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Activation of bone marrow cells treated with *Canova in vitro*

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Abstract

Canova is a Brazilian complex homeopathic medication produced from *Aconitum, Thuya, Bryonia, Lachesis and Arsenicum.* Previous studies demonstrated that *Canova* induces up-regulation in numbers of leukocytes. The bone marrow microenvironment is composed of growth factors, stromal cells, extracellular matrix, and progenitor cells that differentiate into mature blood cells. As it is the major site of blood cell formation, we studied *in vitro Canova* effects on bone marrow cells of mice. Swiss mouse femurs were dissected, cleaned, and the marrow was flushed. The cells were plated, treated or not, incubated for different times and processed for light, scanning electron, and confocal microscopy, and also flow cytometry. The treatment did not modify the expression of the analyzed surface markers or cytokine production. All microscopy techniques showed that a monocytic lineage ($CD11b^+$) and stromal cells (adherent cells) were activated by treatment. *Canova* also increased cell clusters over adherent cells, suggesting proliferation areas.

Keywords: Bone marrow cells; Stromal cells; Activation; *Canova* medication

1. Introduction

The medullar cavity of long bones and the interstices between trabeculae of spongy bones house the soft, gelatinous, highly vascular, and cellular tissue known as marrow. It has a unique anatomic structure that allows survival, proliferation, and differentiation of progenitor cells. Marrow stromal cells, extracellular matrix, growth factors and progenitors cells (which differentiate into mature blood cells) constitute this microenvironment (Kondo et al., 2003). This system allows interactions between stromal cells and haematopoietic stem cells (HSCs) (Haylock et al., 1994). This interaction is dependent, at least in part, on direct cell-to-cell contact or cellular adhesion to extracellular matrix proteins (Paul et al., 1991; Kameoka et al., 1995).

The adherent microenvironment of long-term murine bone marrow cultures is an extensive extracellular network of collagen, glycoproteins, fibronectin, and laminin, as well as the cellular stroma, including endothelial cells, macrophages, fibroblasts, adipocytes and reticular cells (Zuckerman and Wicha, 1983). The stromal cells may provide a microenvironment adequate for rapid expansion of the progenitor cells or by preventing apoptotic cell death of the progenitors (Kameoka et al., 1995). The establishment and maintenance of the extracellular matrix correlates with the production of haematopoietic cells in long-term murine bone marrow culture. According to Zuckerman and Wicha (1983), the extracellular matrix is essential for maintenance of *in vitro* haematopoiesis.

HSCs are defined as cells that are capable of both self-renewal and multilineage reconstitution of the haematopoietic system (Domen and Weissman, 1999). They have the capacity to circulate in the blood and can colonize irradiated haematopoietic tissue (De Gowin and Gibson, 1976). These cells are able to form the myeloid blood cellular lineages (granulocytes, monocytes/macrophages), erythroid (erythrocytes and megakaryocytes), and lymphoid (plasmocytes, T cells, B cells and

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NK cells) (Katsura, 2002). Development of these cells requires adhesion of haematopoietic stem cells and progenitors to the stromal cells and extracellular matrix in the haematopoietic microenvironment (Yanai et al., 1994; Koenigsmann et al., 1992). In normal steady-state haematopoiesis, the adhesion events probably function both to retain primitive progenitor cells within the marrow and to regulate and organize differentiation of lineage-committed progenitors and cells, and release their mature progeny from the marrow (Koenigsmann et al., 1992; Simmons et al., 1992).

Canova is a Brazilian complex homeopathic medicine, used as an immune modulator. Previous studies demonstrated that *Canova* activates macrophages both *in vivo* and *in vitro*. Tumor Necrosis Factor-α (TNFα) *in vitro* production was significantly diminished (Piemonte and Buchi, 2002). NAD(P)H oxidase activity was increased as well as that of inducible nitric oxide synthase (iNOS), consequently producing reactive oxygen species (ROS) and nitric oxide (NO), respectively, (De Oliveira et al., 2006). *Canova* stimulated an increase of the endosomal/lysosomal system and increased phagocytic activity of macrophages of non-infective microorganisms (*Saccharomyces cerevisiae* and *Trypanosoma cruzi* epimastigotes) (Lopes et al., 2006). *Canova* modulatory effects were also observed in experimental infection both *in vivo* and *in vitro* by *Leishmania amazonensis*, controlling infection progression and limiting its dissemination (Pereira et al., 2005). Sarcoma 180-bearing mice treated with *Canova* had a reduction in sarcoma size and a significant infiltration of lymphoid cells, granulation tissue, and fibrosis surrounding the tumor. *Canova* enhanced $CD4^+$ T cells in the untreated group and increased both B and NK cells in S180-treated groups (Sato et al., 2005). Moreover, it was neither toxic nor mutagenic (Seligmann et al., 2003).

