated and the pellet resuspended in 1 mL of RPMI-1640 medium. Cells (5×10^5 cells/mL) samples were

labelled with monoclonal antibodies. Cells were incubated with monoclonal antibodies for 20 min at 25 °C, protected from light. After this period, the samples were centrifuged at 400 ×*g* for 3 min, the supernatant was separated and discarded, and the cell sediment was washed once with PBS (Sigma-Aldrich, USA). The sediment was re-suspended in 250 μL of PBS, and the cells were analyzed in a flow cytometer. The characterization of peritoneal cells was performed using F4/80 (APC; Clone T45-2342, cat. 566787, Becton-Dickinson, Pharmingen, USA), CD11b (FITC; Clone M1/70, cat. 557396, Becton-Dickinson, Pharmingen, USA), TLR-4 (PE; Clone MTS510, cat. 558294, Becton-Dickinson, Pharmingen, USA), CD86 (PE; Clone GL1, cat. 553692, Becton-Dickinson, Pharmingen, USA). Two negative controls sets were performed, unstained and fluorescence-minus-one (FMO) control stain sets. A total 50,000 events were acquired in a FACS Canto II (FACScan®, Becton Dickinson, USA), and data were analyzed with FlowJo® 10 software (Tree Star Inc., USA). The flow cytometry analysis strategy is described in Supplemental Fig. S1.

2.7. Spreading tests on peritoneal macrophages

Peritoneal macrophages obtained as described above were plated at a concentration of 3×10^5 cells/mL in 24-well culture plates, with a 13 × 13 mm sterile cover slip placed on the bottom of each well. The cells were cultured in 1 mL of RPMI 1640 medium (Vitrocell, Brazil), pH 7.4, with 10 % fetal bovine serum (Vitrocell, Brazil), in the presence of 1.25 μg/mL LPS (*Escherichia coli* 026:B6, Sigma-Aldrich, USA) (Fock et al., 2007). The plates were incubated for 48 h at 37 °C, under a 5 % CO_2 atmosphere. After this period, the supernatants were discarded, and the coverslips were washed with PBS, pH 7.4 (Dulbecco) and stained by May-Grünwald-Giemsa solution (Merck, Germany). Using optical microscopy, the adhered cells were distinguished from those which were spread (Santos et al., 2016; Fock et al., 2007).

2.8. Phagocytic and fungicidal activity of peritoneal macrophage

A suspension of *C. albicans* ATCC-40277 was obtained from a 24-h culture in Sabouraud agar (Difco, USA), yeast cells were counted in Neubauer chamber and viability was evaluated using 1 % methylene blue. Only yeast suspensions with >95 % viability were used. The yeast cells were opsonized in 1 mL of homologous serum obtained from normal mice for 30 min with agitation at 0.007 ×*g* at 37 °C. In sterile conical plastic tubes, 3×10^5/mL peritoneal macrophages suspension and 3×10^6 yeast cells/mL 1:10 (vol/vol) of the opsonized *C. albicans* solution were added, maintaining a proportion of 1 cell to 10 yeast cells. The tubes were incubated for 90 min at 37 °C under gentle agitation, and then collected, cytocentrifuged, and immediately fixed and stained with May-Grünwald-Giemsa solution (Merck, Germany). A macrophage suspension without the infectious agent was used as a negative control. For the evaluation of phagocytosis, at least 200 macrophages were counted, and those with one or more internalized *C. albicans* cells were considered as having phagocytic activity, which was expressed as a percentage.

Fungicidal activity was evaluated according to the technique described by Herscowitz et al. (1981) and adapted by us (Fock et al., 2007). Briefly, May-Grünwald-Giemsa (Merck, Germany) stains the live yeast cells, leaving dead cells unstained. As the numbers of phagocytosed yeast vary from cell to cell, the fungicidal activity was scored as follows: the number of macrophages with one phagocytosed dead *C. albicans* was multiplied by one (score 1); with two dead *C. albicans*, multiplied by two (score 2); with three dead *C. albicans* multiplied by three (score 3); and with more than four dead *C. albicans*, multiplied by four (score 4). Fungicidal activity was evaluated by counting at least 200 macrophages with phagocytosed *C. albicans* (Santos et al., 2016; Fock et al., 2007).

2.9. TNF-a, IL-1a, IL-6, and IL-10 determination in vitro

Peritoneal macrophages were collected as described above, and 1×10^6 cells/mL were cultured in 1 mL of RPMI-1640 medium (Vitrocell, Brazil) with 10 % fetal bovine serum (Vitrocell, Brazil) at 37 °C in a humidified atmosphere of 5 % CO_2 for 24 h in the presence of 1.25 μg/mL of LPS (*Escherichia coli*; 026: B6, Sigma-Aldrich, USA). After 24 h, the supernatants were collected for the quantification of cytokines, and the cells were collected for western blot analysis as described below. Quantification of TNF-α, IL-1α, IL-6, and IL-10 was performed in duplicate on the abovementioned supernatants by ELISA (TNF-α, cat. SMTA00B; IL-1α, cat. MLA00; IL-6, cat. SM6000B; IL-10, cat. SM1000B; Quantikine®; R&D Systems, USA), according to the manufacturer's instructions.

