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Short Communication

Chlorella vulgaris treatment ameliorates the suppressive effects of single and repeated stressors on hematopoiesis

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ABSTRACT

The reports regarding the mutual influence between the central nervous system and the immune system constitute a vast and somewhat controversial body of literature. Stress is known to disturb homeostasis, impairing immunological functions. In this study, we investigated the hematopoietic response of *Chlorella vulgaris* (CV)-treated mice exposed to single (SST) and repeated stress (RST). We observed a reduction in the numbers of hematopoietic progenitors (HP) in the bone marrow and long-term bone marrow cultures (LTBMC) using flow cytometry and a coinciding decrease in the number of granulocyte–macrophage colonies (CFU-GM) after treatment with both stressors, but SST caused a more profound suppression. We observed a proportional increase in the colony-stimulating activity (CSA) of the serum of animals subjected to SST or RST. In the bone marrow, SST and RST induced a decrease in both mature myeloid and lymphoid populations but did not affect pluripotent hematopoietic progenitors (**L**in$^-$**S**ca-1$^+$c-**k**it$^+$, LSK), and again, a more profound suppression was observed after SST. We further quantified the levels of interleukin-1α (IL-1α) and interleukin-6 (IL-6) and the number of myeloid cells in LTBMC. Both SST and RST reduced the levels of these cytokines to similar degrees. The myeloid population was also reduced in LTBMC, and SST induced a more intense suppression. Importantly, CV treatment prevented the changes produced by SST and RST in all of the parameters evaluated. Together, our results suggest that CV treatment is an effective tool for the prophylaxis of myelosuppression caused by single or repeated stressors.

1. Introduction

Interdisciplinary collaboration has established psychoneuroimmunology, also known as neuroimmunomodulation, as a field of investigation with the goal of rigorous scientific research into the elusive mind–body connection. The neuroendocrine system is capable of modulating the immune system via a wide breadth of control mechanisms that link these two systems (Blalock, 1994). Evidence for this interaction is derived from the observation that certain neurotransmitters, neuropeptides, and neurohormones affect the immune function both *in vivo* and *in vitro*, and receptors for these molecules are present on lymphocytes and macrophages (Alves et al., 2007; Blalock, 1989; Carvalho-Freitas et al., 2008; Costa-Pinto and Palermo-Neto, 2010; Downing and Miyan, 2000; Nance and Sanders, 2007; Quinteiro-Filho et al., 2012). Since the 1936 studies by Selye (1936), stress induction has been considered a promising method to study the interactions between the nervous and immune systems. Psychological stressors, such as confinement or predator odors, as well as physical stressors, such as low temperature or food shortage, evoke physiological changes that disturb homeostasis by altering the equilibrium of various humoral factors. These factors in turn have a significant impact on the immune response in general (Alves et al., 2007; Besedovsky and Del Rey, 1996; Carvalho-Freitas et al., 2008; Chrousos, 2000; Quinteiro-Filho et al., 2012). Exposing animals to stressful situations activates the hypothalamic–pituitary–adrenal (HPA) axis and the release of glucocorticoids and catecholamines into the blood (Armario et al., 2012; Black, 1994; Blalock, 1994; Dunn, 1995; Glaser and Kiecolt-Glaser, 2005; Stratakis and Chrousos, 1995).

A wide array of physical and psychological stressors alters immunity, and both the qualitative and quantitative features of these stressors markedly influence the immune response. Many differences exist in the ways that short-term and long-term stressors affect physiology and behavior (Dhabhar and McEwen, 1997).

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Several facets of the immune system are differentially influenced by stressors, particularly macrophage activity (Silberman et al., 2003; Palermo-Neto et al., 2003), antibody production (Karp et al., 2000), and sensitivity to the antigen 2,4-dinitro-1-fluorobenzene (DNFB) (Blecha et al., 1982).

Evidence has demonstrated that the nervous system has an important role in the regulation of blood cell production and the selective release of these cells from the bone marrow into the circulation (Afan et al., 1997; Broome et al., 2000; Dhabhar et al., 1995; Maestroni, 2000). Many humoral factors are able to influence the survival, proliferation, and differentiation of the multipotent stem cell and its progeny under stress conditions. In this regard, studies from our laboratory (Malacrida et al., 1997a,b; Souza-Queiroz et al., 2004, 2008) and others (Broome et al., 2000; Dugan et al., 2007; Dygai et al., 1991; Goldberg et al., 1988; Mizobe et al., 1997) have demonstrated hematopoietic alterations after exposure to different experimental models of stressors.

Hematopoiesis is initiated by a rare population of bone marrow (BM)–resident multipotent hematopoietic stem cells (HSC) that are faced at each cell division with the decision to self-renew, differentiate, migrate, or die (Domen and Weissman, 1999). During steady-state hematopoiesis, the HSC population is relatively quiescent, but they give rise, upon cell cycle entry, to a hierarchy of differentiating progenitor populations that undergo the massive proliferative expansion required to replenish the blood system. HSC are recognized to be positive for c-kit, Sca-1 and Thy1.1 and negative for the mature lineage markers (Lin) and FLK2 (Passagué et al., 2005). The HSC-containing **L**in$^-$**S**ca-1$^+$c-**K**it$^+$ (LSK) cell population is able to self-renew and differentiate into a hematopoietic progenitor population (Lin$^-$Sca-1$^-$c-Kit$^+$, HP) that lacks the ability to reconstitute lethally irradiated mice (Peng et al., 2012). Lineage-specific surface antigens such as Gr-1$^+$ and Mac-1$^+$ (Lin$^+$) are known to characterize dedicated myeloid lineage cells (Larsson and Karlsson, 2005), and B220$^+$ and CD3$^+$ have been reported to mark B and T lymphocytes, respectively (Salva et al., 2012).

Long-term bone marrow cultures (LTBMC) appear to embody many of the features of hematopoietic cell regulation *in vivo*, and they closely resemble the environment of hematopoietic tissues (Dexter, 1979; Daniel et al., 1989). *Ex vivo* studies have shown that cells of the adherent layer, either spontaneously or after activation, produce a number of positive soluble factors capable of promoting the maintenance, survival, proliferation, differentiation and extensive cell renewal of hematopoietic cells (Eaves et al., 1991; Fibbe et al., 1988; Herman et al., 1998). Some endogenous positive regulators, such as stem cell factor, IL-6, IL-11, IL-12, and colony-stimulating factors (CSF), among others, are involved in regulating the proliferative activity of primitive hematopoietic cells in LTBMC (Eaves et al., 1991). The fact that hematopoiesis can be maintained for several weeks (Gartner and Kaplan, 1980) makes LTBMC an ideal model for investigating the modulating effects of new compounds on disorders of the hematopoietic tissues.

Chlorella vulgaris (CV) is a microscopic single-celled freshwater green algae that is considered to be a biological response modifier, as demonstrated by its protective activities against viral and bacterial infections in normal and immunosuppressed mice (Dantas and Queiroz, 1999; Hasegawa et al., 1994, 1995; Queiroz et al., 2003; Tanaka et al., 1986) and against tumors (Justo et al., 2001; Konishi et al., 1985; Tanaka et al., 1984, 1998). It is reported to be a rich source of antioxidants, such as lutein, α- and β- carotene, ascorbic acid and tocopherol, and it supplies large quantities of vitamins, minerals and dietary fiber (Gurer and Ercal, 2000; Rodriguez-Garcia and Guil-Guerrero, 2008; Vijayavel et al., 2007). Notably, CV stimulates the pool of hematopoietic stem cells and activates leukocytes, important aspects of CV-mediated modulation of the immune system of immunosuppressed hosts (Hasegawa et al., 1990; Konishi et al., 1990, 1996). Studies from our laboratory have demonstrated that CV significantly prevents the reduced capacity of HP to form granulocyte–macrophage colonies (CFU-GM) observed in tumor-bearing, stressed and infected mice (Dantas and Queiroz, 1999; Justo et al., 2001; Queiroz et al., 2003; Souza-Queiroz et al., 2004, 2008).

To further understand the influence of CV on hematopoiesis, we quantified hematopoietic populations in the bone marrow of mice subjected to a single or repeated stressor using flow cytometry and assessed the clonogenic capacity of myeloid cells to form CFU-GM *in vivo* (bone marrow) and *ex vivo* (LTBMC). LTBMC provided information about the impact of both stressors on functional activity from the medullar stroma and its ability to interact with hematopoietic cells. IL-6 and IL-1α, important hematopoietic regulators, were measured in the cultures. The colony-stimulating activity of the serum (CSA) from these mice provided information about the amount of CSF present in the blood after single and repeated stressors.

2. Materials and methods

2.1. Mice

Male BALB/c mice, 6–8 weeks old, were bred at the Campinas University Central Animal Facilities (Centro de Bioterismo, Universidade Estadual de Campinas, Campinas, SP), raised under specific pathogen-free conditions, and matched for body weight before use. Standard chow and water were freely available. Animal experiments were performed in accordance with institutional protocols and the guidelines of the Institutional Animal Care and Use Committee (Protocol Number 1997-1), which follow the recommendations of the Canadian Council on Animal Care (Olfert et al., 1993). The animals were divided into 6 groups of 6 animals each: Controls (C – gavage with vehicle (warm water) for 5 days before bone marrow removal); *C. vulgaris* (CV – received CV for 5 days before bone marrow removal); single stress/CV + single stress (SST/CV + SST – received vehicle or CV for 5 days before stress protocol); repeated stress/CV + repeated stress (RST/CV + RST – received vehicle or CV for 21 days, i.e., throughout the stress protocol). All experiments were replicated twice.

2.2. Stress model

Single stress consisted of a single 3-h session of restraint stress. Repeated stress consisted of 21 daily sessions that were 2 h each. Restraint stress was performed in plastic 50 mL conical falcon tubes. A hole was made at one extremity of the tubes for the tail of the mouse, and another hole was made in the other extremity to enable the mice to breathe. The animals received no food or water during the stress protocol. After being placed into the tubes, the animals were returned to their home cages inside their room. In all groups, femoral marrow was collected 2 h after either the single or the final repeated stress applications.

2.3. Treatment regimens

Dried CV algae, a unicellular green algae strain, were kindly provided by Dr. Hasegawa (Research Laboratories, Chlorella Industry Co. Ltd., Fukuoka, Japan). Chemical analysis performed by Hasegawa et al. (1990) revealed that CV contains 44.4 g of protein, 39.5 g of carbohydrates and 15.4 g of nucleic acid in 100 g (dry weight) of whole material. No lipids were detected. CV was prepared in distilled water, and a dosage of 50 mg/kg was given orally by gavage in a 0.2 mL volume/mouse for 5 consecutive days before single stress or for the entire period of repeated stress. The selection of doses for CV was based on previous studies performed in

our laboratory (Bincoletto and Queiroz, 1996; Dantas and Queiroz, 1999; Queiroz et al., 2008). In all groups, femoral marrow was collected 24 h after the final administration of CV.

