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Original Article

Reactive oxygen species production triggers green tea-induced anti-leukaemic effects on acute promyelocytic leukaemia model

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ABSTRACT

Green tea (GT) has been consumed as a beverage for thousands of years because of its therapeutic properties observed over time. Because there is no sufficient evidence supporting the protective role of tea intake during the development of acute myeloid leukaemia, we herein study GT extract effects on an acute promyelocytic leukaemia model. Our results demonstrated that GT reduces leucocytosis and immature cells (blasts) in peripheral blood, bone marrow (BM), and spleen of leukaemic mice, parallel with an increase of mature cells in the BM. In addition, GT induces apoptosis of cells in the BM and spleen, confirmed by activation of caspase-3, -8 and -9; GT reduces the malignant clones $CD34^+$ and CD117+ in the BM and reduces CD117+ and Gr1+ immature myeloid cells in the spleen; GT increases intracellular reactive oxygen species (ROS) in the BM Gr1+ cells while reducing $CD34^+$ and CD117+ cells; GT reduces CXCR4 expression on $CD34^+$ and CD117+ cells, and reduces the nuclear translocation of HIF-1α. GT has anti-proliferative effects in leukaemia *in vivo* by inhibiting malignant clone expansion, probably by modulating the intracellular production of ROS.

1. Introduction

Acute myeloid leukaemia (AML) is a form of cancer characterised by infiltration of the bone marrow (BM), blood, and other tissues by proliferative, clonal, abnormally differentiated, and occasionally poorly differentiated cells of the haematopoietic system. AML is generally a disease of older persons and is uncommon before the age of 45 years. Despite cure in 35%–40% of adult patients of age 60 years or younger, the outcome of AML in older patients unable to receive BM transplantation or intensive chemotherapy without the unacceptable side effects remains dismal, with a median survival of only 5- to 10 months [1]. This greatly encourages further discovery of potential new agents, particularly nature products.

Tea is one of the most consumed beverages in the world, only second to water. Green tea (GT) produced from the fresh leaves of *Camellia sinensis* has been largely consumed in China and Japan for more than 4000 years and represents 20% of tea consumption. Epidemiologic studies have already shown that GT consumption is beneficial to health and can reduce the incidence of cancer [2]. Many laboratory studies have shown the inhibition of tumourigenesis by GT and its polyphenols in different animal models [3–5].

Most of the anti-cancer activities of GT are believed to be related to the presence of polyphenols, especially catechins. GT contains

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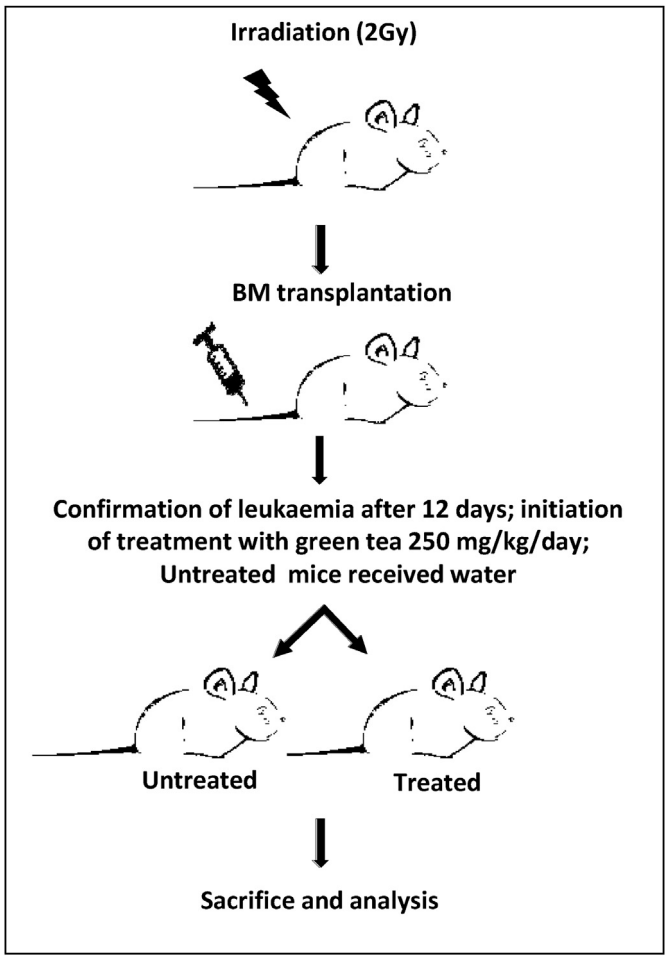

Fig. 1. Leukaemia transplant model. After 4- to 6 h of 2 Gy sublethal cobalt irradiation, 1×10^6 leukaemic cells obtained from PML-RARa transgenic mice were intravenously injected in the tail vein of 12- to 16-week-old NOD.CB17-Prkdc^{scid}/J mice. Haematology counts were monitored weekly, and the following criteria were used for the diagnosis of leukaemia: presence of at least 1% of blasts in peripheral blood associated with leucocytosis above 30,000 cells/L, haemoglobin levels below 10 g/dL, and thrombocytopaenia below 500×10^3 cells/L, as previously described [10]. Twelve days after transplantation, mice were submitted to daily oral treatment (gavage) with 250 mg/kg/day green tea or vehicle only (water) for 5 consecutive days.

four main catechins: epigallocatechin-3-gallate (EGCG), epigallocatechin, epicatechin gallate, and epicatechin [6]. Among these four main catechins, EGCG constitutes the most abundant and effective polyphenolic compound present in tea extracts. This compound is rapidly absorbed and distributed throughout all tissues due to a longer half-life. However, when administered alone, the half-life and bioavailability are reduced. This property is related to its complexation, competition, metabolisation, and interaction with others GT extract compounds [7]. In the present study the whole extract of GT was used, based on the extensive consumption of the beverage by the population, and it might be more effective than EGCG alone.

Because there is no sufficient evidence supporting the protective role of tea intake in the development of AML, we herein investigated the *in vivo* effects of the whole extract of GT using acute promyelocytic leukaemia (APL) model.

2. Materials and methods

2.1. Leukaemia transplant model and treatment

NOD/SCID mice (The Jackson Laboratory) were bred and maintained under pathogen-free conditions at the Universidade Estadual de Campinas (UNICAMP). Leukaemia transplant model (Fig. 1) was performed as previously described [8–10]. Briefly, leukaemic cells obtained from PML/RARa transgenic mice were maintained at - 80 °C. Before transplantation, the cells were thawed and resuspended in RPMI 1640 medium with 10% foetal bovine serum. After 4- to 6 h of sublethal irradiation with 2Gy, 1×10^6 cells were intravenously injected into the tail vein of 12- to 16-week-old mice. Haematology counts were monitored weekly, and the following criteria were used for the diagnosis: presence of at least 1% of blasts in peripheral blood (PB) plus leucocytosis above 30,000 cells/L, haemoglobin levels below 10 g/dL, and thrombocytopaenia below 500×10^3 cells/L. After the confirmation of leukaemia (12th day), mice were submitted to daily oral treatment with 250 mg/kg/day GT containing 50% polyphenols (Galena Química Farmacêutica, Brazil) or vehicle only (water) for 5 consecutive days and named as treated ($n = 5$) and untreated ($n = 4$), respectively. All procedures were conducted according to National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Experimentation Ethics Committee (CEUA/UNICAMP). NOD/SCID non-leukaemic mice were used as wild-type (Wt) controls.

