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ORIGINAL PAPER

Variable impact of three different antiangiogenic drugs alone or in combination with chemotherapy on multiple bone marrow-derived cell populations involved in angiogenesis and immunity

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Abstract
In contrast to VEGF pathway-targeting antibodies, antiangiogenic tyrosine kinase inhibitors (TKIs) have failed to meet primary endpoints in almost all phase III clinical trials when combined with conventional chemotherapy. One exception is the combination of nintedanib and docetaxel as a second-line therapy for rapidly progressing advanced NSCLC. In addition to increased toxicity caused by this type of combination, thus necessitating drug dose reductions or treatment breaks, such phase III trial failures may also be related to the differential impact of host-mediated responses involving mobilization and tumor infiltration of bone marrow-derived cell populations (BMDCs), comprising both pro-angiogenic as well as immune effector cells. Herein, we evaluated two different antiangiogenic TKIs (sunitinib or nintedanib) and a VEGFR-2 antibody (DC101) either alone or combined with maximum tolerated dose paclitaxel for their differential impact on the BMDC host response, evaluating four different cell types. TKIs (in particular sunitinib) induced myelosuppression similar to paclitaxel, whereas DC101 had no such effect. Sunitinib also significantly decreased the number of tumor-infiltrating CD8 + T and B cells, MDSCs, and macrophages. In contrast, the effect of nintedanib on these BMDC populations was less marked, behaving closer to the VEGFR-2 antibody effects than sunitinib. The results raise the possibility that differences observed between antiangiogenic antibodies and TKIs in increasing chemotherapy efficacy could be related, at least in part, to differential effects on cells associated with local immunity within the tumor microenvironment.

Keywords Antiangiogenesis · TKI · Chemotherapy · VEGFR2 antibodies · Bone marrow-derived cells

Background

Based on phase III trial outcomes, both bevacizumab (a VEGF antibody) and ramucirumab (a VEGFR-2 antibody) have been shown to enhance the efficacy of concurrent chemotherapy, depending on the chemotherapy regimen used and type of cancer treated. The use of bevacizumab in combination with chemotherapy was first approved in 2004 for first-line metastatic colorectal cancer [1]. Currently, this type of combination has been approved for other malignancies such as non-small cell lung cancer (NSCLC) [2], ovarian cancer [3], cervical cancer [4, 5], gastric cancer [6], among others. Despite the wide use of this type of drug combination, the mechanism(s) of action of how bevacizumab or ramucirumab enhance the efficacy of chemotherapy is still not well understood, and remains a topic of controversy. Jain proposed in 2005 a hypothesis to explain how VEGF pathway inhibiting therapy may improve the efficacy of cytotoxic chemotherapy, namely by causing the phenomenon of 'vessel normalization,' which is postulated to increase the intra-tumoral delivery and hence concentration of chemotherapy [7].

An alternative hypothesis proposed by our group several years ago involves reactive induction of tumor growth promoting host responses by the chemotherapy treatment, namely the mobilization of different types of pro-angiogenic bone marrow-derived cells (BMDCs), such as circulating endothelial progenitor cells, hemangiocytes, and Tie

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2-expressing monocytes (TEMs), which can be prevented or attenuated by the concurrent administration of the antiangiogenic drug partner [8]. These host-mediated responses can minimize or prevent the desirable antitumor effects of the chemotherapy. The paclitaxel-induced mobilization of pro-angiogenic BMDCs can be suppressed by VEGF pathway-targeting antibodies (such as DC101, a rat antibody directed against mouse VEGFR-2), when both drugs are administered concurrently in combination [8].

There are contradictory preclinical results concerning the effect of antiangiogenic TKIs combined with chemotherapy, which may be related to the type of cancer model studied as well as the treatment schedule [9–15]. However, most of the combinations tested involving an antiangiogenic TKI with various chemotherapeutic drug 'backbone' partners have failed to reach primary efficacy endpoints in numerous phase III clinical trials involving many different types of cancer and different TKIs [16–23]. The combined cost of these failed trials is enormous. The only exception, in terms of marketed approval, is nintedanib combined with docetaxel, as a second-line treatment in rapidly progressing NSCLC, as shown in a phase III trial called LUME Lung-1 [24]. The clinical benefit of this combination may be related to the high affinity of nintedanib for FGFRs, not just VEGFRs and PDGFRs [25] and the more tolerable toxicity profile of nintedanib, compared to most other antiangiogenic TKIs [24, 26–32]. Thus, combination of nintedanib with conventional chemotherapy is more tolerable [24, 27–32], resulting in fewer instances of drug dose reductions or drug 'holidays' of the combination treatment [16, 18–23].

In addition to increased toxicity, another possible reason for the failure of the multiple phase III combination trials of TKIs with chemotherapy may be related to the impact on a host response involving BMDCs. TKIs may have different effects on chemotherapy-induced mobilization and tumor infiltration of BMDCs compared to the more specific targeting antiangiogenic antibodies, involving not only cell types relevant to angiogenesis, but also various immune effector or modulating cells. One of the considerations for the basis of this hypothesis is that antiangiogenic TKIs such as sunitinib can markedly increase not only VEGF but also a number of 'off target' circulating pro-angiogenic host-derived cytokines and chemokines (e.g., G-CSF, SCF, OPN, and SDF-1α) [33]. This effect can be observed even in normal non-tumor-bearing mice [33]. Some of these host factors, including VEGF, are known to recruit various types of pro-angiogenic BMDCs [34–37] as well as having immunosuppressive effects [38–41].

