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Nemorosone inhibits the proliferation and migration of hepatocellular carcinoma cells

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ABSTRACT

Aims: In the tumor microenvironment, dysregulated immune cells could promote tumor progression, invasion and metastasis, by establishing a symbiotic relationship with cancer cells. A pivotal role is played by monocyte recruitment and induction of tumor-associated macrophages (TAMs), which provide immunosuppression and tumorigenesis. The effect of nemorosone, an antiproliferative phytocomponent present in Cuban Propolis, on TAM-induced tumor progression remains to be elucidated. Here we investigated the symbiotic relationship between monocytic leukemia THP-1 and hepatocellular carcinoma HepG2 cells, and the role of nemorosone in preventing TAM-induced tumor growth.

Main methods: Macrophage differentiation induced by HepG2-conditioned medium was assessed by flow cytometry, analysis of secreted molecules and cytokine expression. The effect of nemorosone and/or conditioned THP-1-medium on HepG2 proliferation was evaluated by MTT assay, colony formation, cells cycle and migration assays.

Key findings: HepG2 cells induced THP-1 recruitment and differentiation to macrophages. When compared with control THP-1 cells, differentiated THP-1 showed a significant increase of the matrix metalloproteinases MMP-2 and MMP-9 expression ($P < 0.01$), and slightly induced HepG2 cells growth. This effect was counteracted by nemorosone, which also significantly inhibited colony formation ($P < 0.01$) and migratory capacity of HepG2 cells, driving a high percentage of cells (80%) to the G0/G1 phase.

Significance: HepG2-conditioned medium is a suitable model for THP-1 modulation and differentiation. Moreover, nemorosone significantly inhibits the proliferation of HepG2 cells, both in presence and absence of the soluble factors secreted by TAMs. Further studies are needed to elucidate the role of this natural compound in the HCC-TAM relationship.

1. Introduction

Although immune system cells are involved in several physiological processes, such as the surveillance of tumor cells growth [1,2], several studies have demonstrated that immune cells and inflammatory mediators play also a role in cancer initiation, progression, and invasion [2,3]. In order to avoid immuno-surveillance, tumor cells can influence tumor microenvironment (TME) by releasing extracellular signals able to induce immuno-tolerance, infiltration of inflammatory cells, resistance to therapy, finally leading to the promotion of tumor growth and progression [4,5]. On the basis of these consideration, TME constitutes a focus of interest in cancer immunotherapy research.

Numerous evidences have demonstrated that macrophages are the main infiltrating cells inside TME [6,7]. Macrophages can influence tumor progression by eliciting immuno-suppressive responses, tumor cell migration, invasion, intravasation and angiogenesis [4]. Monocyte differentiation into tumor-associated macrophages (TAMs) is induced by different cytokines secreted by tumor cells, which can change the functional phenotype of macrophage in response to TME [7,8]. Macrophages display two main distinct phenotypes, i.e. M1 macrophages, with a cytotoxic effect toward cancer cells and microorganisms, and M2 macrophages, that have been considered an unfavorable prognosis in patients with cancer [9–11].

There is clear evidence that macrophages can influence hepatocellular carcinoma (HCC) prognosis by accelerating tumor progression, originating metastases and chemoresistance [12–14]. Currently, HCC is

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considered the second cause of cancer-related mortality in the world [15,16]; thus, is important to develop new anticancer agents able to increase HCC patient survival, and one possible target is represented by the inhibition of the symbiotic relationship between infiltrating inflammatory and cancer cells. In this context, natural products are considered excellent antitumor candidates for their anti-proliferative, anti-metastatic and immunomodulators properties with limited side effects [17–21].

Nemorosone (Nem), a poly-isoprenylated benzophenone, is the main phytocompound present in Cuban brown propolis (CBP) and in the resin of *Clusia* species [22–24]. Several studies indicate that Nem is responsible for the biological properties of CBP [22,25–28]. Isolated Nem possesses anti-proliferative activity against different cancer cells including diverse HCC models [25–27,29,30]. However, the effect of Nem against macrophage-induced growth in HCC model has not yet been investigated. In this study, we evaluated the effect of HepG2 conditioned medium on the phenotype of the human monocytic cell line THP-1 cell and HepG2 cell growth modulation induced by macrophages in presence or absence of nemorosone.

2. Materials and methods

2.1. Reagents and antibodies

Nemorosone (1-benzoyl-4-hidroxy-8,8-dimethyl-3,5,7-tris(3-methyl-2-butenyl)-bicyclo-[3.3.1]-non-3-ene-2,9-dione) was provided by Pharmacy and Food Institute, Havana University, Cuba. Stock solution of Nem was prepared in DMSO and diluted in complete medium. DMSO concentration in the medium never exceeded 0.02%. Dulbecco's Modified Eagle's Medium (DMEM), RPMI 1640 medium, Fetal bovine serum (FBS), L-glutamine, and streptomycin/penicillin were provided from Lonza (Belgium, United Kingdon). Dulbecco's Phosphate-Buffered Saline (DPBS) and Trypsin–EDTA were purchased from EuroClone (Milano, Italy). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), gelatin, phorbol-12-myristate-13-acetate (PMA), crystal violet, Coomassie blue (R250) and 2′,7′-diclorofluorescin diacetate (H2DCF-DA) were purchased from Sigma Aldrich (San Louis, USA) and Annexin V-FITC/PI kit from Invitrogen (California, USA). Mouse anti-hCD68 and anti-hCD14 antibodies, Alexa-Fluor 568-conjugated secondary antibody, and Hoechst were purchased from Abcam (Cambridge, UK). Mouse anti-hCD206 antibody (sc-376108) was provided from Santa Cruz Biotechnology (California, USA).

2.2. Cell culture

Human monocytic THP-1 cells derived from an acute monocytic leukemia patient (ATCC, TIB-202) and human hepatocellular carcinoma HepG2 cells (ATCC, HB-8065) were maintained in RPMI 1640 and DMEM medium, respectively. Both culture media were supplemented with 10% FBS, 1% L-glutamine and 1% streptomycin/penicillin. Cell lines were maintained at 37 °C with 5% CO_2.

2.3. Conditioned medium and treatments

To obtain the conditioned medium (CM) from HCC cells, HepG2 (2×10^5 cells/mL) were seeded into 75cm^2 flasks for 48 h. HepG2 supernatant (HepG2-CM) was collected, centrifuged, filtrated and stored at 20 °C until use. For experiments with THP-1 cells were treated as follows: *Control:* 50% DMEM and 50% RPMI-1640 (v/v) for 72 h, *HepG2-CM:* 50% HepG2 supernatant and 50% RPMI-1640 (v/v) for 72 h, *PMA:* 50% DMEM and 50% RPMI-1640 (v/v) with PMA (320 nM) for 48 h followed by another 24 h with fresh medium and *PMA + HepG2-CM:* 50% DMEM and 50% RPMI-1640 (v/v) with PMA (320 nM) for 48 h followed by another 24 h with HepG2-CM (Fig. 1).

