

The influence of protein malnutrition on the production of GM-CSF and M-CSF by macrophages

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It is well established that protein malnutrition (PM) impairs immune defenses and increases susceptibility to infection. Macrophages are cells that play a central role in innate immunity, constituting one of the first barriers against infections. Macrophages produce several soluble factors, including cytokines and growth factors, important to the immune response. Among those growth factors, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF). GM-CSF and M-CSF are important to monocyte and macrophage development and stimulation of the immune response process. Knowing the importance of GM-CSF and M-CSF, we sought to investigate the influence of PM on macrophage production of these growth factors. Two-month-old male BALB/c mice were subjected to PM with a low-protein diet (2%) and compared to a control diet (12%) mouse group. Nutritional status, hemogram and the number of peritoneal cells were evaluated. Additionally, peritoneal macrophages were cultured and the production of GM-CSF and M-CSF and mRNA expression were evaluated. To determine if PM altered macrophage production of GM-CSF and M-CSF, they were stimulated with TNF- α . The PM animals had anemia, leukopenia and a reduced number of peritoneal cells. The production of M-CSF was not different between groups; however, cells from PM animals, stimulated with or without TNF- α , presented reduced capability to produce GM-CSF. These data imply that PM interferes with the production of GM-CSF, and consequently would affect the production and maturation of hematopoietic cells and the immune response.

Uniterms: Protein malnutrition. Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF). Macrophage Colony-Stimulating Factor (M-CSF).

INTRODUCTION

Protein malnutrition (PM) is the most widespread nutritional disorder in developing countries and it is associated with high rates of morbidity and mortality. PM decreases immune defenses, increasing susceptibility to infection. Though all forms of immune response can be affected by PM, non-specific defense and cell-mediated immunity are the most severely affected (Keusch, 2003; Calder, 2013).

The term “cell-mediated immunity” refers to the recognition and/or killing of pathogens by phagocytes, cytotoxic T-lymphocytes and cytokines. Monocytes and macrophages are critical effectors of cell-mediated immunity and regulate cells of the innate and adaptive

immune systems. Monocytes and macrophages also produce important hematopoietic growth factors and immune modulators with profound effects on the functional activities of leukocytes. Among the soluble mediators, granulocyte-macrophage colony-stimulating factor (GM-CSF) and macrophage colony-stimulating factor (M-CSF) play important biological roles in the immune response as well as in the process of blood cell formation and maturation (Metcalf, 2007; Hamilton, Achuthan, 2013; Meshkibaf *et al.*, 2014; Morrison, Scadden, 2014).

GM-CSF and M-CSF mediate cell survival, proliferation and differentiation and also modulate chemotaxis, degranulation, activation, adhesion, cytotoxicity and mRNA phenotype changes of various blood cell populations (Barreda, 2004; Hamilton, Achuthan, 2013). M-CSF is easily detectable in the steady state, but GM-CSF requires an inflammatory stimulation (IL-1, TNF- α or LPS) to reach measurable levels (Hamilton, 2008; Sierra-Filardi *et al.*, 2014).

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Although many different myeloid populations respond to GM-CSF and M-CSF, cells of the monocyte and macrophage lineages are the main target for both of these factors (Hamilton, 2008). GM-CSF is able to stimulate proliferation and differentiation of primitive and early macrophage progenitors while M-CSF is essential to the survival, proliferation, differentiation and maturation of the macrophage myeloid lineage (Rutherford, Schook 1992; Hamilton, 2008).

In this vein, our group has previously shown that PM reduces the proliferation rate of hematopoietic progenitor cells and alters the bone marrow extracellular matrix, and therefore reduces circulating blood cell production. This leads to peripheral leukopenia commonly found in the PM condition (Xavier *et al.*, 2007; Borelli *et al.*, 2009).