Previous studies have shown different if not opposite effects of sunitinib and antiangiogenic antibodies (e.g., bevacizumab or DC101) on immune cells, particularly T lymphocytes. Thus, sunitinib was reported to induce a reversible inhibition of proliferation in human T lymphocytes isolated from both healthy volunteers and cancer patients [42]. Such inhibition was mediated by accumulation of T lymphocytes in G0/G1 phase of the cell cycle. Also, sunitinib was shown to prevent T cell-mediated responses in mice, characterized by reduced delayed-type hypersensitivity (DTH) [42]. On the other hand, it has been reported that the VEGFR2 antibody DC101 can actually promote the intra-tumoral infiltration of CD8 + T lymphocytes after 2 weeks of treatment, contributing to a T cell-mediated tumor regression [43]. Similar effects have been observed in melanoma patients, where the addition of bevacizumab to ipilimumab (a CTLA-4 immune checkpoint inhibiting monoclonal antibody) treatment increased circulating memory T cells and their infiltration into tumors [44]. Furthermore, sunitinib and DC101 seem to have opposite effects on macrophages. Thus, van Dongen et al. observed that sunitinib can induce secretion of anti-inflammatory IL-10 in M1 macrophages [45] (i.e., the classically activated macrophages with antitumor activity [46]), suggesting that it may induce or contribute to an immunosuppressive microenvironment. Conversely, it has been suggested that, depending on the dose of DC101, this agent can polarize the alternatively activated, tumor-promoting M2 macrophages toward the M1-like subtype. Such an effect is maximized when DC101 is administered in vivo at lower than conventional doses [47].

Given this background, we sought, as reported herein, to analyze the role of four different BMDCs involved in angiogenesis and/or immune response, namely myeloid-derived suppressor cells (MDSCs), macrophages, and B and CD8 + T lymphocytes, and hence the possibility that some of the TKI effects on such cells may account for the general failure, at least in part, of antiangiogenic TKIs to enhance chemotherapy efficacy, in contrast to VEGF pathway-targeting antibodies. This study involved three antiangiogenic drugs (two different TKIs and an antibody) and one chemotherapeutic drug, in a head-to-head comparative analysis, evaluating their effects when administered alone and as combinations on four different cell types. To our knowledge, this is the first time this kind of broad comparative analysis has been undertaken, and was done with the goal of determining whether the information may be pertinent to helping explain the numerous and contrasting clinical trial outcomes of antiangiogenic TKIs versus antibodies when combined with standard chemotherapy.

Materials and methods

Tumor and animal models

Male C57Bl/6 mice (6-weeks old, Jackson Labs, Canada) were lethally irradiated and used as recipients of injections with bone marrow cells obtained from UBC GFP + syngeneic Bl6 mice [48]. Experiments were initiated when at

least 95% of cells in peripheral blood of such transplanted bone marrow chimeric mice were assessed to be GFP+. The use of these chimeric mice allowed us to evaluate the effect of the different drugs and combinations on the bone marrow compartment by flow cytometry, as described below. GFP C57Bl/6 mice were used as recipients for a subcutaneous injection of 5×10^5 Lewis lung carcinoma (LLC) cells (ATCC® CRL-1642™). Tumor size was measured regularly with Vernier calipers and tumor volume was calculated using the formula $a^2b/2$, where a is the width and b is the length. Treatment started when the average tumor volume reached 300 mm^3. All mice were randomized just before initiating treatment to obtain similar average tumor burdens among groups. All procedures were in accordance with the animal care guidelines of Sunnybrook Health Science Centre (Canada) and the Canadian Council of Animal Care.

Drugs and treatments

DC101 (Eli Lilly), the rat monoclonal blocking antibody specific for mouse VEGFR2/Flk-1, was administered ip at 800 µg/mouse, considered as an optimal biologic dose [49, 50]. Sunitinib (Sutent, Pfizer) was prepared as recommended by the manufacturer and administered by gavage at 60 mg/kg, which is an optimal dose for causing antitumor effects [33]. Nintedanib was provided by Boehringer Ingelheim (Vienna) and administered by gavage at the recommended preclinical dose (for mice) 50 mg/kg. Paclitaxel was purchased from Sunnybrook Pharmacy Department, Odette Cancer Center (Toronto, Ontario, Canada) at 6 mg/ml and further diluted with sterile normal saline to the appropriate concentration, and administered ip at 30 mg/kg, close to the maximum tolerated dose (MTD) in mice, which induces mobilization and tumor 'homing' of pro-angiogenic BMDCs [8].

Treatment groups and schedules were as follows: (1) control group received relevant vehicles; (2) sunitinib (60 mg/kg) po qd; (3) nintedanib (50 mg/kg) po qd; (4) MTD paclitaxel (30 mg/kg) ip on day 7; (5) DC101 (800 µg/ mouse) ip on day 7; (6) DC101 ip 24 h prior paclitaxel injection; (7) sunitinib (60 mg/kg) po qd and paclitaxel ip on day 7; and (8) nintedanib (50 mg/kg) po qd and paclitaxel ip on day 7.

Flow cytometry analysis

Antibodies used for flow cytometry analysis were the following: CD45-APC Cy7, Gr1-PE Cy7, CD11b PerCP Cy5.5, CD8-APC and CD3e-PE Cy7 were purchased from BD Biosciences; F4/80-PE eF610 and CD19-eF450 were obtained from eBioscience.