Supernatant of THP-1 cells treated with control, HepG2-CM, PMA, PMA + HepG2-CM, namely respectively T1, T2, T3 and T4, were centrifuged, filtrated and stored at 20 °C for further experiments (Fig. 1). HepG2 cells were treated with T1 and T2 in absence or presence of increasing concentration of Nem (5, 10, 25 and 50 μM) for 24 h. All CMs and supernatant of THP-1 were used without additional FBS supplementation.

2.4. Chemotaxis assay

The recruitment of THP-1 cells in response to HepG2-CM was evaluated by a chemotaxis assay according to Chen et al. [31]. Cells (10^6 cells/mL) were loaded into inserts (pore size, 8 μm Falcon®, 353097, MA, USA) with 400 μL of RPMI (1% FBS) or HepG2-CM in the lower chamber. After 1 h at 37 °C and 5% CO_2, migrated cells were fixed with 70% ethanol and stained with 0,2% crystal violet and non-migratory cells were removed. The number of migrating cells was assessed by means of a confocal microscope Zeiss LSM 800 (100× magnification) and quantified using the Image J software.

2.5. Cell viability assay

Cell viability was assessed by MTT assay [32]. THP-1 (10×10^4 cells/mL) and HepG2 (3×10^4 cells/mL) cells were seeded into 96-well plates and treated as described above. MTT solution (0,5 mg/mL) was added into each well and incubated for 4 h 37 °C. After incubation, the produced blue formazan crystals were dissolved with 200 μL of isopropanol acidified with 2% HCl. Absorbance was measured at 570 nm using a Victor X3 multiplate reader (Perkin Elmer). Percentage of cell viability (%) was determined using untreated cells as control.

2.6. Morphology and cell death analysis

The effect of HepG2-CM, PMA, PMA + HepG2-CM treatments on the cellular morphology of THP-1 cells and the mechanism of cell death (apoptosis/necrosis) were assessed by means of flow cytometry analysis using flow cytometer Epics XL, Beckmann Coulter and CXP software (San Diego, USA). Cell death was evaluated using a commercial Annexin V-FITC/PI kit following manufacturers' instructions. THP-1 cells (10×10^5 cells/mL) were seeded into 6-well plates and treated for 72 h as previously described [28].

2.7. Expression of surface markers

The expression of macrophage surface markers was evaluated by immunofluorescence coupled to confocal microscopy. THP-1 cells (10×10^5 cell/mL) were seeded into 24-well plates and after treatment with control, HepG2-CM, PMA, PMA + HepG2-CM were washed with PBS and fixed with 4% paraformaldehyde for 30 min. Cells were washed and incubated with 10% FBS in PBS for 20 min. After incubation, cells were treated 1 h with anti-CD68, anti-CD14 antibody or anti-CD206 antibody (all used at 1:200 dilution). Cells were washed three times with PBS and incubated for 1 h with anti-mouse Alexa Fluor 568 conjugated antibody (dilution 1:200). After three more washes, cells were incubated with the nuclear marker Hoechst (dilution 1:500) for 15 min. Cells were analyzed by immunofluorescence coupled to confocal microscopy and the Image J software was used to analyze the fluorescence associated to the expression of surface markers [33,34].

2.8. Reactive oxygen species (ROS) production

ROS production was evaluated using H_2DCF-DA staining. After seeding into 96-well plates, THP-1cells (10×10^4 cell/mL) were treated with control, HepG2-CM, PMA, PMA + HepG2-CM. Cells were incubated with 10 μM H_2DCF-DA for 30 min. The intensity of fluorescence was measured using a Victor X3 multiplate reader (Perkin Elmer).

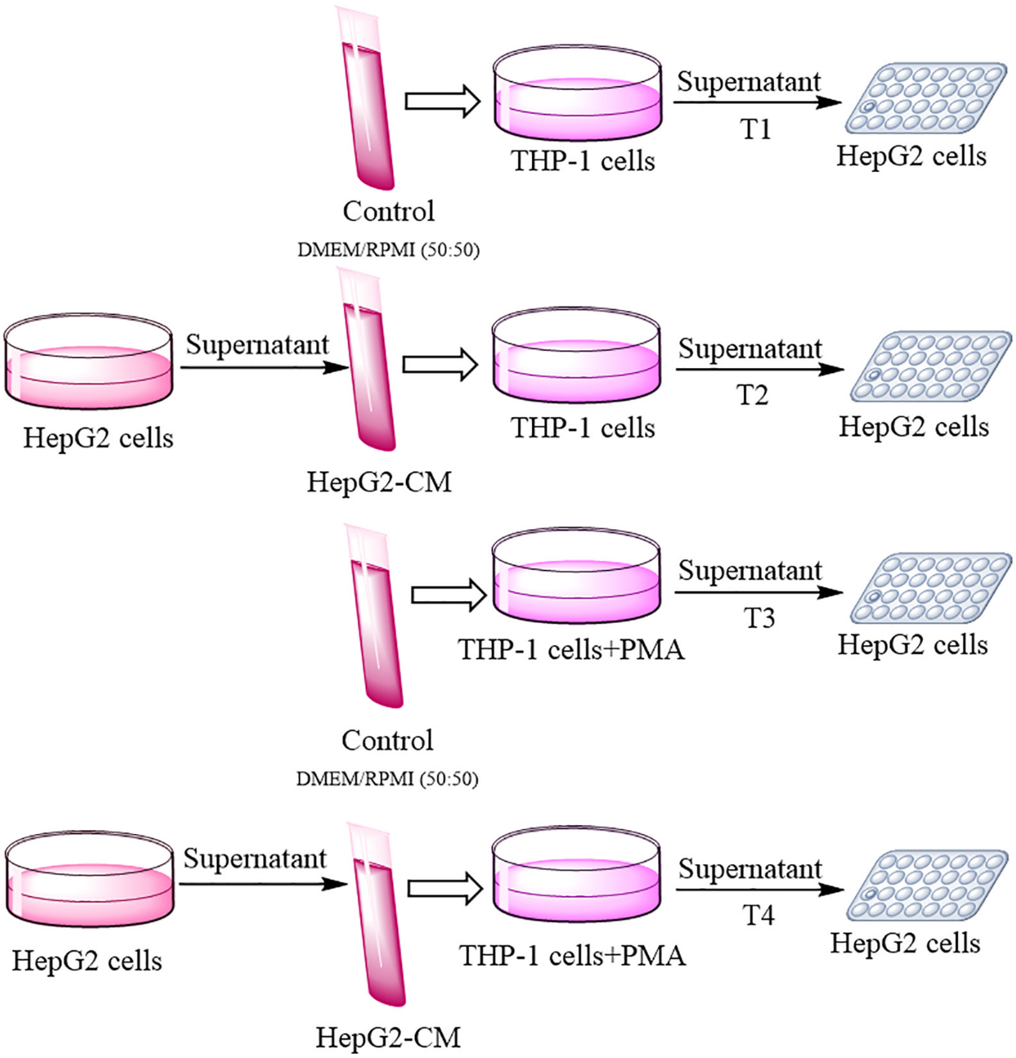

Fig. 1. Conditioned medium and treatments. THP-1 cells were cultured as follows: DMEM/RPMI 50:50 (control), HepG2 supernatant (HepG2-CM), PMA, PMA + HepG2 supernatant (PMA + HepG2-CM). HepG2 cells were cultured as follows: T1 (supernatant of THP-1 cells with control medium), T2 (supernatant of THP-1 cells treated with HepG2-CM), T3 (supernatant of THP-1 cells treated with PMA), T4 (supernatant of THP-1 cells treated with PMA + HepG2-CM).