As *Canova* has no side effects and regulates host defence, it may potentially be used to increase the number of macrophages as a therapeutic drug, which is less toxic than are some reagents, such as M-CSF, or be used as a useful adjuvant or a complementary therapy. With this assay we evaluated the effects of *Canova* treatment on mouse bone marrow cells. We have determined whether *Canova* can promote the differentiation, proliferation, and/or survival of mouse bone marrow cells, thereby increasing the overall quantity of these cells *in vitro*.

2. Materials and methods

2.1. Canova

"Canova do Brasil" is a Brazilian company, located in the State of Paraná, that holds the medication's international patent. *Canova* is produced and sold in drops, inhalant and intravenous forms, only by authorized drugstores, under a very strict and rigorous quality control.

Because of its homeopathic formulation, *Canova* is standardized and authorized by competent agencies for medicinal application. It is currently registered according to Law no. 5,991/73. This commercial medicine represents a new form of immune modulator therapy and follows Hahnemann's homeopathic techniques. Mother tinctures are purchased from authorized agencies indicated by the Brazilian Health Ministry. These matrices have a warrantee certificate, which ensures quality (endotoxin free) and physicochemical composition. The final product, *Canova*, contains 11DH *Aconitum napellus* (Ranunculaceae), 19DH *Thuya occidentalis* (Cupresaceae), 18DH *Bryonia alba* (Curcubitaceae), 18DH *Lachesis muta* (Viperidae), and 19DH *Arsenicum album* (arsenic trioxide) and less than 1% ethanol, all in distilled water. *Canova* is an aqueous, colorless and odorless solution. Experiments were performed with commercial *Canova* (www.canovadobrasil.com.br).

2.2. Animals

Male albino Swiss mice (6–8 week old) weighing 25–30 g were used. Animals were acquired from the Central Animal House of the Universidade Federal do Paraná (UFPR), and received a standard laboratory diet (Purina®) and water *ad libitum*. All recommendations of the Brazilian National Law (no. 6638) for scientific management of animals were respected and the Institutional Animal Care Committee at UFPR approved all related practices. Experiments were carried out at the *Laboratório de Pesquisa em Células Neoplásicas e Inflamatórias*, UFPR, which has a management program for produced residues.

2.3. Cell preparation

Femurs were dissected and cleaned. Epiphyses were removed and the marrow was flushed with Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal bovine serum with 1 U/ml penicillin, 1 μg/ml streptomycin, and 2.5 μg/ml amphotericin.

2.4. Cell culture

Cells were counted in a Neubauer chamber and cell density adjusted depending on the experiment. They were seeded into 24 well culture plates (for adherent cell experiments), 96 well culture plates (for cytokine quantification), or into culture flasks (for supernatant experiments), and maintained at 37 °C and 5% CO_2 atmosphere for 48, 72 and 96 h, depending on the experiment. Three groups were examined. The control did not receive any treatment, the *Canova* group first received 20% medication, and a 1% dose was administered daily to the culture, and the Monocyte-Colony Stimulating Factor (M-CSF) group was used as a positive control. The M-CSF was obtained from L929 cell supernatant.

2.5. Morphology assay

Cells (2.5×10^5) were plated into culture plates with glass slides for morphological analysis (Buchi and De Souza, 1992). They were maintained as described above. After 48, 72 and 96 h they were rinsed with phosphate buffered saline (PBS), fixed in Bouin, stained with May–Grünwald–Giemsa, dehydrated, and mounted with Entellan®. Adhered cells were observed by light microscopy using a Nikon Eclipse E200 microscope.

2.6. Immunophenotyping

An aliquot was used to analyse the cells just after collecting, called zero hour group (t_0). Immunophenotyping was also performed after 48, 72 and 96 h of culture. Cells (10^6) were fixed with 1% paraformaldehyde, washed, and incubated with 0.5 μg/ml biotinylated antibody, listed in item 2.10, in PBS for 40 min. They were then washed with PBS and incubated with 0.5 μg/ml phycoerythrin (PE) labelled secondary antibody in PBS for 30 min (Pearce et al., 2004). Fluorescence was analyzed according to standard procedures using a FACSCalibur flow cytometer (Becton–Dickinson – BD), equipped with an argon ion laser (488 nm). Data were analyzed with a Cell Quest program (BD) and submitted to variance analysis (ANOVA) with a factorial diagram (2×3) to determine the statistical significance. The Tukey test was performed when the effects of interaction was significant. The level of significance was taken at $P < 0.05$ and $P < 0.01$. Data are representative of three independent experiments.

2.7. Scanning electron microscopy (SEM)

Differences among the groups were mainly found after 72 h of culture. Thus cells (2.5×10^5) cultivated for 72 h, were fixed with 2.5% glutaraldehyde (0.1 M cacodylate buffer, pH 7.2), washed, and post-fixed in 1% osmium tetroxide for 30 min in the dark at room temperature (Buchi and De Souza, 1992). After washing, the cells were dehydrated using increasing alcohol concentrations. Cells were CO_2 critical point dehydrated, metallized and observed using a JEOL JSM – 6360 LV SEM Scanning Electron Microscope at the UFPR Electron Microscopy Center.