2.10. NFκB and STAT-3 expression

The expression levels of NFκB and STAT-3 were determined by western blot. Peritoneal macrophages were obtained as described above, washed once with PSB, and lysed with RIPA buffer (25 mM Tris HCL (pH 7.6), 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % SDS) supplemented with protease and phosphatase inhibitor cocktail (150 mM NaCl, 2 μg/mL aprotinin, 1 μg/mL leupeptin, 100 μg/mL PMSF, and 0.5 mM EDTA). Lysates were vortexed for 1 min and centrifuged at 21,913 ×*g* at 4 °C for 15 min. The supernatant was collected; mixed with 5× Laemmli buffer (1 mM Tris HCL (pH 6.8), 10 % 2-mercaptoetanol, 10 % SDS, 50 % glycerol, and 0.01 % bromophenol blue); and boiled for 10 min. The protein concentrations were measured in supernatants using a BCA Protein Assay Kit (Pierce Biotechnology, Inc., USA) and adjusted to the same concentration for loading on the gel. Protein (10 μg per well) was separated on 12 % SDS-polyacrylamide mini-gels and subsequently transferred to nitrocellulose membranes (Hybond® ECL™, GE Healthcare, USA). The membranes were incubated at room temperature overnight with the primary antibodies anti- NF-κB (1:1000; cat. SC-372), anti-STAT-3 (1:1000; cat. SC-482), anti-p-NF-κB (1:1000; cat. SC-33039), and anti-p-STAT-3 (1:1000; cat. SC-8001-R), purchased from Santa Cruz Biotechnology. The secondary antibody (1:1000) conjugated to horseradish peroxidase (cat. 7074S, Cell Signaling, Inc., USA) was incubated for 120 min. Then, the membranes were washed three times with 1× Tris-Buffered Saline, 0.1 % Tween® 20 Detergent (TBST; Sigma-Aldrich, USA) and the immunoreactive bands were visualized using the ECL detection system (Amersham ECL™ Advance Western Blotting Detection Kit, USA). The densitometry analysis of the specific bands was quantified with a digital detection system (ImageQuant™ 400 version 1.0.0, Amersham Biosciences, USA). The results were normalized to the intensity of β-actin (1:5000; cat. A3854, Sigma-Aldrich, USA), and the ratios between phosphorylated and total NF-κB and STAT-3 expression were calculated.

2.11. Statistical analysis

Statistical analyses were performed using GraphPad Prism® (GraphPad Software Inc., La Jolla, USA). Data sets were subjected to normality test. We used two-way ANOVA followed by Tukey's *post hoc* test. The level of significance adopted was 95 % ($p < 0.05$) and n represents the number of mice analyzed in each experiment, as detailed in figure legends and tables. All data are represented as mean ± standard deviation (SD). *p < 0.05, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$.

3. Results

3.1. Nutritional and biochemical evaluation

Diet consumption was similar among all groups; however, both malnourished groups (young and elderly) consumed less protein than

the control groups, which resulted in a body weight loss exceeding 16 % (Table 1). However, no differences in protein consumption between malnourished young and elderly animals were observed (Table 1). The animals from both malnourished groups presented decreased total serum protein and albumin concentrations when compared with the respective control groups, and elderly animals had a lower concentration of serum albumin in comparison with young animals (Table 1).

A significant decrease in the weight of the gastrocnemius muscle was observed in both malnourished groups, as well as in elderly animals in comparison with young control animals (Fig. S2A). The visceral fat weight from the malnourished groups were reduced in comparison with that of the control groups (Fig. S2B), but in both elderly groups, the values for fat weight were increased when compared with those of the respective young groups (Fig. S2B). These data were confirmed by X-ray images of the subcutaneous fat area (Fig. S2C).

Malnourished animals from both groups showed reduced values for serum urea in comparison with the respective control groups, but no differences in serum creatinine concentrations were observed among groups. In addition, AST quantification was higher in the malnourished animals from the young group in comparison with the control groups, whereas ALT quantification was reduced in the elderly animals in comparison with the respective young groups (Table 1).