2.4. Progenitor cell assays

Assays for CFU-GM were performed using bone marrow cells and non-adherent cells collected from LTBMC. The plug of marrow cells was gently extruded into a sterile plastic tube using 1 mL of RPMI medium injected through the femur and then converted to a dispersed cell suspension in 5 mL of RPMI by gently aspirating the suspension up and down 20 times using a sterile 5 mL pipette. The bone marrow cells were placed in duplicate 1 mL semisolid agar cultures in 35 mm Petri dishes using 1×10^5 bone marrow cells per culture for the growth of CFU-GM. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) containing 20% FCS (fetal calf serum) and 0.3% agar. Colony formation was stimulated by the addition of recombinant murine macrophage–granulocyte colony-stimulating factor (rmGM-CSF-Sigma) at a final concentration of 0.5 ng/mL. The cultures were incubated for 7 days in a fully humidified atmosphere of 5% CO_2 in air, and colony formation (clones >50 cells) was scored at 35× magnification using a dissection microscope (Metcalf, 1984).

2.5. Flow cytometric analysis

To evaluate the hematopoietic cell populations, whole BM and LTBMC cells were collected by flushing (1×10^6 cells), fixed and labeled. To the verification of mature cells we used 4 antibodies conjugated with four different fluorocromes: FL1: anti-Gr1-FITC; FL2: anti B220-PE, FL-3:anti-Mac-1-Cy7/PE and FL4: anti-CD3-APC. To analyze the primitive population we used 2 antibodies that recognize the fraction LSK together with a cocktail of mature lineage: FL2: anti-B220, anti-CD3, anti-Ter-119, anti-CD11b and anti-Gr-1, which were all conjugated with PE; FL3: anti Sca-1-Cy7/PE and FL4: anti-c-kit-APC. The data were collected using a FACSCalibur flow cytometer and analyzed using CellQuest software (BD Biosciences). The antibodies were purchased from BD Biosciences.

2.6. Assay for serum colony-stimulating activity

The mice were bled from the heart under deep halothane anesthesia. Within each experimental group, the blood was pooled, left at 37 °C for 30 min, and the clots were allowed to retract overnight at 4 °C. Following centrifugation, the serum was removed and stored at −20 °C. CSA was determined by measuring the ability of serum obtained from control and experimental groups to stimulate HP to form CFU-GM (1×10^5 cells) from normal mice. The results were expressed as units of CSA/mL, where 1 unit/mL was defined as the lowest amount of CSA able to induce the formation of colonies (Van Den Engh and Bol, 1975).

2.7. Long-term bone marrow cultures (LTBMC)

Marrow cells were aseptically collected from two complete femur shafts after killing the animal by cervical dislocation. The plug of marrow cells was gently extruded into a sterile plastic tube using 1 mL of RPMI 1640 medium (Sigma) injected through the femur and then converted to a dispersed cell suspension in 5 mL of RPMI by gently aspirating the suspension up and down 20 times using a sterile 5 mL pipette. To establish the culture, 1×10^7 pooled femoral bone marrow cells were dispensed into T25 tissue culture flasks containing 10 mL of RPMI 1640 supplemented with 25 mM L-glutamine, 25 mM HEPES, 200 UI/mL penicillin, 100 μg/mL streptomycin, 20% horse serum (Sigma), and 0.1 μM hydrocortisone and incubated at 37 °C in 5% CO_2. At 7-day intervals, the cultures were fed by removing half the growth medium (5 mL) and adding an equal volume of fresh growth medium. On the fourth week, the bone marrow cultures were recharged (fed as before, with 5 mL of growth medium containing a further 1×10^7 freshly isolated syngeneic femoral bone marrow cells from comparably aged mice as described by Gartner and Kaplan, 1980). Supernatants from LTBMC were harvested weekly from the 5th to 9th week of culture and frozen at −20 °C until required. The pooled cell suspensions were counted in a hemocytometer and centrifuged at 800g for 10 min, and the clonal growth of non-adherent progenitor cell populations was assayed weekly, as described in Section 2.4.

2.8. Quantification of cytokine levels

The concentrations of IL-1α and IL-6 were evaluated in the supernatant of LTBMC. Cytokines were quantified using a sandwich ELISA (Enzyme-Linked Immunosorbent Assay) in microtiter plates (96-well flat-bottom maxisorp microplate-NUNC, Roskilde, DM) using the following monoclonal antibodies purchased from R&D Systems: DuoSet® ELISA Development System Kit with purified anti-mouse IL-6 (Cat. DY406) and anti-mouse IL-1α/IL-1F1 (Cat. DY40). The cytokine levels were determined according to the R&D Systems cytokine ELISA protocol. Cytokine titers were expressed in pg per mL and were calculated by reference to standard curves constructed with known amounts of recombinant cytokines.

2.9. Statistical analysis

For statistical analysis of changes in the progenitor cell assays, immunophenotyping, cytokine levels and colony-stimulating activity, analysis of variance (ANOVA – two way) followed by the Bonferroni test was used to compare data among all groups. Statistical significance was reached when $P < 0.05$.

3. Results

3.1. CV modulates the clonogenic capacity of primitive cells to form CFU-GM in mice subjected to single or repeated stressors

The effects of CV treatment on the number of bone marrow CFU-GM in animals subjected to SST or RST is demonstrated in Fig. 1A. The application of either SST or RST caused a significant reduction in CFU-GM (CTR: $18 \pm 2 \times 10^3$, SST: $5 \pm 1.5 \times 10^3$ and RST: $10 \pm 1.5 \times 10^3$, $P < 0.05$). This reduction was higher in animals subjected to SST (SST: $5 \pm 1.5 \times 10^3$ and RST: $10 \pm 1.5 \times 10^3$, $P < 0.05$). The oral administration of 50 mg/kg of CV prevented the CFU-GM decrease in mice subjected to stressors, keeping CFU-GM numbers similar to control levels. CV treatment alone produced no changes in the number of CFU-GM in the bone marrow of normal mice.

3.2. CV promotes expansion of the primitive and mature hematopoietic populations in bone marrow

The effects of oral CV treatment were also evaluated on mature myeloid populations in animals subjected to both conditions (Fig. 1B). The percentage of Gr-1$^+$Mac-1$^+$ cells was reduced after SST and RST (CTR: 37 ± 3%, SST: 23 ± 1% and RST: 29 ± 2%, $P < 0.05$) with higher suppression after SST (23 ± 1%, $P < 0.05$). CV treatment prevented the changes induced by SST and RST on the Gr-1$^+$Mac-1$^+$ population, maintaining levels similar to those of the control group (CV + SST: 36 ± 2%, CV + RST: 41 ± 2% and CTR: 37 ± 3%). Representative histogram is demonstrated in Fig. 1C.

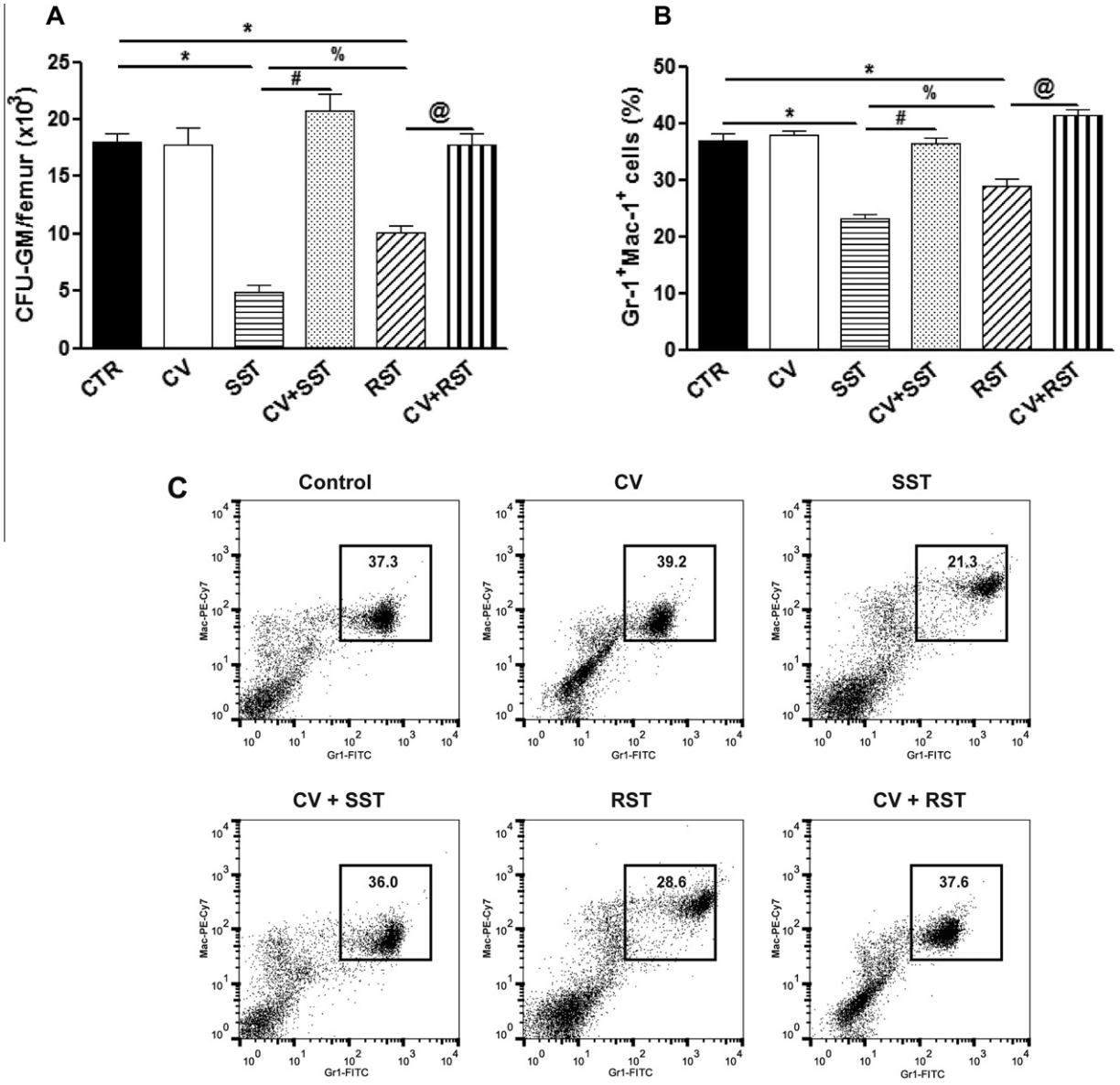

Fig. 1. (A) Number of granulocyte–macrophage progenitor colonies (CFU-GM), (B) quantification of mature myeloid cells ($Gr1^+Mac1^+$) and (C) representative original images from flow cytometric analysis of myeloid cells in mice treated orally with CV at 50 mg/kg/day for 5 days prior to SST or, concomitantly, to RST (21 days). The mice were sacrificed 24 h after the last treatment. Control mice received diluent only. The results represent the means ± SD of six mice per group. $^{*}P < 0.05$ vs. control; $^{\%}P < 0.05$ vs. RST; $^{\#}P < 0.05$ vs. SST; $^{@}P < 0.05$ vs. RST.