2.9. *Zymography assay*

Supernatants of THP-1 cells treated with control, HepG2-CM, PMA, PMA + HepG2-CM were run for 90 min into an 8% polyacrylamide gel containing 1% gelatin. The gel was washed with 2.5% Triton X-100 to remove SDS, as described by Tajhya et al. [35]. Metalloproteinase (MMP) activity was reactivated overnight by incubating the gel in the developing buffer. The gel was stained with Coomassie blue (R250) for 1 h and de-stained with a solution containing 5% acetic acid and 10% methanol until white bands corresponding to MMP molecular weight were clearly visible. The bands were quantified using the Image J software.

2.10. *qRT-PCR analysis*

Total RNA of THP-1cells (10×10^5 cell/mL) seeded into 24-well plates and treated with control, HepG2-CM, PMA, PMA + HepG2-CM was isolated using Isolate II RNA Mini Kit (Bioline, London, UK). qRT-PCR was performed with 8 μg of the extracted RNA using suitable sequences of primers (Table 1) and One Step SYBR Prime Script RT-PCR Kit (Takara, Mountain View, CA, United States) as previously described [36,37]. Relative mRNA expression was calculated using $2^{-\Delta\Delta Ct}$ methods [38].

Table 1
qRT-PCR primer.

Gene	Sequence forward	Sequence reverse
TNFα	AAGCCTGTAGCCCATGTTGT	GAGGTACAGGCCCTCTGATG
IL6	ACTCCTTCTCCACAAGCGCC	TGTGGGGCGGCTACATCTT
IL10	AAGACTTTCTTTCAAATGAA	GTTTTCACAGGGAAGAAATC
GAPDH	ACATCAAGAAGGTGGTGAAGCA	GTCAAAGGTGGAGGAGTGGGT

2.11. *Colony formation assay*

The effect of the supernatants T1 and T2 on the capacity of HepG2 to produce colonies in absence and presence of Nem was evaluated by the colony forming assay [39]. Cells (2×10^2 cells/mL) were seeded into 12-well plates. After treatment, cells were kept in culture for 10 days and subsequently fixed with 4% paraformaldehyde and stained with crystal violet. Colonies with at least 50 cells were counted and surviving fraction (SF) was calculated according to the formula:

$$SF = \frac{Number\ of\ colonies\ formed}{Number\ of\ inoculated\ cells} \times 100.$$

2.12. Wound-healing migration assay

The effect of T1 and T2 in absence or presence of Nem on cell migration was assessed by means of wound healing assay. HepG2 cells (10×10^4 cells/mL) were seeded in 12-well plates until reaching a confluence of 100%. The monolayers were scratched using a pipette tip and the wound was washed to remove non-adherent cells. After 24 h, cell migration into the scratched area was observed by means of a microscope (100× magnification) and quantified using Image J software. The percentage of wound closure was determined as:

$$\%Wound\ closure = \frac{Area\ of\ wound\ (0h) - Area\ ofwound\ (24\ h)}{Area\ of\ wound\ (0\ h)} \times 100$$

2.13. Cell cycle arrest analysis

HepG2 cells (10×10^4 cells/mL) were seeded into 12-well plates and treated for 24 h with T1 and T2 in absence or presence of Nem. The cells were fixed in 70% ethanol at 4 °C for 15 min followed by centrifugation for 5 min at 1800 rpm and staining with 300 μL propidium iodide (PI) for 30 min. DNA content and cell cycle distribution were determined using Epics XL flow cytometer and CXP software (Beckmann, San Diego, USA).

2.14. Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the post hoc Tukey's multiple comparisons test. The experiments were performed in triplicate and results are presented as mean ± SD. $P < 0.05$ was considered statistically significant.

3. Results

3.1. HepG2-CM promoted THP-1 cell migration

Quantitative analysis of cell migration assessed by chemotaxis assay showed that the recruitment of THP-1 cells in response to HepG2-CM was greater compared to that induced by RPMI (1% FBS), used as negative control (Fig. 2). This result suggests that THP-1 monocytes can acquire the ability to migrate in response to signaling molecules secreted by HepG2 cells.

3.2. Effect of HepG2-CM on cell viability and morphology of THP-1 cells

The effect of HepG2 and PMA on THP-1 cells viability was evaluated by MTT assay after a 72 h-exposure. Fig. 3A shows that PMA and PMA + HepG2-CM treatments significantly decreased THP-1 cells proliferation to approximately 85% with respect to control. This slight cytotoxic effect of PMA on THP-1 cells is in accordance with what had been described by other groups [40–42]. The results obtained by flow cytometry analysis are in line with those of the MTT assay, confirming that HepG2-CM did not affect THP-1 cell viability. In contrast, PMA and PMA+ HepG2-CM treatments induced a marked increase in the population of cells in late apoptotic or necrotic stage (Fig. 3B). On the other hand, the morphology of THP-1 cells following HepG2-CM, PMA and PMA+ HepG2-CM treatments was different with respect to control (Fig. 3C), i.e. treated cells displayed an oval or amoeboid morphology; however, this peculiar cell shape was less evident in HepG2-CM-treated THP-1 cells when compared to PMA and PMA+ HepG2-CM treatments (data not shown).

3.3. Analysis of differentiation markers in THP-1 cells

The immunofluorescence staining analysis revealed that treatment with HepG2-CM, PMA and PMA + HepG2-CM lead to the differentiation of THP-1 monocytes. The expression level of CD68 and CD14, markers of THP-1 differentiation in macrophages, and of the TAM marker CD206 were evaluated by immunofluorescence staining after treatment with HepG2-CM, PMA and PMA + HepG2-CM. As shown in Fig. 4A–B, THP-1 cells incubated in the presence of HepG2-CM, PMA and PMA + HepG2-CM became rounded and adherent. The expression of CD14 in THP-1 cells was not influenced by HepG2-CM, whereas treatment with PMA, in absence or presence of HepG2-CM increased significantly CD14 expression. Furthermore, the increase of CD68 expression indicated that differentiation of monocytes to macrophages in all our experimental condition. The expression of CD206 was upregulated by HepG2-CM and PMA + HepG2-CM with respect to control and PMA treatment, indicating that HepG2-CM plays a fundamental role in increasing the expression of this protein, and this is in accordance with its classification as a TAM marker [43].