3.2. Blood cell count

Malnourished animals from the young and elderly groups presented anemia with a decreased erythrocyte number, hemoglobin concentration, and hematocrit when compared with their respective control groups, and these parameters were even lower in malnourished animals from the elderly group when compared with those from the young group (Table 2). Additionally, malnourished animals from the young and elderly groups presented leukopenia with a reduced number of neutrophils and lymphocytes when compared with their respective control groups. The monocyte counts were also different between the control and malnourished groups, besides differences between malnourished young animals and malnourished elderly animals. The number of eosinophils did not show differences among groups (Table 2).

Table 1
The diet and protein consumption, body weight variation, concentrations of serum protein, albumin, urea, creatinine, AST and ALT in control and malnourished animals from young and elderly groups.

Variables	Control Young ($n = 12$)	Malnourished Young (n = 12)	Control Elderly (n = 12)	Malnourished Elderly (n = 12)
Diet consumption (g/day/animal)	3.65 ± 0.33^{a}	3.78 ± 0.58^{a}	3.86 ± 0.41^{a}	3.93 ± 0.33^{a}
Protein consumption (g/day/animal)	0.438 ± 0.04^{a}	0.075 ± 0.01^{b}	0.463 ± 0.05^{a}	0.078 ± 0.01^{b}
Body weight variation (%)	+11.40 ± 5.48^{a}	−21.28 ± 5.35^{b}	12.45 ± 5.37^{a}	−16.67 ± 2.63^{b}
Serum protein (g/dL)	5.40 ± 0.27^{a}	4.71 ± 0.24^{b}	5.14 ± 0.40^{a}	4.41 ± 0.27^{b}
Serum albumin (g/dL)	3.27 ± 0.26^{a}	2.55 ± 0.23^{b}	2.93 ± 0.21^{c}	2.26 ± 0.23^{d}
Urea (g/dL)	29.83 ± 2.32^{a}	15.50 ± 3.84^{b}	34.00 ± 7.79^{a}	19.33 ± 3.47^{b}
Creatinine (g/dL)	0.12 ± 0.03^{a}	0.11 ± 0.02^{a}	0.14 ± 0.03^{a}	0.13 ± 0.04^{a}
AST (U/L)	78.08 ± 7.28^{a}	102.2 ± 8.38^{b}	72.67 ± 17,94^{a}	89.42 ± 25.60^{a,b}
ALT (U/L)	37.92 ± 3.82^{a}	34.17 ± 5.02^{a,b}	29.42 ± 6.99^{b,c}	24.58 ± 6.65^{c}

Results are expressed as mean ± SD. *n* represents the number of animals used in the experiment. Different superscript letters indicate a significant difference between groups ($p < 0.05$).

Table 2
Hemogram of control and malnourished animals from young and elderly groups.

Hematological Parameters	Control Young (n = 12)	Malnourished Young (n = 12)	Control Elderly (n = 12)	Malnourished Elderly (n = 12)
Erythrocytes ($\times 10^6$/mm^3)	6.03 ± 1.44^{a}	4.78 ± 1.25^{b}	5.63 ± 0.83^{a,b}	4.42 ± 0.56^{c}
Hemoglobin (g/dL)	8.6 ± 2.0^{a}	6.7 ± 1.7^{b}	7.9 ± 1.47^{a,b}	6.3 ± 1.48^{c}
Hematocrit (%)	26.2 ± 6.1^{a}	20.9 ± 4.7^{b}	24.5 ± 2.0^{a,b}	19.95 ± 2.8^{c}
Leukocytes (/mm^3)	1648 ± 417^{a}	715 ± 165^{b}	1478 ± 438^{a}	759 ± 281^{b}
Neutrophils (/mm^3)	372 ± 240^{a}	160 ± 63^{b}	374 ± 178^{a}	169 ± 95^{b}
Eosinophils (mm^3)	11 ± 17^{a}	7 ± 25^{a}	19 ± 28^{a}	2 ± 4^{a}
Lymphocytes (/mm^3)	1220 ± 382^{a}	534 ± 156^{b}	1050 ± 454^{a}	588 ± 269^{b}
Monocytes (/mm^3)	45 ± 28^{a}	13 ± 23^{b}	33 ± 38^{a,b}	3 ± 4^{c}

Results are expressed as mean ± SD. n represents the number of animals used in the experiment. Different superscript letters ($p < 0.05$) indicate a significant difference between groups.

3.3. Cellularity of peritoneal exudate

The total number of peritoneal cells and mononuclear cells were significantly reduced in the malnourished animals from young and elderly groups when compared with their respective control groups (Table 3). No differences in the number of mast cells and polymorphonuclear cells were observed among groups (Table 3).

3.4. Phenotypic characterization of peritoneal cells

The peritoneal macrophages and the expression of the TLR-4 and CD86 receptors were characterized by flow cytometry. The results showed that malnourished animals from the young group had a reduced percentage of double-positive cells for CD11b and F4/80 when compared with control animals from the young group. Additionally, elderly animals showed a reduced percentage of F4/80^{+} CD11b^{+} cells in comparison with young animals, but no differences in the percentage of F4/80^{+} CD11b^{+} cells were observed between control and malnourished animals from the elderly group (Fig. 1A).

