

VEGF Receptor Inhibitors Block the Ability of Metronomically Dosed Cyclophosphamide to Activate Innate Immunity–Induced Tumor Regression

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Abstract

In metronomic chemotherapy, frequent drug administration at lower than maximally tolerated doses can improve activity while reducing the dose-limiting toxicity of conventional dosing schedules. Although the antitumor activity produced by metronomic chemotherapy is attributed widely to antiangiogenesis, the significance of this mechanism remains somewhat unclear. In this study, we show that a 6-day repeating metronomic schedule of cyclophosphamide administration activates a potent antitumor immune response associated with brain tumor recruitment of natural killer (NK) cells, macrophages, and dendritic cells that leads to marked tumor regression. Tumor regression was blocked in nonobese diabetic/severe combined immunodeficient (NOD/SCID- γ) mice, which are deficient or dysfunctional in all these immune cell types. Furthermore, regression was blunted by NK cell depletion in immunocompetent syngeneic mice or in perforin-deficient mice, which are compromised for NK, NKT, and T-cell cytolytic functions. Unexpectedly, we found that VEGF receptor inhibitors blocked both innate immune cell recruitment and the associated tumor regression response. Cyclophosphamide administered at a maximum tolerated dose activated a transient, weak innate immune response, arguing that persistent drug-induced cytotoxic damage or associated cytokine and chemokine responses are required for effective innate immunity–based tumor regression. Together, our results reveal an innate immunity–based mechanism of tumor regression that can be activated by a traditional cytotoxic chemotherapy administered on a metronomic schedule. These findings suggest the need to carefully evaluate the clinical effects of combination chemotherapies that incorporate antiangiogenesis drugs targeting VEGF receptor. *Cancer Res*; 72(5): 1103–15. ©2012 AACR.

Introduction

Maximum tolerated dose (MTD) chemotherapy has been a mainstay in the cancer clinic for the past 50 years. However, recent preclinical successes with metronomic chemotherapy, where drug is administered at a regular, more frequent interval, but at a lower dose than MTD chemotherapy, have been rapidly translated into clinical trials, where improved antitumor responses have been observed (1). Metronomic chemotherapy eliminates the need for extended recovery periods between treatment cycles and thus allows for persistent drug treatment in a way that minimizes drug toxicity to the patient (2). Metronomic chemotherapy schedules using cyclophosphamide and other chemotherapeutic drugs induce endothelial cell death in addition to tumor cell death (2–4). Metronomic

chemotherapy also induces the antiangiogenic glycoprotein thrombospondin-1 (TSP1; *Thbs1*; refs. 4, 5), suggesting that antiangiogenesis is an important factor in the superior antitumor profiles of metronomic regimens (6, 7). However, this proposed mechanism is not supported by the finding that bona fide antiangiogenic drugs often show only moderate antitumor activity when used as single agents, despite their effectiveness at inhibiting tumor angiogenesis. Examples of this include non–small cell lung cancer (8) and glioblastomas (9) in human patients, 9L gliosarcoma xenografts treated in severe combined immunodeficient (SCID) mice (4), and metastatic melanomas in C57BL/6 mice (10). Thus, other mechanisms for the improved antitumor effects of metronomic chemotherapy are likely operative.

TSP1, in addition to its antiangiogenic activity, has other actions, including stimulation of chemotaxis, cell proliferation, and protease regulation in healing (11). Moreover, tumors that stably express TSP1 have significantly increased levels of infiltrating antitumor M1 macrophages (12), suggesting a role for the host immune system in the improved tumor responses to metronomic drug treatments.

Presently, we show that a 6-day repeating metronomic schedule of cyclophosphamide activates a potent and sustained antitumor innate immune response that is associated with tumor regression and leads to ablation of large brain

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tumor xenografts. In contrast, MTD cyclophosphamide treatment induces a weak immune response that dissipates during the rest period between treatment cycles. We further show that antitumor innate immunity, and not antiangiogenesis, is the major mechanism for the marked tumor regression seen in these models. Supporting this hypothesis, tumor regression is blocked in natural killer (NK) cell-deficient and macrophage and dendritic cell dysfunctional nonobese diabetic/SCID (NOD/SCID- γ) mice (13) and is blunted by NK cell depletion in an immunocompetent syngeneic mouse model and in mice deficient in the lymphocyte effector molecule perforin, where NK, NKT, and T-cell cytolytic function are compromised (14).