The protective effects of CV oral treatment were also observed in $B220^+$ (B lymphocyte) and $CD3^+$ (T lymphocyte) lymphoid populations. Both stressors decreased the percentage of B cells (CTR: 41 ± 1%, SST: 15 ± 1% and RST: 22%, $P < 0.05$), but the single stress event caused a more intense suppression (15 ± 1%, $P < 0.05$) (Fig. 2A). The number of T cells was also altered during stress (CTR: 1,1 ± 0.1%, SST: 0,4 ± 0.1% and RST: 0.7 ± 0.1%, $P < 0.05$). Similar results were observed in the lymphoid population following CV pretreatment as in myeloid populations, with the pool of cells retaining numbers similar to those seen in controls (CV + SST: 1.1,3 ± 0.1%, CV + RST: 1,2.1 ± 0.1% and C: 1 ± 0.1%) (Fig. 2B). Representative histogram is demonstrated in Fig. 2C.

We also investigated the potential for CV modulation of primitive hematopoietic cells. The LSK cells ($Lin^-Sca1^+c\text{-}Kit^+$) were not altered in these animals (Fig. 3A), but the total number of hematopoietic progenitor cells (HP: $Lin^-Sca1^-c\text{-}kit^+$) was reduced by both stressors (CTR: 0.5% ± 0.007, SST: 0.2% ± 0.001 and RST: 0.3% ± 0.003, $P < 0.05$). Again, the single stress event induced a more robust suppression (0.2% ± 0.001, $P < 0.05$). CV treatment prevented the changes induced by SST and RST in the number of HP, maintaining levels similar to those observed in control animals (CV + SST: 0.5% ± 0.005, CV + RST: 0.5% ± 0.004 and CTR: 0.5% ± 0.007) (Fig. 3B). Representative histogram is demonstrated in Fig. 3C.

3.3. Serum colony-stimulating activity

The effect of oral CV treatment on serum CSA in stressed animals is shown in Fig. 4. The application of both types of stressors led to a significant increase in CSA ($P < 0.05$), with levels reaching amounts 3.5-fold higher in RST animals and 7-fold higher in SST animals compared with control mice. The treatment of these animals with CV further increased CSA by 26% (CV + SST) and 57% (CV + RST) ($P < 0.05$ vs. stressed controls). The treatment of

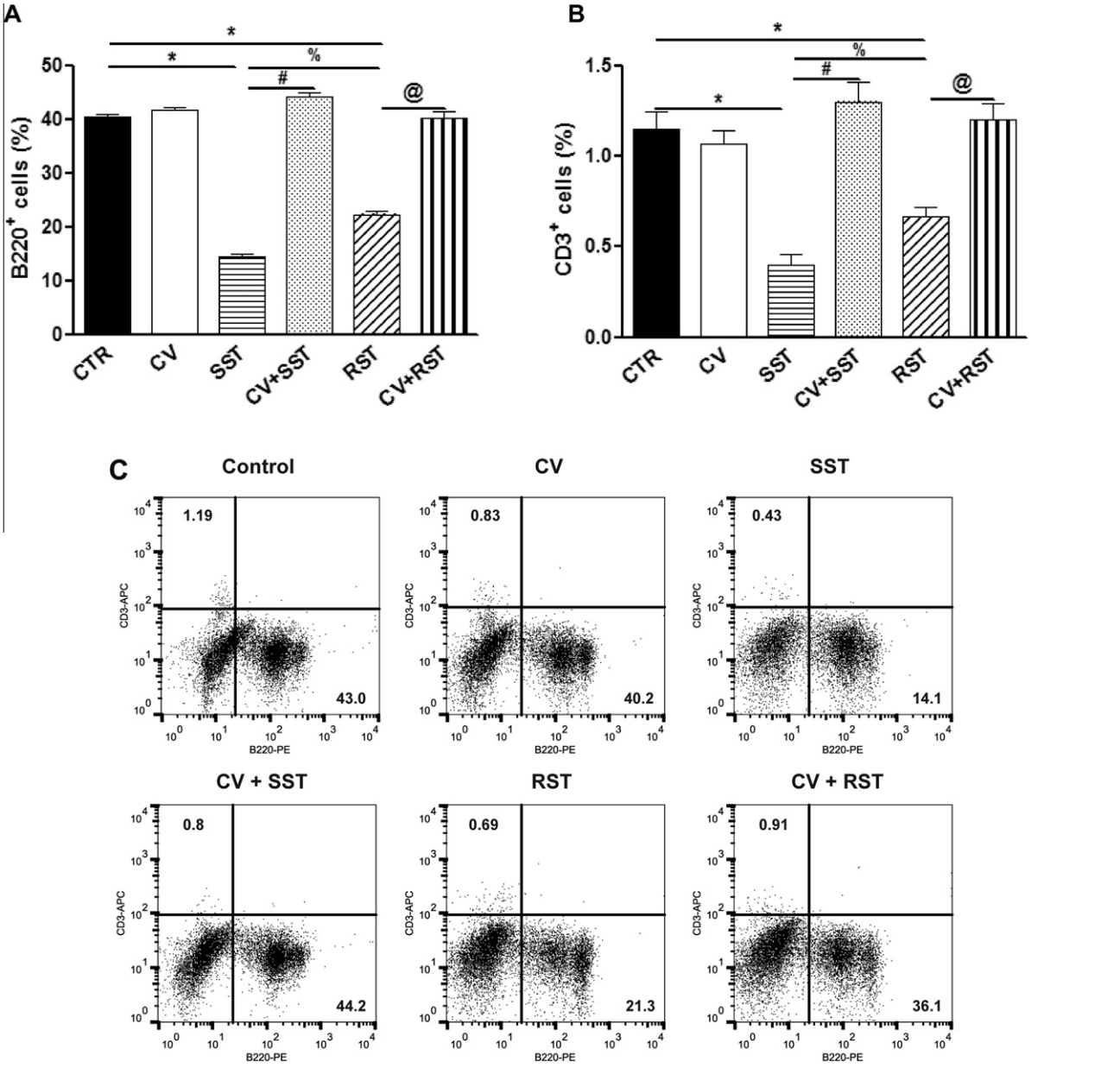

Fig. 2. Quantification of (A) B lymphocytes ($B220^+$) and (B) T lymphocytes ($CD3^+$) in the bone marrow of mice treated orally with CV at 50 mg/kg/day for 5 days prior to SST or, concomitantly, to RST (21 days). The mice were sacrificed 24 h after the final treatment. Control mice received diluent only. (C) Representative original images from flow cytometric analysis of B and T lymphocytes. The results represent the means ± SD of six mice per group. $^*P < 0.05$ in relation to control; $^{\%}P < 0.05$ vs. RST; $^{\#}P < 0.05$ in relation to SST; $^{@}P < 0.05$ in relation to RST.

non-stressed control mice with CV also produced significant increases (2-fold) in CSA levels ($P < 0.05$).

3.4. Long-term bone marrow culture

3.4.1. Pretreatment with CV prevented the suppressive effect promoted by both types of stressors on CFU-GM

The number of bone marrow CFU-GM in the supernatant of LTBMC is presented in Fig. 5. In the fifth week of culture, peak numbers of CFU-GM were produced in all groups as a consequence of repopulation. In SST and RST groups, the crucial feature observed in the cultures was the reduced capacity of cultured cells to support the growth and differentiation of CFU-GM at all time-points evaluated. SST produced a more severe reduction in CFU-GM than RST ($P < 0.05$), with SST reaching levels as low as a 3-fold decrease while RST reached levels as low as a 1.6-fold decrease in the 7th week of culture. However, when these animals were treated with CV, the CFU-GM numbers were maintained at control levels in all time-points studied. No significant changes were observed in CV-treated non-stressed mice. (Fig. 5A). Fig. 5B shows representative original pictures from the cultures.

3.4.2. CV promotes expansion of the primitive and mature hematopoietic populations in LTBMC

The effects of oral CV treatment on mature myeloid cell populations ($Gr1^+Mac1^+$) and the number of HP ($Lin^-c\text{-}Kit^+Sca1^-$) in the LTBMC of animals subjected to SST and RST are shown in Fig. 6. We observed that both stressors decreased the percentage of $Gr\text{-}1^+Mac\text{-}1^+$ cells (CTR: 25 ± 1%, SST: 14 ± 2% and RST: 19 ± 1.8%, $P < 0.05$) (Fig. 6A). The HP number was also altered in this system