Blood was collected in EDTA tubes from anesthetized mice by retro-orbital bleeding on day 8 of treatment for flow cytometry analysis. At day 10 of treatment mice were sacrificed, and tumors collected and dissociated to obtain single cell suspensions. Flow cytometry analysis of BMDCs was undertaken, after lysing red blood cells, on a FACSCalibur (BD). Acquisition of 50 000 events for the different BMDC populations was performed. To analyze the mobilization and infiltration of tumors by BMDCs, the flow cytometry analysis was first based on GFP expression of cells to ensure their bone marrow origin. Different cell populations were enumerated based on the following markers/phenotypes: MDSCs as CD45 + Gr1b + CD11b+ [51], macrophages as CD45 + Gr1-CD11b + F4/80+, B lymphocytes as CD45 + CD19 +, and CD8 T lymphocytes as CD45 + CD3e + CD8+ (Fig. 1). All the BMDC populations were analyzed using fresh tumor samples obtained at the time of terminating the experiment and involved the same set of compensations during flow cytometry analysis.

Histology and immunohistochemistry (IHC)

Tumors were fixed with 10% buffered formalin and embedded in paraffin. Tumor sections (5 µm thick) were deparaffinized and stained with hematoxylin and eosin (Leica) to analyze necrosis. For IHC, sections were quenched in 1% H_2O_2, unmasked in boiling sodium citrate buffer (10 mmol/L, pH 6, 5 min), and stained using the following specific antibodies: CD31 (1:50, Dianova), and α-SMA (1:300, Cell Signaling). Biotin-conjugated secondary antibodies (Jackson ImmunoResearch) were used and detected with Vector Elite HRP kit and DAB chromogen (Dako). Sections were counterstained with hematoxylin (Leica). Sections were visualized with a Leica DM LB2 microscope and digital camera (DFC300FX) and images acquired using AxioVision 3.0 software. Images were analyzed using ImageJ 1.38d software.

Statistical analyses

Statistical analyses were performed using the GraphPad Prism software package version 4.0 (GraphPad Software, Inc, San Diego, CA). Results are reported as mean ± SD and were subjected to ANOVA between groups, followed by Tukey's Multiple Comparison Test. Differences were considered statistically significant when p values were < 0.05.

Results

Effect of the drugs as monotherapy or in combination on leukocyte counts

As mentioned above, the levels of the various BMDC populations were analyzed in peripheral blood 24 h after paclitaxel treatment, based on previous results from our

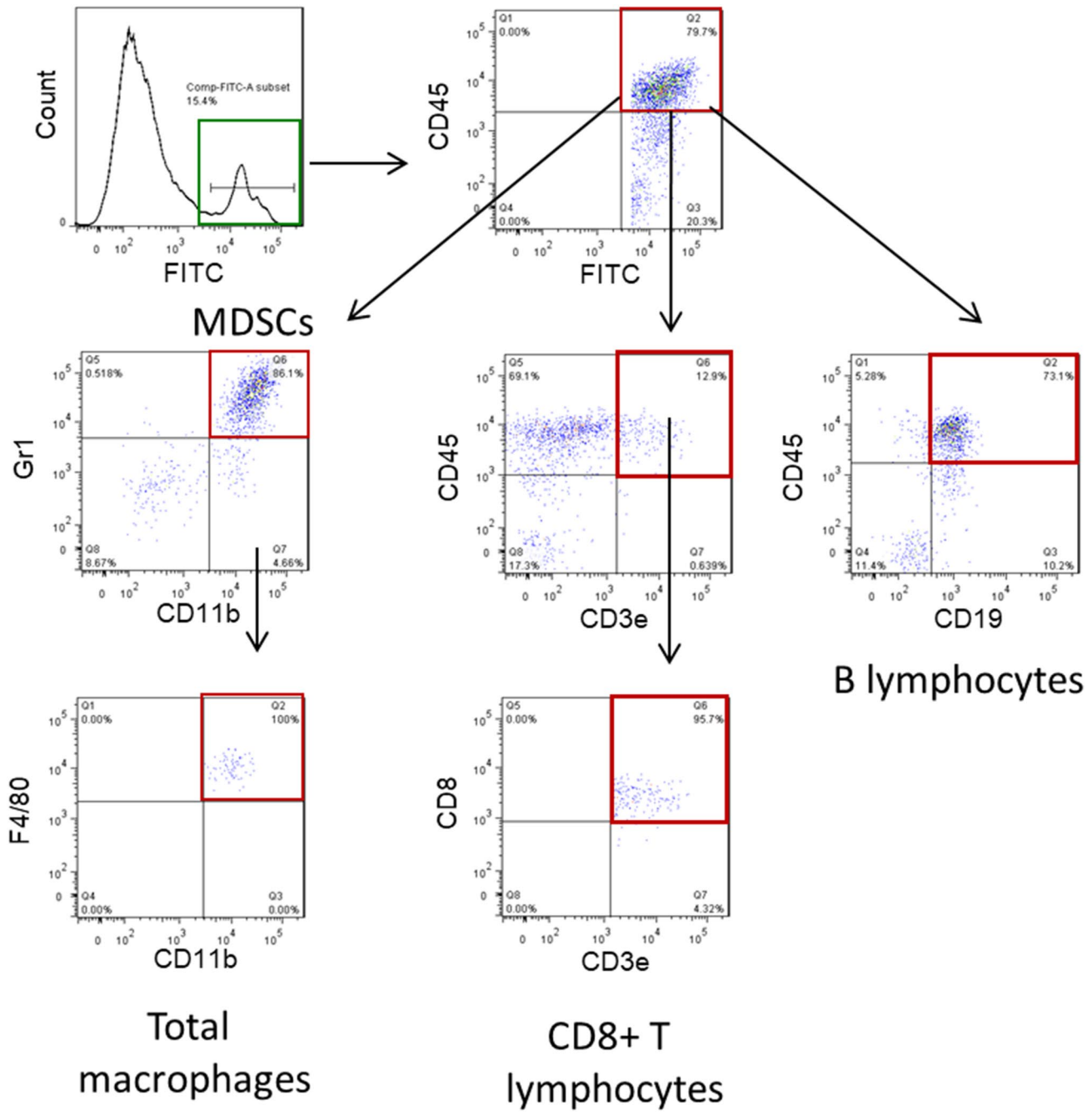

Fig. 1 Gates used to identify and analyze the percentage of different populations of bone marrow-derived cells mobilized to peripheral blood and infiltrating into the tumor following treatment with vehicle, paclitaxel, DC101, sunitinib, nintedanib, and the combinations of the antiangiogenic drugs with paclitaxel