2.8. Cytokine quantification

After 96 h of culture we observed a higher number of adherent cells. Thus at this point, the supernatant of the cell culture was centrifuged and cytokine quantification was performed using a mouse Th1/Th2 cytokine CBA kit (BD Pharmingen), according to the manufacturer's instructions. This kit contains antibodies against TNF-α, interferon-γ (IFN-γ) and interleukins 2, 4 and 5 (IL-2, IL-4, IL-5). The cytokine concentration was obtained by comparing data with a cytokine curve in the CBA program (BD). Fluorescence was measured using a flow cytometer. Data were analyzed with Cell Quest, according to the manufacturer's procedures, ANOVA, and Tukey test ($P < 0.05$) were used to determine the statistical significance of intergroup comparisons.

2.9. Confocal microscopy

Using confocal microscopy we have determined surface markers on adherent cells (2.5×10^5). As this protocol requires various washing steps, we incubated the cells for 12 days to have a complete monolayer on the plate. This assured that at the end of the process we would still have cells adhered.

Immunostaining was performed according to standard protocols using commercially available antibodies, as described below. The cells were maintained on ice, blocked with 1% PBS/BSA (bovine serum albumin), and incubated with 1 μg biotinylated antibody in 1% PBS/BSA for 40 min (Pearce et al., 2004). After washing, they were fixed in 2% paraformaldehyde for 30 min and the aldehyde radicals were blocked with 0.1 M glycine in PBS. The cells were incubated with phycoerythrin (PE) labelled secondary antibody in PBS for 40 min. The nuclei were stained with 300 nM DAPI (4,6-diamidino-2-phenylindole, dihydroxychloride) (Molecular Probes, Eugene, OR, USA), which was added 15 min before cell observation. The cells were washed with PBS, mounted with fluormont-G and the fluorescence was analyzed with a Confocal Microscope Radiance 2001 (BIO-RAD®).

2.10. Surface markers

All antibodies used were from a mouse lineage panel specific to bone marrow purchase from BD Pharmingen.

Antibodies	Main marked cells
CD11b (Mac-1)	Monocytes/macrophages
Ly-6G	Granulocytes
CD45R	B lymphocytes
CD11c	Dendritic cells
CD3	T lymphocytes
TER-119	Erythrocytes

3. Results

3.1. Morphology assay and scanning electron microscopy (SEM)

Fig. 1A, C and E show cells from the control group at 48, 72 and 96 h, respectively, under light microscopy. After cell analysis we clearly observed that the number of adherent cells increased in the *Canova* group. This fact was observed at all tested times but it was mainly found after 72 h (Fig. 1D), and it was enhanced after 96 h (Fig. 1F).

The majority of adherent cells in both groups presented macrophage characteristics. This fact was confirmed by the SEM results. In the control group these cells were mainly resident macrophages with few activated cells (Fig. 2A). Almost all cells from the treated group were activated, as defined by morphological alterations (Fig. 2B). Those cells were more spread, with many cytoplasmic projections around them visualized by SEM, and a larger and lighter nucleus visualized by light microscopy. These morphological characteristics are well known to be typical of activated macrophages (Piemonte and Buchi, 2002). The M-CSF group was used as a positive control group (Fig. 2C).

Cell clusters (cell niches) over the adherent cells were also observed in both groups, but in the *Canova* group it was much more pronounced. These niches were observed in all designated times in the *Canova* group, but after 96 h of culture it was much more evident (Figs. 1F and 2B). In Fig. 2D we observed a detail of the cell niche by SEM, and we also observed interactions between the cells within the cluster.

3.2. Immunophenotyping

Flow cytometric analysis of T_0 showed four cell populations, as described by Civin and Loken (1987). In Graph 1, we can observe erythroid cells (R1 – small and less complex cells), lymphoid cells (R2 – small and less complex), monocytes/macrophages (R3 – large and more complex) and granulocytes (R4 – large and more complex). The mean percentage of each population is shown in Table 1A.

We observed, using flow cytometry, that the number of markers within a cell lineage was not affected by the treatment. Thus, the mean of all groups is shown in Table 1B. We found that after 48 h of culture, the number of cells was very similar to T_0. However, after 72 h of culture, some surface markers floated equally between the groups. For example, CD11c and CD3 expressions increased after 72 h and returned to the 48 h level after 96 h of culture. CD45R expression decreased after 72 h and returned to 48 h values after 96 h. TER-119 expression increased after 72 h and it was maintained after 96 h of culture. The expression of CD11b and Ly-6G did not change during the experiment.

3.3. Cytokine quantification

After 96 h of culture we evaluated the capacity of cells from the control and *Canova* groups to elicit TNF-α, IFN-γ, IL-2, IL-4, and IL-5. The concentrations of these cytokines were assayed in the supernatant of the cells. No statistical differences between the groups were found (data not shown).