2.2. In vivo fluorescence imaging for monitoring the development of the disease

Leukaemic cells were labelled with nanocrystals (Qtracker 800 Cell Labeling Kit, Molecular Probes, Leiden, Netherlands) according to the manufacturer's instructions and transplanted into irradiated mice (2 Gy) via the retro-orbital route (1×10^6 cells/animal), 4 h after irradiation. Cell dissemination was accompanied at different time points using an *in vivo* imaging analyser (In vivo FXBRO, Bruker, USA). Excitation and emission wavelengths were set at 710 and 790 nm, respectively, and the imaging time was 2.5 min. Radiographs were obtained from mice to determine the anatomical localization of the labelling. During imaging procedures, animals were anaesthetized (isoflurane 3%). To exclude possible unspecific labelling, a negative control (non-cell-recipient mouse) was imaged together with transplanted mice. Special care was taken with the fur of the animal and was completely removed from imaging areas, as well as with the skin, that was cleaned to avoid fluorescence interfering materials (food and cage substrate).

2.3. Haematological parameters

At day 16, mice were sacrificed after being subjected to a cardiac puncture to obtain peripheral blood (PB) samples. Automated counts for white blood cells were performed using a CELL-DYN Emerald Haematology System counter (Abbott Laboratories, Lake Bluff, Illinois, USA), and differential counts were obtained from Leishman-Wright-Giemsa–stained smears. For morphological analysis, 100 cells of PB were counted and myeloid cells were classified as immature (promyelocyte plus blast), intermediate (myelocyte plus metamyelocyte), or mature (granulocytes plus monocytes), according to the Bethesda criteria [11]. BM cells were obtained by flushing the bone cavities with RPMI 1640. Spleen cells were obtained after maceration with RPMI 1640. Cytospin slides were prepared and stained with Leishman-Wright-Giemsa.

2.4. Immunophenotyping of BM, spleen, and PB cells

BM and spleen cells were characterised using the following mAbs (BD Pharmingen): CD117-FITC (immature myeloid cells); CD34-Alexa647 (haematopoietic stem cells); CD45-PerCP (pan leukocyte marker); Gr-1-PE (immature myeloid cells). PB cells were characterised using: CD3-APC (T cells); Ly-6G-PE (granulocytes); CD11b-PE (monocytes); CD45-PerCP (pan leukocyte marker). Fluorochrome-conjugated isotype antibodies were used as negative controls. For the exclusion of erythrocytes, cells were selected as the percentage of CD45-positive. A minimum of 10,000 events/sample were acquired with a FACScalibur flow cytometer. Viable CD45-positive cells were gated, and the percentage of each cell subset was determined using the FlowJo software.

2.5. Imaging flow cytometry

Nuclear translocation of HIF-1α was studied using HIF-1α-Alexa 488 and 7-AAD (Biolegend) for nuclei stain. BM cells were stained and fixed in 4% paraformaldehyde prior to the collection of 500–10,000 events per sample. Magnification of 60× was used for all images shown. Data acquisition was performed on an imaging flow cytometer (ImagestreamX; Amnis/EMD Millipore, Seattle, WA, USA). Image analysis was performed with IDEASVR software as previously described [12]. Briefly, the CD117 and CD34 population was hierarchically gated for single cells, focus quality, and their respective relevant fluorescence intensity. To assess nuclear HIF-1α translocation, the corresponding nuclear (7-AAD) image and HIF-1α (Santa Cruz conjugated to Alexa 488 from Molecular Probes) image of each cell was compared and a similarity score was assigned for individual cells.

2.6. Apoptosis detection

Cells were seeded and then resuspended in binding buffer containing 1 mg/mL propidium iodide and 1 mg/mL FITC-labelled Annexin-V (BD Pharmingen). All specimens were analysed using FACScalibur flow cytometry after incubation for 15 min at room temperature in a light-protected area. Ten thousand events were acquired for each sample. Analysis was carried out using the FlowJo software.

2.7. Caspase activity

Cells were incubated with 1 mL of FITC–DEVD–FMK or RED–IETD–FMK or RED–LEHD–FMK (Calbiochem, Merck) for 1 h in a 37 °C incubator with 5% CO_2, and the cells were washed and resuspended with wash buffer. Caspase activity was detected on a FACScalibur flow cytometer and analysis was carried out using the FlowJo software.

2.8. ROS

Intracellular ROS generation was measured by flow cytometry following staining with 25 μmol/L DCFDA (Sigma). Acquisition of cells was performed on a FACScalibur flow cytometer and analysis was carried out using the FlowJo software.

2.9. Plasma polyphenol levels

Total plasma EGCG levels were measured at the end of the fifth day of treatment. Blood was collected into MiniCollect® (Grenier Bio-One) containing sodium heparin, and plasma was removed after centrifugation. Plasma was then aliquoted into cryotubes containing a solution of 20% acid ascorbic with 0.05% EDTA and stored at −80 °C until analysis. Plasma levels of EGCG were then measured using an established high-performance liquid chromatography procedure [13].

2.10. Statistical analysis

For comparison, an appropriate Student *t*-test or analysis of variance was performed. All statistical analyses were performed using Prism version 5.0a software (GraphPad Software). Group comparisons were considered significant for $P < 0.05$.

3. Results

3.1. Evaluation of the leukaemic transplant model

To monitor the progression of the disease, transplanted mice were bled from the retro-orbital plexus 12 days after transplantation. Corroborating obtained parameters [8–10], here again haematological analysis revealed that the transplant was successful as demonstrated by leucocytosis (103 ± 16.9 *vs* Wt 3.9 ± 0.3; $P < 0.0001$), thrombocytopaenia (112 ± 9.8 *vs* Wt 959 ± 68.1; $P < 0.0001$) and anaemia (11.1 ± 0.6 *vs* Wt 13.5 ± 0.5; $P < 0.0001$) observed in the PB (Fig. 2A). There was also an increase in the number of blast cells in the PB (Fig. 2B–C) and an increase in the volume of spleens from leukaemic mice (Fig. 2D). Furthermore, the development of the disease was monitored by *in vivo* fluorescence imaging confirming the infiltration of immature cells in organs and BM of leukaemic animals in different time points (Fig. 2E). One day after transplantation, cell labelling was mostly detected in the spleen and neck, including cervical vertebrae. At day 3, labelling was drastically reduced in the spleen, because part of the cells died and only the survivors were visualised. Weak labelling at day 7 was due to the homing of surviving cells into the niche (bone marrow) to their establishment and proliferation. At this time point, leukopaenia and 1% of blasts were detected in the peripheral blood. At day 11, after the establishment into the niche and consequent proliferation, the cells were disseminated into the peripheral blood (40% of blasts were detected as shown in Fig. 2B) and to the organs (strong labelling areas in the spleen, liver, ribcage bones, jaw, and posterior legs were detected; Fig. 2E).