GM-CSF and M-CSF produced by macrophages are important soluble factors that stimulate blood cell maturation and immune cell production. PM can impair the generation and maturation of blood cells and the immune system. Therefore, we sought to investigate the influence of PM on GM-CSF and M-CSF production by macrophages, since they act as important soluble factors for both processes.

MATERIAL AND METHODS

Diets

Murine diets were prepared in our laboratory. Mineral and vitamin mixtures were prepared according to the recommendations of the American Institute of Nutrition for adult mice (Reeves *et al.*, 1993). Casein (> 85% protein) was used as the protein source. The two diets prepared contained 100 g/kg sucrose, 80 g/kg corn oil, 10 g/kg fiber, 2.5 g/kg choline bitartrate, 1.5 g/kg L-methionine, 40 g/kg mineral mixture and 10 g/kg vitamin mixture. The control diet contained 120 g/kg casein and 636 g/kg cornstarch, whereas the PM diet contained 20 g/kg casein and 736 g/kg cornstarch. Except for the protein content, the two diets were identical and isocaloric; the total amount of casein removed from the formulation of the hypoproteic diet was substituted for the same mass of cornstarch. Both diets provided 1716.3 kJ/100 g. The final protein content was confirmed using the standard micro-Kjeldahl method (Ward, 1963).

Animals

Two-month-old male outbred BALB/c mice were obtained from the animal laboratory at the School of Pharmaceutical Sciences at the University of São Paulo.

The mice were individually placed in cages and received the control diet for 7 days until their body weight stabilized. After this period of adaptation, the animals were divided into two groups and maintained under a regular 12 h light/dark cycle at a temperature of 22–25 °C and a relative humidity of 55 ± 10%. These mice received either the control (n = 12) or the low-protein (n = 12) diet and water *ad libitum*. Body weight and food consumption were monitored every 48 h. The mice were subjected to PM experimental assays after 28 days of eating their respective diets, when the PM group had lost approximately 20% of their original body weight. This study was approved by the Ethics Committee of the School of Pharmaceutical Sciences at the University of São Paulo (Protocol CEUA/FCF/316).

Nutritional evaluation

A nutritional evaluation was performed by measuring body weight, diet, protein consumption and plasma concentrations of protein and albumin. Body weight variation was calculated using the initial (after the adaptation period) and final (on the day of euthanasia) weights of the animals in both groups. For protein and albumin analyses, the animals were anaesthetized with xylazine chlorohydrate (10 mg/kg; Rompun[®], Bayer Animal Health, Brazil) and with ketamine chlorohydrate (100 mg/kg; Ketamina[®], Cristália, Brazil), and whole-blood samples containing EDTA (1 mg/mL) (Sigma-Aldrich[®], St. Louis, MO, USA) were collected. Plasma was separated by centrifugation (1000 × g for 10 min at 4 °C) and total protein content and albumin levels were determined by standard methods (Gornall *et al.*, 1949; Dumas *et al.*, 1997).

Hemogram and peritoneal cell count

Blood samples containing EDTA (1 mg/mL) (Sigma-Aldrich[®]) as an anticoagulant were used to perform a hemogram. The evaluation was performed on ABX Micros ABC Vet equipment (Horiba, Japan). Morphological and leukocyte differentiation analyses were conducted on blood smears stained using the standard May–Grünwald–Giemsa (Sigma-Aldrich[®]) technique.

After being anaesthetized, the animals were euthanized and the peritoneal cavities were washed with 5 mL of sterile RPMI medium (VitroCell[®], Campinas, SP, Brazil) and supplemented with fetal bovine serum (10% v/v, VitroCell[®]), penicillin and streptomycin under aseptic conditions. The resulting cell suspensions were centrifuged for 10 min at 1200 RPM and 4 °C and

re-suspended in 1 mL of sterile RPMI medium. Cell viability was determined by Trypan blue exclusion. Macrophages were obtained by incubating 1×10^6 cells per well in 24-well polystyrene culture plates for 1 h. Non-adherent cells were removed by three vigorous washes with RPMI medium. The entire procedure was executed under aseptic conditions and all of the materials used had been previously sterilized and were pyrogen free. Total cell counts were determined using a Neubauer chamber and the different types of cells were quantified in cytocentrifuge smears stained with standard May–Grünwald–Giemsa solution (Sigma-Aldrich®).