group [8]. Herein, we observed that shortly after treatment with the chemotherapy drug there was a significant reduction in the number of leukocytes detected in peripheral blood (Fig. 2). Interestingly, a similar effect was observed in those mice treated with sunitinib alone. In contrast, the DC101 VEGFR-2 targeting antibody had no such effect, and nintedanib behaved similar to DC101 compared to sunitinib. The suppressive effect on leukocytes by paclitaxel increased when administered in combination with TKIs, compared to the control group. Mice treated with paclitaxel and DC101 showed lower number of leukocytes than those receiving DC101 alone (Fig. 2).

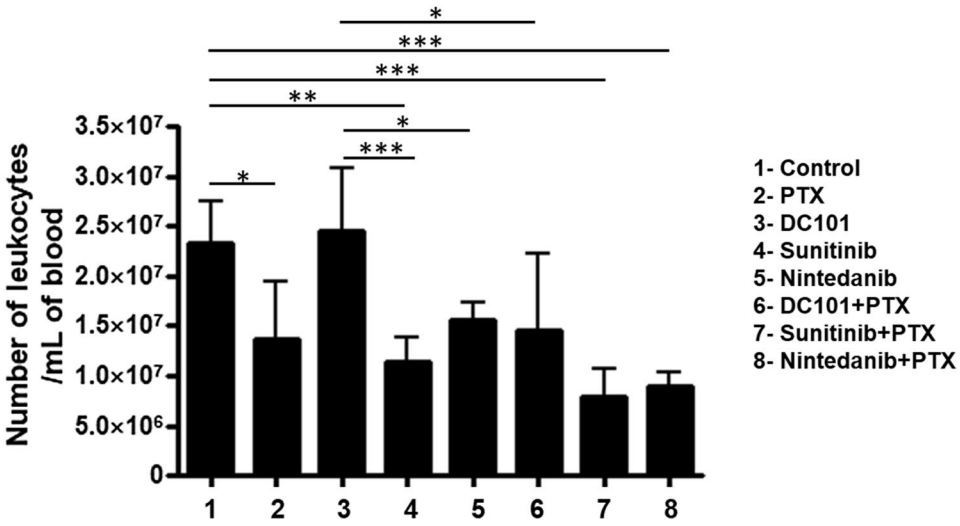

Fig. 2 Effect of different antiangiogenic drugs, or paclitaxel and various combinations on the number of leukocytes in peripheral blood of GFP C57/Bl6 bearing LLC tumors. Leukocytes were counted under the microscope using Turk's solution. All groups $n=5$. Data are presented as means ± SD. ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

Mobilization of BMDCs to peripheral blood

All treatments, except for DC101 alone, significantly decreased the number of MDSCs in peripheral blood, compared to the control group. Such an effect of nintedanib on this cell population was enhanced when it was administered in combination with paclitaxel (Fig. 3a). Interestingly, DC101 promoted the mobilization of CD8 + T lymphocytes