In addition, we show that VEGF receptor-selective antiangiogenic drugs block antitumor immunity and prevent metronomic cyclophosphamide-induced tumor regression. VEGF receptor signaling is important for dendritic cell-endothelial cell cross-talk, transdifferentiation (15), tumor-associated macrophage infiltration (16), and chemokine expression and secretion in proinflammatory responses (17). Furthermore, endothelial cells and immune cells have shared bone marrow-derived stem and progenitor cells regulated by VEGF receptor (18), suggesting that compounds designed to kill tumor blood vessels by inhibiting VEGF receptor signaling may also elicit immunosuppressive responses.

Materials and Methods

Cell lines

Human U251 glioblastoma cells (National Cancer Institute), rat 9L gliosarcoma cells (Neurosurgery Tissue Bank, University of California, San Francisco), and mouse GL261 glioma cells (DCTD, DTP Tumor Repository) were authenticated by and obtained from the indicated sources. Cells were grown at 37°C in a humidified 5% CO₂ atmosphere. U251 and GL261 cells were grown in RPMI-1640 and 9L cells in Dulbecco's Modified Eagle's Media, all of which contained 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.

Mouse models and tumor xenografts

Five-week-old (24–26 g) male ICR/Fox Chase immunodeficient SCID mice (Taconic Farms), 5-week-old male NOD.Cg-*Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ* (NOD-SCID- γ , NSG) mice (The Jackson Laboratory), and 5-week-old (22–24 g) male C57BL/6 (wild-type, immunocompetent; Taconic) and C57BL/6-*Prf1*[−] (perforin knockout; The Jackson Laboratory) mice were housed and treated under approved protocols and federal guidelines. Tumor cells (2×10^6 GL261 glioma cells, 4×10^6 9L gliosarcoma cells, or 6×10^6 U251 glioblastoma cells) were injected s.c. on each posterior flank in 0.2 mL serum-free RPMI using a 0.5-inch 29-gauge needle and a 1-mL insulin syringe. 9L and U251 tumor xenografts were grown s.c. on the flanks of SCID or NSG mice, and GL261 tumors were inoculated into the flanks of C57BL/6 (wild-type or *Prf1*[−]) mice. Tumor areas (length \times width) were measured twice weekly using Vernier calipers (VWR, Cat# 62379-531), and tumor volumes were calculated on the basis of the formula, $\text{Vol} = (\pi/6) \times (L \times W)^{3/2}$. Tumors were monitored and treatment groups were normalized (each

tumor volume set to 100%) once average tumor volumes reached 500 mm³. Mice were treated with cyclophosphamide given on an intermittent metronomic schedule (140 mg cyclophosphamide/kg body weight, repeated every 6 days) or on an MTD schedule (150 mg cyclophosphamide/kg body weight on each of 2 consecutive days, followed by a 19-day rest period) as indicated on each figure using vertical arrows. Axitinib and AG-028262 were administered daily at 25 mg/kg body weight/d intraperitoneally and cediranib at 5 mg/kg body weight/d intraperitoneally for up to 24 days, as indicated in each study. NK cell-depleting monoclonal antibody anti-asialo-GM1 (cat# 986-10001, Wako Chemicals USA) was administered intraperitoneally at a dose of 50 µL (diluted 1:3 in sterile $\times 1$ PBS to final volume of 150 µL for each mouse on the day of injection) and delivered once every 6 days starting 3 days prior to the first metronomic cyclophosphamide treatment (i.e., asialo-GM1 antibody was given 3 days prior to each cyclophosphamide injection). On combination therapy days, cyclophosphamide was administered 4 hours prior to treatment with a VEGF receptor inhibitor to minimize the potential for drug interactions. Tumor sizes and mouse body weights were measured at least twice a week. Tumor growth rates prior to drug treatment were similar among all normalized groups.

