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ORIGINAL ARTICLE

Evidence of bone marrow downregulation in brain-dead rats

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SUMMARY

Experimental findings support the evidence of a persistent leucopenia triggered by brain death (BD). This study aimed to investigate leucocyte behaviour in bone marrow and blood after BD in rats. BD was induced using intracranial balloon catheter inflation. Sham-operated (SH) rats were trepanned only. Thereafter bone marrow cells were harvested every six hours from the femoral cavity and used for total and differential counts. They were analysed further by flow cytometry to characterize lymphocyte subsets, granulocyte adhesion molecules expression and apoptosis/necrosis [annexin V/propidium iodide (PI) protocol]. BD rats exhibited a reduction in bone marrow cells due to a reduction in lymphocytes (40%) and segmented cells (45%). Bone marrow lymphocyte subsets were similar in BD and SH rats (CD3, $P = 0.1$; CD4, $P = 0.4$; CD3/CD4, $P = 0.4$; CD5, $P = 0.4$, CD3/CD5, $P = 0.2$; CD8, $P = 0.8$). Expression of L-selectin and beta$_2$-integrins on granulocytes did not differ (CD11a, $P = 0.9$; CD11b/c, $P = 0.7$; CD62L, $P = 0.1$). There were no differences in the percentage of apoptosis and necrosis (Annexin V, $P = 0.73$; PI, $P = 0.21$; Annexin V/PI, $P = 0.29$). In conclusion, data presented suggest that the downregulation of the bone marrow is triggered by brain death itself, and it is not related to changes in lymphocyte subsets, granulocyte adhesion molecules expression or apoptosis and necrosis.

Keywords
brain death, bone marrow downregulation, rats

Hemodynamic instability, microcirculatory dysfunction and inflammation triggered by brain death (BD) can compromise the viability of organs intended for transplantation (Barklin 2009; Chamorro *et al.* 2009; Barklin *et al.* 2013). The management of brain-dead potential donors aims to preserve the function of peripheral organs minimizing, therefore, the rate and intensity of acute rejection and graft dysfunction in the recipient (Barklin *et al.* 2013).

Acute brain injury can induce immunodeficiency thereby increasing morbidity and mortality after stroke, subarachnoid haemorrhage or trauma (Prass *et al.* 2003; Liesz *et al.* 2009). The bone marrow, an important hematopoietic organ, regulates the production and mobilization of blood cells, especially leucocytes, in physiologic and pathologic conditions (Opferman 2007). Indeed, it has been shown that haematopoiesis depends on the presence of growth factors, and expression of adhesion molecules by bone marrow leucocytes and sinusoidal endothelium (Mercier *et al.* 2012). Experimental findings support the evidence of a persistent leucopenia triggered by brain death (Takada *et al.* 2004; Silva *et al.* 2012; Simas *et al.* 2012). However, the mechanisms underlying this process remain unclear.

Therefore, the aim of this study was to evaluate leucocyte behaviour in both compartments, the bone marrow and peripheral blood. Bone marrow lymphocyte subsets, expression of granulocyte adhesion molecules and leucocyte apoptosis/necrosis in rats submitted to BD, and the influence of the associated trauma were all examined.

Material and methods

Animals and surgical procedures

Male Wistar rats weighing 250–350 g (2–3 months of age) were used. Animals were randomized in brain-dead group, rats in which brain death was induced (BD, $n = 10$), and

sham-operated group, rats trepanned only (SH, $n = 10$). Animals were anesthetized with isoflurane, intubated and mechanically ventilated (10 ml/kg of tidal volume, 70 breaths/min of frequency and 100% FiO_2). The carotid artery was cannulated for continuous mean arterial pressure (MAP) monitoring and blood sampling. The jugular vein was cannulated for infusion of saline solution (2 ml/h) in BD group to minimize dehydration.

Brain death model

Brain death was induced as previously described by Simas *et al.* (2012). Briefly, a balloon catheter (Fogarty 4F, Baxter Health Care, Deerfield, IL, USA) was placed into the intracranial cavity and quickly inflated with 0.5 ml of saline solution to increase intracranial pressure. After BD induction, anaesthesia was stopped, and BD was confirmed by maximal pupil dilatation, apnoea, absence of reflexes and a drop of the MAP. Sham-operated animals were trepanned only, and the anaesthesia was maintained throughout the experiments.

Blood cell counts

Total and differential white blood cell (WBC) counts were determined in blood samples obtained from the cut tip of the tail at baseline (0 min), 3 h and 6 h after surgical procedures. The analyses were performed using a haematologic analyser (Shenzhen Mindray Bio-Medical Electronics Co., Ltd., Shenzhen, China).