Since a number of studies suggest that oxidative stress is involved in the differentiation of macrophages [44,45], to further evaluated the effect of HepG2-CM, PMA and PMA+ HepG2-CM treatments on THP-1 cells differentiation, we measured the production of ROS using H_2DCF-DA staining. All the treatments significantly induced ROS production in the THP-1 cells in comparison to the control, thereby confirming the link between ROS and macrophage differentiation, also in our experimental conditions (Fig. 4D).

3.4. Effect of HepG2-CM on cytokine expression in THP-1cells

The production of cytokines by activated macrophages plays an important role into the tumor microenvironment [46]. The mRNA expression of the cytokines TNFα, IL6 and IL10 in THP-1 cells were examined by qRT-PCR. In cells treated with HepG2-CM, TNFα and IL10 levels increased significantly with respect to control; however, IL6 level was not changed (Fig. 5). In contrast, the exposure to PMA increases significantly TNFα and IL6 expression; whereas, the combined treatment with PMA + HepG2-CM resulted in upregulation of the expression of TNFα and IL10 in THP-1 cells. The data suggest that HepG2-CM treatment can regulate the mRNA expression of the cytokines responsible for THP-1 differentiation into macrophages in a peculiar manner.

3.5. Effect of HepG2-CM on activity of metalloproteinases MMP-2 and MMP-9

The infiltration of macrophages is accompanied by an increase of level expression and release of the gelatinases MMP-2 and MMP-9 [46]. These gelatinases have been extensively studied due to their association with tumor progression, invasion and metastasis [47]. The activity of MMP-2 and MMP-9 was evaluated by gelatin zymography assay, by which a significant increase of MMP-2 activity was detected after treatment with T2 and, to a lesser extent, T4. Meanwhile, MMP-9 activity, detectable in all THP-1 cells, was significantly increased in T2 and T4 (Fig. 6). In accordance with the previous results regarding the cytokine expression, these results demonstrated that HepG2-CM is responsible for the differentiation of THP-1 into macrophages with an increased MMP activity, able to further support tumor progression. On the basis of these results, the following experiments were performed with THP-1 cells stimulated with HepG2-CM.

3.6. HepG2-CM-derived macrophages promote tumor progression

Since immune and tumor cells communicate to each other through secreted soluble factors, we studied the influence of T2 supernatant on HepG2 cell progression. As shown in Fig. 7A, HepG2 cells treated with T2 showed an increase of cell viability when compared to cells treated with T1. Moreover, treatment with T1 caused a significant reduction of cell viability with respect to untreated cells. Additionally, a clonogenic

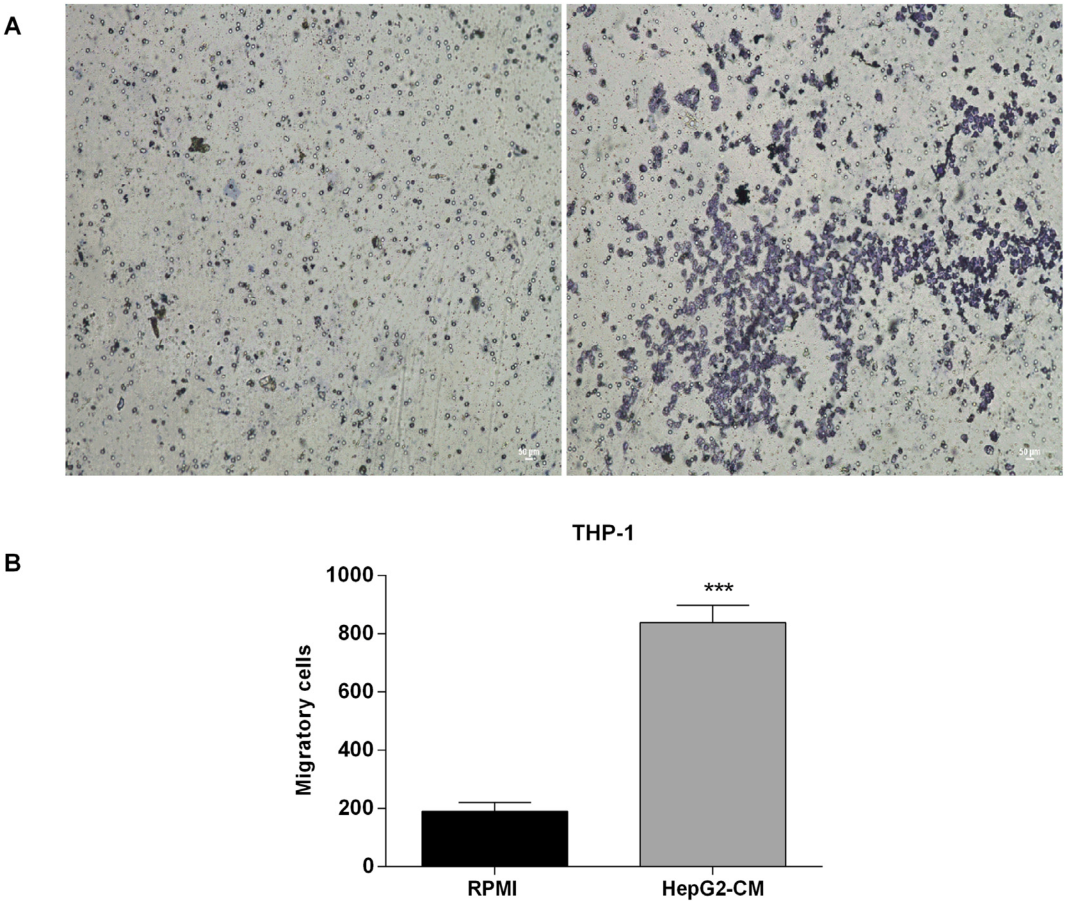

Fig. 2. HepG2-CM promotes THP-1 cell migration. THP-1 migration was evaluated by means of chemiotaxis assay. Magnification: 100×; Scale bar: 50 μm. The results are presented as mean ± SD of three independent experiments, $^{***}P < 0.001$ vs RPMI-cultured control cells.

assay was performed to evaluate proliferation and colony formation of HepG2 cells after 10 days of exposure to T1 and T2 supernatants. The results showed that T2 treatment significantly increased the ability of HepG2 cells of forming colonies (Fig. 7B–C) in comparison with untreated and T1-treated cells. In contrast, treatment with T1 supernatant decreased the clonogenic capacity of HepG2 cells with respect to control cells. Taken together, these data indicate that the soluble factors secreted by THP-1 cells pre-stimulated with HepG2-CM increase the number of colonies of HepG2 cells, further confirming that THP-1 cells, when differentiated to TAMs, concur in tumor proliferation.

3.7. Nemorosone inhibits the proliferation of HepG2 cells

The effect of Nem on HepG2 viability in absence and presence of T1 and T2 was evaluated by the MTT assay. Nem alone or in combination with T1 or T2 for 24 h caused a significant decrease of HepG2 viability in a concentration-dependent manner (Fig. 8A). However, we observed that the effect of Nem alone at the highest concentrations (25 and 50 μM) is more impressive than that of T2-Nem, in accordance with the proliferative effect of T2 (Fig. 7).