3.4. Confocal microscopy

Using light microscopy and SEM we observed that the majority of adhered cells presented macrophage morphological

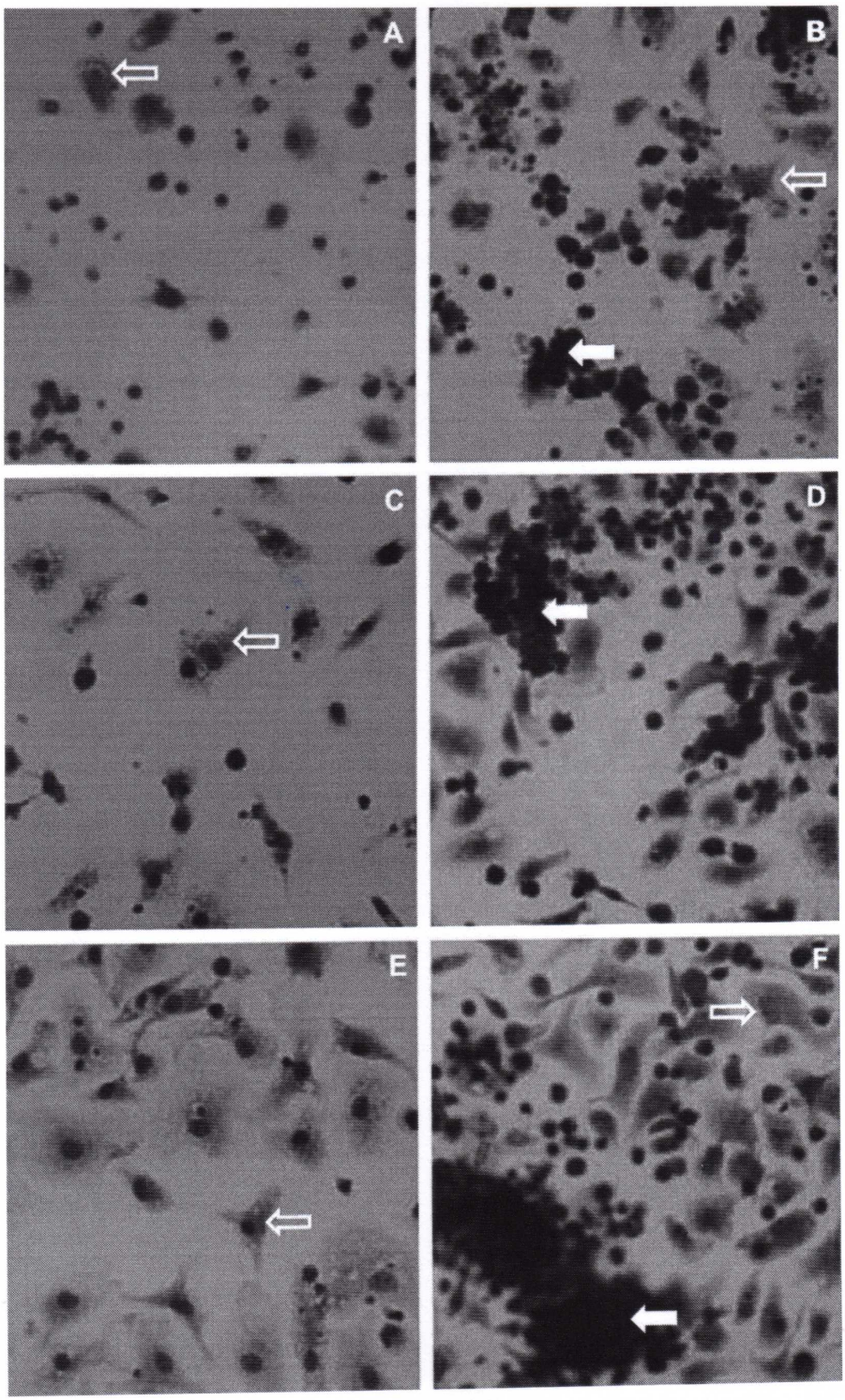


Fig. 1. Light microscopy of adherent bone marrow cells. The cells were flushed from mouse femurs and cultivated for 48, 72 and 96 h. After each time point the cells were fixed, stained with May–Grünwald–Giemsa and observed using light microscopy. We can observe in the *Canova* group an increase in cell number and cell niches after all times tested. (A) Cells from the control group after 48 h of culture. (B) Cells from the *Canova* group after 48 h of culture. (C) Cells from the control group after 72 h. (D) Cells from the *Canova* group after 72 h of culture. (E) Cells from the control group after 96 h. (F) Cells from the *Canova* group after 96 h of culture. All photographs were taken using the microscope 40 × objective. Solid arrow, cell niches; Open arrow, spread cells with activated macrophage morphology.