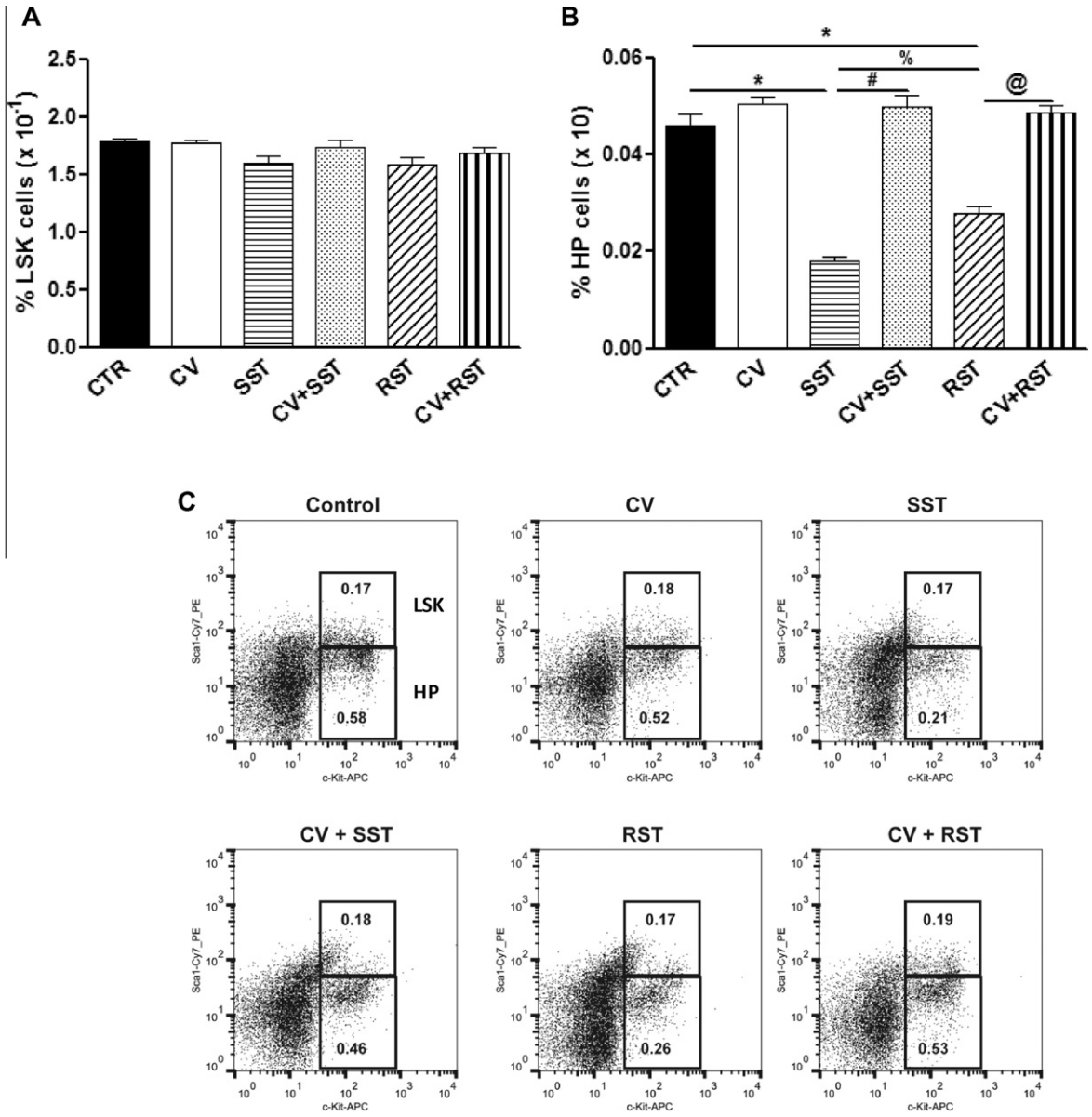

Fig. 3. (A) Gating strategy for analyzing the primitive murine hematopoietic population. (B) Quantification of LSK cells and (C) hematopoietic progenitors (HP) in the bone marrow of mice orally treated with CV at 50 mg/kg/day for 5 days prior to SST or, concomitantly, to RST (21 days). The mice were sacrificed 24 h after the last treatment. Control mice received diluent only. (C) Representative original images from flow cytometric analysis of LSK and HP cells. The results represent the means ± SD of six per group. $^{*}P < 0.05$ vs. control; $^{\%}P < 0.05$ vs. RST; $^{\#}P < 0.05$ vs. SST; $^{@}P < 0.05$ vs. RST.

(CTR: 9 ± 1%, SST: 5 ± 0.5% and RST: 7 ± 0.3%, $P < 0.05$) (Fig. 6B). CV treatment prevented the changes induced by SST and RST in the number of HP and $Gr1^{+}Mac1^{+}$, maintaining levels similar to those observed in control animals (Fig. 6A and B). Representative histogram is demonstrated in Figs. 6C and 6D.

3.4.3. CV induces increase in the levels of the myelocytokines IL-1α and IL-6 in LTBMC

The levels of IL-1α and IL-6 were measured weekly (6–9 weeks) in the supernatants of LTBMC. As shown in Figs. 7 and 8, a progressive decline was observed in the levels of both cytokines in all groups studied. However, SST and RST further reduced the production of IL-1α (Fig. 7 A and B) and IL-6 (Fig. 8 A and B) when compared with controls ($P < 0.05$). Treatment of stressed animals with CV prevented the decrease in the production of both cytokines to control levels ($P < 0.05$). These results are consistent with the increased ability of the stromal cell layer to display CFU-GM *in vitro* (item 3.4.1). Notably, treatment of non-stressed mice with CV caused a 15% increase in the levels of both cytokines.

4. Discussion

Because a variety of stressors may compromise the physiological role of the hematopoietic system in sustaining the proliferation and differentiation of progenitor cells to fulfill the continual cellular demands of the organism, we compared the impact caused by a single stressor (SST) or a repeated stressor (RST) on several parameters of the hematopoietic response in mice treated with CV using both i*n vivo* and *ex vivo* systems. To our knowledge, this is the first study to compare the effects of a single or repeated application of an emotional stressor on the bone marrow (BM) and the functional activity of marrow stroma (measured by LTBMC). The latter is of

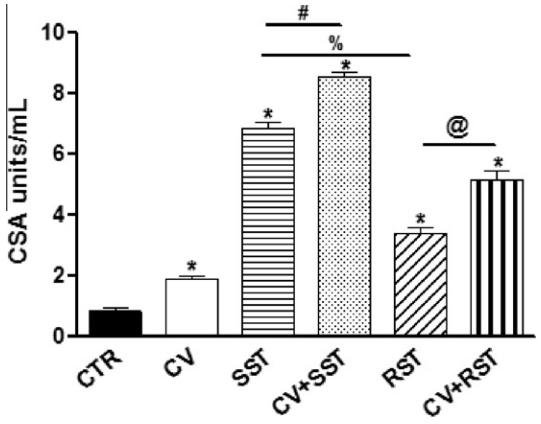

Fig. 4. Colony stimulating activity (CSA) from the serum of mice treated orally with CV at 50 mg/kg/day for 5 days prior to SST or, concomitantly, to RST (21 days). The mice were sacrificed 24 h after the last treatment. Control mice received diluent only. The results represent the means ± SD of six mice per group. $^{*}P < 0.05$ vs. control; $^{\%}P < 0.01$ vs. RST; $^{\#}P < 0.05$ vs. SST; $^{@}P < 0.05$ vs. RST.

great importance, as the hematopoietic microenvironment supports blood and immunocompetent cell generation (Dorschkind, 1990).

Our results showed a reduced number of hematopoietic progenitors (HP) from animals subjected to SST and RST, which corresponded with decreased CFU-GM numbers in both the BM and the LTBMC. In this case, SST induced a stronger suppression. We also measured the serum levels of colony-stimulating factors from plasma (CSA) and observed a significant increase after both stressors, influencing the proliferation and differentiation of BM-derived phagocytes. Persistent elevation of CSA levels during stress events serves as a continuing stimulus that supports the survival, proliferation, differentiation, and end functional activity of granulocytes and monocytes (Cheers et al., 1988; Guleria and Pollard, 2001; Kayashima et al., 1993; Wing et al., 1985; Zhan et al., 1998). Treatment with CV produced a further increase in CSA levels in the BM of stressed mice (both SST and RST) and restored the number of HPs from BM and LTBMC to control levels. Concurrently, the reduction of CFU-GM numbers from BM and LTBMC induced by the stressors was prevented by the treatment with CV.

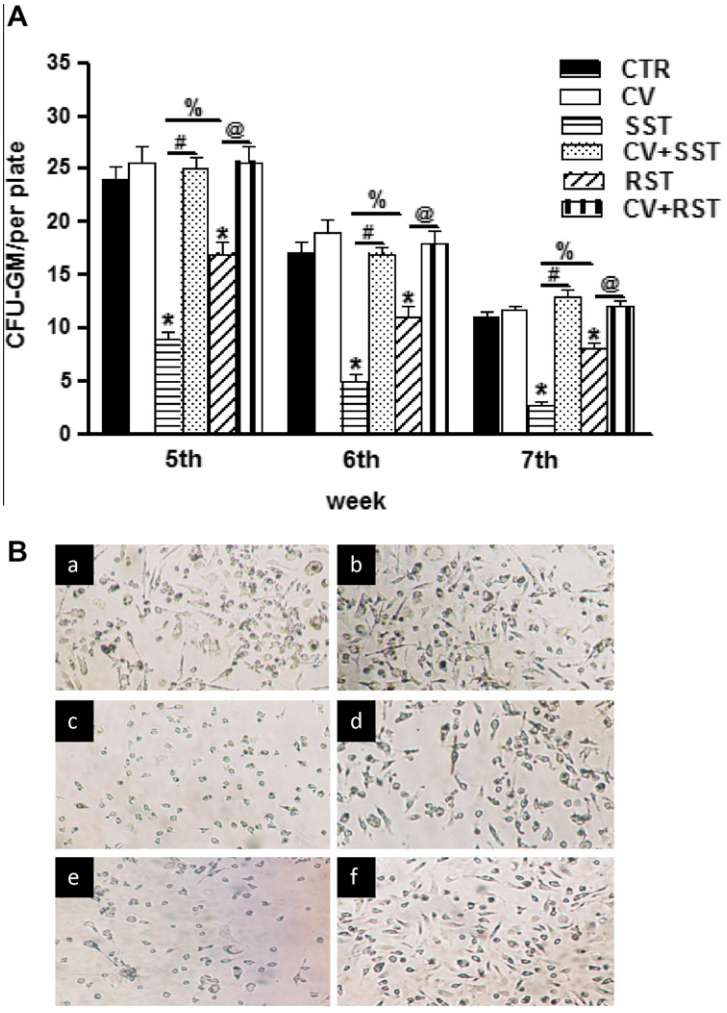

Fig. 5. (A) Number of granulocyte–macrophage progenitor colonies (CFU-GM) collected weekly from LTBMC supernatant. In all treatment schedules, the mice were treated by gavage with 50 mg/kg of CV for 5 days prior to SST or, concomitantly, to RST (21 days). The mice were sacrificed 24 h after the last treatment, and 1×10^7 pooled bone marrow cells were inoculated into flat-bottomed flasks in 10 mL of complete medium to establish LTBMC. At 7-day intervals, the cultures were fed by removing half of the growth medium and adding an equal volume of fresh growth medium. After 4 weeks, the cultures were recharged. Non-adherent cell suspensions of 1×10^5 marrow cells were stimulated by rmGM-CSF for the CFU-GM assays. The results indicate the means ± SD of 3 culture flasks/group. $^{*}P < 0.05$ vs. control; $^{\%}P < 0.05$ vs. RST; $^{\#}P < 0.05$ vs. SST; $^{@}P < 0.05$ vs. RST. (B) Microscopic aspect of LTBMC. (a) Control group, (b) stressed group, **(c)** single or repeated stress + pre-treatment with 50 mg/kg *Chlorella vulgaris*. Increase of 20×.

