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NGR-TNF, a novel vascular-targeting agent, does not induce cytokine recruitment of proangiogenic bone marrow-derived cells

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Background: Administration of certain chemotherapy drugs at the maximum tolerated dose, vascular-disrupting agents (VDAs) and irradiation can induce mobilisation and tumour homing of proangiogenic bone marrow-derived cells (BMDCs). Increases in cytokines and chemokines contribute to such mobilisation that eventually promotes tumour (re)growth. NGR-TNF is a vascular-targeting agent in advanced clinical development, coupling the CNGRCG angiogenic vessel-homing peptide with tumour necrosis factor-alpha (TNF). We investigated whether NGR-TNF mobilises host BMDCs and growth factors.

Methods: Blood was obtained from Lewis lung carcinoma and 4T1 tumour-bearing mice at different time points following NGR-TNF, VDA or anti-VEGFR2/flk-1 antibody treatment. Levels of circulating growth factors were assessed by ELISAs. BMDCs were characterised by FACS. Circulating endothelial progenitor cells were defined as $CD45^-/CD13^+/flk\text{-}1^+/CD117^+/7AAD^-$, Tie2-expressing monocytes as $CD45^+/CD11b^+/Tie2^+$ and myeloid-derived suppressor cells as $CD45^+/CD11b^+/Gr1^+$ cells.

Results: NGR-TNF decreases tumour blood vessel density-inducing apoptosis of tumour and tumour endothelial cells. Unlike VDAs, or high-dose NGR-TNF, lower doses of NGR-TNF, comparable to those used in clinical trials, neither mobilise nor recruit to the tumour site proangiogenic BMDCs or induce host growth factors.

Conclusion: Low-dose NGR-TNF exerts antitumour activity without inducing proangiogenic host responses, conceivably important for preventing/overcoming resistance and designing combination therapeutic strategies.

Currently, there are two main classes of vascular-targeting drugs used in oncology. The first (and main) class is comprised of antiangiogenic drugs, of which a number are thus far approved for clinical use in oncology, for example, bevacizumab, the humanised anti-VEGF monoclonal antibody, and sunitinib, sorafenib, axitinib and pazopanib, all oral small molecule tyrosine kinase inhibitors (TKIs), which target a number of TKIs including VEGF receptors. Registered indications, depending upon the drug, include renal cell cancer (sunitinib, pazopanib, sorafenib, axitinib and bevacizumab), hepatocellular carcinoma (sorafenib), pancreatic neuroendocrine tumours (sunitinib), medullary thyroid cancer (vandetanib), breast, ovarian, non-small cell lung and colorectal carcinomas (bevacizumab) and glioblastoma (bevacizumab) (see http://www.fda.gov). These approvals, especially for historically refractory cancers such as renal cell and hepatocellular carcinomas, represent a genuine breakthrough in medical oncology treatment. Nevertheless, the progression-free survival and overall survival (OS) benefits of these drugs, especially OS, when successful, remain modest.

Vascular-disrupting agents (VDAs) define the second class of vascular-targeting drugs. None of these is currently approved for

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clinical use, although there are more than 10 in various stages of clinical development (see http://www.clinicaltrials.gov). Unlike most neovasculature-targeting antiangiogenic drugs, VDAs directly target the established but abnormal tumour vasculature, causing their very rapid destruction, which leads to profound increases in tumour hypoxia and intra-tumoural necrosis. However, this potent effect is followed by rapid regrowth from the remaining viable tumour rim (Shaked *et al*, 2006).

In this context, NGR-TNF, a new drug generated by the fusion of the CNGRCG peptide to the N-terminal domain of human (h)TNF (Curnis *et al*, 2000), defines a new class of vascular-targeting agent. NGR-TNF is targeted to the tumour vasculature by the CNGRC peptide motif (Arap *et al*, 1998; Curnis *et al*, 2000) that specifically interacts with the CD13 (aminopeptidase N) expressed by endothelial cells of angiogenic vessels (Pasqualini *et al*, 2000; Curnis *et al*, 2002a; Buehler *et al*, 2006). NGR-TNF is able to control tumour growth in several tumour models, either alone or in combination with chemotherapeutics (Curnis *et al*, 2000; Sacchi *et al*, 2006; Gregorc *et al*, 2009; Santoro *et al*, 2010; Gregorc *et al*, 2010b, 2011). In preclinical models, it has been shown that the dose–response curve of NGR-TNF is bell shaped (Curnis *et al*, 2002b). As a matter of fact, NGR-murine(m)TNF has an antitumour effect at doses lower than 1 ng per mouse or equal/higher than 1 μg per mouse. This is due to the induction of counter regulatory mechanisms, including TNF receptor shedding. At doses higher than 1 μg per mouse, NGR-mTNF overcomes these negative feedback mechanisms and shows again its antitumour activity (Curnis *et al*, 2002b). In this context, NGR-mTNF showed a significant antitumour activity at very low doses (0.1 ng per mouse, corresponding to 5 ng per kg) (Curnis *et al*, 2000), equivalent in humans to a dose of 0.2 $\mu g\,m^{-2}$, which was the selected starting dose for phase I clinical development. Dose escalation studies have been performed without reaching a clear MTD (van Laarhoven *et a*l, 2010; Zucali *et al*, 2013). However, it has been observed that the low dose of 0.8 $\mu g\,m^{-2}$ has more pronounced antivascular effects than higher doses, and it was therefore considered as the optimal biological dose (Gregorc *et al*, 2010a). NGR-TNF at the low dose of 0.8 $\mu g\,m^{-2}$ is currently being tested in several phase II and III clinical trials with promising phase II results (Gregorc *et al*, 2009; Santoro *et al*, 2010; Gregorc *et al*, 2010a, b, 2011).