TLR-4 expression was evaluated on CD11b^{+}F4/80^{+} cells. Malnourished animals showed significantly reduced expression of TLR-4 when compared with control animals (Fig. 1B and C); however, no differences between young and elderly animals were observed. Interestingly, the expression of CD86 (percentage and MFI) on CD11b^{+}F4/80^{+} cells was similar between control and malnourished animals but decreased in elderly animals (Fig. 1D and E).

Table 3
Cellularity of peritoneal exudate.

Variables	Control Young (n = 10)	Malnourished Young (n = 10)	Control Elderly (n = 10)	Malnourished Elderly (n = 10)
Total peritoneal cells ($\times 10^6$/mL)	4.81 ± 0.53^{a}	3.35 ± 0.41^{b}	5.59 ± 1.96^{a}	3.98 ± 0.65^{b}
Mononuclear cells ($\times 10^6$/mL)	4.37 ± 0.49^{a}	3.04 ± 0.35^{b}	5.06 ± 1.79^{a}	3.57 ± 0.66^{b}
Mast cells ($\times 10^6$/mL)	0.19 ± 0.07^{a}	0.14 ± 0.06^{a}	0.22 ± 0.20^{a}	0.18 ± 0.06^{a}
Polymorphonuclear cells ($\times 10^6$/mL)	0.25 ± 0.10^{a}	0.16 ± 0.05^{a}	0.31 ± 0.15^{a}	0.22 ± 0.10^{a}

Results are expressed as mean ± SD. n denotes the number of animals used in the experiment. Different superscript letters ($p < 0.05$) indicate a significant difference between groups.

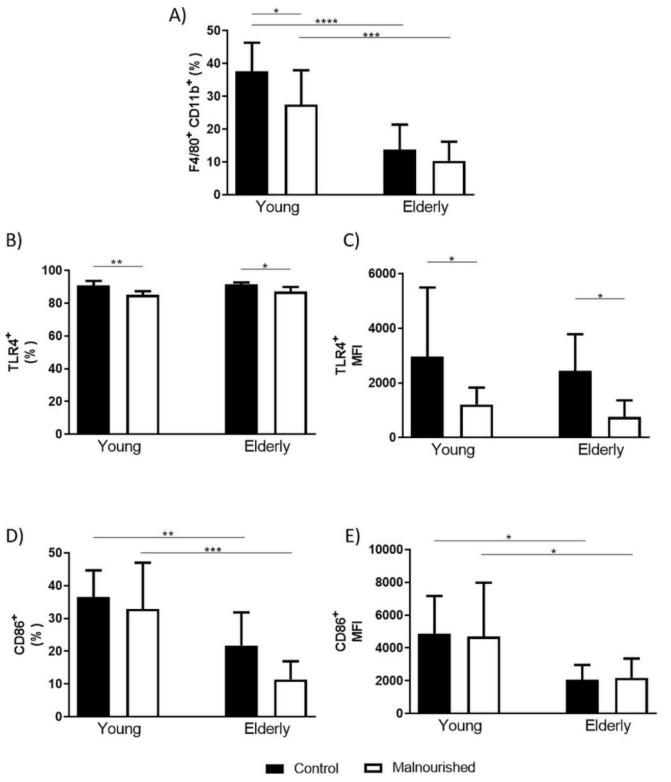

Fig. 1. Results are expressed as mean ± SD of *ex vivo* peritoneal quantification of (A) F4/80 and CD11b double-positive cells, (B) percentage of TLR-4 positive cells, (C) median of fluorescence intensity (MFI) of TLR-4, (D) percentage of CD86 positive cells and (E) median of fluorescence intensity (MFI) of CD86 of control and malnourished animals from the young and elderly groups. $n = 10$ per group. Asterisks indicate significant differences between groups. ($^{*}p < 0.05$; $^{**}p < 0.001$; $^{***}p < 0.0001$; $^{****}p < 0.00001$).

3.5. Spreading and phagocytic and fungicidal activity of peritoneal macrophages

In order to investigate the impact of malnutrition on macrophage function, we investigated their spreading, phagocytic, and fungicidal capacity. Spreading capacity was analyzed in the presence of LPS, and the results showed that the animals responded in a different fashion, depending on their nutritional status (Fig. 2A–E). Malnourished animals from the young group presented significant reduced spreading capacity when compared with animals from the control young group (Fig. 2A, B, and E), and the elderly animals from control the groups also showed significant reduced spreading capacity in comparison with control animals from the young group (Fig. 2A, C, and E). However, when elderly animals from control and malnourished groups were compared, no differences were observed (Fig. 2C, D, and E).