3.2. Effect of GT in the haematological response of leukaemic mice

As mentioned, an important effect observed during the leukaemic phase was an increase in the number of white blood cells in leukaemic mice. The treatment with 250 mg/kg GT for 4 days reduced the percentage of white blood cells (120 ± 56 *vs* untreated 178 ± 42; $P < 0.001$) in the PB (Fig. 3A). Consistent with these haematological changes in the number of white blood cells, analysis by flow cytometry of CD3 (lymphocytes), CD11b (monocytes), and Gr-1 (neutrophils) populations in the PB of leukaemic mice showed a slightly decreased percentage of CD11b and Gr-1 cells after treatment with GT, with no changes in the CD3 cells (Fig. 3C–E). In addition, analysis of Leishman-Wright-Giemsa–-stained smears showed a reduction in the percentage of blasts in the PB of treated mice (78 ± 2.8 *vs* untreated 88 ± 3.2; $P < 0.05$) (Fig. 3F, Table 1). No significant changes were observed in the number of platelets and haemoglobin levels after treatment with GT (Fig. 3B). Another important observation during the leukaemic phase was an increase in the number of immature cells in the BM, typically characteristic of promyelocytes. The results showed a reduction in the percentage of immature cells (promyelocyte plus blast) parallel to an increase of mature cells (granulocytes plus monocytes) after treatment with GT, according to Bethesda criteria [11] for leukaemia in mice (Table 1). Furthermore, analysis of Leishman-Wright-Giemsa staining showed a reduction in the percentage of immature cells (promyelocyte plus blast) in the spleen (81 ± 1.1 *vs* untreated 89 ± 1.4; $P < 0.05$) of leukaemic mice after treatment with GT (Table 1).

3.3. Effects of GT in the apoptosis of total cells of BM and spleen

As leukaemic mice treated with GT presented a reduction of blasts in the BM, spleen, and PB, we then investigated whether this was related to apoptosis, which was studied in BM and spleen because these are the sites where apoptosis, proliferation, self-renewal, and differentiation occur. Fig. 4 shows the percentage of apoptotic cells in the BM of leukaemic mice. Treatment with GT significantly increased apoptotic cells in the BM (29.4 ± 5.2 *vs* untreated 21.0 ± 2.1; $P < 0.05$) and spleen (13.9 ± 3.1 *vs* untreated 9.2 ± 1.9; $P < 0.05$) of mice (Fig. 4A–D). We next evaluated changes in caspase activity. We found that GT treatment induced an increase in the median fluorescence intensity (MFI) of cleaved caspase-3 in the BM (83.9 ± 3.6 *vs* untreated 72.6 ± 4.7, $P < 0.05$) and in the spleen (75.5 ± 28.2 *vs* untreated 55.8 ± 7.3; $P < 0.01$); cleaved caspase-8 in the BM (117.3 ± 9.9 *vs* untreated 89.1 ± 12.3; $P < 0.005$) and in the spleen (118.0 ± 31.5 *vs* untreated 81.5 ± 14.8; $P < 0.001$); and cleaved caspase-9 in the BM (138.2 ± 52.4 *vs* untreated 85.8 ± 12.9; $P < 0.001$) and in the spleen (121.7 ± 49.2 *vs* untreated 76.5 ± 21.9; $P < 0.001$) of leukaemic mice (Fig. 4E–H).

3.4. Effect of GT on CD34, CD117, and Gr-1 cell infiltration in the BM and spleen

We next investigated whether GT had any effect against the malignant clones in the principal haematopoietic compartment, the BM. Treatment with GT reduced the percentage of $CD34^+$ haematopoietic progenitor cells (32.4 ± 2.3 *vs* untreated 41.0 ± 0.5) as well as of CD117+ cells (33.4 ± 3.7 *vs* untreated 44.2 ± 1.8), constituting a notable anti-tumour effect (Fig. 5A). We then evaluated the phenotype of cells infiltrated in the spleen. Interestingly, we found that GT induces a decrease in the percentage of CD117+ (40.7 ± 0.3 *vs* untreated 44.6 ± 0.9) and Gr-1+ cells (60.8 ± 0.2 *vs* untreated 65.6 ± 0.5) present in the spleen (Fig. 5B).

3.5. ROS production triggers GT-induced anti-leukaemic effects

We then analysed the effects of GT in the production of intracellular ROS in BM subpopulations of CD34, CD117, and Gr-1 from leukaemic mice. Fig. 6A showed a significant increase in the MFI of intracellular ROS production by Gr-1+ cells of mice treated with GT when compared with untreated mice (670 ± 103 *vs* untreated 428.5 ± 5.2). Interestingly, GT treatment induced a reduction of MFI of intracellular ROS production in the $CD34^+$ (167.5 ± 27.1 *vs* untreated 405.5 ± 73.3) (Fig. 6B and D) and CD117+ (360 ± 142 *vs* untreated 1635 ± 40.4) cells (Fig. 6C–D).