Bone marrow-derived cells (BMDCs) play an important role in regulating angiogenesis during tumour progression. Over the last 15 years, a number of different subtypes of proangiogenic and protumourigenic BMDCs have been identified, including circulating endothelial progenitor cells (CEPs) (Lyden *et al*, 2001; Shaked *et al*, 2006), tumour-associated macrophages (Lin *et al*, 2006), Tie2-expressing monocytes (TEMs) (De Palma *et al*, 2003, 2005), dendritic cell precursors (Conejo-Garcia *et al*, 2004), mast cells (Coussens *et al*, 1999) and $CD11b^+Gr1^+$ cells, including neutrophils (Nozawa *et al*, 2006) and myeloid-derived suppressor cells (MDSCs) (Yang *et al*, 2004; Shojaei *et al*, 2007). These various cell types are thought to promote tumour angiogenesis by several mechanisms, for example, by producing proangiogenic factors (Murdoch *et al*, 2008; Qian and Pollard, 2010), as well as by their differentiation into endothelial cells in the case of CEPs (Lyden *et al*, 2001; Shaked *et al*, 2006). In addition, it has been shown that several types of BMDCs, such as CEPs, TEMs or $CD11b^+Gr1^+$ cells, are involved in the formation of 'premetastatic niches' (Kaplan *et al*, 2005; Gao *et al*, 2008; Gingis-Velitski *et al*, 2011). There is now evidence that treatment with VDAs (e.g., Oxi4503) or certain chemotherapeutic drugs administered at the maximum tolerated dose (e.g., paclitaxel, cyclophosphamide, 5-FU) or local irradiation can rapidly induce mobilisation and subsequent tumour homing of a number of different proangiogenic BMDCs (Shaked *et al*, 2006; Kerbel, 2008; Shaked *et al*, 2008; De Palma and Lewis, 2011; Denardo *et al*, 2011; Welford *et al*, 2011), exerting in some cases prometastatic effects (Gingis-Velitski *et al*, 2011). Induction of several host growth factors and chemokines, such as granulocyte colony-stimulating factor (G-CSF), stromal cell-derived factor-1 (SDF-1) and osteopontin (OPN), contributes to this drug-induced BMDC mobilisation and migration to the viable tumour rim that remains after treatment (Shaked *et al*, 2008, 2009; Welford *et al*, 2011). This process promoting angiogenesis and rapid tumour regrowth affects the overall antitumour activities of the aforementioned treatments (Shaked *et al*, 2008, 2009; De Palma and Lewis, 2011; Denardo *et al*, 2011; Welford *et al*, 2011).

Based on these observations, we decided to evaluate the impact of NGR-TNF on BMDC mobilisation and its effects on inducing various potential BMDC-mobilizing factors. We therefore tested two different doses of NGR-TNF, a low and a high dose. The rationale for this point is based on the results of several phase I and II clinical trials with NGR-hTNF showing that low doses of the drug, for example, 0.8 $\mu g\,m^{-2}$ (Gregorc *et al*, 2010a) (i.e., 18 ng per kg), are more efficacious than higher doses tested in phase I dose escalation studies (van Laarhoven *et al*, 2010; Gregorc *et al*, 2010a; Zucali *et al*, 2013). We reasoned that this counterintuitive result might be explained, at least in part, by the failure of the lower dose to induce a reactive host BMDC response in contrast to the higher dose.

We report that NGR-mTNF, repetitively administered at low doses to tumour-bearing mice, causes a decrease of tumour blood vessel density and induces apoptosis of both tumour and endothelial cells *in vivo* without inducing a reactive proangiogenic host response.

MATERIALS AND METHODS

Tumour cells and animal models. Mouse Lewis lung carcinoma (LLC; ATCC, Manassas, VA, USA) and 4T1 mammary gland carcinoma cells (ATCC) were cultured in standard conditions. LLC cells (5×10^5) were subcutaneously (s.c.) injected into syngeneic C57BL/6 mice (Jackson) or C57BL/6 mice previously transplanted with green fluorescent protein$^+$ (GFP^+) bone marrow cells from UBI-GFP/BL6 donors (Jackson, Bar Harbor, ME, USA) after lethal irradiation (900 rad). 4T1 cells (7×10^3) were s.c. injected into syngeneic BALB/c mice (Charles River, Calco, Lecco, Italy). Treatments started when tumours were 200–300 mm^3. Mice were sacrificed when tumour sizes reached 1700 mm^3, with the approval and in accordance with the guidelines of the animal care committee of the Sunnybrook Health Sciences Centre and Canadian Council of Animal Care and the Ethical Committee of the San Raffaele Scientific Institute (IACUC 492). Our *in vivo* experiments meet the standards required by the UKCCCR guidelines (Workman *et al*, 2010).

Preparation and characterisation of NGR-mTNF. The cDNA coding for NGR-mTNF was kindly provided by Dr A Corti (San Raffaele Scientific Institute, Milan, Italy). NGR-mTNF expression was induced in transformed BL21 (λD3) *Escherichia Coli* (Novagen, Podenzano, PC, Italy) by 1 mM IPTG (Sigma-Aldrich, St Louis, MO, USA). Bacterial homogenate was clarified by flocculation with polyethyleneimine (Sigma-Aldrich), and soluble NGR-mTNF was purified by three stage of chromatography: ion-exchange chromatography on Q-Sepharose XL (GE Healthcare, Milan, Italy), mixed-mode chromatography on Capto Adhere (GE Healthcare) and ion-exchange chromatography on Q-Sepharose HP (GE Healthcare) in denaturing conditions. The endotoxin content of the purified NGR-mTNF, measured by the quantitative chromogenic *Limulus* amebocyte lysate test (BioWhittaker, Lonza, Walkersville, MD, USA), was 0.12 U μg^{-1}.

Tumour treatments. NGR-mTNF was administered intraperitoneally (i.p.) either at high dose (1 or 10 $\mu g\,100\,\mu l^{-1}$ per mouse, used indifferently because they gave the same results as far as

BMDCs and growth factor release and efficacy are concerned), single-bolus injections, or at low dose (0.1 ng 100 μl^{-1} per mouse), three times a week. A rat monoclonal antibody specific for mouse VEGFR-2/flk-1 (DC101) was administered i.p. at a concentration of 800 μg 200 μl^{-1} per mouse. A second-generation vascular microtubule-disrupting agent, Oxi4503 (designated from now on as 'VDA'), was administered i.p. at 100 $\mu g\,g^{-1}$ as a single dose.

Bone marrow transplantation. Green fluorescent protein$^+$ bone marrow cells (10×10^6) isolated from femurs of UBI-GFP/BL6 donor mice were injected into the tail veins of 6- to 8-week-old lethally irradiated (900 rad) C57BL/6 female mice. After 6–8 weeks, recipient mice were bled from the orbital sinus to evaluate bone marrow transplantation efficiency using flow cytometry. Those that had >99% GFP$^+$ peripheral blood cells were subsequently used as recipients for a subcutaneous injection of LLC cells.