Furthermore, the number of colonies of HepG2 cells after 10 days was drastically decreased by 10 μM Nem (Fig. 8B), and no colonies could be observed when treating HepG2 with Nem concentrations higher than 10 μM (data not shown). In accordance with the data on cell viability (Fig. 8A), the effect of Nem alone on HepG2 survival factor was significantly higher than that of T1-Nem and T2-Nem.

In order to clarify the effect of Nem on HepG2 cells and on their proliferation induced by T2, we evaluated its effect on cell cycle progression and cell migration. As regards cell cycle distribution (Fig. 8C), Nem (25 μM) alone or in combination with T1 and T2 induced a significant shift from the S to the G0/G1 phase compared to control and T2-treated cells. Notably, T1 caused a significant shift to the G0/G1 phase with respect to both to control and T2-treated cells, in accordance with what observed for cell viability (Fig. 7).

The migratory capacity of HepG2 cells incubated with T1 and T2 in presence or absence of 25 μM Nem was evaluated by the wound-healing assay after 24-h of treatment. As indicated in Fig. 8D–E, only T2 treatment significantly increased the migratory capacity of HepG2 cells with respect to control cells and T1-treated cells, further confirming that HepG2-CM led to differentiated THP-1 cells with the characteristics of TAMs, able to promote tumor invasiveness. Interestingly, Nem induced an inhibition of cell migration in both T1- and T2-treated cells, and this effect is particularly evident in T2-treated cells, in which a significant Nem-induced decrease of wound closure could be observed with respect to T2 treatment.

4. Discussion

Recruitment and differentiation of immune system cells are pivotal processes occurring during tumor growth and progression. Indeed, it has been demonstrated that immune cells and inflammatory mediators integrate an immune-regulatory network in the TME, favoring tumor escape from immuno-surveillance and promoting tumor invasion, intravasation and angiogenesis [2–5]. In this regard, the symbiotic relationship between cancer cells and monocytes differentiating into TAMs constitutes one of the main targets of cancer immunotherapy research.

In the present study, we evaluated the interaction between THP-1 monocytes, used as a macrophage differentiation model, and hepatocellular carcinoma HepG2 cells. Additionally, we investigated for the

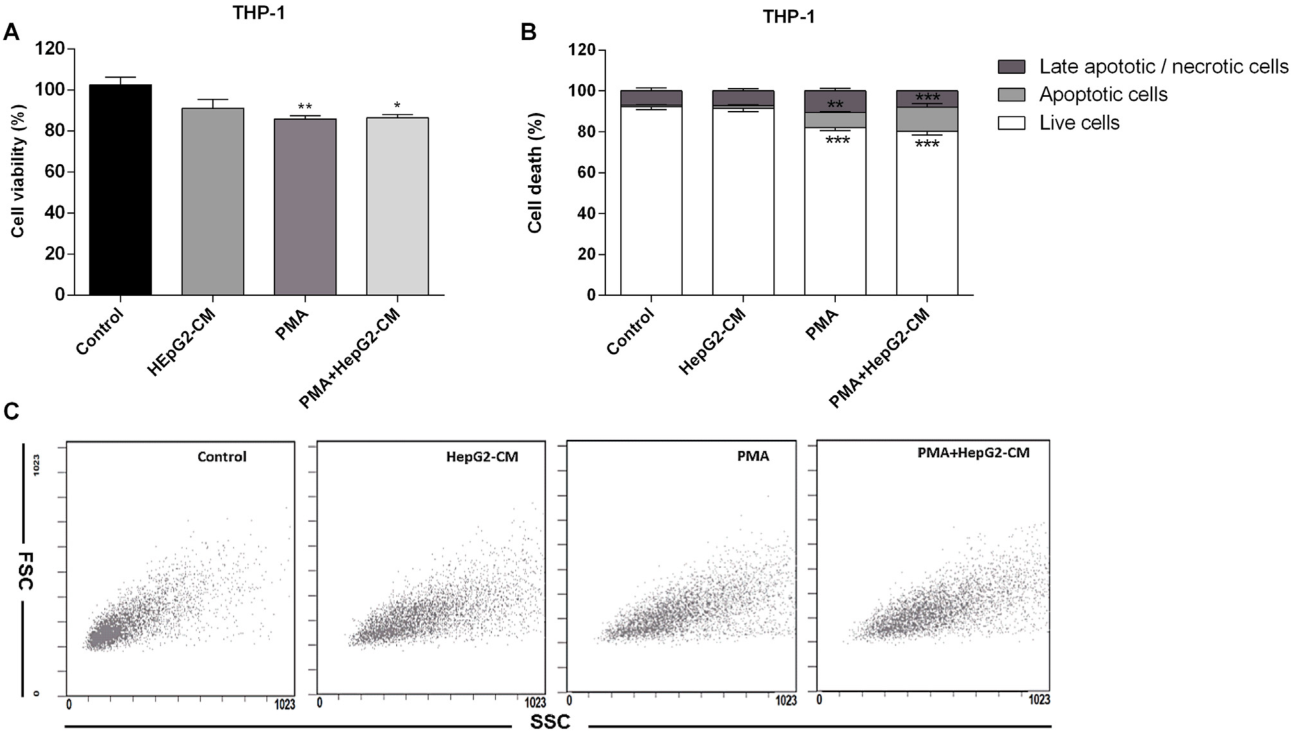

Fig. 3. Effect of HepG2-CM, PMA and PMA + HepG2-CM on proliferation, cell death and morphology of THP-1 cells. (A) Cell proliferation was determined by MTT assay after 72 h of exposure. (B) Effect on cell death of THP-1 cells. Apoptosis/necrosis cell death was measured by flow cytometry using Annexin V-FITC/PI staining. **(C)** Influence on THP-1 morphology evaluated by flow cytometry. Dot blots are representative of three independent experiments. Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs control cells.

first time the ability of nemorosone, a poly-isoprenylated benzophenone present in brown Cuban propolis and in the resin of *Clusia* species, of interfering with the macrophage-mediated hepatocellular carcinoma cell growth.

Our study demonstrates that the soluble factors secreted by HepG2 cells are able to influence the migratory ability and the differentiation of THP-1 into macrophages, without affecting their viability. As already described, this increased migratory ability of monocytes in response to tumor microenvironmental signals is induced and regulated by several chemokines secreted by tumor cells [2,3]. Our results are in line with previous findings suggesting the ability of different HCC models to induce monocytes recruitment [48]. The concentration of PMA (320 nM) used in our study, either alone or in combination with HepG2-CM, induced only limited apoptosis and necrosis to THP-1 cells. The proinflammatory stimulator PMA is a widely used to differentiate THP-1 into macrophages which are non-polarized or in resting state (M0) [10,11,42,49,50]. Nevertheless, high concentrations of this substance can affect cell viability and cause aberrant gene expression during the differentiation process [51,52].