Quantitative PCR and statistical analysis

RNA isolation, quantitative PCR (qPCR) primer design, and qPCR analyses were done as described in Supplementary Materials and Methods. qPCR data are expressed as mean values \pm SE for $n = 5$ to 6 individual tumors from 3 mice per time point per treatment group unless indicated otherwise. Statistically significant differences between mean values of different treatment groups were determined by 2-tailed Student *t* test (*, $P < 0.05$; **, $P < 0.001$; ***, $P < 0.0001$).

Other methods

Sources of reagents and drugs, fluorescence-activated cell sorting (FACS) analysis, and immunohistochemical methods are described in Supplementary Materials and Methods.

Results

Metronomic cyclophosphamide-induced regression of brain tumor xenografts does not involve antiangiogenesis

Although antiangiogenesis is considered a key mechanistic feature of metronomic chemotherapy, metronomic cyclophosphamide treatment of SCID mice bearing U251 glioblastoma xenografts induces tumor regression that is sustained and complete (Fig. 1A) in the absence of antiangiogenesis (Fig. 1B). In contrast, the VEGF receptor-selective inhibitor axitinib (19) is strongly antiangiogenic (Fig. 1B) yet primarily shows a growth static response, followed by rapid tumor regrowth upon discontinuation of treatment (Fig. 1A). Axitinib initially increased antitumor activity when combined with metronomic cyclophosphamide treatment but ultimately blocked the tumor regression seen with metronomic therapy