Analysis of circulating cells by flow cytometry. Blood was drawn from the retro-orbital sinus of anaesthetised mice 4, 24 and 72 h after a single treatment or 24 or 72 h after repeated (three) treatments with NGR-mTNF. Cell suspensions were analysed on an LSRII or a FACSCanto (BD Biosciences, San Diego, CA, USA) after red cell lysis. At least 100 000 cells per sample were acquired, and percentages of stained cells were determined and compared with appropriate negative controls. Viable CECs (vCECs) and viable CEPs (vCEPs) were counted using five-colour flow cytometry. Briefly, monoclonal antibodies specific for CD45 were used to exclude CD45$^+$ haematopoietic cells, and CECs or CEPs were detected as expressing the murine endothelial markers fetal liver kinase 1/VEGF receptor 2 (*flk-1*/VEGFR-2), CD13 and (only) for CEPs, CD117 (c-kit) (BD Biosciences). After acquisition of at least 100 000 cells per sample, analyses were considered informative when an adequate number of events (i.e., >25, typically 50–150) were collected in the CEP enumeration gate in untreated control animals (Bertolini *et al*, 2006). Positive staining was defined as being greater than nonspecific background staining, and 7-aminoactinomycin D (7AAD) was used to distinguish apoptotic and dead cells from viable cells (Bertolini *et al*, 2006). TEMs (De Palma *et al*, 2005) and CD11b$^+$/Gr1$^+$ MDSCs were detected as being positive for CD45, CD11b (BD Biosciences), Tie2 (eBioscience, San Diego, CA, USA) and CD45, CD11b, Gr1 (Ly6G, BD Biosciences) markers, respectively.

Evaluation of circulating growth factor plasma levels by ELISA. Mice were treated when tumours were 250–350 mm^3. Blood was drawn from the retro-orbital sinuses of anaesthetised mice or by cardiac puncture 4, 24 or 72 h after treatment with either NGR-mTNF or VDA. Plasma samples were obtained by centrifugation (3000 revolutions per min (r.p.m.), 5 min) and stored at −80 °C. Levels of G-CSF, SDF-1 and OPN were assessed with commercially available DuoSet R&D System ELISA assays following the manufacturer's instructions. Standard curve for G-CSF started from 1000 pg ml^{-1} and samples were diluted 1 : 2; a standard curve for SDF-1 started from 1000 pg ml^{-1} and samples were diluted 1 : 1; and a standard curve for OPN started from 2 ng ml^{-1} and samples were diluted 1 : 450.

Tumour immunofluorescence and immunohistochemistry. Tumour tissues were collected 72 h after a single treatment or 24 h after repeated (three) treatments, snap frozen and stored at −80 °C. Tumour cryosections (15–20 μm) were stained for vessels with an anti-CD31 antibody (1 : 200, BD Biosciences) and its secondary Dylight649-conjugated donkey anti-rat antibody (1 : 200, Jackson ImmunoResearch, West Grove, PA, USA) or Cy3-conjugated donkey anti-rat antibody (1 : 200, Jackson ImmunoResearch), for apoptosis with an anticleaved caspase-3 antibody (Cell Signaling Technology, Boston, MA, USA) and its secondary Cy3-conjugated donkey anti-rabbit antibody (1 : 200, Jackson ImmunoResearch) and for GFP positivity with an anti-GFP fluorescein isothiocyanate (FITC)-conjugated antibody (1 : 500, BD Biosciences). All sections were counterstained with DAPI for identifying nuclei. Controls were immunostained with a secondary antibody alone. For detecting hypoxia, mice received an i.p. injection of pimonidazole hydrochloride (60 mg per kg) (Chemicon International, Temecula, CA, USA) 90 min before euthanasia (Daenen *et al*, 2009). Tumours were then removed, snap frozen and stored at −80 °C. Hypoxia immunostaining was carried out using the anti-pimonidazole antibody Hypoxyprobe-1 (1 : 200, Chemicon International) and its secondary Cy3-conjugated rat anti-mouse antibody (1 : 200, Jackson ImmunoResearch). Necrosis was detected as autofluorescence in the FITC channel on sections (5 μm) obtained from formalin-fixed, paraffin-embedded tumours (Daenen *et al*, 2009). Proliferation was determined by immunostaining with a rabbit polyclonal Ki-67 antibody (Vector, Burlington, ON, Canada) on sections (5 μm) obtained from formalin-fixed, paraffin-embedded tumours. Histological data were collected in a blind manner. Images were captured with a Zeiss Axiocam digital camera connected to the microscope using AxioVision 3.0 software. At least two non-consecutive slides were analysed per tumour, and at least 10 pictures (magnification ×100) were taken for each slide to represent the whole tumour section. Pictures were analysed with ImageJ and Prism. For each colour (green, red, blue and cyan), the number of pixels per image was divided by the total number of pixels of the tumour area. Results were expressed as fold increase/decrease in comparison with control group (100%). All the experiments were repeated at least twice. Pictures shown are representative of the most frequently seen fields per slide.

Statistical analysis. GraphPad Prism version 5.0b was used to assess the statistical significance of differences in mean values. The two-tailed Student's *t*-test or the one-way Anova test was used to assess the significance of the mean difference. Difference between designated groups compared with saline-treated group (unless indicated otherwise) was considered significant at values of $0.05 > P > 0.01$ (*) or $0.01 > P > 0.001$ (**) or $P < 0.001$ (***). The absence of symbols means that no statistically significant differences were detected. Data are expressed as mean ± s.d.