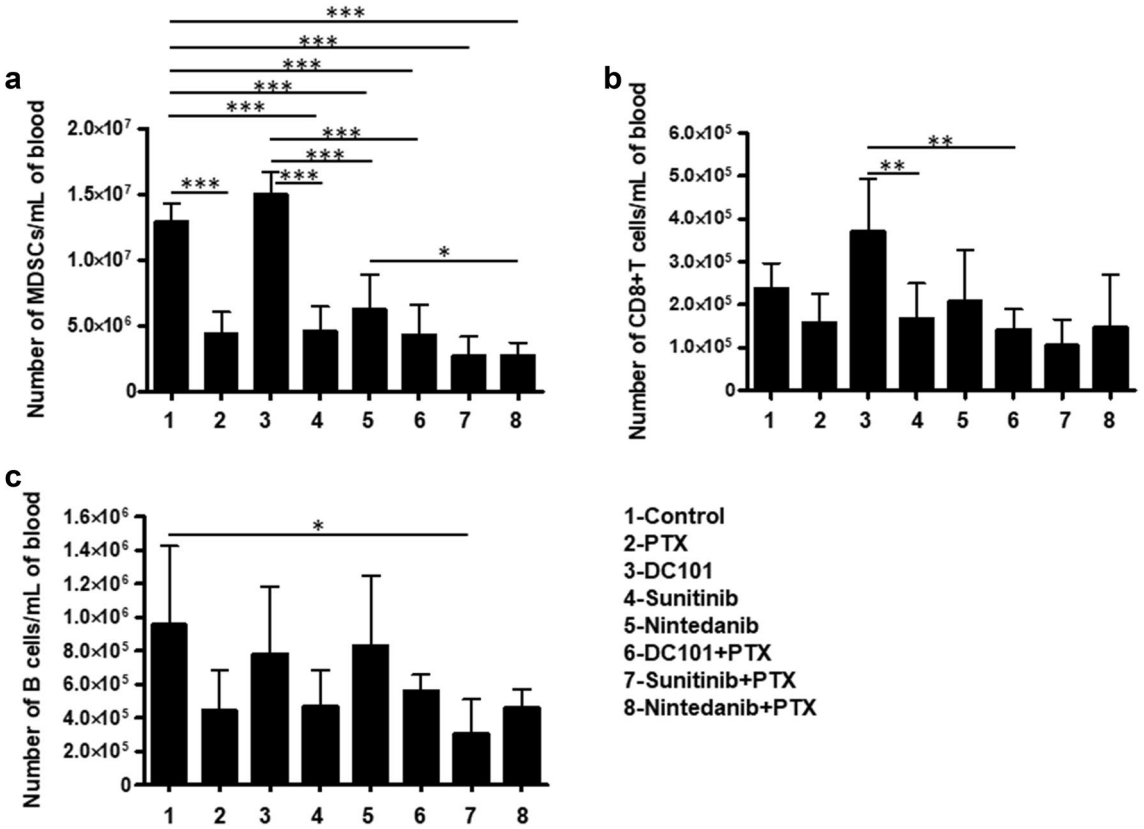

Fig. 3 Effect of different antiangiogenic drugs, paclitaxel, and combinations on the number of BMDCs in peripheral blood of GFP C57/Bl6 bearing LLC tumors. **a** Myeloid-derived suppressor cells (MDSCs); **b** CD8 + T lymphocytes; **c** B lymphocytes. All groups $n=5$. Data are presented as means ± SD. ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. $^{*}p<0.05$; $^{**}p<0.01$; $^{***}p<0.001$

into peripheral blood, which was abolished when mice were treated with the antibody combined with paclitaxel (Fig. 3b), whereas mice treated with sunitinib monotherapy had fewer CD8 + T cells compared to DC101 alone. On the other hand, and similar to nintedanib alone, DC101 had no effect on the number of B lymphocytes in peripheral blood (Fig. 3c). In contrast, both paclitaxel and sunitinib seemed to decrease the number of B lymphocytes in peripheral blood compared to the control group, and such effect increased when both drugs were administered in combination (Fig. 3c).

Tumor infiltration of BMDCs

We analyzed whether the effects observed in peripheral blood with the antiangiogenic drugs and/or paclitaxel corresponded with differences in the number of BMDCs infiltrating established primary tumors. Sunitinib alone or when combined with paclitaxel decreased the number of MDSCs in the tumor compared to the control group (Fig. 4a). In contrast, we did not observe any effect of DC101 or nintedanib on this cell population, being significantly different from sunitinib. The combination of nintedanib with paclitaxel resulted in fewer MDSCs in the tumor compared to mice treated with nintedanib alone (Fig. 4a).

Only sunitinib significantly affected the infiltration of total macrophages, either alone or when combined with paclitaxel (Fig. 4b) compared to the control group. Also, mice treated with DC101 had levels of macrophages significantly higher compared to those treated with sunitinib, either alone or when the antiangiogenic drugs were combined with paclitaxel (Fig. 4b). Sunitinib significantly suppressed the levels of CD8 + T lymphocytes, either alone or combined with paclitaxel, compared the control group (Fig. 4c). Furthermore, adding sunitinib to paclitaxel affected the tumor infiltration of CD8 + T cells compared to paclitaxel monotherapy. In contrast, mice treated with either DC101, nintedanib, or paclitaxel monotherapy had numbers of CD8 + T lymphocytes in the tumor similar to the control group (Fig. 4c). When we analyzed the levels of B lymphocytes in the tumor (which we assessed considering that this cell population may also play an important role in the antitumor effect mediated by the immune system [52]) mice treated with sunitinib had much lower levels of B lymphocytes compared to the control group (Fig. 4d), and to mice treated with DC101 or with nintedanib monotherapy (similar to the effect