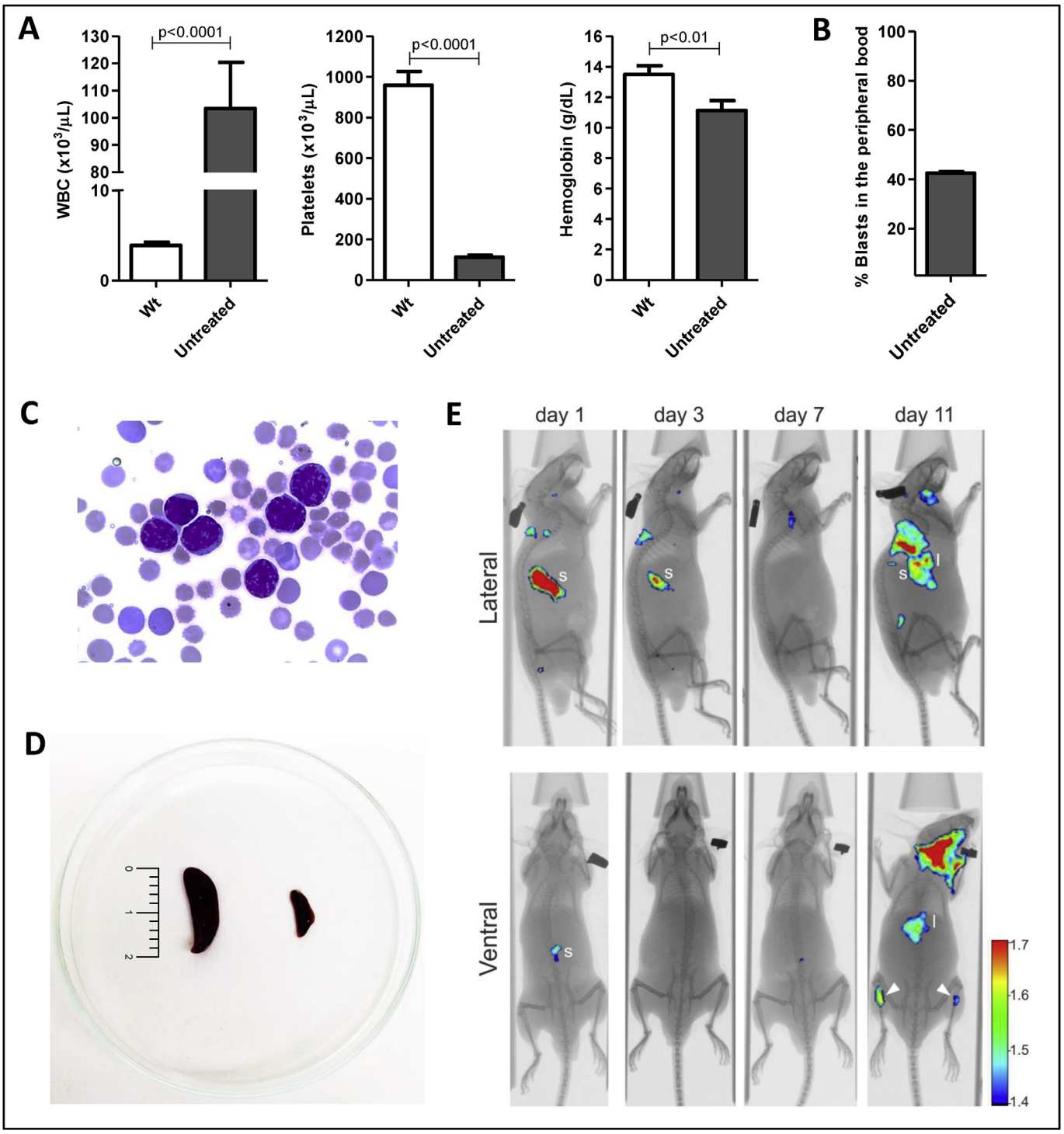

Fig. 2. Development of leukaemia transplant model. To confirm the success of the transplant, the mice were bled from the retro-orbital plexus 12 days after transplantation. (**A**) The increase in the total number of leukocytes, thrombocytopaenia and anaemia, is evidence of the progression of the disease. NOD/SCID mice non-leukaemic were used as wild-type (Wt) controls. (**B**) An increase in the number of undifferentiated cells, characteristic of blast cells, in the peripheral blood was observed. (**C**) Representative photomicrograph of Leishman-Wright-Giemsa–stained smears from peripheral blood of untreated mice (100 ×); evidence of the presence of blasts. (**D**) Representative photo of spleens from untreated (left) and Wt (right) mice. (**E**) *In vivo* imaging of leukaemic cell dissemination in untreated mice. Nano-crystal-labelled leukaemic cells were transferred to irradiated mice and followed by *in vivo* imaging. Lateral and ventral planes are shown. s, spleen; l, liver; arrowheads indicate posterior leg labelling. Intensity scale: x 10^7 photons/s/mm^2. Images are representative of multiple experiments (n = 4).

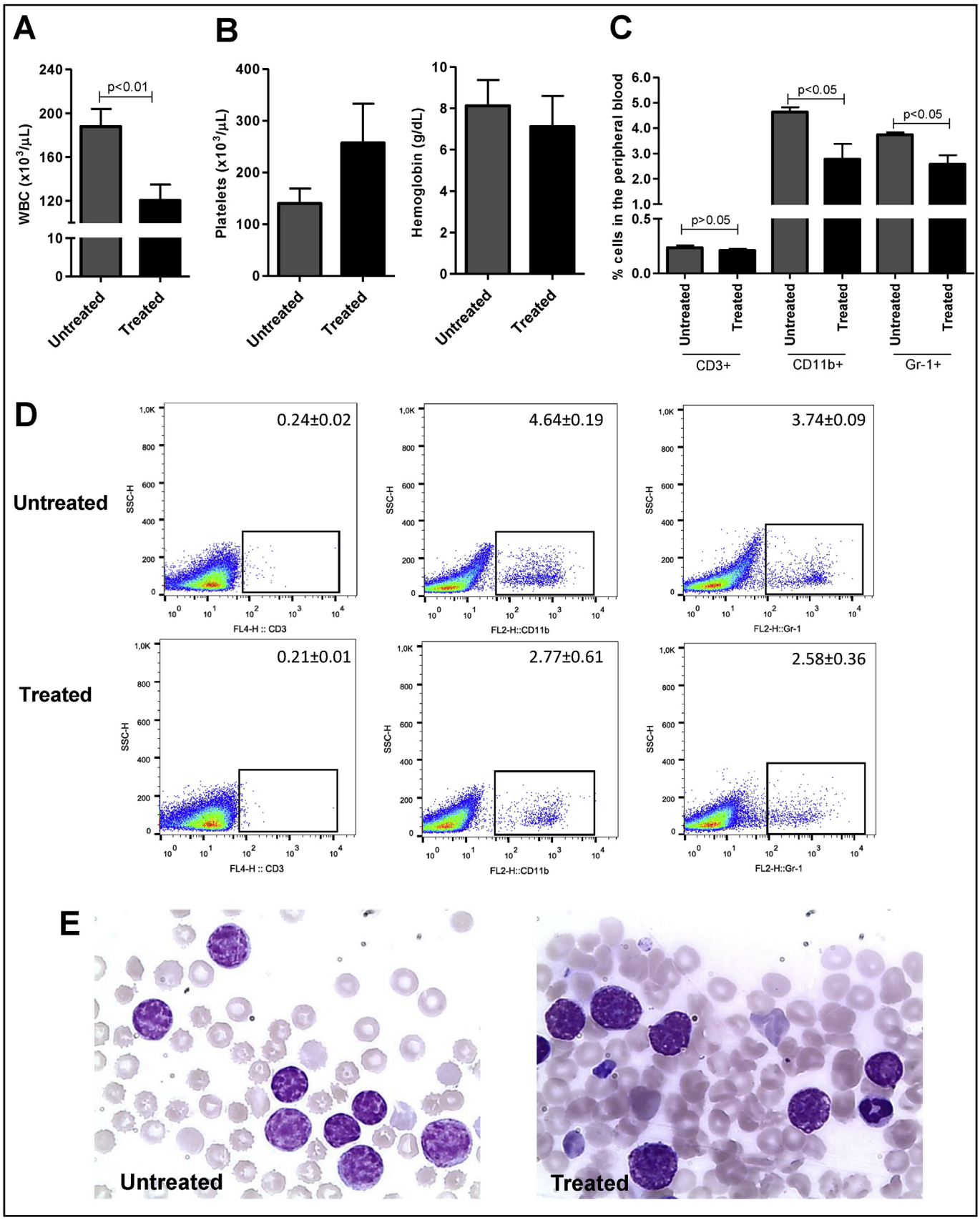

Fig. 3. Haematological and cytological analysis of treated leukaemic mice. (**A**) Green tea reduces the total number of leukocytes of leukaemic mice. (**B**) Green tea not affected thrombocytopaenia and anaemia in the leukaemic mice. (**C**) Percentage of $CD3^{+}$, CD11b+ and Gr-1+ cells in the peripheral blood of mice treated with green tea. (**D**) Representative dot plots of cell subsets generated and evaluated for the expression of CD3, CD11b and Gr-1 markers. (**E**) Representative dot plots of live gated cells (left); cells were selected as the percentage of CD45-positive to exclude mature erythrocytes (right) (**F**) Representative photomicrograph of Leishman-Wright-Giemsa–stained smears from peripheral blood of untreated and green tea treated leukaemic mice (100 ×).

Table 1
Counting and classification of cells according to the Bethesda criteria [11] for **leukaemia** in mice.