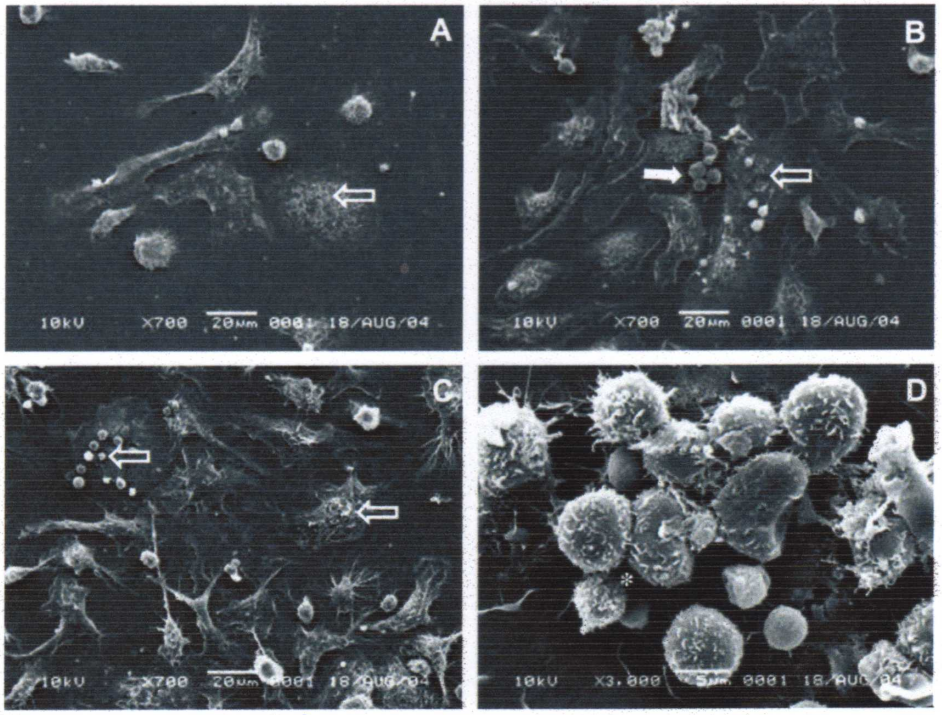
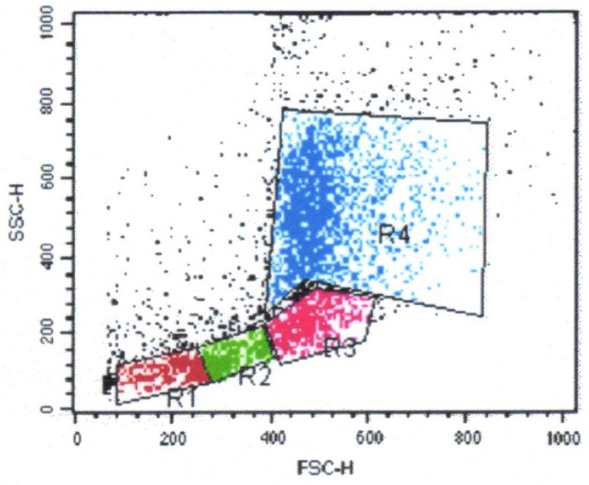


Fig. 2. Scanning electron microscopy of adherent bone marrow cells. The cells were flushed from mouse femurs and cultivated for 72 h. After this time of culture the cells were fixed and observed using SEM. We can observe an increase of adherent cells in the *Canova* group when compared to the control group. The M-CSF group was used as a positive control. (A) Cells from the control group after 72 h. Bar 20 µm. (B) Cells from the *Canova* group after 72 h of culture. Bar 20 µm. (C) Cells from the M-CSF group after 72 h. Bar 20 µm. (D) Detail of a cell niche. Bar 5 µm. We can observe the interaction between the cells in the cluster. Solid arrow, cell niches; Open arrow, spread cells with activated macrophage morphology; *, adhesion sites with cell interactions.

characteristics. These observations were confirmed by CD11b staining and confocal microscopy. We found that almost all adherent cells, from both the control and the *Canova* groups, were positively stained for CD11b. Fig. 3A shows adherent cells from the control group representative of macrophages. Apparently, the *Canova* group increased the number of macrophages (Fig. 3B) as also observed using light microscopy and SEM. Clusters of CD11b were found on the surface of most cells as seen in Fig. 3Ba.

Few adherent cells also expressed CD11c on the surface (Fig. 3D) and this marker presented a net-like distribution (Fig. 3Da). No differences between the groups were observed. The markers CD45R, CD3, Ly-6G and TER-119 were not observed in the adherent cells from either group (data not shown). Fig. 3C shows the fluorescence control of all markers.



Graph 1. *x* Axis, forward scatter (FSC); *y* axis, side scatter (SSC). The graph represents the bone marrow supernatant populations analyzed using flow cytometry at t_0. The size and complexity of cells were used to separate the populations. The gate R1 corresponds to the localization of erythroid cells, R2 lymphoid cells, R3 monocytes/macrophages and R4 granulocytes.