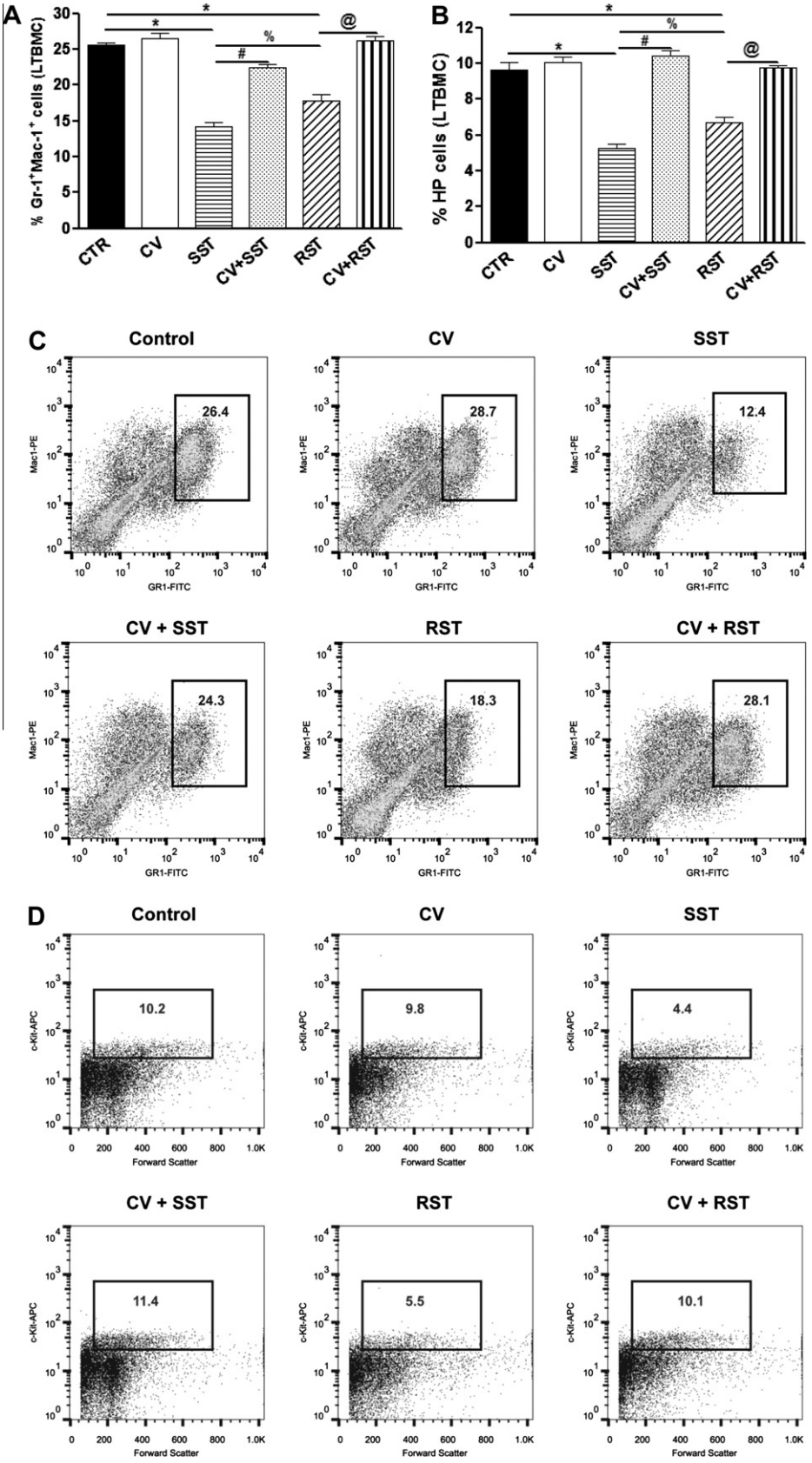

Fig. 6. Quantification of (A) myeloid cells (Gr1+Mac1+) and (B) hematopoietic progenitors (HP) from the LTBMC of mice treated orally with CV at 50 mg/kg/day for 5 days prior to SST or, concomitantly, to RST (21 days). Representative original images from flow cytometric analysis of (C) myeloid cells and (D) hematopoietic progenitors are demonstrated. The mice were sacrificed 24 h after the last treatment. Control mice received diluent only. The results represent the means ± SD of six mice per group. $^{*}P < 0.05$ vs. control; $^{\%}P < 0.05$ vs. RST; $^{\#}P < 0.05$ vs. SST; $^{@}P < 0.05$ vs. RST.

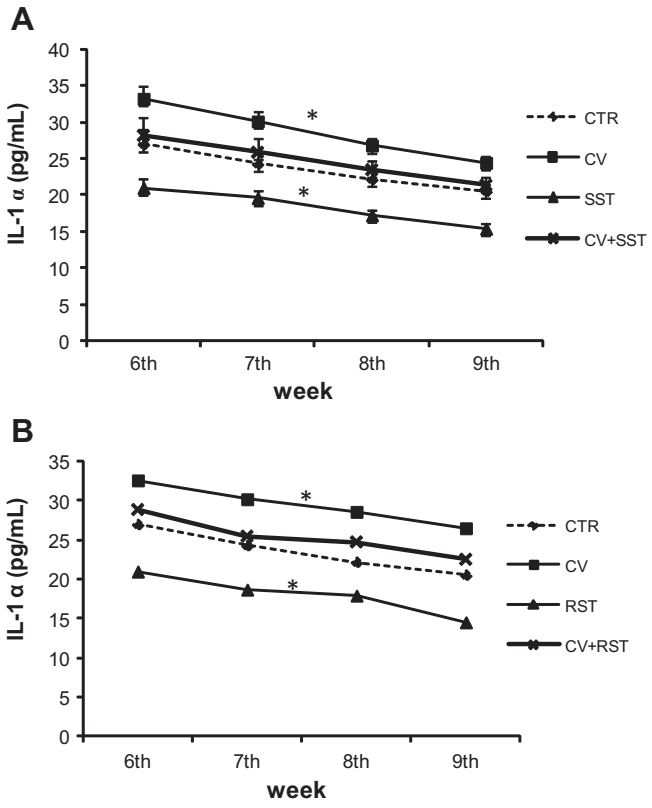

Fig. 7. Levels of IL-1α collected weekly from the supernatant of LTBMC. Mice were treated by gavage with CV at 50 mg/kg for 5 days prior to (A) SST or, concomitantly, to (B) RST (21 days). The mice were sacrificed 24 h after the last treatment. Control mice received diluent only. The results represent the means ± SD of six mice per group. $^{*}P < 0.05$ vs. control.

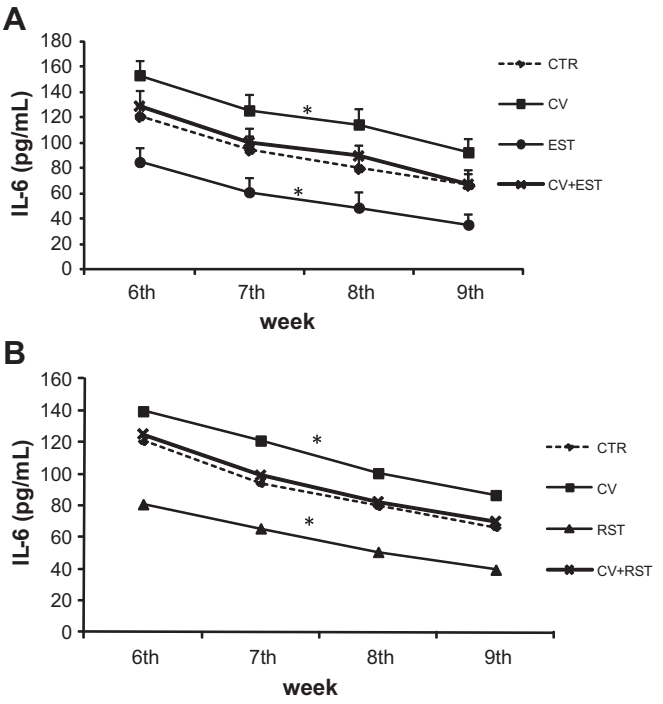

Fig. 8. Levels of IL-6 collected weekly from the supernatant of LTBMC. The mice were treated by gavage with CV at 50 mg/kg for 5 days prior to (A) SST or, concomitantly, to (B) RST (21 days). The mice were sacrificed 24 h after the last treatment. Control mice received diluent only. The results represent the means ± SD of six mice per group. $^{*}P < 0.05$ vs. control.

These findings agree with previous studies (Hasegawa et al., 1997, 2000) showing that CV treatment increased mRNA levels for granulocyte–macrophage colony-stimulating factor (GM-CSF). This stimulus can be attributed to the presence of a glycoprotein, which is purified from CV, is soluble in water and has been reported to be a hematopoietic stimulator that increases CSF levels and promotes progenitor cell migration from the bone marrow to the spleen followed by an expansion of CFU-GM in this organ after chemotherapy (Konishi et al., 1996). The presence of α-tocopherol in CV, the former of which is a member of the vitamin E family and possesses numerous biological properties including significant effects on inflammation, cell proliferation, and apoptosis (Azzi, 2007; Lemaire-Ewing et al., 2010; Singh et al., 2006), may also be important here, as it has been shown to increase the number of HP as demonstrated by CFU-GM assays in the bone marrow of irradiated mice after treatment (Bichay and Roy, 1986; Cherdyntseva et al., 2005; Roy et al., 1982). The presence of these components in CV can explain, in part, the fact that we observed a small but significant increase in CSA in the BM of non-stressed animals after CV treatment; however, this increase did not interfere with the number of HP or with the CFU-GM.

The reduced capacity of cultured cells to support the growth and differentiation of CFU-GM following the application of SST or RST was consistent throughout the duration of the cultures (7 weeks), and the suppression caused by SST was more severe until the 7th week. From the 1st to the 4th weeks of culture, the stromal layer is formed in the flasks. In the 5th week, the cultures are repopulated with cells from the respective groups of mice. These cells interact with the stroma, demonstrating their capability to maintain hematopoiesis. Therefore, we propose that SST and RST directly interfere with the physical contacts between stromal and hematopoietic cells. This hypothesis is in agreement with a significant reduction in the local production of IL-6 and IL-1α by stromal cells after stressor application, as observed in this study. IL-6 plays a critical role in the generation and maintenance of myelopoiesis in murine LTBMC (Hauser et al., 1997) and is a survival factor for hematopoietic stem cells (Bernard et al., 1994). Both IL-6 and IL-1α have synergistic activity with CSFs in stimulating hematopoiesis, thus contributing to the maintenance of neutrophil maturation and viability (Eaves et al., 1991; Dinarello, 1996; Muench et al., 1992). Studies in the literature demonstrate that IL-1α accelerates both granulopoietic and thrombopoietic recovery in 5-fluorouracil myelosuppressed mice (Kovacs et al., 1997). However, in contrast to what we observed with HP and CFU-GM numbers, the decrease caused by SST and RST on the levels of these cytokines was of equal magnitude. Thus, the modulation of other important environmental regulators by SST is responsible for causing the increased suppression of HP and CFU-GM. IL-3 is also a significant cytokine during hematopoiesis, and it participates in the host response to various types of stressors (Bessler et al., 2000). Treatment with CV increased the ability of stromal cells from stressed animals to produce IL-6 and IL-1α, which is consistent with the increased numbers of HP and the increased ability of the stromal cell layer to support CFU-GM *ex vivo*.