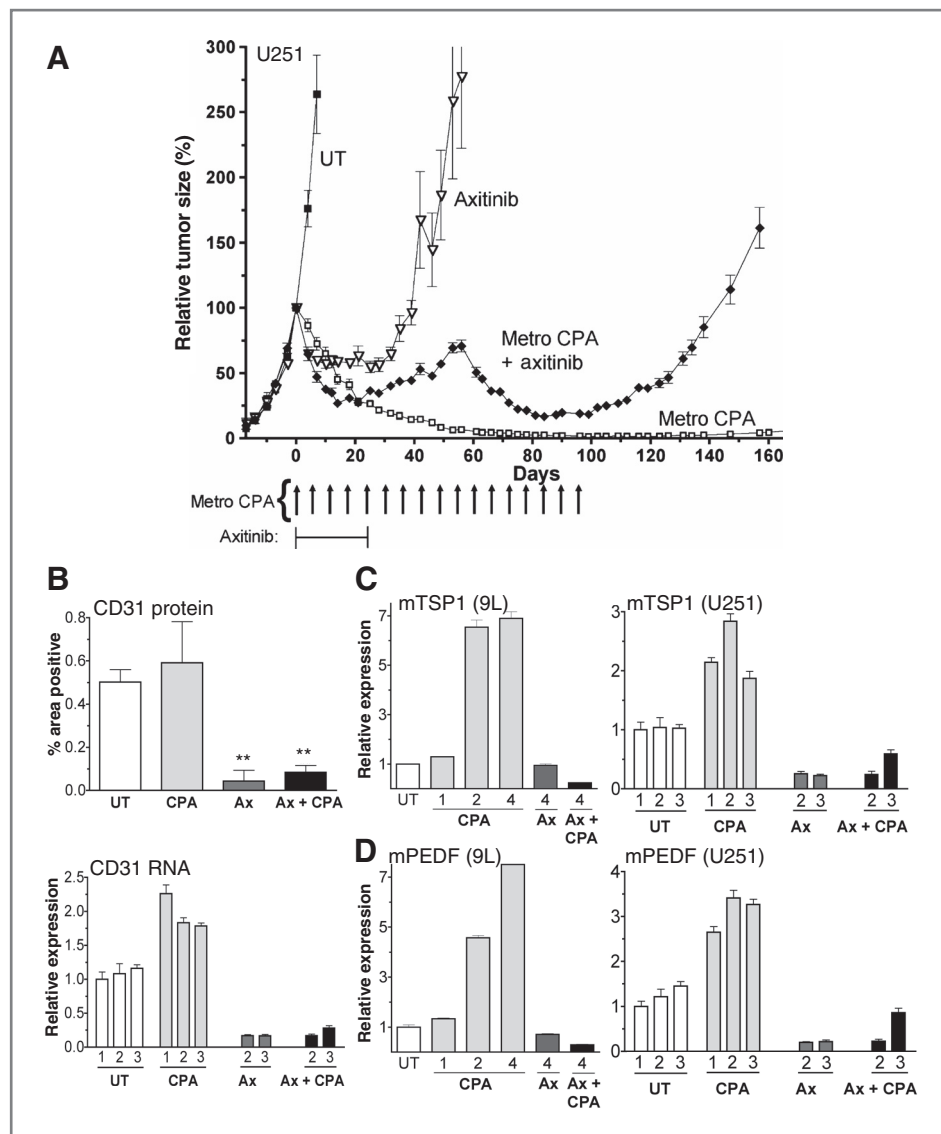


Figure 1. Metronomic cyclophosphamide (CPA)-induced regression of U251 tumors and inhibitory effects of the VEGF receptor-selective inhibitor axitinib (Ax). **A**, human U251 glioblastoma xenografts grown in SCID mice were untreated (UT) or were treated with metronomic (metro) cyclophosphamide (140 mg/kg intraperitoneally, every 6 days for 17 cycles; arrows along x-axis), daily axitinib (25 mg/kg, intraperitoneally daily for 24 days), or metronomic cyclophosphamide in combination with daily axitinib. Tumor volumes were normalized to 100% on the day of first drug treatment (day 0), when group averages reached about 500 mm³ ($n = 12$ tumors per group). Tumors were inoculated 32 days prior to the start of drug treatment. **B**, U251 tumors treated as indicated were immunohistochemically stained for the endothelial cell marker CD31 protein (top; 12 days after initiating drug treatment; **, $P < 0.001$ compared with UT control group) or assayed by qPCR for CD31 RNA (bottom; 6 days after the first, second, and third cyclophosphamide treatments, as marked along the x-axis). CD31 immunohistochemical staining was quantified as described under Supplementary Materials and Methods. qPCR analysis of host (m, mouse) TSP1 (**C**) and PEDF expression (**D**) in 9L rat (left) and U251 human (right) tumors grown in SCID mice, treated as in (**A**) and isolated 6 days after the indicated number of cyclophosphamide injections. For each comparison, qPCR data were normalized to the first untreated (UT) tumor group, whose relative RNA level was set to 1. Where indicated ("m"), qPCR analysis was carried out using mouse (host)-specific primers. Bars, mean \pm SE for $n = 5$ –6 tumors/group. See also Supplementary Fig. S1. PEDF, pigment epithelium-derived factor.