RESULTS

Low-dose NGR-TNF causes a decrease in tumour blood vessel counts and induces apoptosis. Antitumour activity of NGR-mTNF has been reported in different tumour models following the administration of low doses of NGR-mTNF alone or in combination with chemotherapeutic agents (Curnis *et al*, 2000, 2002b; Sacchi *et al*, 2006; Calcinotto *et al*, 2012). To assess whether the tumour inhibitory activity of NGR-TNF may involve a local effect on tumour vessels, LLC-bearing mice were treated with either high- or low-dose NGR-mTNF ($n = 4$–6 mice per group). Tumour tissues were collected 72 h after a single treatment or 24 h after repeated treatments and stained for CD31 and cleaved caspase-3 (ClCasp3) as a marker of apoptosis. The low-dose treatment and, to a greater extent, the high-dose treatment caused a decrease in tumour-associated blood vessel density 72 h after treatment (Figures 1A and B). Similar results were obtained on tumours collected after repeated low-dose drug administration (Supplementary Figures 1A and B). As expected, increased levels of ClCasp3 expression were detected in both high-dose- and low-dose-treated tumours (Figure 1C). Indeed, we observed similar levels of apoptosis of both tumour cells and tumour endothelial cells, thus suggesting that even at low doses NGR-TNF has antivascular effects, which could contribute to its antitumour therapeutic activity. This biological activity of NGR-mTNF is translated into a reduction of tumour growth (Supplementary Figure 2A).

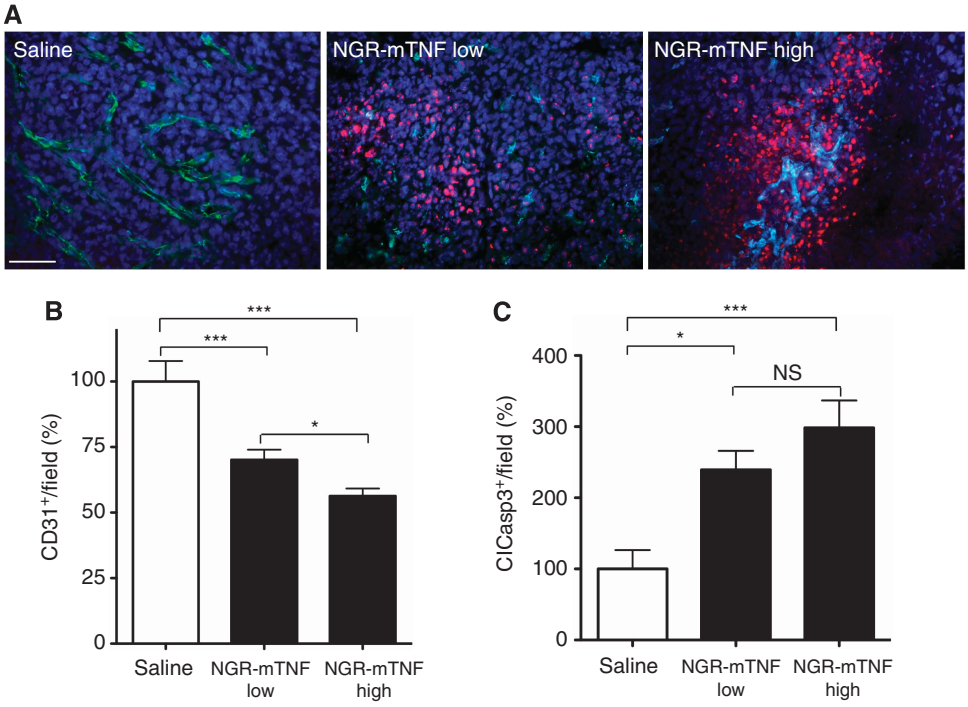

Figure 1. Low-dose NGR-mTNF decreases vascular density and induces apoptosis of tumour cells and tumour endothelial cells. LLC tumours were harvested 72 h after a single treatment and processed as described in the Materials and Methods section. (**A**) Cleaved caspase-3 (ClCasp3; red) and CD31 (cyan) immunostaining of LLC tumour sections. Nuclei were stained with DAPI (blue). Results are representative of three independent experiments, and 4–6 mice per group were analysed. For each tumour, 2–10 non-consecutive sections were analysed. At least 10 pictures per slide were taken. Magnification $\times 100$; scale bar, 200 μm. (**B** and **C**) The plots show mean ratio of CD31 and ClCasp3 expression analysed as the measure of vascular density and apoptosis, respectively.

In particular, 72 h after treatment, VDA and high-dose NGR-mTNF exert antitumour effects. However, at sacrifice (usually around day 22 after tumour injection, 12 days after the first treatment) the most potent antitumour agent we tested is low-dose NGR-mTNF (Supplementary Figure 2A).

Low-dose NGR-TNF does not induce the mobilisation of proangiogenic BMDCs and proangiogenic growth factors. As VDA treatment has been shown to provoke the induction of multiple host cytokines such as G-CSF (Shaked *et al*, 2009) or SDF-1 (Welford *et al*, 2011), which can then act to mobilise several types of BMDC populations, we investigated whether NGR-TNF can induce cytokine-driven recruitment of proangiogenic BMDCs. We analysed blood from LLC-bearing mice obtained in an early phase (4 and 24 h) (Shaked *et al*, 2006) or in a late phase (72 h) (Taylor *et al*, 2012) after a single treatment or 24 or 72 h after repeated (three) treatments with either NGR-mTNF or VDA or an anti-VEGFR2/flk-1 antibody ($n = 5$–6 mice per group) for the presence of circulating BMDCs by FACS. In particular, vCECs were defined as $CD45^-/CD13^+/flk\text{-}1^+/7AAD^-$, vCEPs as $CD45^-/CD13^+/flk\text{-}1^+/CD117^+/7AAD^-$, TEMs as $CD45^+/CD11b^+/Tie2^+$ and MDSCs as $CD45^+/CD11b^+/Gr1^+$ cells. VDA treatment induced the mobilisation of vCEPs, vCECs, TEMs and $CD11b^+Gr1^+$ cells (Figure 2A). Furthermore, we observed increased levels of circulating proangiogenic BMDCs after treatment with high doses of NGR-mTNF (Figure 2A), whereas both single (Figure 2A) and repeated (Supplementary Figure 3) treatments with NGR-mTNF at low dose did not induce such cell mobilisation. Indeed, in the latter setting, increased levels of circulating $CD45^+/CD11b^+/Gr1^+$ cells were observed after repeated administration of both saline and NGR-mTNF at low dose probably because of the inflammation and the stress associated to the manipulations (Everds, 2007). The results obtained were confirmed in the 4T1 model, where increases in circulating proangiogenic BMDCs were observed after high-dose treatments but not after low-dose NGR-mTNF treatments (Figure 3). To in deep characterise the phenomenon, we also looked for circulating proangiogenic BMDCs after treatment with intermediate doses of NGR-mTNF, 1 and 10 ng per mouse (Supplementary Figure 4). At 10 ng per mouse, we observed the release of proangiogenic BMDCs into circulation, thus suggesting that this phenomenon is already active in the nanogram range (Supplementary Figure 4).