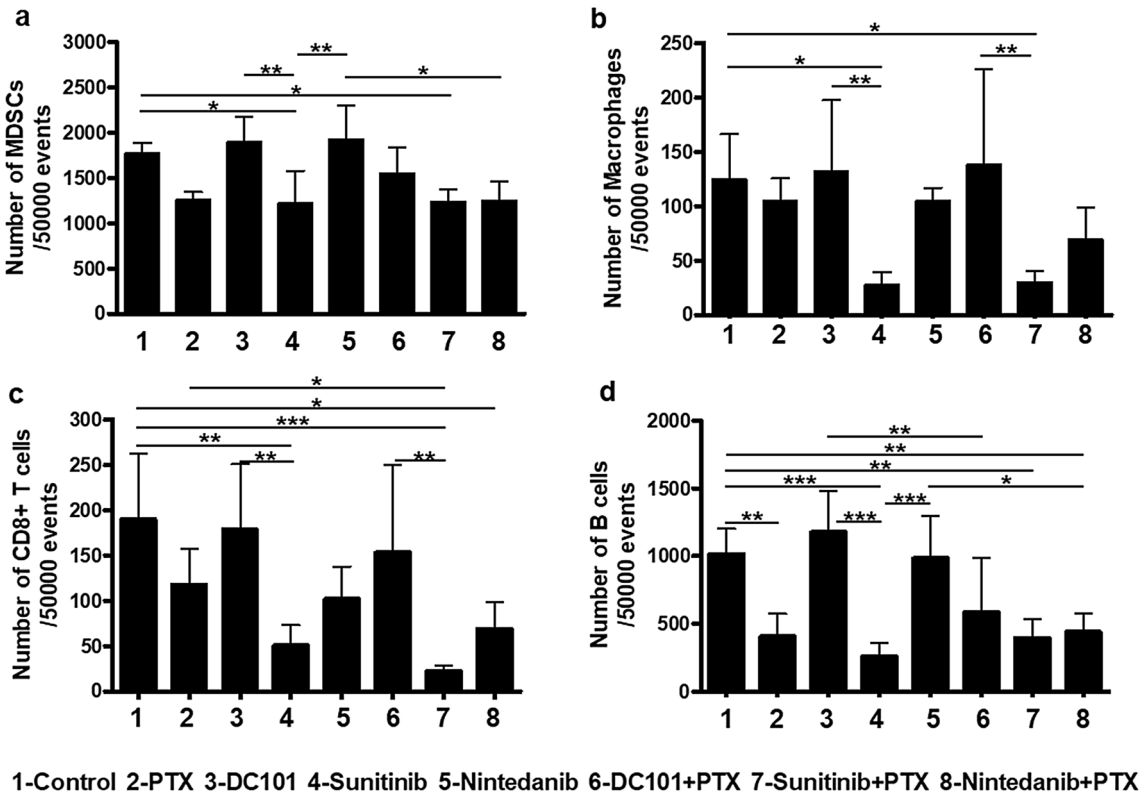

Fig. 4 Effect of different antiangiogenic drugs, paclitaxel, and combinations on the number of BMDCs invading the tumors of GFP C57/Bl6 mice bearing LLC. **a** Myeloid-derived suppressor cells (MDSCs); **b** Macrophages; **c** CD8 + T lymphocytes; **d** B lymphocytes. All groups $n = 5$. Data are presented as means ± SD. ANOVA followed by Tukey's multiple comparison test was used for statistical analysis. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

observed on CD8 + T lymphocytes). In general, B lymphocytes seem to be sensitive to treatment with paclitaxel, since all the mice receiving the drug had lower intra-tumoral levels of these cells, as previously reported [53–57]. However, neither DC101 nor nintedanib had an effect on B lymphocyte infiltration (Fig. 4d) similar to what was observed when we analyzed levels in the peripheral blood (Fig. 3c).

The number of BMDCs infiltrating the tumors may be influenced not only by the effect of the treatment on the mobilization and tumor homing of the BMDC populations studied, but also by differences in vascularity, necrosis, and fibrosis in the tumors. Thus, we also analyzed these features in tumors treated with the different drugs and their combinations (Fig. 5). As expected, and in line with flow cytometry data for endothelial cells (data not shown), all tumors treated with antiangiogenic drugs were less vascularized, compared to the control group and paclitaxel monotherapy (Fig. 5a). However, this did not translate into different levels of necrosis (Fig. 5b), presumably because this tumor model is highly necrotic in nature. We also did not observe differences in terms of number of α-SMA + fibroblasts in the tumors (used as a cell marker of fibrosis) (Fig. 5c) among treatments that could explain fewer BMDCs infiltrating the tumors when treated with antiangiogenic TKIs (particularly sunitinib) when combined with paclitaxel. Only tumors treated with sunitinib plus paclitaxel showed lower levels of α-SMA + fibroblasts compared to DC101 plus paclitaxel (Fig. 5c).

Discussion

Despite the approval of more than ten antiangiogenic drugs for cancer therapy, both antiangiogenic antibodies (e.g., bevacizumab and ramucirumab) and TKIs (e.g. ,sunitinib, pazopanib, sorafenib, axitinib, nintedanib) [58], a key question that remains unanswered is how antiangiogenic VEGF pathway-targeting antibodies improve chemotherapy efficacy (when they do so), whereas TKIs