alone. Partial tumor regression was eventually seen after discontinuation of axitinib treatment, followed by rapid tumor regrowth after termination of metronomic chemotherapy. An initial improvement in tumor response followed by inhibition of metronomic cyclophosphamide-induced tumor regression was also observed with axitinib cotreatment in the 9L gliosarcoma model (4).

Metronomic cyclophosphamide activates antitumor innate immunity

To better understand why metronomic cyclophosphamide fully regresses U251 tumors, even in the absence of antiangiogenesis, we investigated other potential mechanisms and found that several macrophage-associated host factors are increased in the metronomic cyclophosphamide-

treated tumors, including TSP1 (Fig. 1C). While TSP1 has antiangiogenic activity (5), it has also been linked to tumor infiltration by antitumor M1 macrophages (12). Metronomic cyclophosphamide also increased host (mouse cell) expression of pigment epithelium–derived factor (PEDF; *Serpinf1*; Fig. 1D), an antiangiogenic factor that also stimulates M1 macrophage recruitment to tumors (20). These responses were seen in both rat 9L and human U251 tumors grown in SCID mice, where TSP1 and PEDF levels were strongly correlated ($r = 0.89$) in large sets of untreated and drug-treated tumors isolated various times after initiation of metronomic cyclophosphamide treatment (Supplementary Fig. S1). Metronomic cyclophosphamide also induced the host macrophage markers CD68 and F4/80 (Fig. 2A;