Similar results were observed regarding phagocytic and fungicidal activities. Malnourished animals from the young group presented reduced phagocytic and fungicidal activity in comparison with animals from the control young group (Fig. 2F, G, J, and K) and elderly animals from the control groups showed reduced phagocytic and fungicidal activities in comparison with control animals from the young groups (Fig. 2F, H, J, and K). In addition, no differences in phagocytic and fungicidal activities were observed between control and malnourished animals from the elderly groups (Fig. 2H, I, J, and K).

3.6. IL-1α, IL-6, TNF-α, and IL-10 production in vitro

Cytokines were quantified in the supernatants of peritoneal macrophages cultured in the presence of LPS. As shown in Fig. 3A, cells from malnourished animals from both groups as well as cells from elderly animals from the control group produced reduced concentration of TNF-α when compared with cells from control young animals. Although differences in TNF-α production were observed between control and malnourished cells from the young group, no differences were observed between cells from the control and malnourished elderly groups (Fig. 3A). Regarding IL-6 quantification, age, but not malnutrition, decreased its production by peritoneal macrophages *in vitro* (Fig. 3B). On the other hand, the IL-1α production was reduced by malnutrition, but not by age, in both the young and the elderly groups (Fig. 3C).

IL-10 production showed the inverse trend to that of pro-inflammatory cytokines (Fig. 3D). Cells from the malnourished groups showed increased production of IL-10 when compared with the control groups. In addition, malnourished animals of the elderly group presented higher amounts of this cytokine than animals of the other groups (Fig. 3D).

3.7. Quantification of NFκB and STAT 3

The expression of phosphorylated NFκB (p-NFκB) and STAT-3 (p-STAT-3) were measured in peritoneal macrophages from control and malnourished animals of young and elderly groups following LPS stimulus. Malnourished animals from the young and elderly groups presented reduced expression of p-NFκB/NFκB in comparison with control animals; interestingly, no differences were observed between age groups (Fig. 4A and B). Regarding p-STAT-3, a slight increase was observed in malnourished animals, but no statistically significant differences were observed among all groups (Fig. 4C and D).

4. Discussion

Protein malnutrition, especially in vulnerable populations such as the elderly, can disrupt the immune system. In the last decade, great efforts have been made to elucidate the mechanisms that could be disrupted in the presence of inadequate food consumption (Abd Aziz et al., 2017; Rush, 1997; Yeung et al., 2020). Along these lines, this study aimed to evaluate some of the mechanisms related to the functions of macrophages, the effector cells of the innate immune system (Bonnardel and Guilliams, 2018). To study the isolated impact of protein deficiency intake in elderly, we established a protein malnutrition model using male mice.

It is important to assert that we opted to use male animals since the literature has already reported that fluctuations in female hormones levels can have effect on the number of granulocyte/macrophage progenitor cell colonies development and, on the differentiation of macrophage/dendritic population, suggesting that estrogen could increase the number of potent antigen-presenting cells (Maoz et al., 1985; Paharkova-Vatchkova et al., 2004). Additionally, our model is exclusively focused on reduced protein intake and can thus be used to make inferences about protein malnutrition specifically because other macronutrients or micronutrients are consumed in adequate amounts. Accordingly, as both groups consumed the same amount of food, and the levels of micronutrient consumption were in accordance with the minimum daily recommendations (Reeves et al., 1993; Elinav et al., 2006), we can conclude that the changes observed in our experimental model were mainly due to the decrease in protein intake.

The malnourished young animals lost approximately 21 % of their initial body weight, while the malnourished elderly animals lost about 16 %, after 30 days consuming a protein-deficient diet. This weight loss was accompanied by skeletal muscle mass loss in both malnourished groups; however, elderly malnourished animals showed more body fat than young malnourished animals. These results are in accordance with the literature, where changes in body composition usually occur with advancing age (Kyle et al., 2001; Hickson, 2006), as the adipocyte tissue can relocate in the body and muscle mass loss can be due to loss of fibers and a decrease in fiber size, especially that of type II muscle fibers