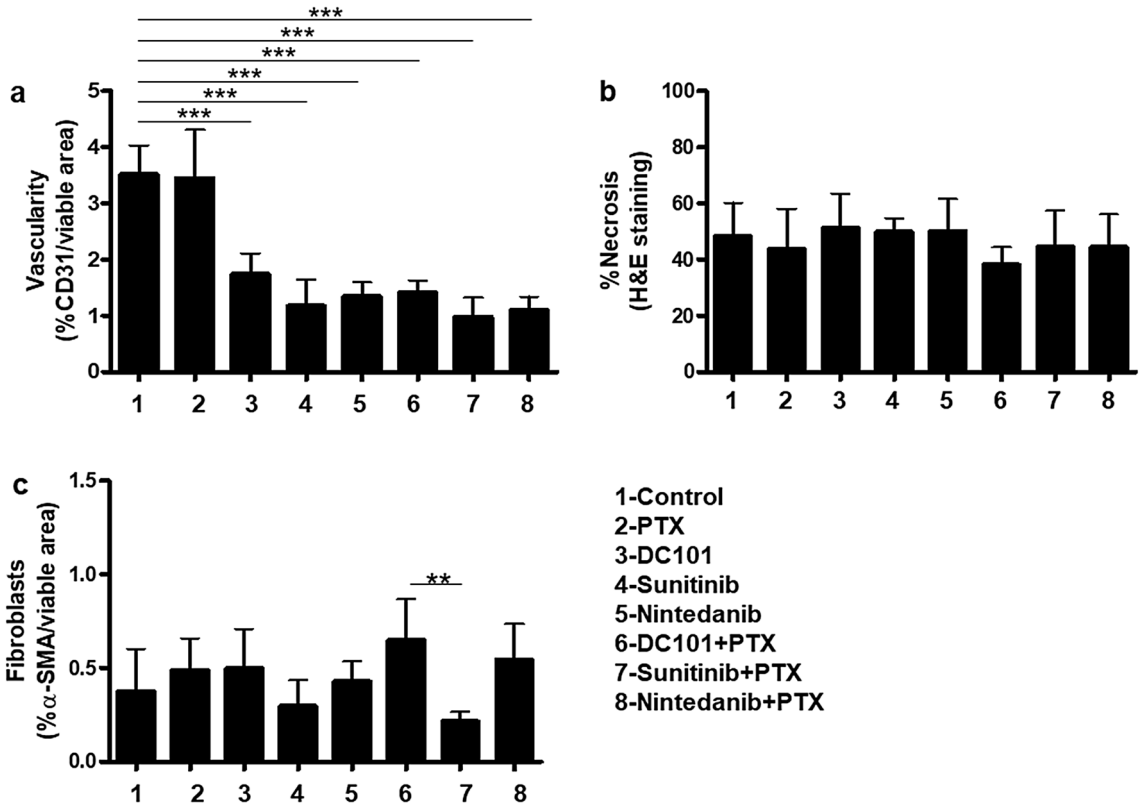

Fig. 5 Effect of different antiangiogenic drugs, paclitaxel, and combinations on the vascularity (**a**), necrosis (**b**), and levels of fibroblasts (**c**) in the tumors of C57/Bl6 mice bearing LLC. **a** All the treatments involving an antiangiogenic drug significantly decreased the vascularity compared to the control group. Treatments involving antiangiogenic drugs and PTX significantly decreased vascularity compared to PTX ($p < 0.001$, not shown). **b** Tumors were very necrotic in general, despite treatment with cytotoxic or antiangiogenic drugs. **c** No significant difference in the levels of fibroblasts among the different treatments and combinations, only tumors treated with sunitinib + PTX had significantly less fibroblasts that DC101 + PTX. All groups $n = 5$. Data are presented as means ± SD. Tukey's multiple comparison test was used for statistical analysis. $^{**}p < 0.01$; $^{***}p < 0.001$

almost always fail to do so. Currently, it is believed that such contrasting phase III failures of TKIs combined with chemotherapy is mainly due to the increased toxicity of such combinations, which leads to treatment interruptions or drug dose reductions, which in turn may compromise antitumor efficacy, as mentioned above. However, we hypothesized that increased toxicity may not be the only explanation for the common failure of antiangiogenic TKI/chemotherapy combinations, in contrast to antiangiogenic antibodies, but that certain biologic mechanisms such as differences in the intra-tumoral levels of various BMDCs induced by chemotherapy and how these levels are differentially altered by antiangiogenic antibodies versus TKIs may also be involved.

Bone marrow progenitor cells divide rapidly with a doubling time of 15–24 h, explaining their high sensitivity to cytotoxic drugs and resultant myelosuppression [59], as observed in this study 24 h after treatment of mice with MTD paclitaxel (Fig. 2). Myelosuppression is also a side effect frequently observed in patients treated with antiangiogenic TKIs, although the frequency and severity vary among the different TKIs [60]. Some of TKI-induced myelosuppression has been ascribed to inhibition of the intracellular autocrine loop of VEGF signaling mediated by VEGFR1/2 [61]. This may explain, at least in part, the decrease in leukocyte counts induced by TKIs in this study (particularly sunitinib), as well as the lack of such an effect of DC101 (Fig. 2).

In addition, the c-Kit and Flt3 signaling pathways also play a crucial role in the cell cycle of hematopoietic stem/progenitor cells [62], by mediating both proliferation and differentiation [60]. The differential affinities of sunitinib versus nintedanib for these RTKs may also explain myelosuppressive effect induced by sunitinib (Fig. 2) in contrast to nintedanib, as previously reported when compared to pazopanib and sorafenib [60], as well as their different toxicity profiles [19–22, 24, 27–32]. The potent myelosuppressive effects induced by TKIs combined with paclitaxel (Fig. 2) may also be related to the suppressive effects of TKIs on cell cycle progression of hematopoietic progenitor cells during recovery from chemotherapy mediated by VEGFR1/2 inhibition, as previously reported for SU5416 combined with 5-FU in C57/Bl6 mice [62].

We analyzed the effect of sunitinib, nintedanib, or DC101 (as monotherapies or combined with paclitaxel) on certain BMDC populations that promote angiogenesis (endothelial cells, TEMs, and hemangiocytes) (data not shown). Results suggested that the contrasting clinical results between antiangiogenic TKIs and antibodies combined with chemotherapy may involve mechanisms in addition to, or other than, inhibition of paclitaxel-induced mobilization and infiltration of pro-angiogenic BMDCs. Thus, considering the relevance of the immune system, we decided to evaluate the effect of such antiangiogenic drugs, paclitaxel, and their combinations on cell populations that contribute both to angiogenesis and to an immunosuppressive tumor microenvironment (MDSCs and tumor-associated macrophages, TAMs) and on immune effector cells such as T and B lymphocytes.

We observed differences in therapy-induced levels of various BMDC populations detected in peripheral blood versus tumor homing/infiltration of such cells, which are likely due to different RTKs involved in mobilization versus infiltration and the differential affinity of DC101, sunitinib, or nintedanib for such RTKs, as described below.

Some of the RTKs involved in mobilization of MDSCs (e.g., Bv8 and endocrine gland-derived VEGF) [63, 64] can be inhibited by antiangiogenic TKIs. Herein, we report that sunitinib decreased significantly levels of MDSCs both in peripheral blood (Fig. 3a) and in the treated tumors (Fig. 4a), as previously reported [63, 65–70]. In contrast, nintedanib only inhibited the mobilization of MDSCs into peripheral blood (Fig. 3a), but had no significant effect on the number of MDSCs infiltrating into the tumors (Fig. 4a). Such differential effects of nintedanib and sunitinib on tumor infiltration of MDSCs may be related to differences in their respective RTK targets and their role in recruitment of MDSCs to the tumor (e.g., SCF/c-Kit pathway mediate tumor infiltration of this BMDC population) [63, 64]. Furthermore, DC101 had no effect in the levels of MDSCs in peripheral blood or in the tumors, similar to previously reported results [47, 71], highlighting the importance of targeting RTKs in MDSCs other than VEGFR-2. Similar results have been reported for peripheral blood of glioblastoma (GBM) and renal cell cancer (RCC) patients treated with bevacizumab [72, 73].