Almost all immune cells have receptors for one or more of the hormones associated with HPA and SNAS activation (Black, 1994; Glaser and Kiecolt-Glaser, 2005; Heyworth et al., 1992; Miyan et al., 1998; Spiegel et al., 2007). To further understand the effects of CV treatment on the hematopoiesis of animals subjected to SST or RST, we evaluated the mature cell populations from bone marrow and LTBMC samples. Both stressors had a suppressive effect on lymphoid lineage cells (B and T cells) in the BM, with a more significant suppression after SST. The reduction in the number of

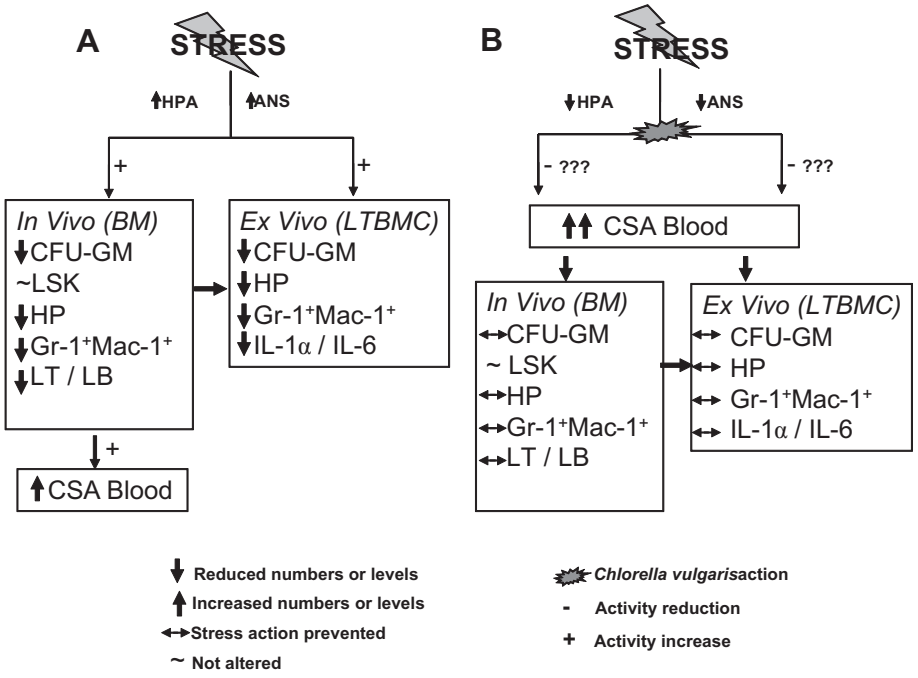

Fig. 9. (A) Effects of single and repeated stress application on myelopoiesis. (B) Mechanisms of action from CV on stress induced myelosuppression. HPA: hypothalamus-pituitary axis; ANS: Autonomic Nervous System; BM: bone marrow; LTBMC: long term bone marrow culture; CFU-GM: number of colony-forming-units of granulocytes and macrophages; CSA: colony stimulating activity from blood; HP: hematopoietic progenitors; LSK: pluripotent hematopoietic progenitors; $Gr1^+Mac1^+$: mature myeloid cells; LB: B lymphocytes; LT: T lymphocytes; IL: interleukins.

lymphocytes, together with thymic atrophy, is considered to be a hallmark of the stress response (Edgar et al., 2003; Souza-Queiroz et al., 2008). Elevated glucocorticoids lead to rapid apoptotic loss of lymphoid cells both peripherally and in the bone marrow (Black, 1994). Mature myeloid cell population ($Gr1^+Mac1^+$) was also reduced after SST and RST in both the BM and LTBMC, with further reductions in the SST group. Elevation of noradrenaline and adrenaline levels may produce changes in lymphocyte, monocyte, and leukocyte function (Dunn, 1990). The primitive hematopoietic population (LSK) was also evaluated in the BM. No alteration in the number of LSK cells was observed after stress, a fact that can be explained, at least in part, by the fact that the blood-forming system should be able to respond efficiently to hematological stressors by expanding the LSK population, mainly through increased self-renewing divisions (Morrison et al., 1997; Wright et al., 2001). Thus, LSK proliferation must be highly adaptive to ensure durable production of progenitor populations during steady-state hematopoiesis and extensive, stress-induced, self-renewal proliferation without depleting the stem cell pool (Passagué et al., 2005).

Relevant to our present findings is the fact that nerve fibers containing noradrenaline enter the hematopoietic tissue of bone marrow and terminate at synapses on hematopoietic cells. They promote negative regulation of hematopoietic activity, affecting both hematopoiesis and the release of mature cells from the marrow (Heyworth et al., 1992). These observations acquire additional significance in view of the fact that adrenoreceptors are expressed on Th1 cells, but not Th2 cells (Sarders et al., 1997; Elenkov et al., 2000), thus providing a mechanistic basis for the differential effects on Th1/Th2 function. An increasing body of evidence indicates that suppression of cellular immunity through selective inhibition of Th1, in favor of Th2 responses, is an important feature of stress (Elenkov et al., 1996, 2000; Woiciechowsky et al., 1998; Zhang et al., 2005; Souza-Queiroz et al., 2008). B_2-agonists inhibit IL-12 production (Panina-Bordignon et al., 1997), which is known to have a central role in the immune system by skewing the immune response towards Th1-type responses. In this respect, studies from our laboratory and others (Hasegawa et al., 1997; Queiroz et al., 2002, 2011; Souza-Queiroz et al., 2008; Torello et al., 2010) have proposed that CV has a direct myelostimulating outcome through inducing the Th1 response via activation of macrophages to produce IL-12 and IFN-γ. Previous findings from our laboratory demonstrated that pre-treatment with CV prevented this decrease in IFN-γ (Th1) and increase in IL-10 (Th2) after an acute foot-shock stressor (Souza-Queiroz et al., 2008). This reduction in IL-1 and TNF-α was prevented by treating mice with CV that were inoculated with tumors (Ramos et al., 2010) or exposed to lead (Queiroz et al., 2008, 2011). These cytokines are known to stimulate the production of neutrophils from the bone marrow and to mediate chemoattraction of granulocytes from the circulation to peripheral sites of injury.

In the present study, we observed that the effects produced by both single and repeated stressors were suppressive, however, SST had a stronger impact on most of the parameters evaluated. This could be explained by a decrease in hormone release due to glandular exhaustion or down-regulation of receptors, among other possibilities, or it could also be explained by a reduction in the emotional impact initially caused by the stressful situation, thus leading to a decreased endocrine response over time (Armario, 2001).

Delineating how stress influences hematopoiesis is important for developing potential pharmacological interventions to decrease the incidence of stress-induced immune dysfunction. Irrespective of the mechanisms involved, the immunomodulatory effect of CV on stressed mice may have an important role in protecting hosts from stressful situations, leading to an increase in the ability of the immune system to respond to this challenge (for an overview of the mechanisms of action from CV on stressed mice observed in this study, see Fig. 9).

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References

Afan, A.M., Broome, C.S., Nicholls, S.E., Whetton, A.D., Miyan, J.A., 1997. Bone marrow inervation regulates cellular retention in the murine haemopoietic system. Br. J. Haematol. 98, 569–577.

Alves, G.J., Vismari, L., Palermo-Neto, J., 2007. Cohabitation with a sick cage mate: effects on ascitic form of Ehrlich tumor growth and macrophage activity. Neuroimmunomodulation 4 (6), 297–303.

Armario, A., 2001. Neurobiología del estrés: una perspectiva desde el eje hipotálamo-pituitatio-adrenal. In: Sandi, C., Clés, J.M., Sans y Torres (Eds.), Estrés: consecuencias psicologicas, fisiologicas y clínicas.

Armario, A., Daviu, N., Muñoz-Abellán, C., Rabasa, C., Fuentes, S., Belda, X., Gagliano, H., Nadal, R., 2012. What can we know from pituitary-adrenal hormones about the nature and consequences of exposure to emotional stressors? Cell. Mol. Neurobiol. 32 (5), 749–758.

Azzi, A., 2007. Molecular mechanism of α-tocopherol action. Free Radic. Biol. Med. 43, 16–21.

Bernard, A., Kopf, M., Kulbacki, R., Weich, N., Koehler, G., Gutierrez-Ramos, J.C., 1994. Interleukin-6 is required in vivo for the regulation of stem cells and committed progenitors of the hematopoietic system. Immunity 1, 725–731.

Besedovsky, H.O., Del Rey, A., 1996. Immune–neuro-endocrine interactions: facts and hypotheses. Endocr. Rev. 17, 64–102.

Bessler, H., Bergman, M., Salman, H., 2000. Interleukin-3 and stress. Biomed. Pharmacother. 54, 299–304.

Bichay, T.J., Roy, R.M., 1986. Modification of survival and hematopoiesis in mice by tocopherol injection following irradiation. Strahlenther. Onkol. 162, 391–399.

Bincoletto, C., Queiroz, M.L.S., 1996. The effect of lead on the bone marrow stem cells of mice infected with Listeria monocytogenes. Vet. Hum. Toxicol. 38, 186–190.

Black, P.H., 1994. Central nervous system–immune system interactions: psychoneuroendocrinology of stress and its immune consequences. Antimicrob. Agents Chemother. 38 (1), 1–6.

Blalock, J.E., 1989. A molecular basis for bidirectional communication between the immune and neuroendocrine systems. Physiol. Rev. 69, 1–32.

Blalock, J.E., 1994. The syntax of immune-neuroendocrine communication. Immunol. Today 15 (11), 504–510.

Blecha, F., Barry, R.A., Kelley, K.W., 1982. Stress-induced alterations in delayed-type hypersensitivity to SRBC and contact sensitivity to DNFB in mice. Proc. Soc. Exp. Biol. Med. 169, 239–246.

Broome, C.S., Whetton, A.D., Miyan, J.A., 2000. Neuropeptide control of bone marrow neutrophil production is mediated by both direct and indirect effects on CFU-GM. Br. J. Haematol. 108, 140–150.

Carvalho-Freitas, M.I., Rodrigues-Costa, E.C., Nasello, A.G., Palermo-Neto, J., Felicio, L.F., 2008. In vitro macrophage activity: biphasic effect of prolactin and indirect evidence of dopaminergic modulation. Neuroimmunomodulation 15 (2), 131–139.

Cheers, C., Haigh, A.M., Kelso, A., Metcalf, D., Stanley, E.R., Young, A.M., 1988. Production of colony-stimulating factors (CSFs) during infection: separate determinations of macrophage-, granulocyte-, granulocyte–macrophage-, and multi-CSFs. Infect. Immun. 56, 247–251.