Bone marrow cell counts

Six hours after surgical procedures animals were exsanguinated, via the abdominal aorta, and the femur and the sternum were removed. Analyses of bone marrow cellularity were performed as follows: (i) in the femoral cavity lavage fluid on the basis of leucocyte morphological criteria; (ii) in H&E sections of the sternum bone marrow by histomorphometry. Bone marrow cells were harvested by flushing the femur cavity with 10 ml of Iscove's medium (Sigma Chemical Co., St. Louis, MO, USA) supplemented with 10% foetal calf serum (Cultilab, Campinas, SP, Brazil). After centrifugation at 500 ***g*** and 4°C for 10 min, cells were resuspended in a final volume of 2 ml Iscove's medium. Total cell counts were determined using a haemocytometer. Differential cell counts were performed on smears stained with May-Grünwald Giemsa solution (Sigma Chemical Co.). A total of 300 cells were counted and classified on the basis of normal morphological criteria. Results are presented as the number of cells $\times$ $10^3/mm^3$. For bone marrow histology, the sternum was fixed in 4% paraformaldehyde for 48 h, at room temperature and decalcified in 4% EDTA solution (pH 7.2) for 60 days. After decalcification, the sternum was processed by standard histological techniques (paraffin-embedding), and 3 μm sections were stained by H&E. Sections were examined at 200 $\times$ magnification in three randomly chosen areas (10^4 μm^2/area) in each of 10 non-successive microscopic fields of each slide (randomly chosen and coded). The number of leucocytes in the cellular compartment of the bone marrow was determined after the digital acquisition of the image of each microscopic field used for the analyses. The system used for image acquisition included a digital camera DS-Ri1 (Nikon, Tokyo, Japan) connected to the microscope (Nikon) and a computer. The images were processed using the software NIS-Elements-BR (Nikon). Results are presented as the number of cells/ 10^4 μm^2.

Flow cytometric analyses

After erythrocyte lysis, performed using sequential 0.2% and 1.6% NaCl solutions, aliquots (1×10^6 cells) of the femoral cavity lavage fluid were added to mAbs against: anti-rat CD3 phycoerythrin (PE) conjugated (clone G4.18, Abcam, Inc., MA, USA), anti-rat CD4 allophycocyanin (APC) conjugated (clone W3/25, AbD Serotec, Oxford, UK), anti-rat CD5 fluorescein isothiocyanate (FITC) conjugated (clone OX-19, Abcam), anti-rat CD8-PE (clone 341, Abcam), anti-rat CD11a-FITC (clone WT.1, Abcam), anti-rat CD11b/c-PE (clone MRC OX-42, Abcam) and anti-rat CD62L-FITC (clone OX-85, Abcam). Isotype controls were mouse immunoglobulin (Ig) G3κ-PE for CD3, IgG1-APC for CD4, IgG1-FITC for CD5, IgG1κ-PE for CD8, IgG2a-FITC for CD11a, IgG2a-PE for CD11b/c and IgG1-FITC for CD62L (Abcam, Inc., MA, USA). The working concentration for each antibody was determined in a preliminary titration experiment. Bone marrow cells were incubated with each antibody for 30 min at 4°C in the dark. Then, cells were washed twice with phosphate-buffered saline (PBS), and the sediment was resuspended in PBS containing 1% paraformaldehyde. Cells were acquired using FACSCanto II (Becton Dickinson, San Diego, CA, USA). Data from 10,000 cells were analysed by FlowJo (Becton Dickinson). Apoptosis and necrosis were characterized using annexin V-FITC (BD-Pharmingen, San Diego, CA, USA) and propidium iodide (PI, BD-Pharmingen). Briefly, 1×10^5 cells suspended in annexin V binding buffer (BD-Pharmingen) were stained with 5 μl annexin V-FITC and 5 μl PI. As positive apoptosis and positive necrosis controls 5 μM camptothecin (Sigma-Aldrich, Saint Louis, MO, USA) and 100 μl ethanol were used respectively. Double labelling was performed at room temperature for 25 min in the dark. To evaluate cell viability it was used the 7-AAD (7-Amino-Actinomycin D) Kit (BD-Pharmigen), according to manufacturer protocol. Cells were immediately acquired using FACSCanto II (Becton Dickinson). Data from 10,000 cells were analysed by FlowJo (Becton Dickinson). Viable cells were negative for both PI and annexin V; apoptotic cells were positive for annexin V and negative for PI, and late apoptotic cells or necrotic cells were positive for both annexin V and PI.

Statistical analysis

All data are presented as the mean ± standard error of the mean (SEM). Statistical analyses were performed using two-way analysis of variance complemented by Tukey's multiple comparisons test or the Student's unpaired *t* test using GraphPad Prism software version 6.07 (GraphPad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Ethical approval

The experimental protocol was approved by the Animal Subject Committee of the Heart Institute (InCor), University of São Paulo Medical School (approval number 3742/11/160).

Results

Hemodynamic parameters

The induction of BD triggered a sudden increase in MAP immediately after the catheter inflation. This increase was followed by a decrease in MAP below the baseline level returning to baseline after 1 h (Figure 1). In SH rats, no differences were observed in the MAP over time. No differences were observed in arterial blood gases, electrolytes, lactate and haematocrit values between BD and SH groups at baseline, 3 h and 6 h after surgical procedures (data not shown).