Next, we assessed the presence of circulating factors, previously shown to be involved in mobilisation of CEPs and some other BMDC types such as TEMs (e.g., G-CSF, SDF-1 and OPN) by ELISAs. VDA treatment, as well as high-dose NGR-mTNF, induced a significant increase of the plasma level of G-CSF, OPN and SDF-1, which was not detected either 4 or 24 h after treatment with NGR-mTNF at low dose (Figure 2B). The increase of growth factors is transient as 72 h after treatment all the growth factors tested returned to basal levels (data not shown). These results suggested that low-dose NGR-mTNF, comparable to the doses being used in the ongoing clinical trials (mouse: about 6 ng per kg; human: 0.8 $\mu g\,m^{-2}$ corresponding to about 18 ng per kg) (Gregorc *et al*, 2010a), does not cause a rebound of the potentially proangiogenic circulating growth factors that we assessed, nor the associated mobilisation of proangiogenic BMDCs.

Low-dose NGR-TNF does not recruit BMDCs to the tumour site. To investigate the presence of potential tumour growth-promoting proangiogenic BMDCs at the tumour site, we treated LLC-bearing GFP^+ bone marrow chimaeric mice with either NGR-TNF or VDA or an anti-VEGFR2/flk-1 antibody ($n = 4$–6 mice per group). Tumour tissues were collected 72 h after treatment and analysed for the presence of GFP^+ BMDCs (Figure 4A). GFP^+ cells were observed in all the groups at variable levels, with a statistically significant increase detectable only in the VDA-treated tumours (Figures 4A and B). In line with

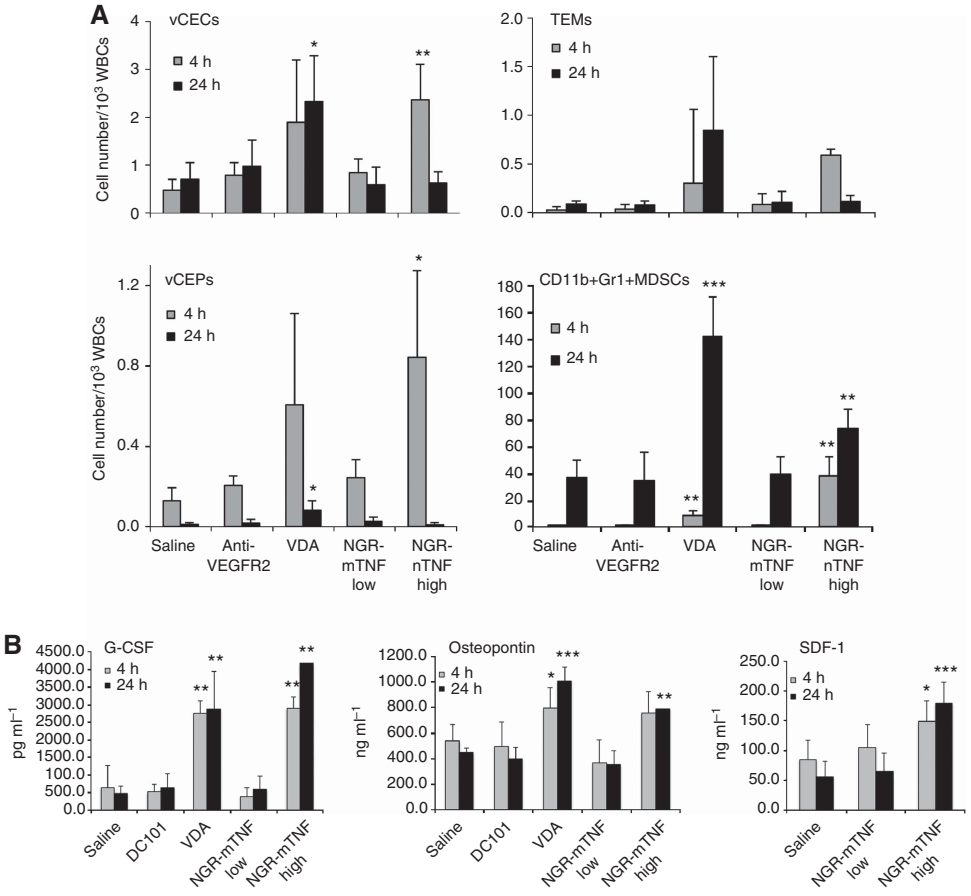

Figure 2. Low-dose NGR-mTNF does not modify the levels of circulating potentially tumour-promoting BMDCs and circulating GFs. (**A**) Levels of circulating vCECs, vCEPs, TEMs and CD11b$^+$Gr1$^+$ MDSCs in LLC tumour-bearing mice ($n=5$) were evaluated by FACS analysis 4 and 24 h after treatment with the indicated drugs. (**B**) Plasma levels of G-CSF, OPN and SDF-1 were obtained from LLC tumour-bearing mice ($n=5$) 4 and 24 h after treatment with the indicated drugs.