It has already been reported that the differentiation of monocytes into macrophages is accompanied by stereotypic alterations in the expression of several cell surface markers [4,9–11]. Our results are in line with these previous observations, since we demonstrated that THP-1 stimulated with HepG2-CM, PMA and PMA + HepG2-CM showed an increase of CD68 expression. Conversely, the expression of CD14 was unchanged after HepG2-CM treatment, whereas PMA, in absence or presence of HepG2-CM, increased its expression with respect to untreated cells. This result is not surprising, since inconclusive findings can be found about CD14 expression in cultured THP-1: previous reports showed that treating these cells with different conditioned mediums did not cause variations in CD14 expression [53], probably because of experimental limitations, i.e., the small dimension of cells and/or cellular low density [54]. Furthermore, it has been noticed that CD14 expression by THP-1 varied in response to PMA from no response or poor to increased levels, depending on PMA concentration and incubation time [54,55].

Moreover, we observed an increase of the expression of the mannose receptor CD206 caused by HepG2-CM both in absence and presence of PMA treatment. This CM-dependent increase is in line with the fact that CD206 is considered a marker of TAMs and, therefore, is mainly expressed by M2 macrophages [43]. On the other hand, the high production of ROS by THP-1 observed in our in vitro differentiation model is also suggestive of monocyte differentiation into macrophages, mainly into M1 macrophages [43,45]. Several studies have indicated that conditioned medium of different tumor cell lines can influence the plasticity of macrophage functional state [53,56]. Thus, our results also support the hypothesis that the phenotype of THP-1 monocytes can be influenced by soluble factors secreted by HepG2.

Macrophages are known to produce epidermal growth and angiogenic factors, as well as activators of signal transducer through the expression and release of cytokines [46,49,53,57]. To obtain further information about THP-1 differentiation and polarization, we decided to evaluate the mRNA expression of different cytokines typically released by both M1 and M2 phenotypes. In fact, according to their phenotype, polarized macrophages can produce different cytokines. M1 macrophages produce low IL10 levels, whereas M2 macrophages produce high levels of this cytokine. The M1 phenotype is also characterized by the expression of the pro-inflammatory cytokines TNF and IL6, meanwhile M2 cells generally express these cytokines in low concentration [50]. Our study showed that THP-1 cells incubated with tumor HepG2-CM increased m-RNA expression of IL10 and TNFα compared to control. These data, although limited by the fact that we analyzed only mRNA expression of these cytokines in THP-1, suggest that the soluble factors released by HepG2 could induce THP-1 cells differentiation into a mixed population of M1 and M2 macrophages, as also suggested by the expression of the surface markers we analyzed. Our results are in line with other studies showing that THP-1 cells exposed to supernatants obtained from different tumor cell lines were

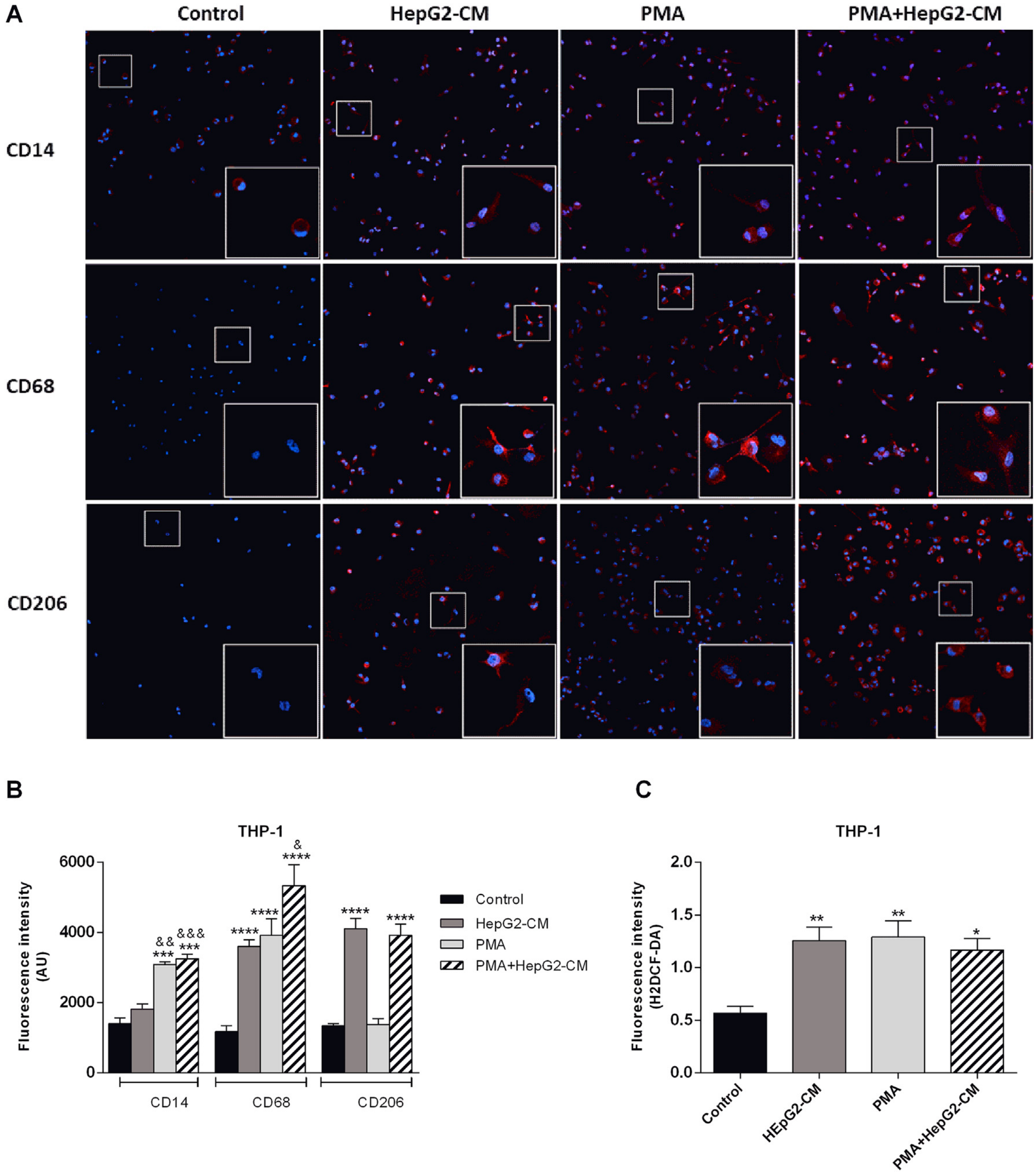

Fig. 4. Influence of HepG2-CM, PMA and PMA + HepG2-CM on monocyte differentiation to macrophages. (A, B) CD14, CD68, CD206 expression was evaluated by immunofluorescence analysis. Immunofluorescence images of surface markers expression are in red. Nuclei were stained with Hoechst (blue). Magnification: 10×. (D) ROS production induction was evaluated by means of H2DCF-DA assay. Results are representative of three independent experiments ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$vs control cells. $^{\&}P < 0.5$, $^{\&\&}P < 0.01$, $^{\&\&\&}P < 0.001$ vs HepG2-CM-treated cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

differentiated into a mixed population composed by both M1 and M2 macrophages [53,56]. In our experimental model, PMA treatment increased TNFα and IL6 gene expression, whereas HepG2-CM increase TNFα and IL10. Therefore, the upregulation of IL10 gene by HepG2-CM, both in absence and presence of PMA, is peculiar of THP-1 stimulated with the soluble factors released by HepG2, whereas the significant increase of IL6 gene expression observed only in PMA-stimulated cells could be related to this reagent. According to other studies indicating that THP-1 differentiation by PMA at a concentration higher than 200 nM induces the aberrant expression of different cytokines, chemokines, as well as matrix metalloproteinases [41,51], our findings confirm that treatment with PMA (320 nM) could affect or mask the expression of genes involved in inflammation and recruitment of immune cells.