White blood cell counts

Total and differential WBC counts were performed at baseline, 3 h and 6 h after surgical procedures. As illustrated in Figure 2, in the SH group an increase in total WBC counts was observed at 3 and 6 h after trepanation compared to basal values, due to an increase in the number of circulating granulocytes. Lymphocyte counts decreased, and monocyte counts did not change significantly in SH group over time. In contrast, in the BD group a leucopenia was observed due to reduced numbers of lymphocytes and monocytes. Furthermore, granulocyte counts did not change at 3 and 6 h compared to basal values. When compared to SH rats, BD rats exhibited a reduction in the number of WBC counts from 44% at 3 h to 53% at 6 h after the surgical procedure. Numbers of granulocytes decreased approximately 55%, and monocytes 75%. Despite the fact that there were no differences in lymphocyte counts between BD and SH, granulocyte/lymphocyte ratios differed significantly. Values were (mean ± SEM): 2.35 ± 0.32 in the BD group, and 5.73 ± 0.48 in SH group ($P < 0.0001$).

Evaluation of bone marrow cells

At the end of the experiment, cells were harvested from the femur, and the sternum of the animals was prepared for histological analysis. Results, illustrated in Figure 3, showed that BD rats exhibited a reduction in bone marrow cellularity as depicted by the number of cells harvested from the femoral cavity (Figure 3a), and by the number of cells obtained by histomorphometric analysis (Figure 3b), compared to values attained in SH rats. As summarized in Table 1, a reduction in the number of lymphocytes (40%) and segmented cells (45%) was observed in BD rats, as demonstrated by the differential counting of bone marrow cells in the femoral cavity lavage fluid. There were no differences in the number of blast cells, granulocyte precursors, band cells, macrophages and eosinophils between BD and SH groups.

Bone marrow lymphocyte subsets

Flow cytometric analyses of lymphocyte subsets showed that, despite a 40% reduction in the number of bone marrow lymphocytes in BD rats, there were no differences in

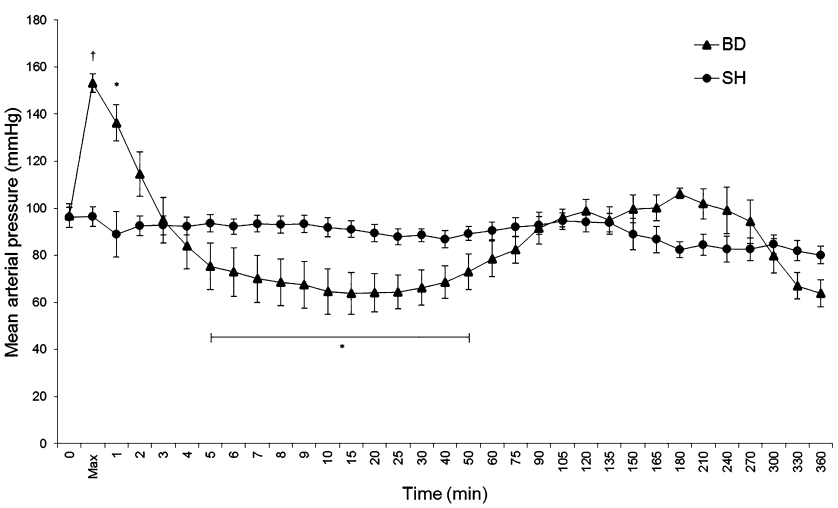


Figure 1 Mean arterial pressure of brain-dead (BD, $n = 10$) and sham-operated (SH, $n = 10$) rats 360 min after the surgical procedure. The animals were monitored over time. Data are presented as the mean ± SEM. $^{*}P < 0.05$, $^{\dagger}P < 0.0001$ *vs.* SH.

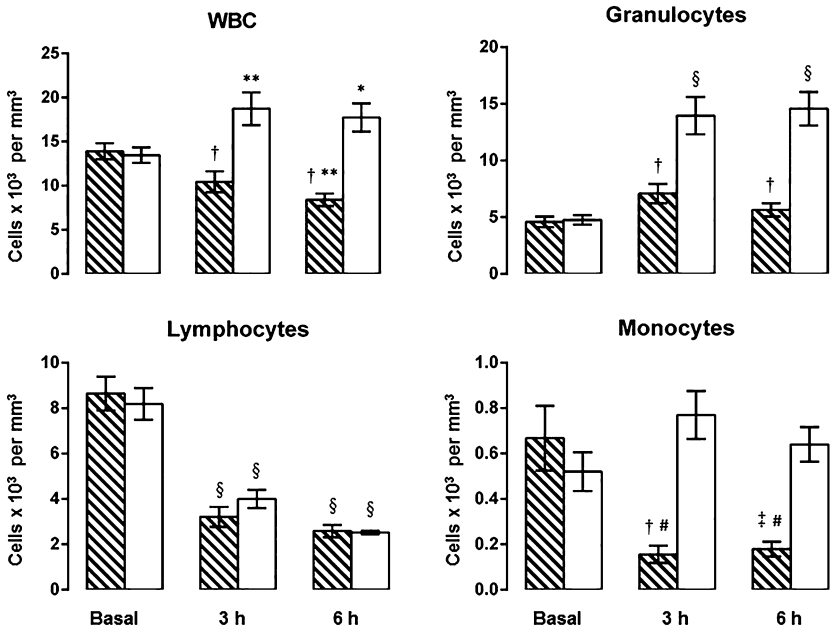


Figure 2 White blood cell (WBC) counts of brain-dead (hatched column, $n = 10$) and sham-operated (open column, $n = 10$) rats obtained at baseline, 3 h and 6 h after the surgical procedure. Data are presented as the mean ± SEM. $^{\ddagger}P < 0.01$, $^{\dagger}P < 0.0001$ *vs.* SH at the same time point. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{\#}P < 0.001$, $^{\S}P < 0.0001$ *vs.* Baseline.