Cherdyntseva, N., Shishkina, A., Butorin, I., Murase, H., Gervas, P., Kagiya, T.V., 2005. Effect of tocopherol-monoglucoside (TMG), a watersoluble glycosylated derivate of vitamin E, on hematopoietic recovery in irradiated mice. J. Radiat. Res. (Tokyo) 46, 37–41.

Chrousos, G.P., 2000. The stress response and immune function: clinical implications. The 1999 Novera H. Spector. Lecture. Ann. NY Acad. Sci. 917, 38–67.

Costa-Pinto, F., Palermo-Neto, J., 2010. Neuroimmune interactions in stress. Neuroimmunomodulation 17, 196–199.

Daniel, C.P., Ponting, I.L., Dexter, T.M., 1989. Growth and development of haemopoietic cells: a deterministic process? Hamatol. Bluttransfus. 32, 172–177.

Dantas, D.C., Queiroz, M.L.S., 1999. Effects of *Chlorella vulgaris* on bone marrow progenitor cells of mice infected with Listeria monocytogenes. Int. J. Immunopharmacol. 21, 499–508.

Dexter, T.M., 1979. Haemopoiesis in long-term bone marrow cultures. Acta Haematol. 62, 299–305.

Dhabhar, F.S., McEwen, B.S., 1997. Acute stress enhances while chronic stress suppresses cell-mediated immunity in vivo: a potential role for leukocyte trafficking. Brain Behav. Immun. 11 (4), 286–306.

Dhabhar, F.S., Millar, A.H., McEwen, B.S., Spencer, R.L., 1995. Effects of stress on immune cell distribution: dynamics and hormonal mechanisms. J. Immunol. 154, 5511–5527.

Dinarello, C.A., 1996. Biologic basis for interleukin-1 in disease. Blood 87, 2095–2147.

Domen, J., Weissman, I.L., 1999. Self-renewal, differentiation or death: regulation and manipulation of hematopoietic stem cell fate. Mol. Med. Today 5, 201–208.

Dorschkind, K., 1990. Regulation of hematopoiesis by bone marrow stromal cell and their products. Annu. Rev. Immunol. 8, 11–137.

Downing, J.E.G., Miyan, J.A., 2000. Neural immunoregulation: emerging roles for nerves in immune homeostasis and disease. Immunol. Today 21, 281–289.

Dugan, A.L., Schwemberger, S., Noel, G.J., Babcock, G., Ogle, C.K., Horseman, N.D., 2007. Psychogenic stress prior to burn injury has differential effects on bone marrow and cytokine responses. Exp. Biol. Med. 232, 253–261.

Dunn, A.J., 1990. Interleukin-1 as a stimulator of hormone secretion. Prog. Neuroendocrinimmunol. 3, 26–34.

Dunn, A.J., 1995. Interactions between the nervous system and the immune system: implications for psychopharmacology. Psychopharmacology (Chapter 63), 719–731.

Dygai, A.M., Shakhov, V.P., Mikhlenko, A.V., Goldberg, E.D., 1991. Role of glucocorticoids in the regulation of bone marrow hemopoiesis in stress reaction. Biomed. Pharmacother. 45, 9–14.

Eaves, C.J., Cashman, J.D., Kay, R.J., Dougherty, G.J., Otsuka, T., Gabory, L.A., Hogge, D.E., Lansdorp, P.M., Eaves, A.C., Humphries, R.K., 1991. Mechanisms that regulate the cell cycle status of very primitive hematopoietic cells in long-term human marrow cultures. II. Analysis of positive and negative regulators produced by stromal cells within the adherent layer. Blood 78, 110–117.

Edgar, V.A., Silberman, D.M., Cremaschi, G.A., Zieher, L.M., Genaro, A.M., 2003. Altered lymphocyte catecholamine reactivity in mice subjected to chronic mild stress. Biochem Pharmacol. 65 (1), 15–23.

Elenkov, I.J., Papanicolaou, D.A., Wilder, R.L., Chrousos, G.P., 1996. Modulatory effects of glucocorticoids and catecholamines on human interleukin-12 and interleukin-10 production: clinical implications. Proc. Assoc. Am. Phys. 108, 374–381.

Elenkov, I.J., Wilder, R.L., Chrousos, G.P., Vizi, E.S., 2000. The sympathetic nerve—an integrative interface between two supersystems: the brain and the immune system. Pharmacol. Rev. 52, 595–638.

Fibbe, W.E, can Damme, J., Billiau, A., Goselink, H.M., Voogt, P.J., Van Eeden, G., Ralph, P., Altrock, B.W., Falkenburg, J.H., 1988. Interleukin 1 induces human marrow stromal cells in long-term culture to produce granulocyte colony-stimulating factor. Blood 71, 430–435.

Gartner, S., Kaplan, H., 1980. Long-term culture of human bone marrow. Proc. Natl. Acad. Sci. USA 77 (8), 4756–4759.

Glaser, R., Kiecolt-Glaser, 2005. Stress-induced immune dysfunction: implications for health. Nat. Rev. Immunol. 5 (3), 243–251.

Goldberg, E.D., Zakharova, O., Dygai, A.M., 1988. Modulating effect of opioid peptides on hemopoiesis in stress. Bull. Eksp. Biol. Med. 106, 23–26.

Guleria, I., Pollard, J.W., 2001. Aberrant macrophage and neutrophil population dynamics and impaired Th1 response to Listeria monocytogenes in colony-stimulating factor 1-deficient mice. Infect Immun. 69 (3), 1795–1807.

Gurer, H., Ercal, N., 2000. Can antioxidants be beneficial in the treatment of lead poisoning? Free Radic. Biol. Med. 29, 927–945.

Hasegawa, T., Yoshikai, Y., Okuda, M., Nomoto, K., 1990. Accelerated restoration of the leukocyte number and augmentated resistance against *Escherichia coli* in cyclophosphamide-treated rats orally administered with a hot water extract of *Chlorella vulgaris*. Int. J. Immunopharmacol. 12, 883–891.

Hasegawa, T., Okuda, M., Nomoto, K., Yoshikai, Y., 1994. Augmentation of the resistance against Listeria monocytogenes by oral administration of a hot water extract of *Chlorella vulgaris* in mice. Immunopharmacol. Immunotoxicol. 16, 191–202.

Hasegawa, T., Okuda, M., Makino, M., Hiromatsu, K., Nomoto, K., Yoshikai, Y., 1995. Hot water extracts of *Chlorella vulgaris* reduce opportunistic infection with Listeria monocytogenes in C57BL/6 mice infected with LP-BM5 murine leukemia viruses. Int. J. Immunopharmacol. 17, 505–512.

Hasegawa, T., Kimura, Y., Hiromatsu, K., Kobayashi, N., Yamada, A., Makino, M., Okuda, M., Sano, T., Nomoto, K., Yoshikai, Y., 1997. Effect of hot water extract of *Chlorella vulgaris* on cytokine expression patterns in mice with murine acquired immunodeficiency syndrome after infection with Listeria monocytogenes. Immunopharmacology 35, 273–282.

Hasegawa, T., Noda, K., Kumamoto, S., Ando, Y., Yamada, A., Yoshikai, Y., 2000. *Chlorella vulgaris* culture supernatant (CVS) reduces psychological stress induced apoptosis in thymocytes of mice. Int. J. Immunopharmacol. 22, 877–885.

Hauser, S.P., Kajkenova, O., Lipschitz, D.A., 1997. The pivotal role of interleukin 6 in formation and function of hematopoietically active murine long-term bone marrow cultures. Stem Cells 15, 125–132.

Herman, P., Ferrant, A., De Bruyere, M., Straetmans, N., 1998. Stromal factors support the expansion of the whole hematopoietic spectrum from bone marrow CD34 + DR – cell and of some hematopoietic subset from CD34+ and CD34+DR+ cells. Leukemia 12, 735–745.

Heyworth, C.M., Whetton, A.D., Nicholls, S.E., Zsebo, K., Dexter, T.M., 1992. Stem cell factor directly stimulates the development of enriched granulocyte–macrophage colony-forming cells and promotes the effects of other colony stimulating factors. Blood 80, 2230–2236.

Justo, G.Z., Silva, M.R., Queiroz, M.L., 2001. Effects of the green algae *Chlorella vulgaris* on the response of the host hematopoietic system to intraperitoneal