Mice	Bone marrow			Spleen	Peripheral blood
	Immature	Intermediate	Mature	Immature	Immature
	%	%	%	%	%
Untreated	75.0 ± 3.0	15.4 ± 0.5	3.0 ± 1.0	89.0 ± 1.4	88.0 ± 3.2
Treated	69.7 ± 1.4*	15.7 ± 2.7	7.4 ± 1.3£	81.0 ± 1.1§	78.0 ± 2.8§

Immature: promyelocyte plus blast; Intermediate: myelocyte plus metamyelocyte; Mature: granulocytes plus monocytes; *P < 0.0001; £ P < 0.001; §P < 0.05; Anova-Tukey.

3.6. Anti-leukaemic effects of GT in ROS production by CD34 and CD117 cells reduces CXCR4 expression and nuclear translocation of HIF-1α

To clarify the reduction of ROS induced by GT in $CD34^+$ and CD117+ cells in the BM of leukaemic mice, we then study the proteins CXCR4 and HIF-1α. Studies have shown that ROS increased the expression of CXCR4 in cancer and immune cells [13–16] through nuclear translocation of HIF-1α [17]. Our results showed that GT decreased the expression of CXCR4 in the surface of CD34 (197 ± 8 *vs* untreated 142 ± 20) and CD117 cells (9028 ± 1367 *vs* untreated 4196 ± 970) from leukaemic mice detected by fluorescence intensity (Fig. 7A–B). Reduction of the nuclear translocation of HIF-1α in these cells was also observed, as demonstrated by the co-localization of HIF-1α with the nuclei using the ImageStream imaging flow (Fig. 8).

3.7. Plasma polyphenol levels

After 5 days of treatment with 250 mg/kg/daily GT the total plasma EGCG levels detected were 2.15 μg/mL (4.6 μM).

4. Discussion

As mentioned previously, GT is a popular beverage worldwide and its consumption could be beneficial to health [2]. Extracts of GT, which are rich in polyphenols, have been shown to inhibit the formation and growth of tumours at different organ sites in animal models [18]. However, how GT promotes these cancer-preventive effects remains to be elucidated. Herein, we used a well-established transgenic APL model in which leukaemia develops in approximately 12 days after transplantation [8,9,19,20]. Our results are in agreement with this transgenic model, which is characterised by pronounced leucocytosis accompanied by anaemia and/or thrombocytopaenia during the leukaemic phase. Furthermore, the development of the disease was monitored by *in vivo* fluorescence imaging confirming the infiltration of immature cells in the spleen and BM of leukaemic animals, which was corroborated by the increase in the volume of spleens.

After confirmation of the leukaemia acute phase, the effects of GT were evaluated in these animals. Studying the haematological parameters, we first showed that treatment of leukaemic mice with 250 mg/kg GT for 4 days reduced the number of white blood cells, which was confirmed by the reduction of CD11b (monocytes) and Gr-1 (neutrophils) subpopulations in the PB evaluated by flow cytometry. The reduction of immature cells (blasts) in Leishman-Wright-Giemsa–stained smears of the PB also corroborated this effect of GT on the white blood cells. In addition, according to Bethesda criteria for leukaemia in mice [11], GT treatment caused a decrease of immature cells (promyelocyte plus blast) in the BM compartment, parallel to an increase of mature cells (granulocytes plus monocytes). A reduction of blasts in the spleen of these leukaemic animals after GT treatment was also observed. These results, although preliminary, suggests that GT exhibits some antiproliferative effect in a leukaemia model, blocking the dissemination of malignant clones. To better understand these findings, we next investigated the apoptotic effect of GT in the BM and spleen cells of leukaemic mice. The results showed an increase of apoptotic cells and cleaved caspase-3, -8, and -9, both in the BM and spleen of leukaemic mice after treatment with GT. These findings are in accordance with literature data showing that GT polyphenols can induce apoptosis in a mitochondrial-dependent manner, with activation of caspases, and remarkably inhibit tumour growth after the establishment of a murine xenograft model with these leukaemic cell lines [3,4]. Two main pathways act as signs of death sensors and can activate the cell death program apoptosis. Extrinsic or via receptor-mediated involves members of the tumour necrosis factor receptor superfamily, which caspase-8 active via adapter proteins FAS associated FADD death domain and are activated by cytokines and extracellular signals. In the intrinsic pathway, mitochondria play an essential role in death signal transduction such that permeability transition pore opening and collapse of the mitochondrial membrane potential result in the rapid release of caspase activators such as cytochrome *c* in the cytosol, and caspase-9 activation, which subsequently cleaves and activates procaspase-3 [21]. Both these pathways control the activation of caspases, which are well known as the executors of apoptosis and allow the dismantling of cells. Therefore, GT seems to be capable of activating both cell death pathways.

We then evaluated how GT inhibits the progression of leukaemogenesis by performing an immunophenotypic analysis of malignant clones in the BM and spleen of leukaemic animals. The expression of the CD34 and CD117 antigens on the leukaemic t(15; 17) cell constitute the phenotype used as a marker for the diagnosis and prognosis of APL [10]. CD34 is an important marker of human haematopoietic stem cells and is typically observed in acute myelogenous leukaemia [20]. CD117 is a c-kit proto-oncogene that encodes a transmembrane tyrosine kinase receptor involved in the proliferation of leukaemic cells, which is present in the early stages of myeloid differentiation [22]. Our results are in agreement with these data indicating that GT could inhibit the increase of malignant clones in the BM as evidenced by the reduction of CD34 and CD117 antigens in this organ. In relation to the spleen, reduction of CD117 and Gr-1 were observed after treatment with GT. Studies have shown an accumulation of Gr-1 immature myeloid cells in lymphoid organs and spleens of cancer patients and tumour-bearing mice [23,24]. The presence of these cells in the spleen can be associated with immune suppression [25,26]. Thus far, these results suggest that GT impaired the malignant clone expansion and proliferation. However, the mechanism may be related to some immunological improvement.

To further evaluate how GT promotes the elimination of the malignant clones, we analysed the production of intracellular ROS in the BM subpopulations of CD34, CD117, and Gr-1 cells from leukaemic mice. Interestingly, GT increased intracellular ROS in the Gr-1 cells while reducing ROS in the CD34 and CD117 cells. ROS are chemically reactive molecules containing oxygen free radicals and are involved in modulating biological cell functions, cell signalling,