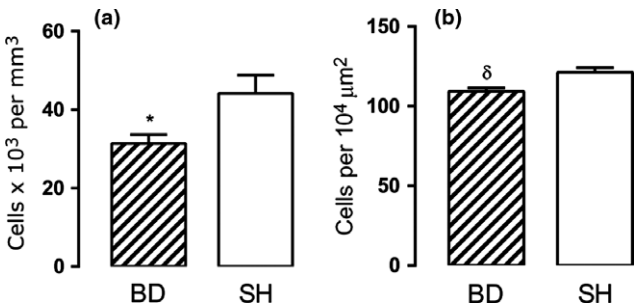
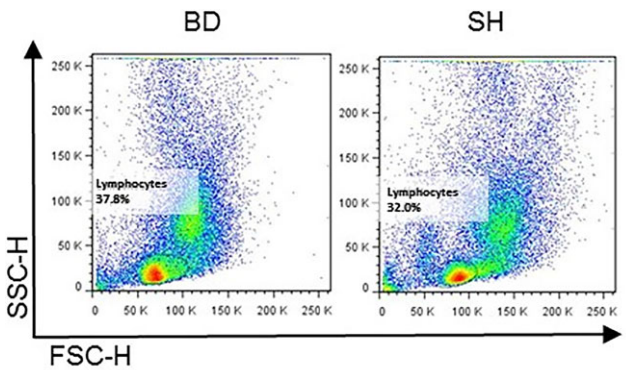


Figure 3 Evaluation of bone marrow cellularity in brain-dead (BD) and sham-operated (SH) rats 6 h after the surgical procedure. (a) bone marrow cell counts in the femoral cavity lavage fluid (10 animals/group). (b) bone marrow cell counts in the sternum (three areas/field; 10 fields/sample; seven rats/group). Data are presented as the mean ± SEM. $^{*}P = 0.0235$, $^{\delta}P = 0.0061$.

Table 1 Bone marrow cell counts

	BD	SH	*P* value
Lymphocytes (10^3/mm^3)	7.26 ± 1.44	12.08 ± 1.65	0.0410
Blast cells (10^3/mm^3)	2.30 ± 0.53	1.76 ± 0.36	0.4112
Granulocyte precursors (10^3/mm^3)	3.03 ± 0.50	3.37 ± 0.84	0.7255
Band cells (10^3/mm^3)	3.04 ± 0.62	4.44 ± 1.41	0.3763
Segmented cells (10^3/mm^3)	1.97 ± 0.25	3.57 ± 0.62	0.0287
Macrophages (10^3/mm^3)	0.34 ± 0.10	0.49 ± 0.13	0.3505
Eosinophils (10^3/mm^3)	1.99 ± 0.28	2.74 ± 0.44	0.1679

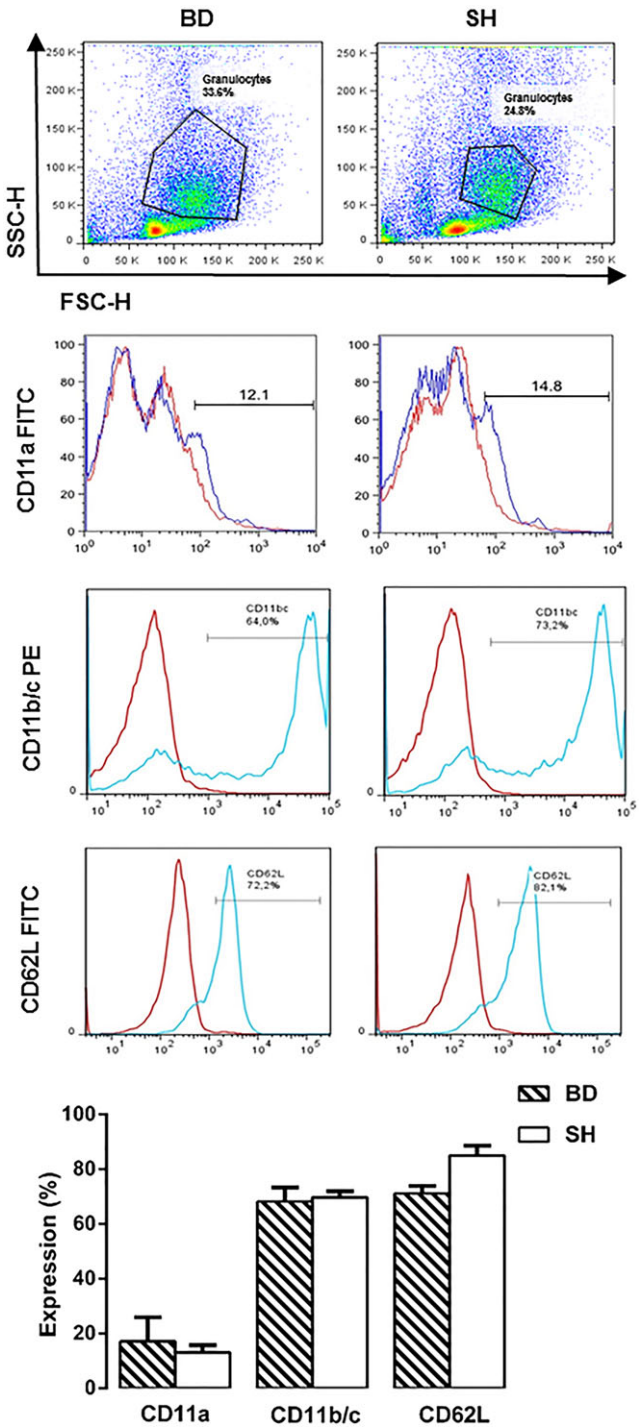
Differential counts of bone marrow cells harvested from the femoral cavity of brain-dead (BD) and sham-operated (SH) rats 6 h after the surgical procedure. Data are presented as the mean ± SEM for 10 animals/group.