Supplementary Fig. S2A), macrophage cytolytic effectors lysozyme 1 and 2 (Supplementary Fig. S2B), and the death receptor Fas (Fig. 2B), which activates macrophages and increases their tumor cytotoxicity (21).

The above findings suggested that metronomic cyclophosphamide activates an innate immune response in the regressing tumors. To investigate this possibility, we considered NK cells, given the role of Fas in mediating interactions between NK cells and cells marked for destruction (22). In metronomic cyclophosphamide–treated tumors, we observed large increases in the host NK cell marker NK1.1 and in the NK cell–associated cytotoxic granzymes A, B, and C and perforin (Fig. 2A and C; Supplementary Figs. S2C and S3A–S3C), which are essential for cytotoxic lymphocyte–

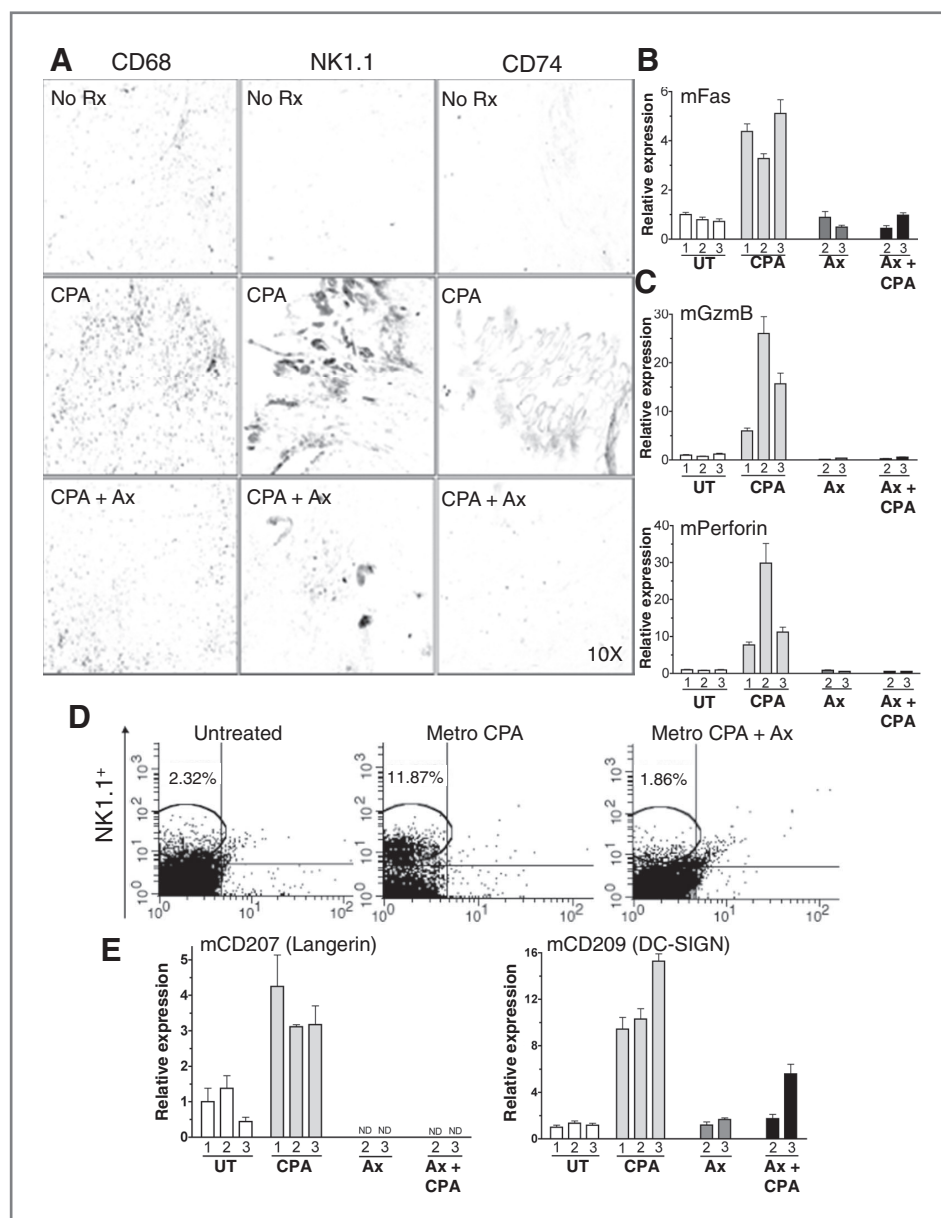


Figure 2. Metronomic cyclophosphamide (Metro CPA) induces and axitinib (Ax) blocks recruitment of macrophages, NK cells, and dendritic cells to U251 tumors. **A**, immunostaining of macrophage (left), NK cell (middle), and dendritic cell markers (right) in U251 tumors, untreated (no Rx) or treated with metronomic cyclophosphamide \pm axitinib and excised on treatment day 12, as shown in Fig. 1A. Representative images are shown with signal intensities equivalent to group mean ImageJ quantification data (i.e., Supplementary Fig. S2). **B**, **C**, and **E**, expression of the indicated host factors (m, mouse) in U251 tumors 6 days after the first, second, or third metronomic cyclophosphamide injection \pm axitinib treatment, as marked. qPCR data were normalized to the first untreated (UT) tumor group, whose relative RNA level was set to 1. Bars, mean \pm SE for $n = 5$ –6 tumors/group. **D**, FACS analysis of NK1.1 $^{+}$ cells (%) in single-cell suspensions prepared from untreated and cyclophosphamide-treated and/or axitinib-treated 9L tumors grown in SCID mice. Immunoglobulin G background control, 0.54%. CD49b (no change with treatment) was the marker along the x-axis. See also Supplementary Fig. S2.