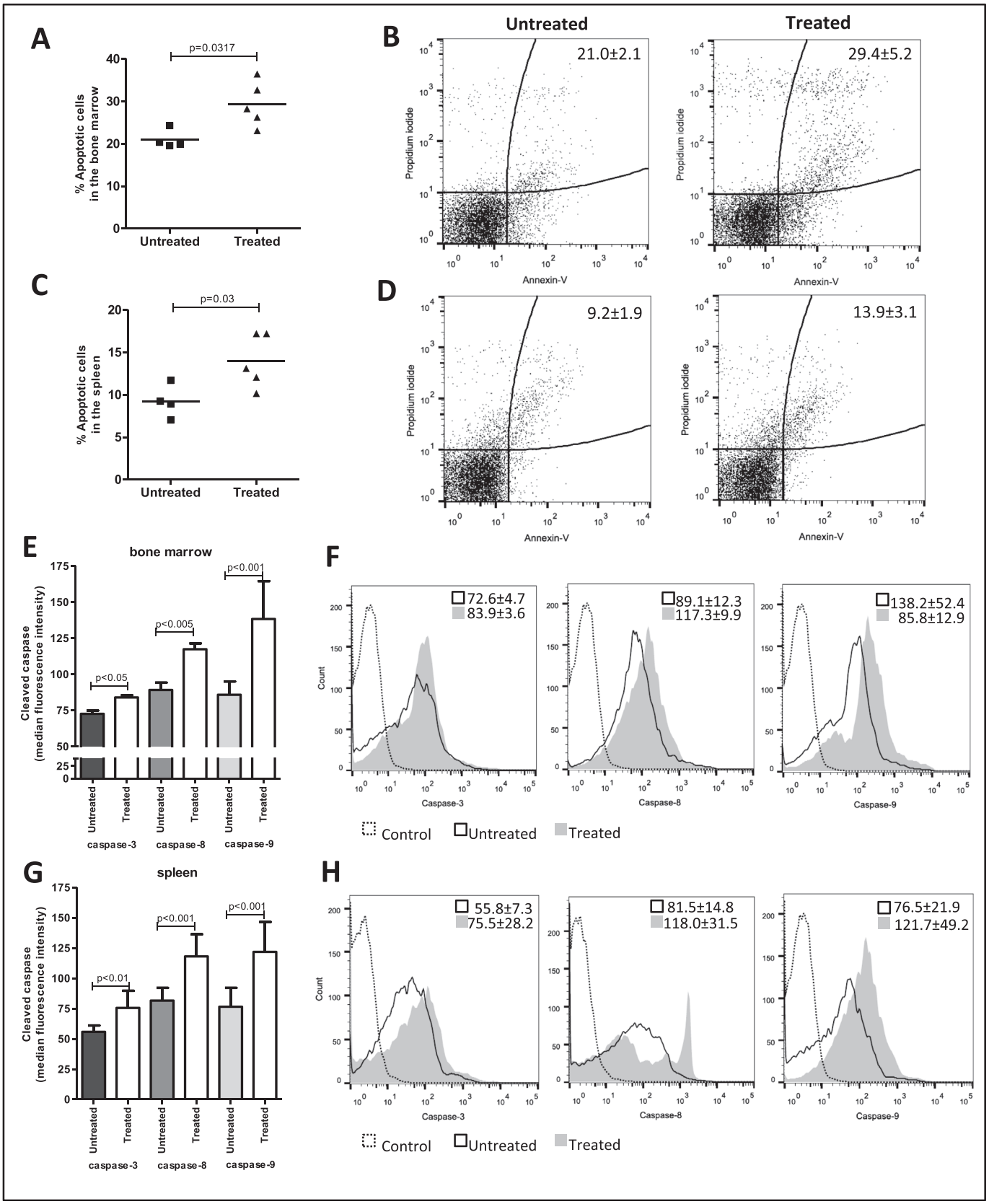

Fig. 4. Green tea induces apoptosis in leukaemic mice. (**A**) Percentage of apoptotic cells in the bone marrow of mice determined by flow cytometry using annexin V-FITC/PI staining. (**B**) Representative dot plots of bone marrow cells. (**C**) Percentage of apoptotic cells in the spleen of mice determined by flow cytometry using annexin V-FITC/PI staining. (**D**) Representative dot plots of spleen cells. (**E**) Median fluorescence intensity (MFI) of cleaved caspase-3, -8 and -9 in the bone marrow cells. (**F**) Representative histogram of bone marrow cells. (**G**) MFI of cleaved caspase-3, -8 and -9 in the spleen cells. (**H**) Representative histogram of spleen cells.

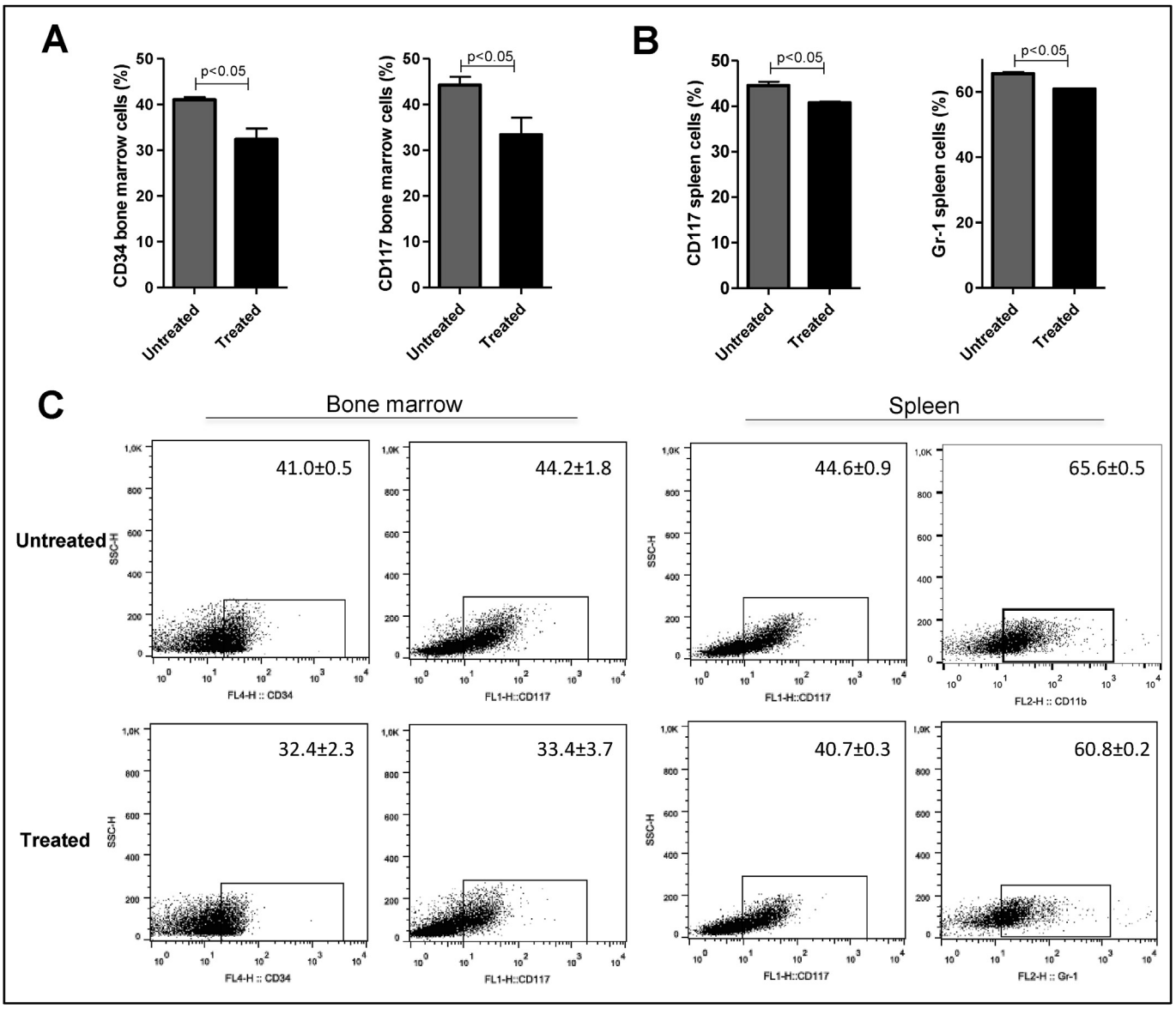

Fig. 5. Immunophenotyping by flow cytometry of CD34, CD117 and Gr-1 cells infiltrated in the bone marrow and spleen of leukaemic mice treated with green tea. (**A**) Percentage of CD34 and CD117 cells infiltrated in the bone marrow. (**B**) Percentage of CD117 and Gr-1 cells infiltrated in the spleen. (**C**) Representative dot plots selected as the percentage of CD45-positive cells to exclude mature erythrocytes and CD34, CD117 and Gr-1 markers.