Several evidences demonstrate that macrophage-induced angiogenesis is driven by the activated zymogen of matrix metalloproteinases [7,9]. Here, we showed that both MMP-2 and MMP-9 activities were significantly increased in supernatant of THP-1 cells stimulated with HepG2-CM and PMA + HepG2-CM. The metalloproteins MMP2/9, used for classification of TAMs, has long been associated with M2 polarization [43], and the results we obtained further confirmed the role of HepG2-CM on macrophage differentiation, together with the observed increase in the expression of CD206 and IL10, which occurred only in HepG2-CM-based treatments. Numerous MMPs are expressed by monocytes to let them migrate into tissues where they can differentiate into macrophages, which are also able to produce many proangiogenic

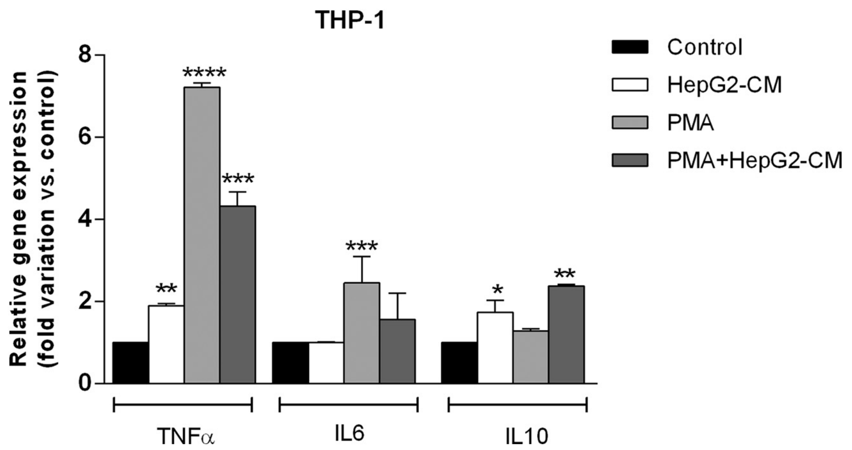

Fig. 5. Effect of HepG2-CM, PMA and PMA + HepG2-CM treatment on mRNA expression of TNFα, IL6 and IL10. Results are presented as mean ± SD of three independent experiments. $^{*}P < 0.5$ $^{**}P < 0.01$ $^{***}P < 0.001$ $^{****}P < 0.0001$ vs control cells.

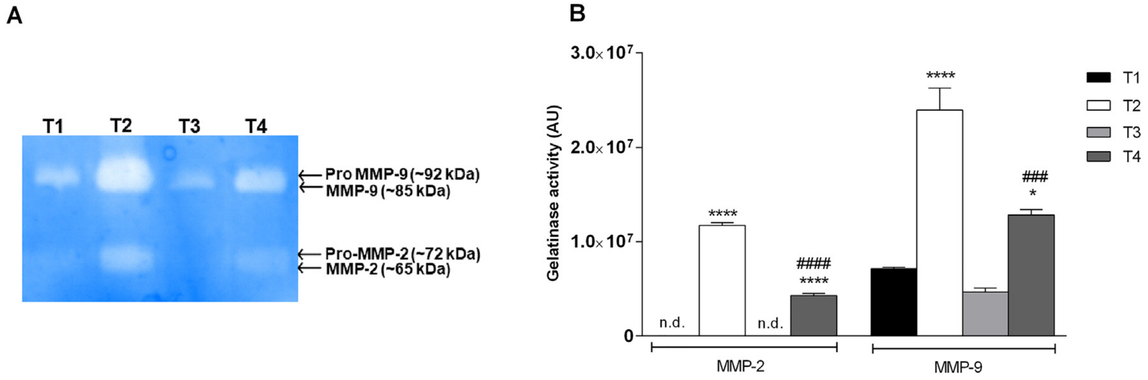

Fig. 6. Effect of HepG2-CM, PMA and PMA + HepG2-CM on gelatinase activity in THP-1 cells. (A) MMP-9 and MMP-2 activity in THP-1 supernatant was evaluated by zymography after 72 h (T1: supernatant of unstimulated THP-1 cells, T2: supernatant of THP-1 cells stimulated with HepG2-CM, T3: supernatant of THP-1 cells stimulated with PMA, T4: supernatant of THP-1 cells stimulated with PMA + HepG2-CM). (B) AU: arbitrary units. Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$ $^{**}P < 0.01$, $^{****}P < 0.0001$ vs T1, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ vs T3.

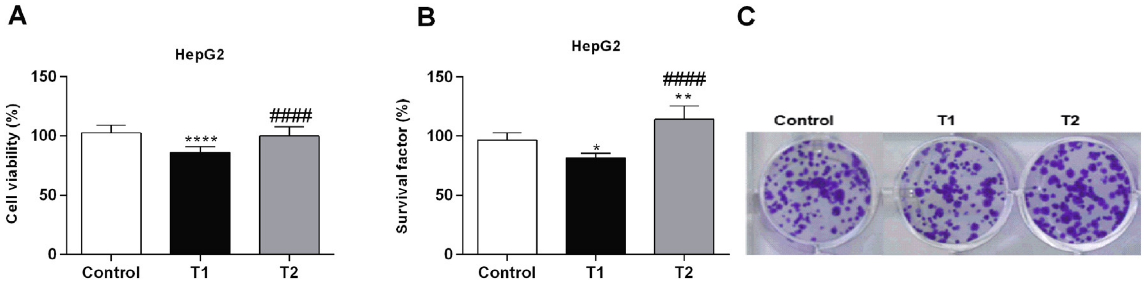

Fig. 7. Effect of T1, T2, T3 and T4 on viability and proliferation of HepG2 cells. T1: supernatant of unstimulated THP-1 cells, T2: supernatant of THP-1 cells stimulated with HepG2-CM. (A) Cell viability was determined by MTT assay after 24 h of exposure. (B, C) Cell proliferation was assessed by colony formation assay. $^{*}P < 0.05$, $^{****}P < 0.0001$ vs control (untreated cells), $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ vs T1.

and growth factors, as, for instance, MMP-2, MMP-9, TNFα and VEGF [9,58,59]. In our in vitro model, we observed a slight activity induced by MMP-9 in the supernatant of unstimulated THP-1 cells (T1) and in T3 supernatant, the latter probably due to the concentration of PMA used in this study (> 200 nM) which is known to induce MMPs expression [41]. On this basis, we decided to perform the analysis of the effect of Nem only with T1 and T2, treatments in which PMA has not been used.