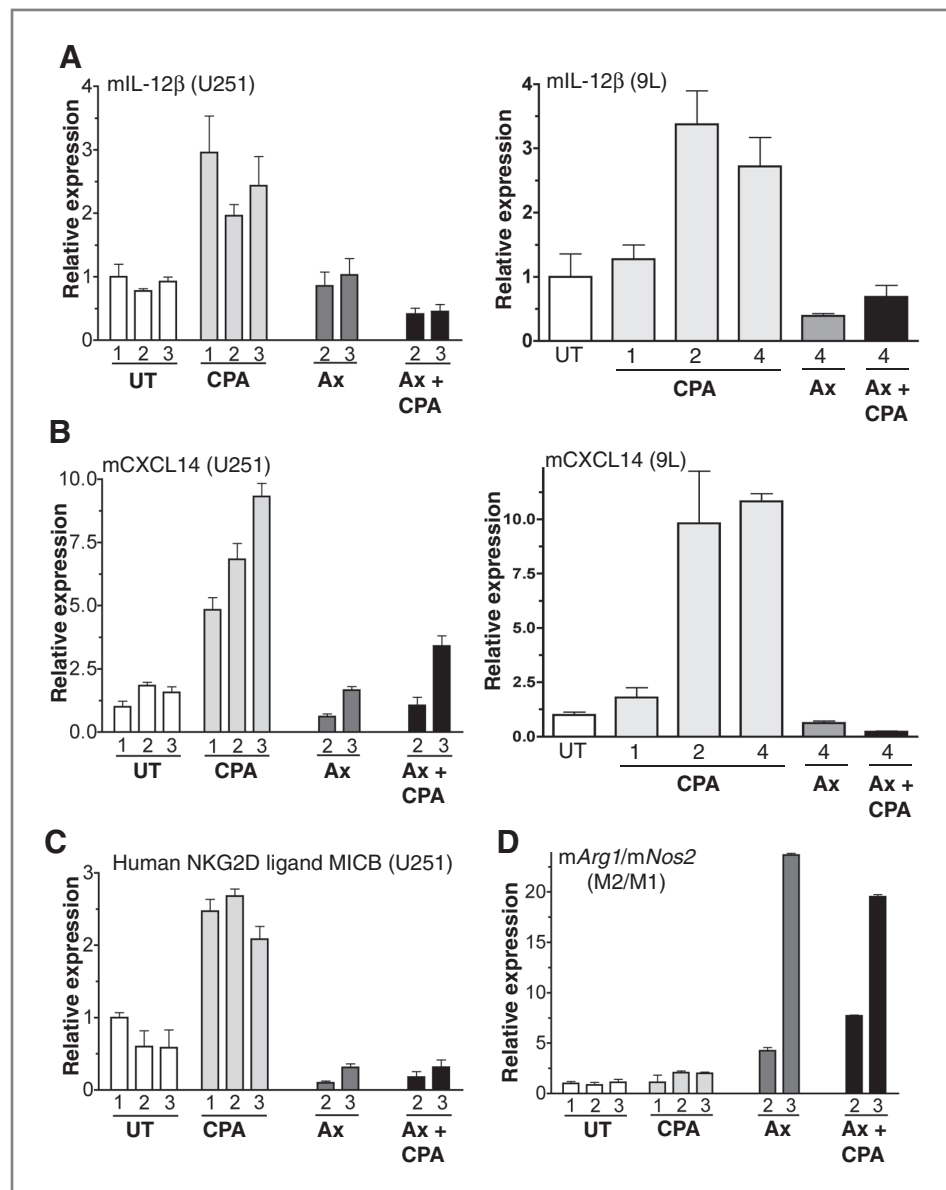
mediated cell death (22). FACS analysis confirmed the influx of NK1.1⁺ cells into the metronomic cyclophosphamide-treated tumors (Fig. 2D; Supplementary Fig. S3D). Dendritic cells were also recruited to the tumors, as shown by the activation of host dendritic cell markers important for cell-target interactions, and antigen presentation to the adaptive immune system (Fig. 2E). CD74, implicated in the regulation of dendritic cell migration (23), was also increased (Fig. 2A; Supplementary Figs. S2D and S3A and S3B). Because NK1.1 and dendritic cell markers are both increased in the cyclophosphamide-treated tumors, other NK1.1⁺ cells, such as IFN-producing killer dendritic cells (24), could also be involved. Host cytokine and chemokine immune attractants, such as interleukin (IL)-12 β and CXCL14, which can influence leukocyte and lymphocyte activation and migration

(25), were also increased in the metronomic cyclophosphamide-treated U251 and 9L tumors (Fig. 3A and B), suggesting their role in mobilizing the host innate immune response.

VEGF receptor-targeted inhibitors block metronomic cyclophosphamide-activated antitumor immunity

We next sought to determine why the VEGF receptor inhibitor axitinib blocks tumor regression induced by metronomic cyclophosphamide treatment (Fig. 1A; ref. 4). In both U251 and 9L tumors, axitinib blocked tumor infiltration of NK cells, macrophages, and dendritic cells (Fig. 2; Supplementary Figs. S2 and S3A–S3C). Axitinib also blocked the formation of chemokine and cytokine gradients that may mobilize these cells into tumors (Fig. 3A and B). Importantly, axitinib

Figure 3. Axitinib (Ax) blocks cytokine, chemokine, and other responses to metronomic cyclophosphamide (CPA). qPCR analysis of host (m, mouse) macrophage-associated IL-12 β (A), NK cell-associated CXCL14 (B), and human tumor cell-specific MICB, an activating ligand for the immune cell receptor NKG2D (C), in metronomic cyclophosphamide-treated U251 and 9L tumor xenografts grown in SCID mice. D, axitinib, both alone and in combination with metronomic cyclophosphamide, shifted the tumor-infiltrating macrophage subpopulations to the protumor M2 subtype, as indicated by the ratio of protumor (M2) macrophage marker (arginase-1; *Arg1*) to antitumor (M1) macrophage marker [inducible nitric oxide synthase (*iNOS*); *Nos2*] expression in the same set of U251 tumors. qPCR data were normalized to the first untreated (UT) tumor group, whose relative RNA level was set to 1. Bars, mean \pm SE for $n = 5$ –6 tumors/group.