Lymphocyte subsets (%)

	BD	SH	*P*
CD3⁺	30.3 ± 13.4	61.9 ± 3.4	0.100
CD4⁺	5.0 ± 2.7	1.2 ± 0.2	0.400
CD3⁺/CD4⁺	6.5 ± 2.1	11.6 ± 4.1	0.400
CD3⁺	28.2 ± 12.7	60.7 ± 5.1	0.100
CD5⁺	4.3 ± 2.6	1.0 ± 0.2	0.400
CD3⁺/CD5⁺	7.5 ± 1.9	11.6 ± 1.9	0.200
CD8⁺	0.9 ± 0.2	1.1 ± 0.1	0.800

Figure 4 Detection of CD3⁺, CD4⁺, CD5⁺ and CD8⁺ lymphocytes on bone marrow of brain-dead (BD) and sham-operated (SH) rats through flow cytometry. Dot plots of bone marrow cells depicting FSC and SSC scatter are presented. Cells gated as lymphocytes are encircled. Evaluation of lymphocyte subsets are presented as percentage of positive cells. Data are the mean ± SEM for five animals/group. [Colour figure can be viewed at wileyonlinelibrary.com].



CD3⁺, CD4⁺, CD5⁺ and CD8⁺ lymphocyte subpopulations between BD and SH groups (Figure 4).

Expression of adhesion molecules on bone marrow granulocytes

Expression of the adhesion molecules CD11a, CD11b/c and CD62L on bone marrow granulocytes was similar in both groups, BD and SH, as depicted by flow cytometric analyses (Figure 5).

Figure 5 Adhesion molecules expression on bone marrow granulocytes of brain-dead (BD) and sham-operated (SH) rats through flow cytometry. Dot plots of bone marrow cells depicting FSC and SSC scatter are presented in upper line. Granulocytes were gated by their FSCint–high/SSChigh phenotype. The total granulocyte population was selected from the FSCxSSC dot plot and subsequently plotted in a new histogram to calculate the percentages of CD11a, CD11b/c and CD62L-positive cells. Representative histograms of adhesion molecules expression (blue line) and isotype controls (red line) are presented in the following lines. Evaluation of the expression of CD11a, CD11b/c and CD62L in BD and SH groups is presented as the mean ± SEM for five animals/group. [Colour figure can be viewed at wileyonlinelibrary.com].

Analysis of apoptosis and necrosis

Viability of bone marrow cells was ≥95% in both groups of animals, as demonstrated by the percentage of 7-AAD-positive cells in bone marrow of BD (8.9 ± 0.5%) and SH (5.7 ± 1.3%, $P = 0.05$) rats. As illustrated in Figure 6, there were no significant differences in the percentage of Annexin V, PI and Annexin/PI-positive cells between BD and SH rats.

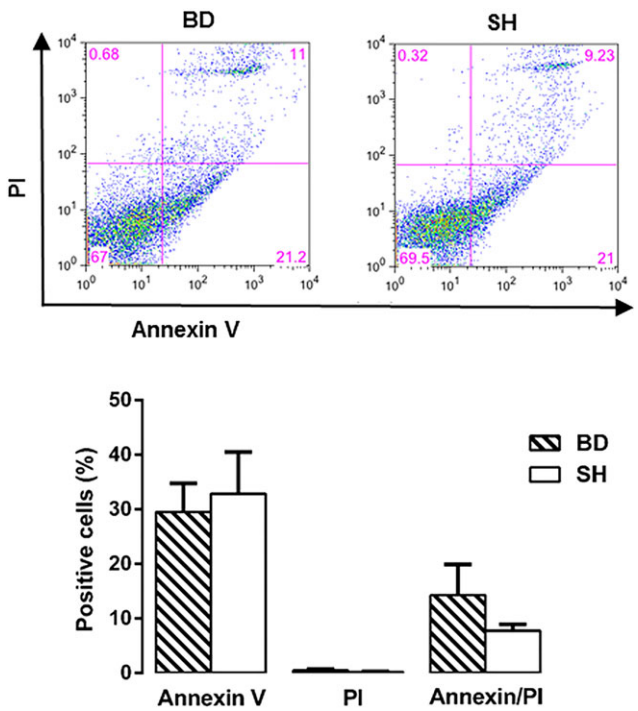


Figure 6 Flow cytometric analyses of apoptosis and necrosis in bone marrow cells of brain-dead (BD) and sham-operated (SH) rats using double labelling with annexin V and propidium iodide (PI). Data are presented as the mean ± SEM for five animals/group. [Colour figure can be viewed at wileyonlinelibrary.com].