In this study, from the analysis of MMPs activity, we found an increase in cell proliferation, colonies formation and cell migration in T2 supernatant-treated HepG2 cells. Since MMP-2 and MMP-9 can promote tumor progression through cellular proliferation and migration, matrix remodeling and formation of stabilized vessels [9], our findings suggest that HepG2-CM, even in absence of PMA, is able to differentiate monocytes into macrophages, which induce the release of MMPs that are involved in HepG2 progression.

Another aim of this study was the analysis of the possible effect of Nem in TME. Here we demonstrated that Nem causes the reduction HepG2 cells proliferation and the cell cycle arrest in Go/G1 phase. Additionally, the number of colonies and the migratory capacity of HepG2, which were increased by the soluble factors secreted by THP-1, were significantly reduced by Nem, and this effect on tumor invasiveness could not be observed in T1-treated cells. Nem had already been found to reduce cell viability of different tumor model cells [25,28,30]. Our in vitro study indicates a possible scenario in which a heterogeneous population of TAMs establishes a mutual crosstalk with HepG2

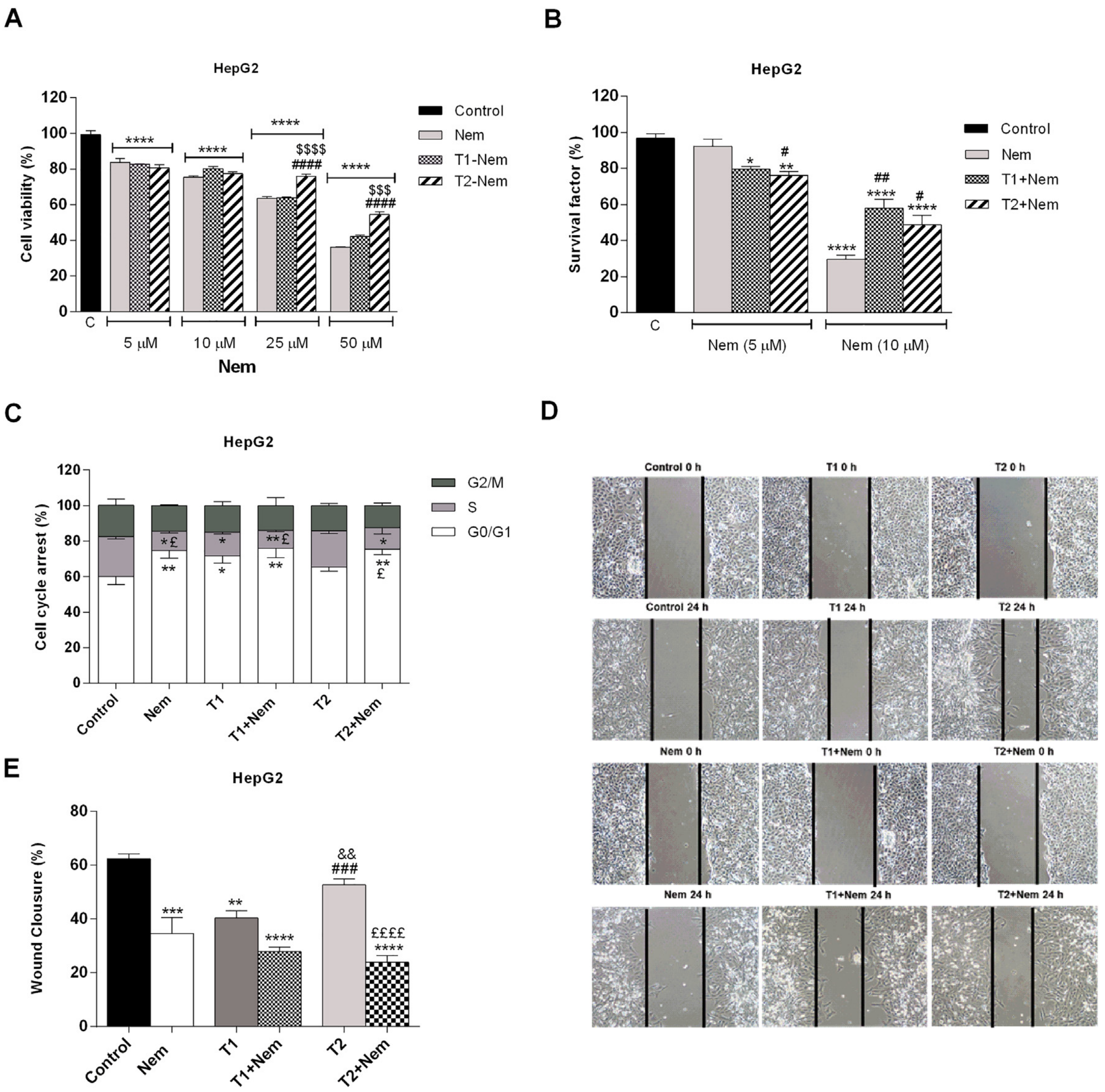

Fig. 8. Effect of T1, T2 and Nem on proliferation, cell cycle distribution and cell migration of HepG2 cells for 24 h. Cell viability was assessed by MTT assay (A), cell proliferation was assessed by colony formation assay (B), cell cycle distribution by flow cytometry (C) and cell migration by wound-healing migration assay (D, E). Control: untreated cells, Nem: Nemorosone (25 μM), T1: supernatant of unstimulated THP-1 cells, T2: supernatant of THP-1 cells stimulated with HepG2-CM. Data are presented as mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$ vs control cells, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$, $^{\#\#\#\#}P < 0.0001$ vs Nem group, $^{\&\&}P < 0.01$ vs T1 group, $^{£}P < 0.05$, $^{££££}P < 0.0001$ vs T2 group, $^{\$\$\$\$}P < 0.0001$, $^{\$\$\$}P < 0.001$ vs T1-Nem group.

cells, increasing their proliferation. This was counteracted by Nem. Our results could encourage further studies about the effect of Nem on the tumorigenic soluble factors released by TAMs into the tumor microenvironment.

5. Conclusion

In conclusion, our findings show that HepG2 cells secrete factors acting as chemoattractant and induce the differentiation of monocytes into a mixed population of M1 and M2 macrophages, which in turn increase cell proliferation, number of colonies and migration of HepG2. Nem exerts an antiproliferative effect on hepatocellular carcinoma cells, by decreasing the number of colonies and cell migration. Further studies are needed to ascertain whether Nem could also actively modulate the HCC-TAMs crosstalk.

Declaration of competing interest

The authors have no conflict of interest to declare.

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