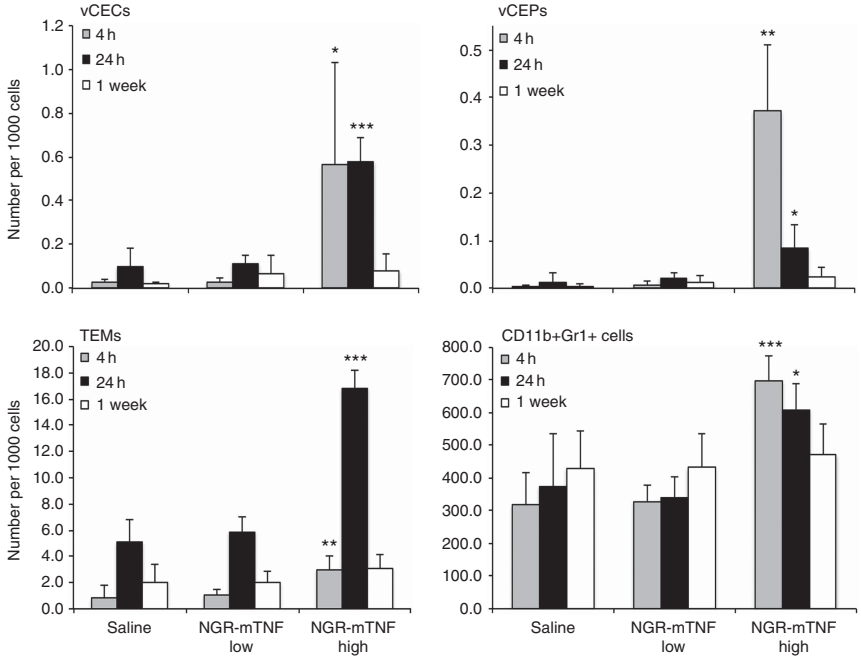

Figure 3. NGR-mTNF does not increase the levels of circulating tumour-promoting BMDCs in a mouse model of breast cancer. Levels of circulating vCEPs, vCECs, TEMs and CD11b$^+$Gr1$^+$ MDSCs in 4T1 tumour-bearing mice ($n=5$) were evaluated by FACS analysis 4 or 24 h or 1 week after the first treatment with the indicated drugs.

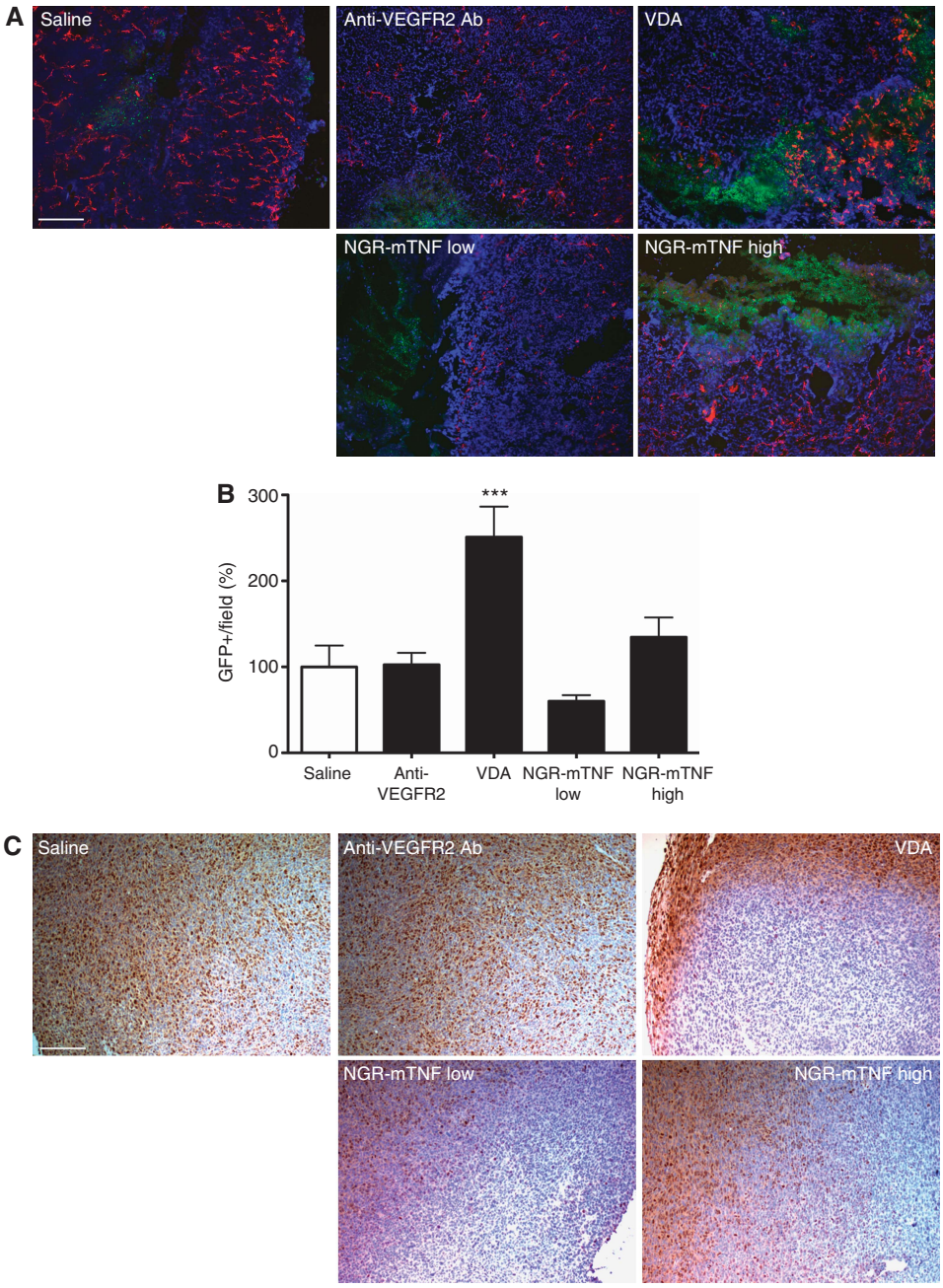

Figure 4. Low-dose NGR-mTNF does not support the BMDC-induced viable and proliferating tumour rim. LLC tumours grown in GFP$^+$ bone marrow chimaeric mice were harvested 72 h after a single treatment and processed as described in the Materials and Methods section. (**A**) GFP$^+$ BM-derived cells (green) and CD31 (red) immunostaining of LLC tumour sections. Nuclei were stained with DAPI (blue). Results are representative of two independent experiments, and 4–6 mice per group were analysed. For each tumour, 2–10 non-consecutive sections were analysed. At least 10 pictures per slide were taken. Magnification $\times$ 100; scale bar, 200 μm. (**B**) The plot illustrates quantification of tumour-infiltrating GFP$^+$ cells. The plot shows mean ratio of GFP staining analysed. (**C**) Ki67 (brown) immunostaining of paraffin-embedded LLC tumour sections. Nuclei were stained with haematoxylin (blue). Results are representative of two independent experiments, and 4–6 mice per group were analysed. For each tumour, 2–10 non-consecutive sections were analysed. At least 10 pictures per slide were taken. Magnification $\times$ 100; scale bar, 200 μm.

the rapid tumour regrowth previously described to occur after VDA therapy, we observed GFP$^+$ BMDC localisation at the viable tumour rim in VDA-treated tumours and to a lesser extent in the high-dose NGR-mTNF-treated tumours. Significantly, no viable tumour rim was observed after administration of low-dose NGR-mTNF.