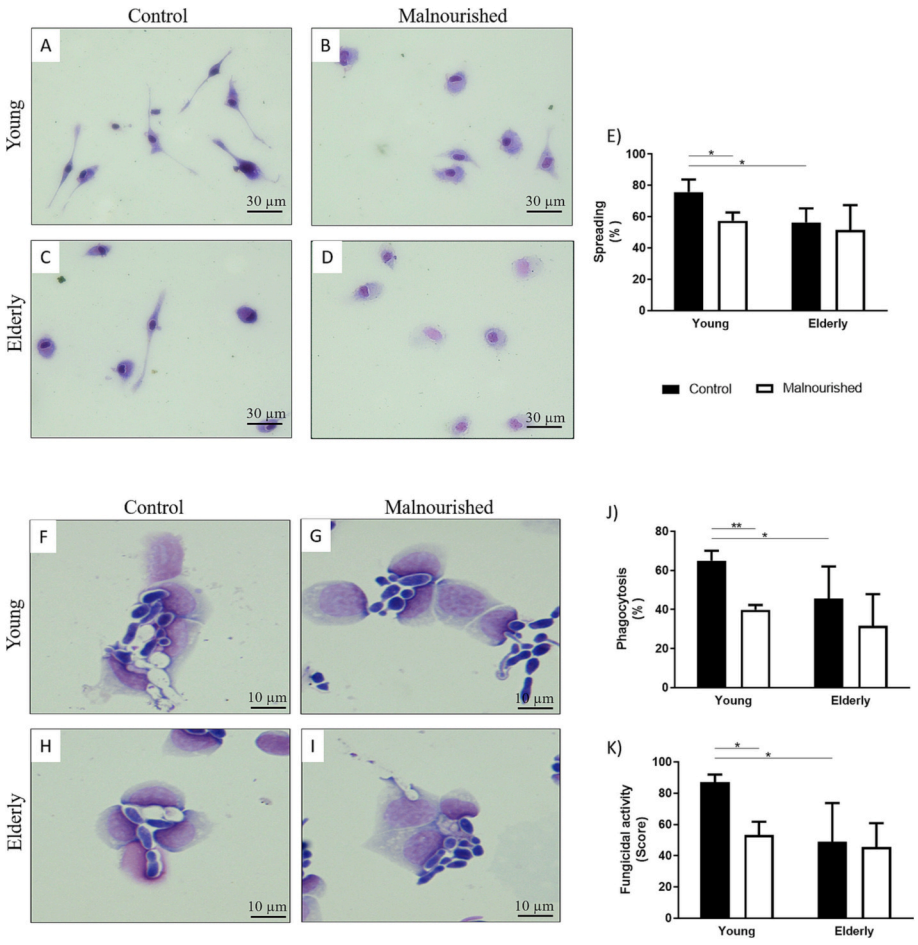

Fig. 2. Results of the spreading capacity and the phagocytic and fungicidal activities of control and malnourished animals from young and elderly groups. (A–D) Representative images of the spreading capacity from groups. (E) Results are represented by mean ± SD of the percentage of spread cells of peritoneal exudate macrophages of control and malnourished animals from the young and elderly groups. (F–I) Representative images of the phagocytic and fungicidal activities from groups. (J) Results of the percentage of phagocytosis (mean ± SD) and (K) the fungicidal activity (mean ± SD) of peritoneal macrophages of control and malnourished animals from young and elderly groups. $n = 6$ per group. All samples were processed in duplicate. Asterisks indicate significant differences between groups ($^{*}p < 0.05$ and $^{**}p < 0.01$).

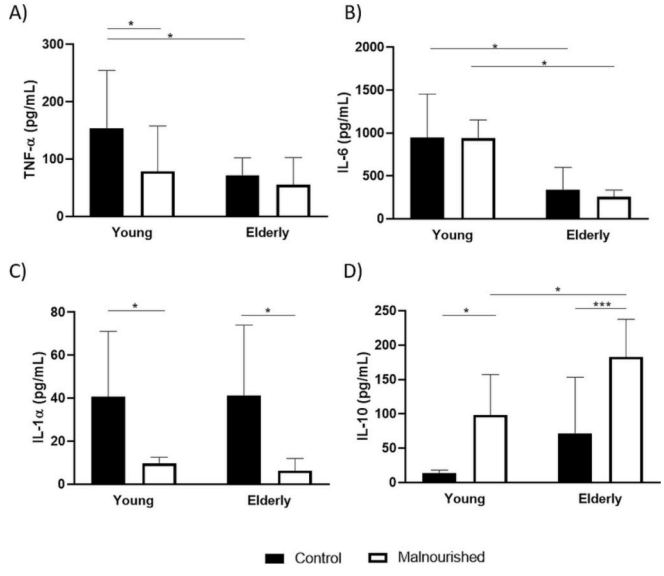

Fig. 3. TNF-α, IL-6, IL-1α and IL-10 quantification *in vitro*. Results of the *in vitro* production of (A) TNF-α, (B) IL-6, (C) IL-1α and (D) IL-10 in the supernatant of peritoneal macrophages of control and malnourished animals from young and elderly groups, in the presence of LPS. Results are expressed as means ± SD ($n \geq 10$, per group). Asterisks indicate significant differences between groups ($^{*}p < 0.05$ and $^{***}p < 0.001$).