Discussion

Data presented suggest that BD-induced leucopenia is associated with a downregulation of the bone marrow. This is supported by the following observations. First, reduced numbers of blood lymphocytes, monocytes and granulocytes, accounted for BD-induced leucopenia. Second, the total number of bone marrow cells decreased in BD rats, due to a reduction in lymphocyte and segmented counts. As leucocytosis with increased granulocyte/lymphocyte ratio exhibited by SH rats characterizes the response to trauma, the suggestion is that bone marrow downregulation was triggered by BD itself.

Experimental evidence and clinical evidence demonstrate that brain tissue damage leads to alterations of the peripheral immune system (Prass *et al.* 2003; Urra *et al.* 2009; Denes *et al.* 2011). As demonstrated herein, the bone marrow downregulation induced by BD was due to a significant reduction in the number of both lymphocytes and segmented cells. Lymphocytes accounted for the majority of bone marrow cells, being the dominant WBC. Indeed, the composition of rodents blood is dominated by B- and T-lymphocytes, whereas neutrophils are the most abundant leucocyte in the human blood (Rankin 2010; Rongvaux *et al.* 2013). In order to further investigate the reduced number of bone marrow cells, lymphocyte subpopulations were characterized by the expression of CD3 subunits of the T-cell receptor (TCR) complex; expression of the major histocompatibility complex (MHC)-binding co-receptors CD4 and CD8 on T cells (Wang & Reinherz 2012); and CD5 expression on naive T cells used to engage key TCR signalling pathways (Azzam *et al.* 2001; Fulton *et al.* 2015). Despite the fact that brain death induced a significant reduction in the number of bone marrow lymphocytes, there were no differences in the percentage of T lymphocyte subsets between BD and SH groups, as depicted by flow cytometry analyses. It has been shown that the acute-phase response to ischaemic stroke, characterized by increases in WBC counts and expression of pro-inflammatory cytokines (Emsley *et al.* 2003), is followed by a marked immunodepression, which includes lymphopenia, monocyte deactivation, upregulation of anti-inflammatory cytokines, lymphocyte apoptosis and splenic atrophy (Offner *et al.* 2006; Iadecola & Anrather 2011). In general, changes in cellular immune responses correlate with the severity of brain injury, occur within hours after the ischaemic insult and can last for up several weeks (Meisel *et al.* 2005).

As demonstrated in the present study, BD rats exhibited neutropenia and reduced numbers of bone marrow segmented cells. It has been shown that the major determinants of the number of circulating neutrophils are production, bone marrow egress, margination and extravasation/clearance (Bugl *et al.* 2012; Manz & Boettcher 2014). The release from the bone marrow depends on neutrophil migration across the sinusoidal endothelium which expresses constitutive adhesion molecules including P-selectin, intercellular adhesion molecule (ICAM)-1 and vascular cell adhesion molecule (VCAM)-1 (Schweitzer *et al.* 1996; Ley *et al.* 2007; Rankin 2010). In the attempt to investigate BD-induced decrease in segmented cells, the expression of L-selectin (CD62L) and beta$_2$ integrins (CD11a, CD11bc) on bone marrow granulocytes were analysed through flow cytometry. As demonstrated herein, there were no differences in the expression of these adhesion molecules on bone marrow granulocytes between BD and SH groups.

In addition, flow cytometric analyses of apoptosis and necrosis in bone marrow cells showed no differences between BD and SH rats. Apoptosis plays a critical role in maintaining an appropriate balance between progenitor cells or differentiated cells and elimination of damaged or non-functional cells. Many of these events are regulated by growth factors, cytokines and cellular interactions that promote the survival, proliferation and differentiation of all cells and progenitor subsets of blood cells (Opferman 2007). Reduced levels of hematopoietic growth factors, cytokines and chemokines could be associated with BD-induced downregulation of the bone marrow and leucopenia. Further investigation will be required to identify the underlying mechanism.

Vascular sinusoids allow communication of the hematopoietic tissue with the peripheral circulation. The bone marrow provides a framework of niches that support the number and function of hematopoietic stem cells and immune cells, integrating inputs from the organism such as innervation, vascular perfusion and oxygenation (Mercier *et al.* 2012). It has been shown that the extensive apoptotic loss of lymphocytes in lymphoid organs and peripheral blood induced by stroke is related to the activation of the sympathetic nervous system, and the hypothalamic–pituitary–adrenal axis, resulting in a systemic release of catecholamines and steroid hormones (Prass *et al.* 2003; Mracsko *et al.* 2014). Indeed, interactions among the central nervous system, the hypothalamic–pituitary–adrenal axis and components of the innate and adaptive immune system play key roles in the regulation of inflammation (Webster *et al.* 2002). The rapid activation of these neuroendocrine pathways during inflammation protects the individual against an overwhelming immune response, but can inappropriately suppress the immune system when activated without a systemic inflammation (Prass *et al.* 2003).