Flow cytometric analysis using a FACSCalibur flow cytometer (Becton & Dickinson, San Jose, CA, USA) was used to determine the fraction of peritoneal F4/80⁺ cells labeled with the F4/80 FITC antibody. Peritoneal exudate cells consisted of more than 80% F4/80⁺ macrophages whereas more than 90% of the cells adhering to the wells were F4/80⁺ macrophages (data not shown).

Macrophage cell cultures

Peritoneal macrophage cultures were plated in 24-well polystyrene culture plates (Falcon, Lincoln Park, NJ, USA) at a final concentration of 1×10^6 cells/mL and cultured (at 37 °C in a humidified atmosphere with 5% CO₂) for 2 h in the presence or absence of TNF- α (10 ng/mL) stimulation. The incubation time and TNF- α concentration had been previously standardized. After this period, the supernatants were collected for quantification of the levels of GM-CSF (cat #DY415) and M-CSF (cat #DY417) by ELISA. The tests were performed using the commercially available reagent Quantikine® M Mouse (R&D Systems, Inc., Minneapolis, MN, USA), following the procedures recommended by the manufacturer.

Macrophages and quantitative PCR (qPCR) for CSF1 and CSF2

Peritoneal macrophages from the control and PM groups were isolated as described above. Total RNA was obtained using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) according to the manufacturer's protocol. The total RNA was reverse transcribed into cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The cDNA samples were then amplified using TaqMan Universal Master Mix with an optimized concentration of the primer set for GM-CSF (CSF2,

Mm01290062_m1, Applied Biosystems, Foster City, CA, USA) and M-CSF (CSF1, Mm00432686_m1, Applied Biosystems, Foster City, CA, USA). The internal control used was ACTB (Mm00607939_s1, Applied Biosystems, Foster City, CA, USA). The expression of CSF 1 and CSF2 was evaluated by qPCR using StepOnePlus™ (Applied Biosystems, Foster City, CA, USA). The amplification conditions were as follows: an initial 5 min at 95 °C, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing and extension at 60 °C for 30 s. The relative quantification of GM-CSF and M-CSF was conducted according to the $\Delta\Delta C_t$ method (Livak, Schmittgen, 2001).

Statistical analysis

All statistical analyses were performed with GraphPad Prism® software (version 5.03 for Windows; GraphPad Software, San Diego, CA, USA) and the data are expressed as mean values with their standard deviations. The dependent variables were normally distributed. The results were analyzed by analysis of variance (ANOVA), followed by Bonferroni's multiple comparison test when three or more groups were compared and by parametric Student's *t*-test when two groups were compared. Statistically significant differences were considered at $p \leq 0.05$.

RESULTS

Body weight variation, diet and protein consumption, total plasma protein and albumin concentrations and hemogram

The amount of food and calories consumed by the PM and control groups were similar. However, the PM experimental diet with lower protein content resulted in significantly reduced protein ingestion in the PM group as expected. Animals from control and PM groups had body weight around 21 g at the beginning of the experimental protocol. After 28 days of the experimental protocol the body weight mean of control animals was around 25 g and of PM animals was around 17 g. Mice fed with the hypoproteic diet exhibited significant body weight loss, amounting to approximately 20% of their initial weight, and a significant decrease in total plasma protein and albumin concentrations was observed (Table I).