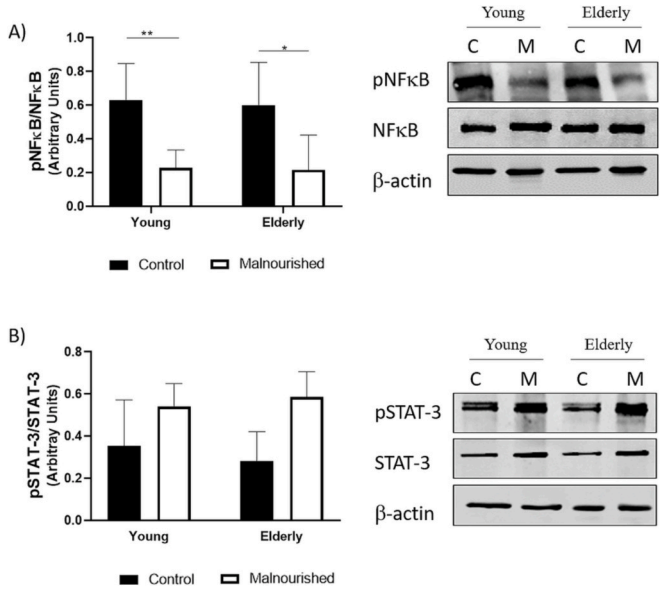

Fig. 4. The results for the *in vitro* expression levels (expressed by mean ± SD), of the ratio of (A) p-NFκB/NFκB and (B) p-STAT-3/STAT-3 in peritoneal macrophages of control (C) and malnourished (M) animals from young and elderly groups, in the presence of LPS, determined by western blot analysis. The western blot images are representative of six independent experiments with similar results. The protein expression was normalized to that of β-actin. Asterisks indicate significant differences between groups ($^{*}p < 0.05$ and $^{**}p < 0.01$).

(Hickson, 2006).

In our study, both groups of malnourished animals presented reduced levels of serum total proteins and albumin. Moreover, control elderly animals showed reduced levels of albumin in comparison to control young animals. Albumin is the most abundant protein in serum, and it has been used as an indicator of malnutrition; accordingly, serum albumin concentrations decrease with increasing age (Keller, 2019). Biochemical alteration parameters observed in malnourished animals, such as urea, creatinine, AST, and ALT, correlate with lower protein consumption in the diet, which was also reported in the literature (Nunes-Souza et al., 2016; Couto et al., 2008). The reduced levels of ALT observed in our control elderly animals in comparison with control young can be associated with aging and reduction in hepatic functions including gluconeogenesis. Therefore, low circulating activity of ALT might reflect liver aging (Elinav et al., 2006; Le Couteur et al., 2010), but this mechanism has not been completely elucidated.

The red blood cell count and number of peripherical leucocytes were not affected by age; however, malnourished animals presented anemia and leukopenia, independently of age. These results are in accordance with the literature, and the evidence also indicates that these findings are common in situations in which malnutrition is not associated with other diseases (Santos et al., 2017; Grimble, 1996; Catchatourian et al., 1980).

Malnourished animals also presented decreased peritoneal cell numbers in both the young and the elderly groups. The literature has already reported that malnutrition affects bone marrow cellularity also reducing cellularity of peritoneal cavity with a reduction in the number of macrophages (Cunha et al., 2013; Papadimitriou and van Bruggen, 1988). Moreover, the decreased peritoneal cellularity observed in malnourished animals were accompanied by a decrease in population of F4/80 and CD11b double-positive cells. Additionally, control elderly animals also showed a reduced population of F4/80 and CD11b double-positive cells in comparison with control young animals. Also, cells from elderly animals showed reduced values for CD86 markers. CD86 is one of the most representative surface molecules of M1 macrophages (Orecchioni et al., 2019). M1 macrophages defend against external pathogens by releasing cytotoxic and inflammatory mediators, such as TNF-α, IL-1, and IL-6, also forming a link between innate and adaptive immunity (Orecchioni et al., 2019), which, in part, can explain the results discussed below.

Interestingly, results were also observed in relation to the expression of TLR4 where a reduced percentage was observed only in malnourished animals. TLR-4 is a receptor that, in response to an appropriate pathogen-associated molecular patterns (PAMP), induces signal transduction pathways, thus activating genes and the synthesis of molecules such as cytokines, chemokines, cell adhesion molecules, and immunoreceptors, all of which are needed to mount an adequate immune response (Krabbe et al., 2004; Medzhitov and Janeway, 2000), which, in part, can interfere with the host's ability to respond adequately to infection in a malnourished population.