Taken into account the neuroendocrine-mediated systemic immune-supression after acute brain injury, two major points should be addressed when evaluating the role of BD in graft outcomes in both experimental and clinical settings. First, it is known that a strong stimulation of the sympathetic nervous system is usually seen with BD. Marked tachycardia and hypertension documented for a few minutes are replaced by vasoplegia and hypotension (Novitzky *et al.* 1986; Woiciechowsky *et al.* 1998; Kutsogiannis *et al.* 2006; Silva *et al.* 2012; Simas *et al.* 2012). Second, BD impairs the endocrine system and decreases the release of hormones, such as triiodothyronine (T3), thyroxine (T4), cortisol and anti-diuretic hormone, thereby suggesting an interruption

along the hypothalamic–pituitary–adrenal axis (Pratschke *et al.* 1999; Wilhelm *et al.* 2000; Simas *et al.* 2012). Although the release of high levels of catecholamines and adrenal steroids hormones is an essential component of the response to BD, these stress mediators do not have long-lasting effects, as previously registered in brain-dead patients and demonstrated in experimental models of BD (Herijgers *et al.* 1996; Floerchinger *et al.* 2012; Simas *et al.* 2012). Notwithstanding this, the initial response to these stress mediators is characterized by vasoconstriction, hypoperfusion, hypovolaemia and tissue hypoxia. Indeed, it has been demonstrated that early after the induction of BD, by inflation of an intracranial balloon in rats, brain perfusion is virtually absent as well regional flow in several vascular beds (Herijgers *et al.* 1996). Using intravital microscopy to observe the rat mesenteric microcirculation an immediate hypoperfusion of mesenteric microvessels, triggered by BD itself (Simas *et al.* 2012) was observed. Accordingly, the initial feature of BD includes hemodynamic instability with compromised organ perfusion and reduced oxygenation, contributing to a reduced quality of organs from BD donors. Finally, studies focused on stroke have demonstrated the importance of impaired cellular immune responses for the increased susceptibility to infection after cerebral injury (Prass *et al.* 2003; Liesz *et al.* 2009). A better understanding of the pathophysiology of BD-associated bone marrow downregulation will allow define novel and effective treatments of the BD donor.

In conclusion, data presented suggest that the downregulation of the bone marrow is triggered by BD itself, and it is not related to changes in lymphocyte subsets, granulocyte adhesion molecules expression or apoptosis and necrosis.

Conflict of interest

There is no conflict of interest from any of the authors. The sponsor had no role on the design of the study, the collection and analysis of the data, or in the preparation of the manuscript.

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Author contribution

LM involved in surgical procedures, data analysis and interpretation, draft and review of the manuscript. RS, FLZ involved in surgical procedures and interpretation of data. JFJ involved in flow cytometry assays. LFFS, JMC involved in histological analysis. PB involved in study design, histological data analysis and interpretation. LFPM involved in study conception and design, statistical analysis and interpretation of data. PS involved in study conception and design, data analysis and interpretation, draft and review of the manuscript. All authors involved in approval of manuscript.

References

Azzam H.S., DeJarnette J.B., Huang K. *et al.* (2001) Fine tuning of TCR signaling by CD5. *J. Immunol.* **166**(9), 5464–5472.

Barklin A. (2009) Systemic inflammation in the brain-dead organ donor. *Acta Anaesthesiol. Scand.* **53**, 425–435.

Barklin A., Hvas C.L., Toennesen E. (2013) The inflammatory response to brain death. In: *The Brain-Dead Organ Donor: Pathophysiology and Management*, pp. 107–119 (eds D. Novitzky & D.K. C. Cooper), New York: Springer Science+Business Media.

Bugl S., Wirths S., Müller M.R. *et al.* (2012) Current insights into neutrophil homeostasis. *Ann. N. Y. Acad. Sci.* **1266**, 171–178.

Chamorro C., Falcón J.A. & Michelena J.C. (2009) Controversial Points in Organ Donor Management. *Transplant. Proc.* **41**, 3473–3475.

Denes A., McColl B.W., Leow-Dyke S.F. *et al.* (2011) Experimental stroke-induced changes in the bone marrow reveal complex regulation of leukocyte responses. *J. Cereb. Blood Flow Metab.* **31**, 1036–1050.

Emsley H.C.A., Smith C.J., Gavin C.M. *et al.* (2003) An early and sustained peripheral inflammatory response in acute ischemic stroke: relationships with infection and atherosclerosis. *J. Neuroimmunol.* **139**, 93–101.

Floerchinger B., Oberhuber R. & Tullius S.G. (2012) Effects of brain death on organ quality and transplant outcome. *Transplant. Rev.* **26**, 54–59.

Fulton R.B., Hamilton S.E. & Xing Y. (2015) The TCR's sensitivity to self peptide-MHC dictates the ability of naive CD8(+) T cells to respond to foreign antigens. *Nat. Immunol.* **16**(1), 107–117.