The effect of antiangiogenic therapy on the number of tumor-infiltrating macrophages, and their phenotype/function, seems be related to their targets/specificity and dose/schedule, as well as the sensitivity of the tumor [45, 47, 74–77]. It has been suggested that DC101 can polarize tumor-associated M2 macrophages toward the M1-like subtype in vivo, but only when administered at doses below the conventional antiangiogenic dose [47]. Herein, conventional doses of DC101 had no effect on the number of total macrophages infiltrating LLC tumors (Fig. 4b) or their expression of M2 phenotype (data not shown), similar to previously reported results [47]. Also, nintedanib had no effect on the number of macrophages invading the tumors, whereas sunitinib significantly decreased this cell population, compared to the control group or DC101 monotherapy (Fig. 4b). This effect on macrophages of sunitinib could be related to the inhibition of CSF1 pathway [78], which mediates recruitment of this cell population to the tumor [79] as well as the development of monocytes from hematopoietic progenitor cells mediated, as previously reported for another (non-antiangiogenic) TKI, imatinib [80]. This is in line with results obtained in this study showing fewer MDSCs (Figs. 3a, 4a) and TEMs infiltrating sunitinib-treated tumors (data not shown).

Bevacizumab has been shown to promote an increase of CD8 + T lymphocytes not only in peripheral blood but also in tumors when combined with different therapeutic drugs such as conventional chemotherapy [81–83], immune checkpoint inhibitors (e.g., ipilimumab and atezolizumab) [44, 84, 85], and non-antiangiogenic TKIs (e.g., EGFR TKI) [86]. Similar to bevacizumab, it has been shown by others that DC101 promoted infiltration of CD8 + T cells into mammary tumors growing in FVB mice after 2 weeks of treatment [43, 47]. This effect seems to be related to the dose and duration of therapy, being more marked using doses below the conventional antiangiogenic dose which promote 'vessel normalization' and tumor infiltration of T cells [47]. Herein, the number of CD8 + T cells in the tumors 3 days after DC101 treatment was similar to the control group (Fig. 4c), despite the trend showing increase of this cell population in peripheral blood (Fig. 3b).

On the other hand, controversy remains about the immune modulatory effects of antiangiogenic TKIs, particularly with respect to T lymphocytes, and this seems to depend not only on their target specificity profile [87], but also on the tumor treatment setting [88, 89]. In this study, sunitinib inhibited the intra-tumoral infiltration of CD8 + T cells (Fig. 4c) providing evidence for the inhibitory effect of sunitinib on T cells in general, as previously reported by Gu and colleagues [42]. Another line of evidence suggesting that the effect of antiangiogenic TKIs on T lymphocytes (particularly CD8 + T cells) may be related to their target specificity (as mentioned above) is that in this study nintedanib had no significant suppressive intra-tumoral effect on this cell population (Fig. 4c), in contrast to sunitinib. Also, tumors exposed to sunitinib combined with paclitaxel contained less CD8 + T lymphocytes, compared to the control group or those treated with DC101 or nintedanib plus paclitaxel. This may have contributed, at least in part, to the positive clinical results of nintedanib plus docetaxel in NSCLC [24], in contrast with the clinical failures of sunitinib combined with different chemotherapeutic drug partners [19–23].

Sunitinib has been associated with a decrease in the number of B lymphocytes infiltrating the tumors in colon cancer mouse models [89]. Herein, sunitinib therapy significantly decreased the number of bone marrow-derived B cells infiltrating tumors, compared to the control group, DC101, or nintedanib monotherapy, in contrast to the lack of such an effect by nintedanib (or DC101) (Fig. 4d). Such a suppressive effect of sunitinib on the number of B lymphocytes may be related, at least in part, to its inhibitory effect on Flt3, which is expressed by B cell progenitors and plays an important role during their development [90, 91].

We acknowledge that this study, which is the first to undertake a comparative analysis of multiple and different types of antiangiogenic drugs on multiple BMDC populations in peripheral blood and tumors, has some limitations such as the short duration of the treatments and the lack of therapeutic efficacy analysis, in part because of the characteristics of the tumor model used in mice receiving bone marrow transplant (of GFP + bone marrow cells) after lethal irradiation. Such procedure allowed us to evaluate the effect of the different drugs on the bone marrow compartment; however, it makes the mice more sensitive in terms of toxicity to the drugs evaluated than the normal mouse strain and limits their use in long-term studies (e.g., efficacy analysis). A second limitation of this study is that there are additional BMDC population types that would likely impact the antitumor effect of the drugs, which were not included in the analysis, e.g., NK cells and T or B regulatory cells. Also, information regarding the activation status and localization in the tumor (e.g., rim vs core) of the effector cells analyzed may provide valuable information to help explain clinical results.

Despite these limitations, our results broadly indicate differences in VEGF pathway-targeting antibodies versus antiangiogenic TKIs and between different TKIs with respect to their effects on various BMDC populations relevant to tumor immunity. Based on our overall results, we suggest that the differences observed in the clinic between antiangiogenic antibodies and TKIs in enhancing chemotherapy efficacy could conceivably be related, at least in part, to different effects on immunity within the local tumor microenvironment. Future studies will be required to support this hypothesis.

All procedures performed in studies involving animals were in accordance with the ethical standards of the institution or practice at which the studies were conducted.

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Compliance with ethical standards

Conflict of interest FH works for Boehringer Ingelheim RCV, Vienna, Austria. The other authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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