Furthermore, our current study showed that protein malnutrition or aging, as well as the association of protein malnutrition and aging, can modify the peritoneal macrophages functions affecting the processes of spreading, phagocytosis, and killing. These processes are important for macrophages to engulf and kill microorganisms (Bonnardel and Guilliams, 2018; Locati et al., 2013). If on the one hand, the literature reports, in protein malnourished states, changes in the enzyme system responsible for the respiratory burst of phagocytes which is required for the killing of microorganisms (Santos et al., 2016; Fock et al., 2007; Prestes-Carneiro et al., 2006), by the other hand, the impact of aging on this system is conflicted since different macrophage sources, different age protocols, low-grade inflammation, associated diseases, and others can directly affect these processes (Mancuso et al., 2001; Sadeghi et al., 1999; Linehan and Fitzgerald, 2015).

Additionally, the literature reports a low-grade inflammatory activity in elderly populations (Krabbe et al., 2004). However, conflicting results can be observed especially when cells from elderly are challenged (Sadeghi et al., 1999; Bruunsgaard et al., 1999; Gabriel et al., 2002; Krabbe et al., 2004). Then, different results in basal or upon stimulation conditions can be found in the literature as well as different cells sources and stimulus which can lead to different results. Nonetheless, many studies have shown that monocytes of older population produce reduced amounts of cytokines than those of young population upon Toll-like receptor (TLR) stimulation (Puchta et al., 2016; Hearps et al., 2012; Metcalf et al., 2017). In this way, our results are in agreement with the literature (Boehmer et al., 2004; Renshaw et al., 2002), where was found that expression of pro-inflammatory cytokines was reduced and IL-10 was increased in macrophages from elderly mice, when challenged with LPS.

Macrophages from malnourished animals also showed reduced production of IL-1α and TNF-α in association with the reduced ratio of pNFκB/NFκB, which is an important transcription factor involved in the process of pro-inflammatory gene induction (Vijay, 2018; Mitchell et al., 2016). However, the key point of the current work is related to cells from elderly malnourished animals, and at this point, the changes in the production of the cytokines studied were more evident in this group in comparison with the others.

As mentioned above, macrophages from malnourished animals, independently of age, presented reduced expression of TLR-4, which, in part, can explain the reduced production of pro-inflammatory cytokines, since TLR-4 activation mediated by LPS activates a series of intracellular events, ending with the activation of NFκB (Vijay, 2018; Mitchell et al., 2016). Our group has already shown reduced expression of TLR-4 in macrophages from malnourished mice (Fock et al., 2007), but here we show, for the first time, that this reduction also occurs in macrophages from malnourished elderly animals, implying that nutritional status and not age is a limiting point to mount an adequate response mediated by TLR-4 activation. In this sense, and although we did not find differences in the expression of TLR-4 in cells from control elderly animals, these cells showed reduced expression of CD86, which also could explain the reduced production of pro-inflammatory cytokines by this group.

Furthermore, no differences in STAT-3 expression were observed among groups, but an increased trend in the ratio of pSTAT3/STAT3 was observed in the malnourished groups. STAT-3 is a transcriptional factor that plays a key role in suppressing signal transduction mediated by Toll-like receptors, also mediating the anti-inflammatory effects of IL-10 (Levy and Lee, 2002; Hillmer et al., 2016). At this point, increased production of IL-10 was observed in all experimental groups when compared with cells from control young animals. IL-10 is an anti-inflammatory cytokine able to inhibit the action of pro-inflammatory cytokines such as IL-1 and TNF-α (Medzhitov and Janeway, 1997; Linehan and Fitzgerald, 2015).

However, some limitations of this study should be noted as the work was based in an animal model using exclusively male mice, which do not reflect how different sexes of mice react since both aging and the immune system are extremely sex dimorphic. Additionally, the experiments of the cells´ mechanisms were performed in *ex vivo* and the parameters evaluated do not reflect all the mechanisms that can modulate the macrophages response, especially mediated by other receptors such as TLR-2, dectin-1, mannose, and others or else the effects on the antigen presentation. In this way, the effects of protein restriction intake and aging to modulate other immune parameters cannot be discarded.

To summarize, it is clear that aging or protein malnutrition affects host defense and that the immune system in aged and malnourished animals does not respond with the same intensity or in the same manner as that of well-nourished young animals. Therefore, the balance of proinflammatory and anti-inflammatory signaling is clearly altered in malnourished or elderly animals, biased toward the anti-inflammatory profile, especially in cells from elderly malnourished animals, which sustains the adverse impact on the immune response.

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CRediT authorship contribution statement

Conceptualization, GKV and RAF; methodology, GKV, ROS, ACAS, AAH, CCD, ENM and COR; software, GKV and AAH; formal analysis, GKV, ROS, AAH and ENM; investigation, GKV, ROS, AAH and ENM; data curation, GKV; writing—original draft preparation, GKV, PB and RAF; writing—review and editing, GKV, PB, COR and RAF; supervision, RAF and PB; project administration, RAF; funding acquisition, RAF.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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