Herijgers P., Leunens V., Tjandra-Maga T.B. *et al.* (1996) Changes in organ perfusion after brain death in the rat and its relation to circulating catecholamines. *Transplantation* **62**(3), 330–335.

Iadecola C. & Anrather J. (2011) The immunology of stroke: from mechanisms to translation. *Nat. Med.* **17**(7), 796–808.

Kutsogiannis D.J., Pagliarello G., Doig C. *et al.* (2006) Medical management to optimize donor organ potential: review of the literature. *Can. J. Anaesth.* **53**, 820–830.

Ley K., Laudanna C., Cybulsky M.I. *et al.* (2007) Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat. Rev. Immunol.* **7**(9), 678–689.

Liesz A., Hagmann S., Zschoche C. *et al.* (2009) The spectrum of systemic immune alterations after murine focal ischemia. Immunodepression versus immunomodulation. *Stroke.* **40**, 2849–2858.

Manz M.G. & Boettcher S. (2014) Emergency granulopoiesis. *Nat. Rev. Immunol.* **14**(5), 302–314.

Meisel C., Schwab J.M., Prass K. *et al.* (2005) Central nervous system injury-induced immune deficiency syndrome. *Nat. Rev. Neurosci.* **6**, 775–786.

Mercier F.E., Ragu C. & Scadden D.T. (2012) The bone marrow at the crossroads of blood and immunity. *Nat. Rev. Immunol.* **12**, 49–60.

Mracsko E., Liesz A., Karcher S. *et al.* (2014) Differential effects of sympathetic nervous system and hypothalamic–pituitary–adrenal axis on systemic immune cells after severe experimental stroke. *Brain Behav. Immun.* **41**, 200–209.

Novitzky D., Wicomb W.N., Cooper D.K. *et al.* (1986) Prevention of myocardial injury during brain death by total cardiac

sympathectomy in the Chacma baboon. *Ann. Thorac. Surg.* **41**, 520–524.

Offner H., Subramanian S., Parker S.M. *et al.* (2006) Splenic atrophy in experimental stroke is accompanied by increased regulatory T cells and circulating macrophages. *J. Immunol.* **176**, 6523–6531.

Opferman J.T. (2007) Life and death during hematopoietic differentiation. *Curr. Opin. Immunol.* **19**, 497–502.

Prass K., Meisel C., Höflich C. *et al.* (2003) Stroke-induced immunodeficiency promotes spontaneous bacterial infections and is mediated by sympathetic activation reversal by poststroke T helper cell type 1-like immunostimulation. *J. Exp. Med.* **198**(5), 725–736.

Pratschke J., Wilhelm M.J., Kusaka M. *et al.* (1999) Brain death and its influence on donor organ quality and outcome after transplantation. *Transplantation* **67**, 343–348.

Rankin S.M. (2010) The bone marrow: a site of neutrophil clearance. *J. Leukoc. Biol.* **88**(2), 241–251.

Rongvaux A., Takizawa H., Strowig T. *et al.* (2013) Human hemato-lymphoid system mice: current use and future potential for medicine. *Annu. Rev. Immunol.* **31**, 635–674.

Schweitzer K.M., Dräger A.M., van der Valk P. *et al.* (1996) Constitutive expression of E-selectin and vascular cell adhesion molecule-1 on endothelial cells of hematopoietic tissues. *Am. J. Pathol.* **148**(1), 165–175.

Silva I.A., Correia C.J., Simas R. *et al.* (2012) Inhibition of autonomic storm by epidural anesthesia does not influence cardiac inflammatory response after brain death in rats. *Transplant. Proc.* **44**(7), 2213–2218.

Simas R., Sannomiya P., Cruz J.W.M.C. *et al.* (2012) Paradoxical effects of brain death and associated trauma on rat mesenteric microcirculation: an intravital microscopic study. *Clinics* **67**(1), 69–75.

Takada M., Toyama H., Tanaka T. *et al.* (2004) Augmentation of interleukin-10 in pancreatic islets after brain death. *Transplant. Proc.* **36**(5), 1534–1536.

Urra X., Cervera A., Obach V. *et al.* (2009) Monocytes are major players in the prognosis and risk of infection after acute stroke. *Stroke* **40**, 1262–1268.

Wang J.H. & Reinherz E.L. (2012) The structural basis of αβ T-lineage immune recognition: TCR docking topologies, mechanotransduction, and co-receptor function. *Immunol. Rev.* **250**(1), 102–119.

Webster J.I., Tonelli L. & Sternberg E.M. (2002) Neuroendocrine regulation of immunity. *Annu. Rev. Immunol.* **20**, 125–163.

Wilhelm M.J., Pratschke J., Laskowski I.A. *et al.* (2000) Brain death and its impact on the donor heart–lessons from animal models. *J. Heart Lung Transplant.* **19**, 414–418.

Woiciechowsky C., Asadullah K., Nestler D. *et al.* (1998) Sympathetic activation triggers systemic interleukin-10 release in immunodepression induced by brain injury. *Nat. Med.* **4**(7), 808–813.