4. Discussion

Previous studies demonstrated that treatment with *Canova* induces an up-regulation in the number of total leukocytes and increases the number of TCD4 lymphocytes, as well as NK cells in peripheral blood (Sato et al., 2005). As the major site of blood cell formation is the bone marrow, we were prompted to consider that *Canova* might act on these cells. Thus *in vitro* studies are very important to show how *Canova* is acting.

Our assays with mouse bone marrow showed that the number of adherent, larger, and activated cells increased in the *Canova* group. Dexter et al. (1976) showed that after 72 h culture of mouse bone marrow, the majority of adherent cells were phagocytic mononuclear cells with numerous cytoplasmic extensions, and an overall tendency to spread. The adherent cells found in our experiments were mainly macrophages

Table 1A
Marker expression at t_0

Markers	t_0 (%)
Ly-6G	66.04
CD11b	69.85
CD45R	72.80
CD11c	22.80
CD3	59.60
TER-119	27.30

(CD11b$^+$), based on our SEM and confocal microscopy results. Zuckerman and Wicha (1983) showed that after 4 days a small number of adherent cells remained in culture, but the *Canova* group showed different results. After 96 h we observed a cell monolayer (Fig. 1F), different from the control group, that gave a small number of adherent cells after 4 days, in agreement with the literature.

Macrophages become activated when treated with *Canova* (Piemonte and Buchi, 2002; De Oliveira et al., 2006; Lopes et al., 2006; Pereira et al., 2005). We also observed cell clusters over the adherent cells (Fig. 1F), suggesting sites of multiplication and differentiation called cell niches by Li et al. (2004). This condition was observed at all designated times, but after 96 h of culture it was more evident. These niches are known to contain stem cells (Fuchs et al., 2004) and/or leukocytes committed with some cellular lineage (Li et al., 2004). With SEM it was possible to observe communication between the cells in the niches (Fig. 2D). The existence of an *in vitro* functional gap junction between stromal and bone marrow leukocytes has been described as being rare (Cancelas et al., 2000), but the image in Fig. 1F suggests the presence of some kind of communication between them, and these mechanisms may be involved in the regulation of haematopoietic cell development and differentiation.

The adherent cell clusters are dependent on direct cell-to-cell communication, as well as cytokines and growth factors (colony forming) (Paul et al., 1991). While the production and release of various cytokines represents one aspect of haematopoietic regulation by the stroma, it is clear that cellular interactions, both cell-to-cell and cell-to-extracellular matrix, between haematopoietic cells and stromal cells are also of major importance. Such adhesive interactions are believed to be responsible for the homing of haematopoietic stem cells to marrow after marrow transplantation (Simmons et al., 1992).

Li et al. (2004) verified by SEM that macrophages were in close contact with erythroblasts and that they formed a three-dimensional network. Several lymphocytes adhered to the central macrophages in these assemblies. These arrangements of cells were confirmed *in vivo* by transmission electron microscopy: erythroblasts and lymphocytes formed close and firm contacts with macrophages via their cytoplasmic membranes. We observed this interaction in the *Canova* group (Fig. 2B). In the control group few niches occurred. In the M-CSF group the majority of cells were spread and many more clusters were observed (Fig. 2C). In fact, this was anticipated since this group was a positive control.

The immunophenotyping analysis showed some surface markers floating during the culture period and it was found equally in the control and *Canova* groups. The expression of CD11b and Ly-6G did not change during the experiment.

When the cultured cells were analyzed, we observed differences from t_0 (Tables 1A and 1B). This was verified by differences in cell size and complexity in the graphs. The CD11b$^+$ SSC/FSC graphs showed large and complex cells, characteristic of monocytes and macrophages. The Ly-6G$^+$ cells were large, but a little more complex than Ly-6G$^+$ cells at t_0, indicating the presence of immature cells. The CD45Rs were large and more complex when compared to t_0 cells, being also a characteristic of immature B cells. The size of CD3$^+$ cells varied, but the majority were large and somewhat complex (lymphoblasts), different from t_0, where we found differentiated cells that were small and less complex. Dendritic cells (CD11c$^+$) were large and complex. TER119$^+$ cells (characteristic of the erythroid lineage) were small and less complex, as observed in the SSC/FSC graphs (data not shown).

After immunophenotyping analysis for CD11b$^+$ and Ly-6G$^+$ supernatant cells, no differences were found between the groups and the times tested. These cells neither proliferated nor died, but were maintained in culture. Separation of neutrophils from monocytes is particularly difficult because they share many surface antigens. Immature monocytes may express the Ly-6G marker at a higher level in bone marrow than monocytes in the circulation, and this marker expression increases on neutrophils as they differentiate (Lagasse and Weissman, 1996). The Ly-6G$^+$ cells were bigger and showed little complexity, when compared to CD11b$^+$ cells, indicating that they were maintained in an immature state (blast cells). Kameoka et al. (1995) showed that the exogenously added granulocyte colony stimulate factor (G-CSF) did not affect the number of granulocyte colonies.