and homeostasis [27,28]. The fate of cells depends on the levels of ROS in the redox microenvironment [29]. Endogenous ROS levels are elevated in tumour cells [30]. Particularly in leukaemia, several studies support the role of ROS [31–33]. ROS are also essential components of the innate immune response [34]. Unlike the leukaemic cell, the increased ROS in immune cells (mature cells) represents a positive aspect of the disease. Immune cells have efficient immune-modulatory and cytotoxic properties that are essential for recognising and attacking AML cells [35]. Apparently, GT increases ROS in cells in an advanced stage of differentiation, as observed in the BM Gr-1 cells of GT-treated leukaemic mice. Our findings are in accordance with these concepts and suggest that the increased intracellular ROS in Gr-1 cells can be related to the intense metabolism necessary for the differentiation of these cells [36], and this was corroborated by the increase of mature cells (granulocytes plus monocytes) in the Leishman-Wright-Giemsa staining of BM cells from mice treated with GT.

Interestingly, unlike Gr-1 cells, the results showed a reduction of intracellular ROS in the CD34 and CD117 cells of the BM after treatment with GT. To clarify this effect of GT, we then study the proteins CXCR4 and HIF-1α. Studies have shown that ROS increases the expression of CXCR4 in cancer cells [13–16] through HIF-1 activation [17]. A potential underlying mechanism for this may result from alterations that occur within the vasculature or O2-carrying capacity of cells leading to hypoxic conditions during tumour progression. Hypoxia induces the activation of HIF-1, which promotes expression of several target genes including CXCR4 [37]. The HIF-1α subunit acts as a transcription factor for CXCR4 by binding to the HIF-1α response element found in the promoter region of CXCR4 [13]. Therefore, a tumour microenvironment that is rich in ROS may critically influence CXCR4-mediated expression and functions, ultimately encouraging tumour progression [16]. As expected, leukaemia led to increased expression of CXCR4 in the BM CD34 and CD117 cells of mice, with nuclear translocation of

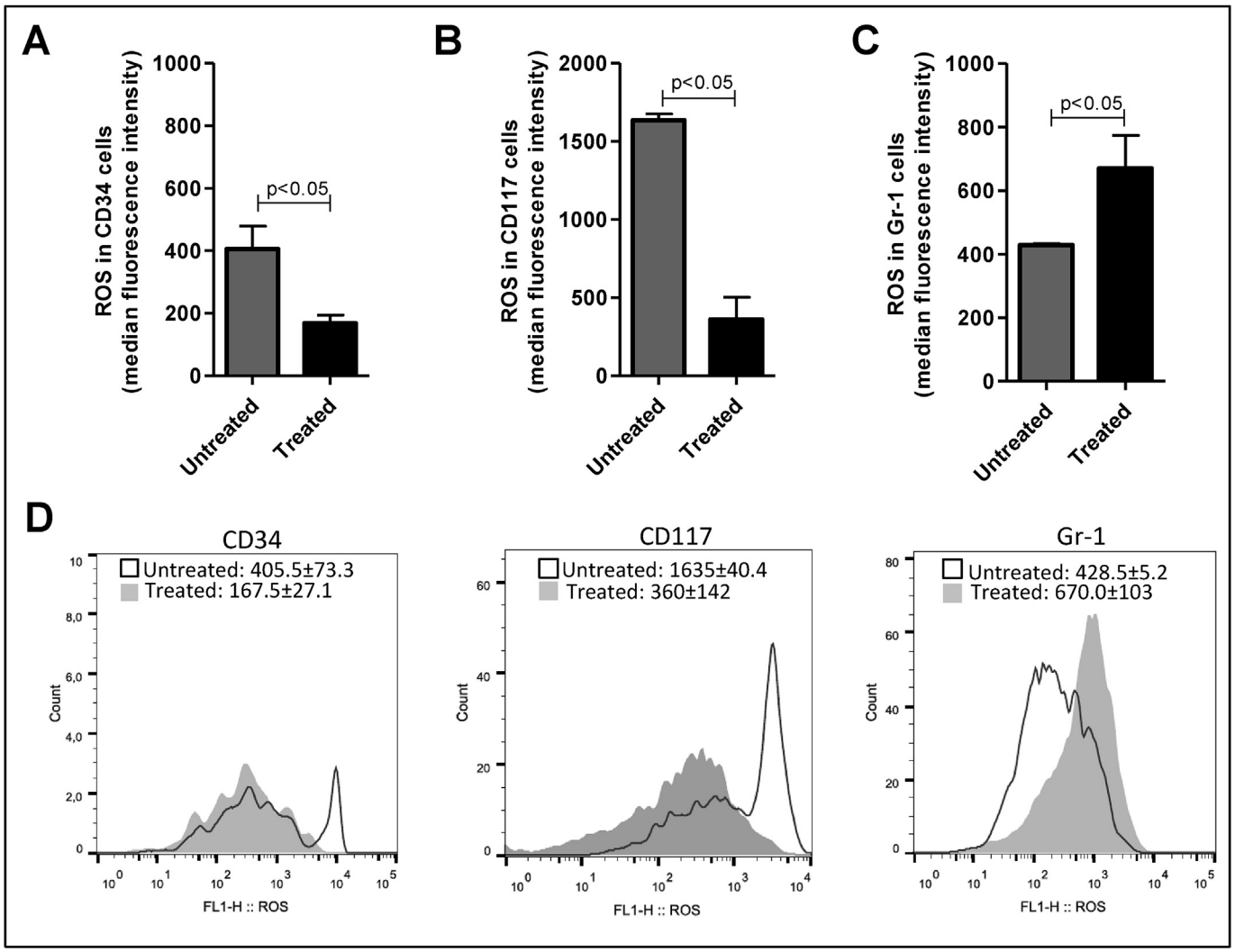

Fig. 6. Production of intracellular ROS by flow cytometry in subpopulations of CD34, CD117 and Gr-1 bone marrow cells of leukaemic mice treated with green tea. (**A**) Median fluorescence intensity (MFI) of intracellular ROS production in the CD34 bone marrow cells. (**B**) MFI of intracellular ROS production in the CD117 bone marrow cells. (**C**) MFI of intracellular ROS production in the Gr-1 bone marrow cells. (**D**) Representative histograms of intracellular ROS production in the subpopulations.