Ehrlich ascites tumor transplantation in mice. Immunopharmacol. Immunotoxicol. 23, 119–132.
Karp, J.D., Smith, J., Hawk, K., 2000. Restraint stress augments antibody production in cyclophosphamide-treated mice. Physiol. Behav. 70 (3–4), 271–278.
Kayashima, S., Tsuru, S., Hata, N., Rokutanda, M., 1993. Therapeutic effect of granulocyte colony-stimulating factor (G-CSF) on the protection against Listeria infection in SCID mice. Immunology 80 (3), 471–476.
Konishi, F., Tanaka, K., Himeno, K., Taniguchi, K., Nomoto, K., 1985. Antitumor effect induced by a hot water extract of *Chlorella vulgaris* (CE): resistance to Meth-A tumor growth mediated by CE-induced polymorphonuclear leukocytes. Cancer Immunol. Immunother. 19, 73–78.
Konishi, F., Tanaka, K., Kumamoto, S., Hasegawa, T., Okuda, M., Yano, Y., Yoshikai, Y., Nomoto, K., 1990. Enhanced resistance against *Escherichia coli* infection by subcutaneous administration of the hot-water extract of *Chlorella vulgaris* in cyclophosphamide-treated mice. Cancer Immunol. Immunother. 32, 1–7.
Konishi, F., Mitsuyama, M., Okuda, M., Tanaka, K., Hasegawa, T., Nomoto, K., 1996. Protective effect of an acidic glycoprotein obtained from culture of *Chlorella vulgaris* against myelosuppression by 5-fluorouracil. Cancer Immunol. Immunother. 42, 268–274.
Kovacs, C.J., Evans, M.J., Daly, B.M., Thomas-Patterson, D., Johnke, R.M., et al., 1997. Secondary cytokines interact in sequence with interleukin-1alpha (IL-1alpha) with or without macrophage colony-stimulating factor (M-CSF) to further accelerate granulopoietic recovery in myelosuppressed animals. J. Interf. Cytok. Res. 17, 453–460.
Larsson, J., Karlsson, S., 2005. The role of Smad signaling in hematopoiesis. Oncogene 24, 5676–5692.
Lemaire-Ewing, S., Desrumaux, C., Neel, D., Lagrost, L., 2010. Vitamin E transport, membrane incorporation and cell metabolism: is α-tocopherol in lipid rafts an oar in the lifeboat? Mol. Nutr. Food Res. 54, 631–640.
Maestroni, G.J., 2000. Neurohormones and catecholamines as functional components of the bone marrow microenvironment. Ann. NY Acad. Sci. 917, 29–37.
Malacrida, S.A., Teixeira, N.A., Queiroz, M.L., 1997a. Hematopoietic changes in rats after inescapable and escapable shocks. Immunopharmacol. Immunotoxicol. 19, 523–533.
Malacrida, S.A., Teixeira, N.A., Queiroz, M.L., 1997b. Regulation of stress-induced reduced myelopoiesis in rats. Int. J. Immunopharmacol. 19, 227–233.
Metcalf, D., 1984. The bioassay of colony-stimulating factors. In: Hematopoietic colony stimulating factors, Elsevier, Amsterdam, pp. 187–212.
Miyan, J.A., Broome, C.S., Afan, A.M., 1998. Coordinated host defense through an integration of the neural, immune and haemopoietic systems. Domest. Anim. Endocrinol. 15 (5), 297–304.
Mizobe, K., Kishihara, K., Ezz-Din El-Naggar, R., Madkour, G.A., Kubo, C., Nomoto, K., 1997. Restraint stress-induced elevation of endogenous glucocorticoid suppresses migration of granulocytes and macrophages to an inflammatory locus. J. Neuroimmunol. 73, 81–89.
Morrison, S.J., Wright, D.E., Weissman, I.L., 1997. Cyclophosphamide/granulocyte colony-stimulating factor induces hematopoietic stem cells to proliferate prior to mobilization. Proc. Natl. Acad. Sci. USA 94, 1908–1913.
Muench, M.O., Schneider, J.G., Moore, M.A., 1992. Interactions among colony-stimulating factors, IL-1 beta, IL-6, and kit-ligand in the regulation of primitive murine hematopoietic cells. Exp. Hematol. 20, 339–349.
Nance, D.M., Sanders, V.M., 2007. Autonomic innervation and regulation of the immune system. Brain. Behav. Immun. 6, 736–745.
Olfert, E.D., Cross, B.M., McWilliam, A.A., 1993. Guide to the care and use of experimental animals, vol. 1. Canadian Council on Animal Care, Ottawa.
Palermo-Neto, J., Massoco, C.O., Souza, W.R., 2003. Effects of physical and psychological stressors on behavior, macrophage activity, and Ehrlich tumor growth. Brain Behav. Immun. 17, 43–54.
Panina-Bordignon, P., Mazzeo, D., Lucia, P.D., D'Ambrosio, D., Lang, R., Fabbri, L., Self, C., Sinigaglia, F., 1997. Beta2-agonists prevent Th1 development by selective inhibition of interleukin 12. J. Clin. Invest. 100 (6), 1513–1519.
Passagué, E., Wagers, A.J., Giuriato, S., Anderson, W.C., Weissman, I.L., 2005. Global analysis of proliferation and cell cycle gene expression in the regulation of hematopoietic stem and progenitor cell fates. J. Exp. Med. 202 (11), 1599–1611.
Peng, C., Chen, Y., Shan, Y., Zhang, H., Guo, Z., Li, D., Li, S., 2012. LSK derived LSK-cells have a high apoptotic rate related to survival regulation of hematopoietic and leukemic stem cells. PLoS One 7(6), e38614.
Queiroz, M.L.S., Bincoletto, C., Valadares, M.C., Dantas, D.C.M., Santos, L.M.B., 2002. Effects of *Chlorella vulgaris* extract on cytokines production in Listeria monocytogenes infected mice. Immunopharmacol. Immunotoxicol. 24, 483–496.
Queiroz, M.L.S., Rodrigues, A.P.O., Bincoletto, C., Figueiredo, C.A.V., Malacrida, S., 2003. Protective effects of *Chlorella vulgaris* in lead-exposed mice infected with Listeria monocytogenes. Int. Immunopharmacol. 3, 889–900.
Queiroz, M.L.S., Torello, C.O., Perhs, S.M.C., Rocha, M.C., Bechara, E.J.H., Morgano, M.A., Valadares, M.C., Rodrigues, A.P.O., Ramos, A.L., 2008. Food Chem. Toxicol. 46, 3147–3154.
Queiroz, M.L., da Rocha, M.C., Torello, C.O., de Souza Queiroz, J., Bincoletto, C., Morgano, M.A., Romano, M.R., Paredes-Gamero, E.J., Barbosa, C.M., Calgarotto, A.K., 2011. *Chlorella vulgaris* restores bone marrow cellularity and cytokine production in lead-exposed mice. Food Chem. Toxicol. 49 (11), 2934–2941.
Quinteiro-Filho, W.M., Rodrigues, M.V., Ribeiro, A., Ferraz-de-Paula, V., Pinheiro, M.L., Sá, L.R., Ferreira, A.J., Palermo-Neto, J., 2012. Acute heat stress impairs performance parameters and induces mild intestinal enteritis in broiler chickens: role of acute hypothalamic-pituitary-adrenal axis activation. J. Anim. Sci. 90 (6), 1986–1994.
Ramos, A.L., Torello, C.O., Queiroz, M.L.S., 2010. *Chlorella vulgaris* modulates immunomyelopoietic activity and enhances the resistance of tumor-bearing mice. Nutr. Cancer 62 (8), 1170–1180.
Rodriguez-Garcia, I., Guil-Guerrero, J.L., 2008. Evaluation of the antioxidant activity of three microalgal species for use as dietary supplements and in the preservation of foods. Food Chem. 108, 1023–1026.
Roy, R.M., Malick, M.A., Clark, G.M., 1982. Increased haematopoietic stem cell survival in mice injected with tocopherol after X-irradiation. Strahlentherapie 158, 312–318.
Salva, S., Merino, M.C., Aguero, G., Gruppi, A., Alvarez, S., 2012. Dietary supplementation with probiotics improves hematopoiesis in malnourished mice. PLoS One 7 (2), e31171.
Sarders, V.M., Baker, R.A., Ranner-Quinn, D.S., Kasprowicz, D.J., Fuchs, B.A., Street, N.E., 1997. Differential expression of the beta2-adrenergic receptor by Th1 and Th2 clones: implications for cytokine production and B cell help. J. Immunol. 158, 4200–4210.
Selye, H., 1936. A syndrome produced by diverse nocuus agents. Nature 138, 32–34.
Silberman, D.M., Wald, M.R., Genaro, A.M., 2003. Acute and chronic stress exert opposing effects on antibody responses associated with changes in stress hormone regulation of T-lymphocyte reactivity. J. Neuroimmunol. 144 (1–2), 53–60.
Singh, V.K., Shafran, R.L., Jackson III, W.E., Seed, T.M., Kumar, K.S., 2006. Induction of cytokines by radioprotective tocopherol analogs. Exp. Mol. Pathol. 81, 55–61.
Souza-Queiroz, J., Malacrida, S.A., Justo, G.Z., Queiroz, M.L.S., 2004. Myelopoietic response in mice exposed to acute cold/restraint stress: modulation by *Chlorella vulgaris* prophylactic treatment. Immunopharmacol. Immunotoxicol. 26, 455–467.
Souza-Queiroz, J., Torello, C.O., Palermo-Neto, J., Valadares, M.C., Queiroz, M.L.S., 2008. Hematopoietic response of rats exposed to the impact of an acute psychophysiological stressor on responsiveness to an in vivo challenge with Listeria monocytogenes: modulation by *Chlorella vulgaris* prophylactic treatment. Brain Behav. Immun. 20, 1056–1065.
Spiegel, A., Shivtiel, S., Kalinkovich, A., Ludin, A., Netzer, N., Goichberg, P., Azaria, Y., Resnick, I., Hardan, I., Ben-Hur, H., Nagler, A., Rubinstein, M., Lapidot, T., 2007. Catecholaminergic neurotransmitters regulate migration and repopulation of immature human CD34+ cells through Wnt signaling. Nat. Immunol. 8 (10), 1123–1131.
Stratakis, C.A., Chrousos, G.P., 1995. Neuroendocrinology and pathophysiology of the stress system. Ann. NY Acad. Sci. 771, 1–2.
Tanaka, K., Konishi, F., Himeno, K., Taniguchi, K., Nomoto, K., 1984. Augmentation of antitumor resistance by a strain of unicellular green-algae, *Chlorella vulgaris*. Cancer Immunol. Immunother. 17, 90–94.
Tanaka, K., Koga, T., Konishi, F., Nakamura, M., Mitsuyama, M., Himeno, K., Nomoto, K., 1986. Augmentation of host defense by a unicellular green alga, *Chlorella vulgaris*, to *Escherichia coli* infection. Infect. Immun. 53, 267–271.
Tanaka, K., Yamada, A., Noda, K., Hasegawa, T., Okuda, M., Shoyama, Y., Nomoto, K., 1998. A novel glycoprotein obtained from *Chlorella vulgaris* strain CK22 shows antimetastatic immunopotentiation. Cancer Immunol. Immunother. 45, 313–320.
Torello, C.O., Souza-Queiroz, J., Oliveira, S.C., Queiroz, M.L.S., 2010. Immunohematopoietic modulation by oral B-1,3-glucan in mice infected with Listeria monocytogenes. Int. Immunopharmacol. 10 (12), 1573–1579.
Van Den Engh, G., Bol, S., 1975. The presence of a CSF enhancing activity in the serum of endotoxin-treated mice. Cell Tissue Kinet. 8, 579–587.
Vijayavel, K., Anbuselvam, C., Balasubramanian, M.P., 2007. Antioxidant effect of the marine algae *Chlorella vulgaris* against naphthalene-induced oxidative stress in the albino rats. Mol. Cell. Biochem. 303, 39–44.
Wing, E.J., Barczynski, L.C., Waheed, A., Shadduck, R.K., 1985. Effect of Listeria monocytogenes infection on serum levels of colony-stimulating factor and number of progenitor cells in immune and nonimmune mice. Infect. Immun. 49, 325–328.
Woiciechowsky, C., Asadullah, K., Nestler, D., Eberhardt, B., Platzer, C., Schoning, B., Glockner, F., Lanksch, W.R., Volk, H.D., Docke, W.D., 1998. Sympathetic activation triggers systemic interleukin-10 release in immunodepression induced by brain injury. Nat. Med. 4, 808–813.
Wright, D.E., Cheshier, S.H., Wagers, A.J., Randall, T.D., Christensen, J.L., Weissman, I.L., 2001. Cyclophosphamide/granulocyte colony-stimulating factor causes selective mobilization of bone marrow hematopoietic stem cells into the blood after M phase of the cell cycle. Blood 97, 2278–2285.
Zhan, Y., Lieschke, G.J., Grail, D., Dunn, A.R., Cheers, C., 1998. Essential roles for granulocyte–macrophage colony-stimulating factor (GM-CSF) and G-CSF in the sustained hematopoietic response of Listeria monocytogenes-infected mice. Blood 91, 863–869.
Zhang, X., Okutsu, M., Kanemi, O., Nagatomi, R., 2005. Effect of foot shock stress on the interferon-c production of murine intestinal intraepithelial lymphocytes. Immunol. Lett. 100, 170–176.