The hemograms showed significantly reduced erythrocyte counts, hemoglobin concentrations and hematocrit in PM animals compared with control animals. PM animals also exhibited leukopenia, with depletion

TABLE I - The body weight variation, diet and protein consumption, concentrations of plasmatic protein and albumin, and hemogram in control (C) and protein malnourished (PM) animals

Variables	C (n=12)	PM (n=12)
Body weight variation (%)	+20.49±1.65	-18.59±1.15***
Diet consumption (g/day/animal)	5.16±0.20	4.98±0.14
Protein consumption (g/day/animal)	0.62±0.02	0.09±0.01***
Plasma protein (g/dL)	6.53±0.30	5.24±0.23**
Albumin (g/dL)	2.82±0.14	1.88±0.16***
Erythrocytes (x10 ⁶ /mm ³)	9.30±0.31	7.46±0.31**
Hematocrit (%)	41.6±0.95	35.8±1.12**
Hemoglobin (g/dL)	13.2±0.52	11.0±0.42**
Peripheral leukocytes (/mm ³)	2833±273.1	1283 ± 107.9***
Peripheral neutrophils (/mm ³)	563.8±67.2	160.6± 16.7***
Peripheral eosinophils (/mm ³)	13.42±7.26	2.33±1.64
Peripheral lymphocytes (/mm ³)	2153±216.8	1089±99.5***
Peripheral monocytes (/mm ³)	106.2±17.78	31.9±5.09**

Results are expressed as the mean with standard deviation of body weight variation; diet and protein consumption; concentrations of plasma protein and albumin; hemoglobin levels; total number of erythrocytes and peripheral leukocytes; and the different leukocyte subsets in the blood of control (C) and protein-malnourished (PM) animals. Numbers in parentheses denote the number of animals used in the experiments. ** $p \leq 0.01$ and *** $p \leq 0.0001$ where there was a significant difference between the PM and C groups.

of polymorphonuclear granulocytes, lymphocytes and monocytes (Table II).

Cellularity of the peritoneal exudate

PM animals displayed a significant reduction in the number of peritoneal cavity cells and mononuclear cells in comparison with the control group (Table II).

M-CSF (CSF1) and GM-CSF (CSF2) qPCR

qPCR was performed to determine expression of

M-CSF (CSF1) and GM-CSF (CSF2) in the peritoneal cells *ex vivo*. There was no difference between M-CSF mRNA expression in the control and PM groups (Figure 1A). However, the expression of GM-CSF mRNA was reduced in the PM group when compared with the control group (Figure 1B).

Determination of M-CSF and GM-CSF production *in vitro* by peritoneal cells

ELISA was used to determine cytokine levels in the supernatants of peritoneal cells cultured with or without

TABLE II - Total cell numbers and different types of cells in peritoneal exudates from control (C) and protein malnourished (PM) animals

Variables (x10 ⁶ /mL)	C (n=12)	PM (n=12)
Peritoneal total cells	2.42±0.14	1.86±0.16*
Peritoneal mononuclear cells	2.31±0.13	1.77±0.15*
Peritoneal granulocytes	0.08±0.02	0.06±0.01
Peritoneal mastocytes	0.026±0.006	0.026±0.01

Results are expressed as the mean with standard deviation of the total number of peritoneal exudate cells from control and malnourished animals. Numbers in parentheses denote the number of animals used in the experiments. * $p \leq 0.05$ where there was a significant difference between the malnourished and control groups.