Thus, the number of granulocytes and granulocyte/monocyte-macrophages colonies was mostly dependent on supportive activities of stroma cells. Macrophages can produce a large amount of cytokines and growth factors and since these cells are the target of *Canova*, a partial supply of the necessary molecules may be occurring with this treatment. All cells were maintained in a DMEM medium supplemented with fetal

Table 1B
Marker expression during culture

	48 h		72 h		96 h	
Markers	Mean (%)	SD (%)	Mean (%)	SD (%)	Mean (%)	SD (%)
CD11b	65.00	7.29	65.00	7.29	65.00	7.29
Ly-6G	60.00	8.9	60.00	8.9	60.00	8.9
CD45R	73.00	6.06	58.7*	11.98	67.6	6.70
CD11c	21.76	5.27	35.11*	11.57	24.25	9.86
CD3	61.84	3.85	75.77*	11.69	57.35	7.83
TER-119	25.32	1.83	56.24*	7.98	61.48*	6.39

These results were obtained from cells of bone marrow culture supernatant stained with several specific antibodies and analyzed using flow cytometry at 48, 72 and 96 h of culture. t_0 was used to compare with the first time of culture. These results are presented as mean ± S.D. The significance of differences between mean values was evaluated by two-away analysis of variance (ANOVA) followed by Tukey test. *P values <0.05 were considered statistically significant.

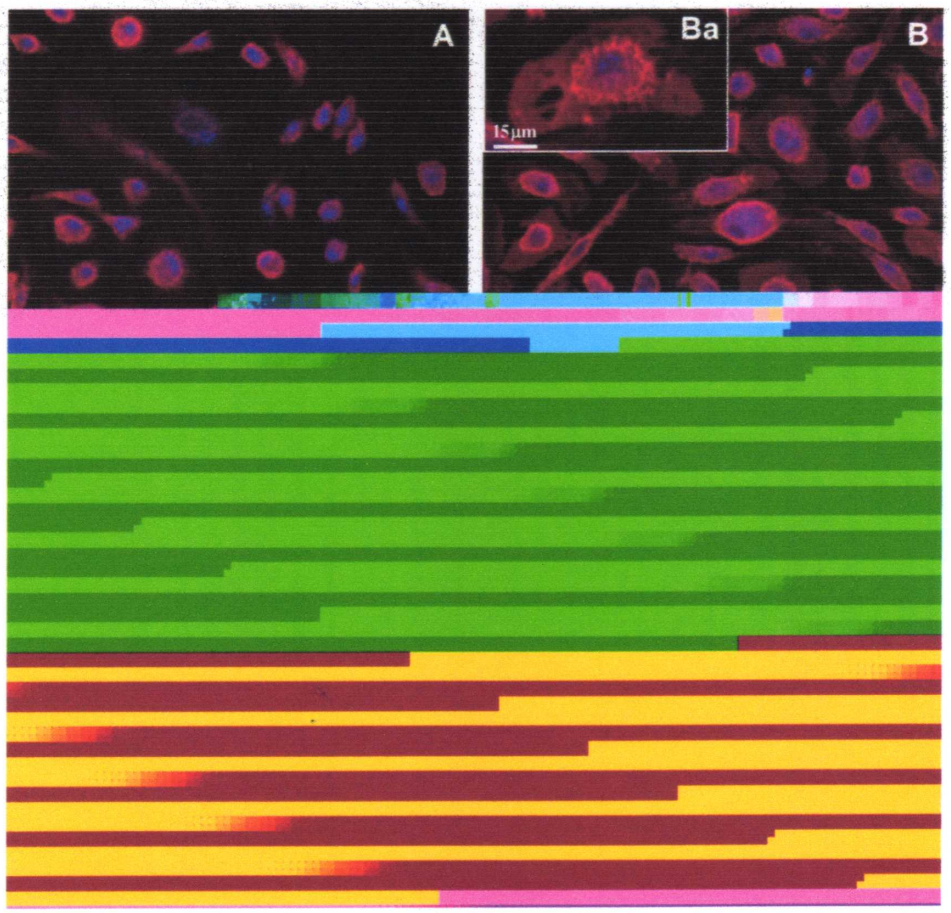


Fig. 3. Confocal microscopy of adherent bone marrow cells. The cells were flushed from mouse femurs and cultivated for 12 days. The adherent cells were stained with several specific antibodies and DAPI fixed and observed using confocal microscopy. We can observe that the majority of adherent cells were macrophages (CD11b positive), but CD11c cells were also found. (A) Adherent cells from the control group where the CD11b is stained in red and the nucleus in blue (DAPI). Bar 30 μm. (B) Adherent cells from the *Canova* group stained for CD11b (red) and nucleus (blue). Bar 30 μm. (Ba) Cells showing the observed clusters of CD11b receptors. Bar 15 μm. (C) Fluorescence control of all markers. Bar 30 μm. (D) Adherent cells where the CD11c is stained in red and the nucleus in blue (DAPI). Bar 30 μm. (Da) Cells showing the net-like distribution of CD11c receptors. Bar 15 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

bovine serum, but no extra growth factor was added to the medium. Probably for this reason, even without additional growth factors, the monocytes-macrophages and granulocytes survived in the culture until the end of the experiment, indicating that the treated stromal cells supplied the need of these factors, allowing their survival. We observed that these adherent cells are mainly $CD11b^+$, macrophages, and previous results showed that the production of some cytokines could be modified after treatment (Piemonte and Buchi, 2002).