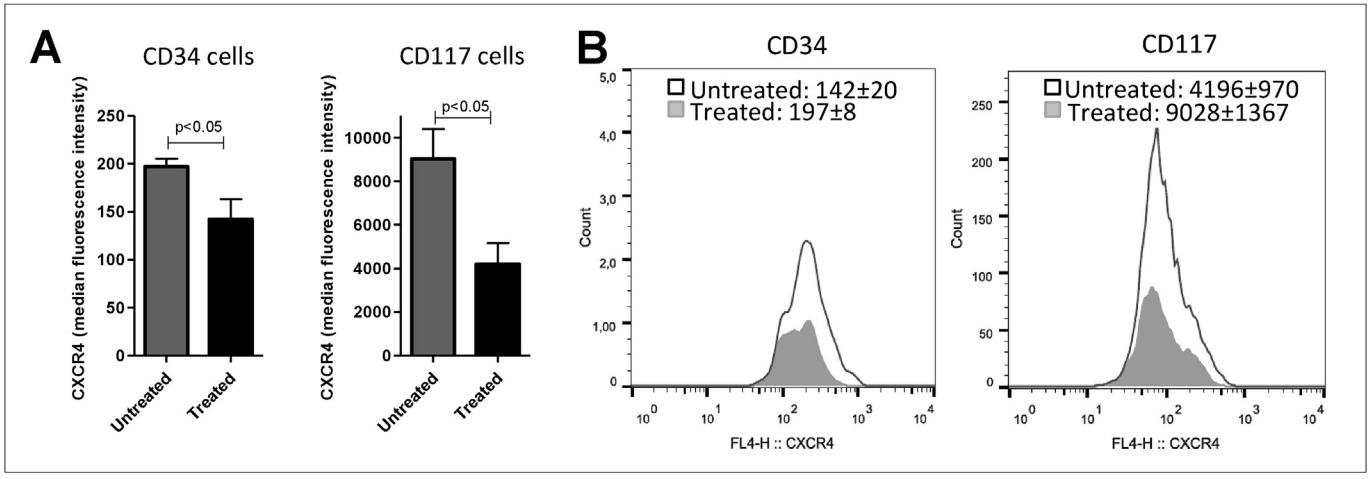

Fig. 7. CXCR4 expression on CD34 and CD117 bone marrow cells of leukaemic mice treated with green tea. (**A**) Median fluorescence intensity (MFI) of CXCR4 expression on CD34 and CD117 cells. (**B**) Representative histograms of CXCR4 expression.

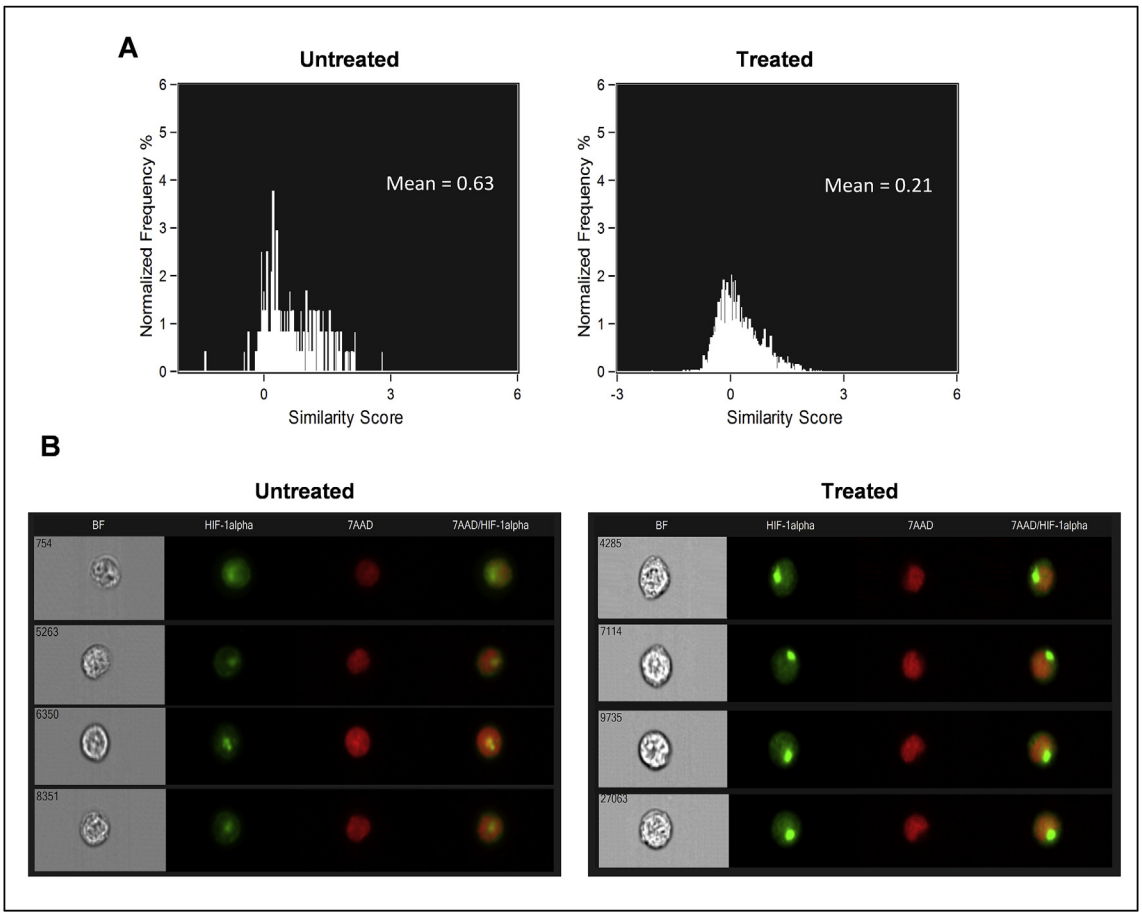

Fig. 8. Nuclear translocation of HIF-1α in the bone marrow cells of leukaemic mice treated with green tea, using imaging flow cytometry. The anti-mouse antibody for HIF-1α Alexa 488 (green fluorescence) and 7-AAD for nucleus (red fluorescence) were used. (**A**) Similarity score distribution of HIF-1α. Results represent the mean of two experiments. (**B**) Representative images of both untreated and treated leukaemic bone marrow cells showing HIF-1α co-localization with the nucleus.

HIF-1α. Activation of CXCR4 induces leukaemia cell trafficking and homing to the marrow microenvironment, where CXCL12, the ligand for CXCR4, retains leukaemia cells in close contact with marrow stromal cells that provide growth and drug resistance signals [38]. The treatment of leukaemic animals with GT reduced CXCR4 expression and nuclear translocation of HIF-1α. Literature data have shown that GT polyphenols inhibit the activation of HIF in cancer cells [39,40]. Therefore, GT could possibly impair ROS production on CD34 and CD117 cells, which could inhibit the activation of HIF-1α, subsequently reducing CXCR4 expression and cell survival. The impairment of CXCL12/CXCR4 interactions induced by GT could prompt the loss of adhesion of leukaemic cells in the medullar microenvironment, leading to mobilization out of their niche and rendering these cells more vulnerable to chemotherapy agents.

In summary, GT treatment in APL mice induces apoptosis of leukaemic cells in the BM and spleen, confirmed by activation of caspase-3, -8, and -9, probably by modulating the production of intracellular ROS in these cells. Despite being well known as antioxidants, there is evidence that some of the effects of these compounds may be related to induction of oxidative stress in immune cells, which might be responsible for the induction of apoptosis of tumour cells. These pro-oxidant properties may also induce endogenous antioxidant systems in normal tissues that offer protection against cancer. However, as leukaemic cells have excessive ROS, the antioxidant effect of GT could become more evident. Several potential mechanisms have been proposed, including both antioxidant and pro-oxidant effects to polyphenol compounds; however, questions remain concerning the relevance of these mechanisms to cancer prevention.

Conflicts of interest

None declared.

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