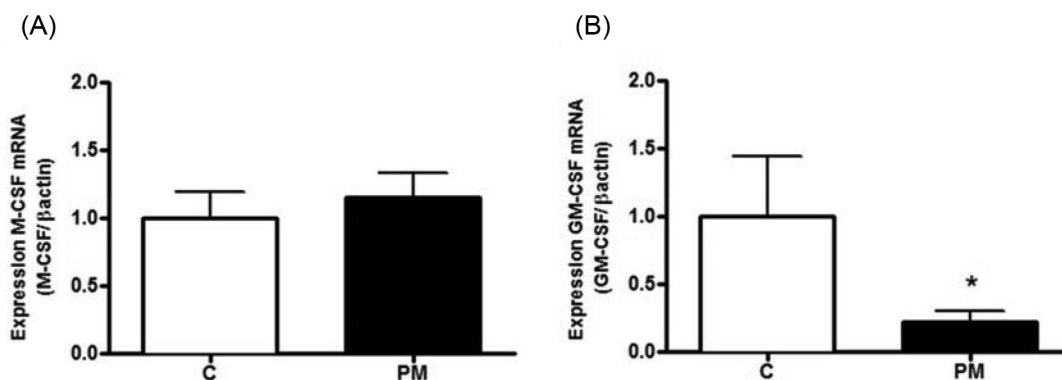


FIGURE 1 - Expression of M-CSF (A) and GM-CSF (B) mRNA in peritoneal cells. Results are expressed as the mean and standard deviation for the control (C) (n = 6) and protein-malnourished (PM) (n = 6) groups. * Significant difference between the PM and control groups (p ≤ 0.05).

10 ng/mL TNF-α for 2 h. There was no difference in the levels of M-CSF produced by peritoneal cells with or without stimulation by TNF-α (Figure 2A).

The results showed that the level of GM-CSF was diminished in the PM group without TNF-α stimulation when compared with the control group level (Figure 2B). TNF-α stimulation for 2 h increased GM-CSF production in the control group but not the PM group (Figure 2B).

DISCUSSION

In this study, we evaluated the capacity of macrophages to produce GM-CSF and M-CSF in a PM model. Additionally, we evaluated GM-CSF and M-CSF production by macrophages after TNF-α stimulation. Our primary finding from this study is that PM results in reduced capability of macrophages to produce GM-CSF, as we observed reduced CSF2 (GM-CSF) mRNA as well as reduced GM-CSF protein in the supernatant of

peritoneal macrophages.

The control group received a diet containing adequate protein, while the PM group received a protein-deficient diet. The PM animals consumed the same amount of food as control animals; however, a markedly lower protein intake and weight loss were observed, with mice losing approximately 20% of their initial weight in a period of 28 days after the introduction of the hypoprotein diet. The levels of micronutrient consumption were in accordance with the minimum daily recommendations (Reeves *et al.*, 1993). Considering the fact that both groups consumed the same amount of food, we can conclude that the changes observed in our PM experimental model were mainly due to the decrease in protein intake.

In our study, the PM animals exhibited reduced protein and albumin concentrations as well as leukopenia associated with anemia and reduction of peritoneal cell number. These results are in accordance with the literature and the evidence also indicates that, even in situations in

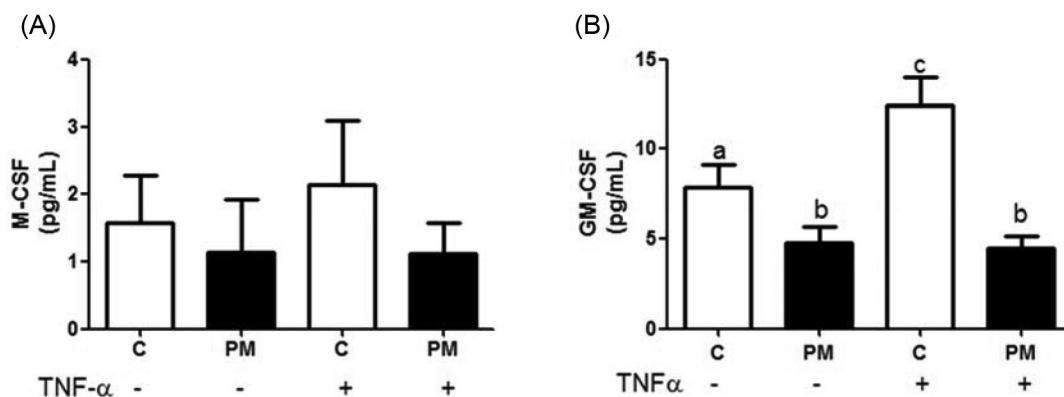


FIGURE 2 - Results are expressed as the mean and standard deviation of the levels of M-CSF (A) and GM-CSF (B) in the supernatant of a 2 h peritoneal cell culture stimulated or not stimulated with 10 ng/mL TNF-α and from the control (C) (n = 6) or protein-malnourished (PM) (n = 6) groups. Different superscript letters indicate significant differences among the groups (p ≤ 0.05).