To further investigate the effects of the treatments on tumour cell proliferation, we analysed LLC tumours recovered 72 h after treatment for Ki67 staining. In agreement with the localisation of GPF$^+$ BMDCs at the viable tumour rim, tumour cell proliferation was clearly observed at the edge of VDA- and high-dose NGR-mTNF-treated tumours, further supporting the presence of a viable regrowing (repopulating) tumour rim (Figure 4C). In contrast, treatment with low-dose NGR-mTNF modestly reduced tumour cell proliferation and, more importantly, did not lead to the formation of highly proliferative viable tumour rims (Figure 4C), thus avoiding or minimizing rapid tumour regrowth.

Low-dose NGR-TNF induces low-grade hypoxia and necrosis. Several factors may contribute to VDA-mediated mobilisation

of BMDCs, including host-mediated, tumour-independent effects that may be related to direct toxicity of the drugs on bone marrow cells, as well as the elevated levels of tumour hypoxia and tumour cell necrosis induced by drug treatment (Bergers and Hanahan, 2008; Du *et al*, 2008). To further investigate the mechanism of action of low-dose NGR-mTNF, we evaluated its ability to induce tumour hypoxia and necrosis in LLC tumours, using pimonidazole staining as a marker of hypoxia and autofluorescence as a marker of necrosis (Daenen *et al*, 2009). As expected, treatments with VDA or high-dose NGR-mTNF increased tumour hypoxia and necrosis (Figures 5A–C). Low-dose NGR-mTNF only slightly increased these two parameters

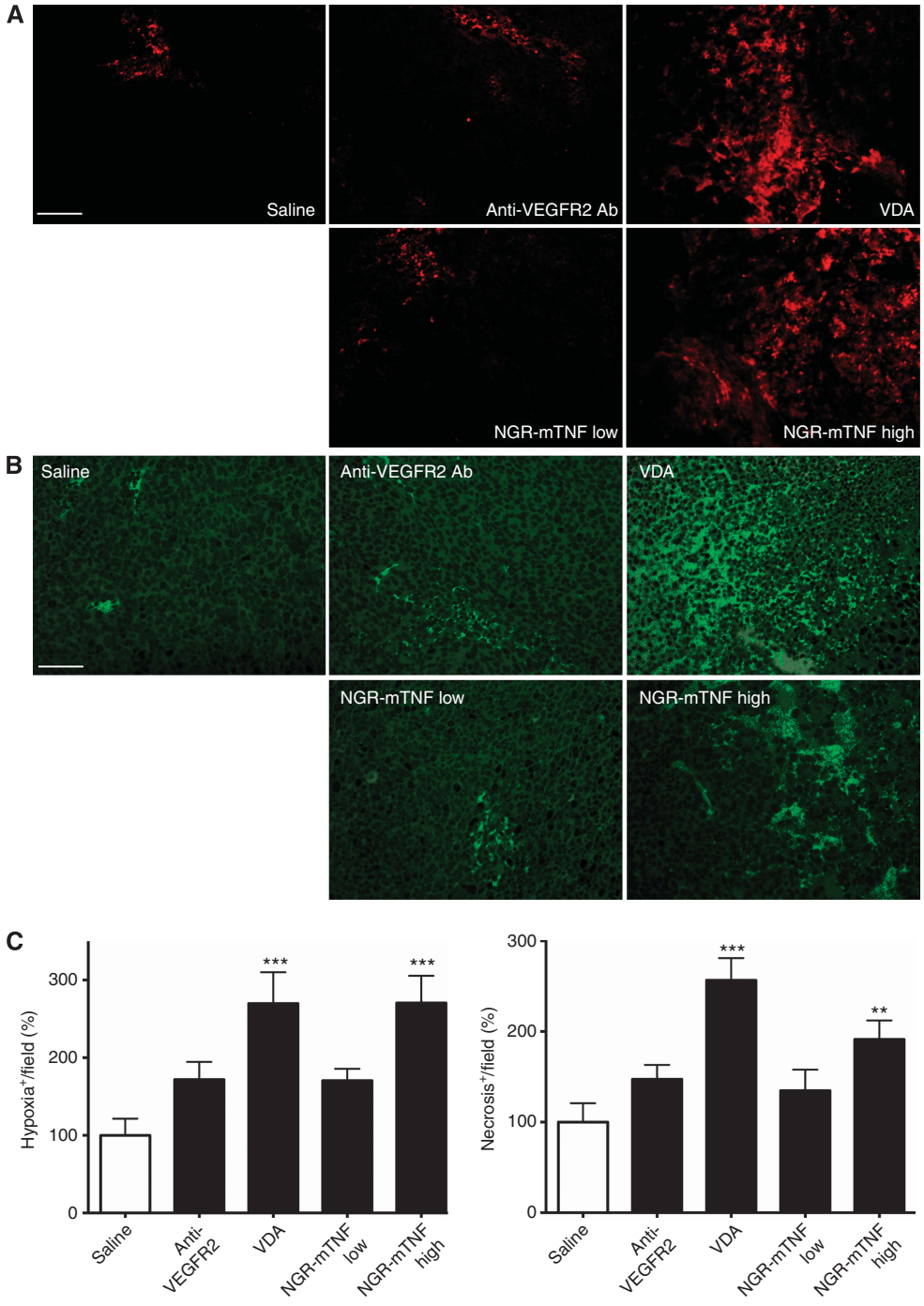

Figure 5. Low-dose NGR-mTNF effects on tumour hypoxia and necrosis. LLC tumours were harvested 72 h after a single treatment and processed as described in the Materials and Methods section. (**A**) The level of hypoxia was evaluated as pimonidazole (red) immunostaining. Results are representative of two independent experiments, and 4–6 mice per group were analysed. For each tumour, 2–10 non-consecutive sections were analysed. At least 10 pictures per slide were taken. Magnification × 100; scale bar, 200 μm. (**B**) The level of necrosis was evaluated as autofluorescence (green) of paraffin-embedded sections. Results are representative of two independent experiments, and 4–6 mice per group were analysed. For each tumour, 2–20 non-consecutive sections were analysed. At least 10 pictures per slide were taken. Magnification × 100; scale bar, 200 μm. (**C**) Left panel illustrates quantification of tumour hypoxia. The plot shows mean ratio of pimonidazole immunostaining analysed; right panel illustrates quantification of tumour necrosis. The plot shows the mean ratio of green pixels/field analysed.