The other expression markers changed with time, indicating cell differentiation. The CD45R marker is present in large quantities on $CD11b^+$ cells (Lagasse and Weissman, 1996). This is an explanation for the presence of large cells in these graphs. We found by flow cytometry a reduction of $CD11c^+$ cells in the supernatant after 96 h. Confocal microscopy showed few $CD11c^+$ cells adhering to the glass slides. Probably these cells are immature dendritic cells. These are adherent and are released on maturation (Inaba et al., 1992), but their maturation requires the presence of cytokines and growth factors (Citterio et al., 1999) The absence of the correct level of cytokines and growth factors rapidly revert $CD11c^+$ to an adherent and less stimulatory phenotype (Citterio et al., 1999).

Bone marrow-derived dendritic cells have the ability to activate and induce clonal expansion of naive and memory T cells (Rutella and Lemoli, 2004). These data justify the maintenance of cells in culture without additional growth factors. We found a decrease of CD11c and CD3 after 72 h, probably due to their dependence. The great diversity in cells shown in the SSC/FSC graphs indicates that immature T cells (lymphoblasts) as well as mature cells exist in this culture up to 96 h. Mouse blood has a large number of $CD3^+$ cells (Sato et al., 2005). Although the thymus is the predominant source of T cells, extrathymic sources also contribute to the T cell pool and the bone marrow has this capacity (Dejbakhsh-Jones and Strober, 1999). The bone marrow is very irrigated allowing a reflux of $CD3^+$ cells. This may be an explanation for the large quantity of these cells in the bone marrow.

TER-119 is absent in cells that have typical erythroid blast-forming or erythroid colony-forming units (undifferentiated cells). When $TER\text{-}119^+$ cells were cultured, they were able

to form colonies, suggesting that the earliest TER-119$^+$ cells still possessed a proliferative potential *in vitro* (Kina et al., 2000). We found increased expression of TER-119 after 72 h. We also observed, by light microscopy, niches with erythroblast-like cells in the *Canova* group. Vogt et al. (1991) suggested that erythroblast-macrophage contact promotes proliferation and terminal maturation of erythroid cells. Dörmer et al. (2004) showed that the erythroid differentiation regulator (EDR) is released from stromal cells and can be a substitute for a soluble stromal factor, and under its action bone marrow cells survive better in culture. We can suggest that *Canova* is acting on macrophages, facilitating the arrangement of cells and promoting the development of niches. As macrophages are activated, EDR production is probably occurring. EDR stimulates cell nest formations constituted by cell-like erythroblasts, maintains mature cells, and probably stimulates differentiation of immature cells. This factor, thus, does not stimulate the maturation of immature cells. This hypothesis would justify the existence of a larger number of cell niches in the *Canova* group.

Canova medication did not alter the quantity of surface markers on supernatant cells of bone marrow culture, but acted on adherent cells, suggesting a specific action. This was expected since the adherent cells were mostly macrophages and *Canova* is a macrophage activator (Piemonte and Buchi, 2002).

At present, we know that for every 10,000–15,000 cells in the bone marrow, there is only one haematopoietic stem cell (HSC). Terminally differentiated cells of the haematopoietic system produce at least eight lines, each having different functions, morphologies and cellular kinetics (Weissman, 2000). Some of these cells (0.01–0.1%) do not develop haematopoietic stem cells directly, but by a proliferating progenitor population. Those are the totipotent stem cells, which, under suitable conditions, have the ability to produce a whole spectrum of cell types. This phenomenon is called stem cell plasticity (Filip et al., 2005).

Our assays showing the *Canova* effects on bone marrow cells are very important. Firstly, because it is a non-toxic medication (Seligmann et al., 2003) and secondly because there is the possibility of an *in vitro* cell therapy that can be directed at several diseases that affect bone marrow cells. One possibility is to treat bone marrow stem cells with *Canova* and transplant them into weakened organs, facilitating their regeneration. Because of cell plasticity, bone marrow cells have the ability to repopulate injured livers (Dahlke et al., 2006), promote angiogenesis, affect cardiac function (Yokoyama et al., 2006), cardiac muscles (Orlic et al., 2001), skeletal muscles (Ferrari et al., 1998), and neurons (Mezey et al., 2000). *Canova* could also be used to treat haematopoietic stem cells, inducing their differentiation *in vitro*, and then transplanting them into leukemic patients, increasing their quality of life.

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