which malnutrition is not associated with other diseases, leukopenia always occurs (Catchatourian *et al.*, 1980; Grimble, 1996; Borelli *et al.*, 2009; Cunha *et al.*, 2013). However, the exact mechanism underlying protein-malnutrition leukopenia remains unknown.

Innate immunity is the first barrier against infections and plays a pivotal role in the induction of adaptive immunity (Chandra, Kumari, 1994; Grimble, 1996; Fock *et al.*, 2007). Macrophages are involved in innate immunity, and are capable of phagocytosing and killing fungi and other microorganisms. Furthermore, macrophages are also responsible for the secretion of soluble stimulatory factors, such as cytokines and growth factors. The up-regulation of these soluble factors is an important mechanism of host defense against infection and has an important function in the production and maturation of hematopoietic cells (Ulevitch, Tobias, 1995; Katholnig *et al.*, 2013).

Among these growth factors, the evaluation of M-CSF and GM-CSF production by macrophages is not just important to understand the immune response, but also to understand the production and maturation of hematopoietic cells, especially monocytes and macrophage lineage. M-CSF is the primary regulator of mononuclear phagocyte production *in vivo* and plays an essential role in the survival, proliferation, differentiation and maturation of the macrophage myeloid lineage. GM-CSF has been shown to be involved in maturation and mobilization of macrophages and antigen presentation to these cells, and acts as a pro-inflammatory cytokine (Burgess, Metcalf 1980; Gonzalez-Juarrero *et al.*, 2005).

As described above, M-CSF levels can be detectable in steady states; however, GM-CSF requires an additional stimulus to be detected (Hamilton, 2008; Sierra-Filardi *et al.*, 2014) and, because of that, in the current study we decided to stimulate the peritoneal macrophages with TNF- α . Our results revealed no difference in M-CSF production; however, macrophages from PM animals showed reduced GM-CSF mRNA expression, which led to a reduced capability to produce GM-CSF. In addition, macrophages from PM animals stimulated with TNF- α did not show an increase in GM-CSF production as observed in the control group in which, after TNF- α stimulation, the macrophage production of GM-CSF increased two-fold in comparison to unstimulated cells. These results can be explained, in part, by reduced expression of the TNF receptor as previously reported by our group (Oliveira *et al.*, 2014).

GM-CSF supports the differentiation and maturation of hematopoietic cells, activates mature blood cells, stimulates phagocytosis and antigen-induced immune responses and alters the Th1/Th2 cytokine balance

(Burgess, Metcalf, 1980; Gonzalez-Juarrero *et al.*, 2005). Studies blocking GM-CSF with a neutralizing monoclonal antibody (mAb) in inflammation and autoimmune models demonstrate that endogenous GM-CSF can contribute to mobilization of monocytes and neutrophils, presumably from the bone marrow. Thus, its pro-inflammatory effects may lead to a systemic and local increase of myeloid cell numbers (Hamilton, Tak, 2009; Hamilton, Achuthan, 2013).

CONCLUSIONS

Based on previous literature results (Vinolo *et al.*, 2008; Cunha *et al.*, 2013; Oliveira *et al.*, 2014) and with the current results, we can conclude that PM impacts GM-CSF production, leading to alterations in hematopoietic cell production and immune response. This pattern of cytokine production could partially explain immunodeficiency and the hematopoietic changes presented in protein-malnourished organisms.

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