(Figures 5A–C), in agreement with the observation that low-dose NGR-TNF treatment fails to induce robust cytokine/growth factor or BMDC responses.

DISCUSSION

Various angiogenesis inhibitors targeting the VEGF pathway have demonstrated proven efficacy in both preclinical tumour models and in clinical trials. However, in both settings the therapeutic effects are transient, and after an initial phase of tumour growth control, for example, tumour regression in some cases, or induction of stable disease, the tumours progress. As such, there is considerable interest in elucidating the reasons for their transient clinical benefits, for example, understanding the basis of intrinsic or acquired resistance mechanisms, and devising strategies to delay the onset of resistance or treat overt recurrent resistant disease with second-line therapies–including with new and different antiangiogenic drugs or other types of vascular-targeting agents/strategies such as VDAs (Bergers and Hanahan, 2008; Ebos and Kerbel, 2011). In this context, the recruitment of BM-derived proangiogenic cells at the tumour site has been identified as one possible reason of the transient efficacy of some of these treatments, such as VDAs, in preclinical models (Shaked *et al*, 2006, 2008; Welford *et al*, 2011). Moreover, the relevance of this mechanism in patients treated with VDAs is supported by several clinical studies (Farace *et al*, 2007; Shaked *et al*, 2009; Taylor *et al*, 2012).

The combination properties of NGR-TNF define a new class of vascular-targeting agent. For example, unlike both antiangiogenic VEGF pathway-targeting drugs and VDAs, NGR-TNF neither induces hypertension nor causes proteinuria. Like VDAs, it can directly attack the tumour vasculature, but the molecular target for NGR-TNF expressed by endothelial cells – CD13 – is known. Unlike VDAs or antiangiogenic VEGF pathway inhibitors, it has an additional mechanism of action, namely, potent stimulation of host antitumour immune mechanisms (Curnis *et al*, 2000; Sacchi *et al*, 2004; Balza *et al*, 2006; Calcinotto *et al*, 2012). In addition, NGR-TNF therapy has been shown to rapidly increase the intratumour uptake of chemotherapy drugs such as doxorubicin as a consequence of the damage inflicted upon the integrity of the tumour vasculature resulting in increased vascular permeability (Sacchi *et al*, 2006; Gregorc *et al*, 2009).

One of the properties of conventionally dosed direct vascular-targeting VDAs, which has been preclinically shown to limit its otherwise potent initial antitumour efficacy, is its effect on inducing a rapid systemic mobilisation of BMDCs including CEPs and/or TEMs that then home to and colonise the drug-treated tumours (Shaked *et al*, 2006; Welford *et al*, 2011; Taylor *et al*, 2012). The CEP host response can be partially blocked by cotreatment with VEGF- or VEGF receptor-2-neutralizing antibodies, which results in improved antitumour effects mediated by the VDA (Shaked *et al*, 2006). Based on these observations, mimicking the doses used in the clinical setting, we investigated whether NGR-TNF, compared with other vascular-targeting agents, causes an increase of circulating host-derived cytokines or growth factors and mobilises/recruits BMDCs to the tumour site. We tested two different doses of NGR-TNF, a low and a high dose. Clinical trials showed that low doses of NGR-hTNF, for example, 0.8 $\mu g\,m^{-2}$ (i.e., 18 ng per kg), are more efficacious than higher doses (Gregorc *et al*, 2010a; Zucali *et al*, 2013). Our results indeed indicate that the low dose of NGR-TNF neither induces the robust mobilisation of several types of BMDCs, including CEPs, TEMs and MDSCs, nor increases the levels of G-CSF, SDF-1 and OPN. In contrast, treatments with high-dose NGR-TNF resulted in BMDC mobilisation and in increased levels of growth factors. These responses are similar in nature to those observed with conventionally dosed VDAs or certain chemotherapy drugs administered at maximum tolerated doses (Bertolini *et al*, 2003; Shaked *et al*, 2006, 2008; Welford *et al*, 2011).

Whether lower doses of VDAs administered in a metronomic scheduling manner would also avoid induction of BMDC responses and would show similar or even increased antitumour efficacy is presently unknown, and is worth investigating. Another open question, deserving deeper investigation in suitable tumour models, is the effects that NGR-TNF treatment may have on the metastatic process, which it is known to be enhanced by BMDC mobilisation (Gingis-Velitski *et al*, 2011).

We observed that, as already reported by others (Shaked *et al*, 2006; Taylor *et al*, 2012), mice treated with VDA showed a significant increase of tumour-infiltrating GFP^+ BMDCs compared with that in the saline-treated mice. Similar results, even if not statistically significant, were also observed in the high-dose NGR-TNF group, thus suggesting a correlation between the raise of CEPs, TEMs and MDSCs detected in peripheral blood and the level of GFP^+ BMDCs observed in tumours. In agreement, mice administered with low-dose NGR-TNF and anti-VEGFR2, agents that do not mobilise proangiogenic BMDCs, showed mild incorporation of GFP^+ BMDCs in LLC tumours, comparable with that observed in saline-treated mice.

Our results add to a growing body of evidence showing the potential benefits of using cytotoxic drugs, including chemotherapy, in more metronomic-like scheduling and dosing protocols. In addition, the properties and mechanisms of action of NGR-TNF suggest that it would be a particularly promising drug to consider not only for front-line therapy in combination with chemotherapy but also as a second-line therapy when resistance to antiangiogenic drugs such as bevacizumab or small molecular TKIs has developed. In such situations, the use of a direct-acting molecular vascular-targeting agent could be particularly effective because its action does not depend on VEGF or VEGF receptors as a target and also because it exploits additional antitumour mechanisms, such as stimulation of host immunity. In summary, our results provide a rationale for using repetitive low-dose NGR-TNF administration schedules in the clinic, in addition to the expected reduced toxicities that would ensue for such protocols.

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