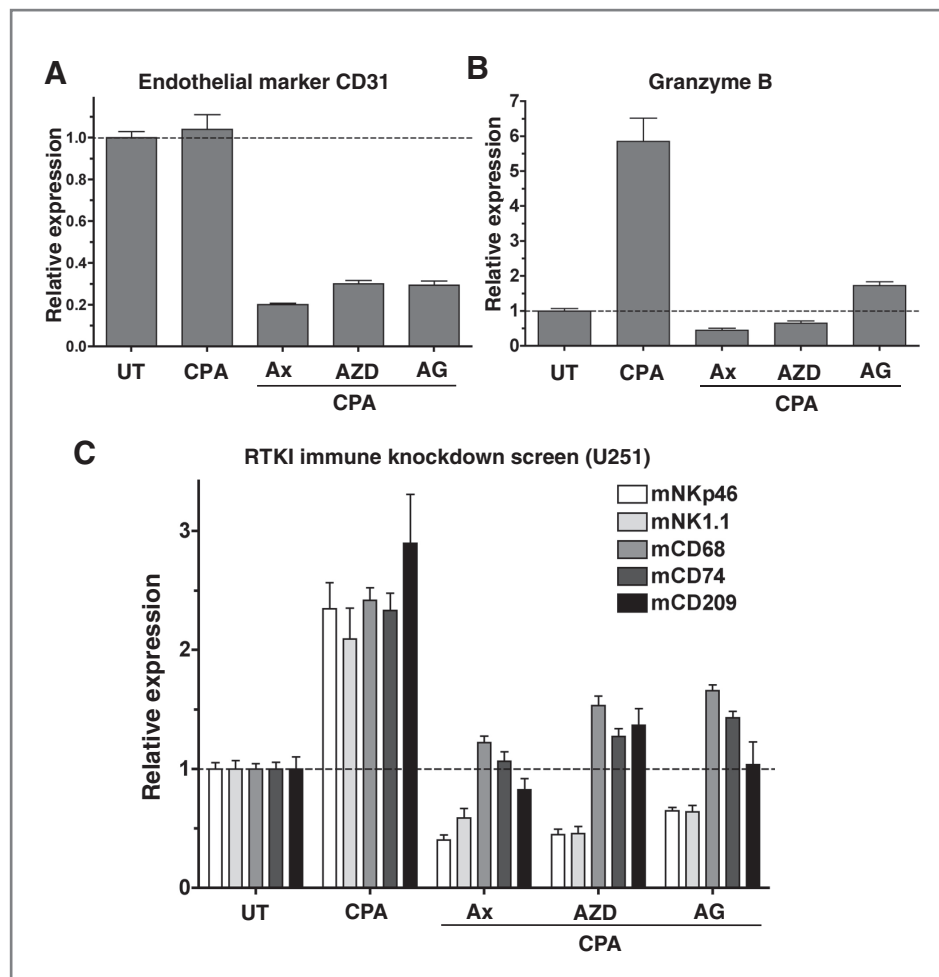


Figure 4. Immunosuppressive effects of VEGF receptor tyrosine kinase inhibitors (RTKI). U251 tumors grown in SCID mice were treated with metronomic cyclophosphamide (CPA) given alone or in combination with daily axitinib (Ax, 25 mg/kg/d, intraperitoneally), cediranib (AZD, AZD2171; 5 mg/kg/d, intraperitoneally), or AG-028262 (AG, 25 mg/kg/d, intraperitoneally). Each antiangiogenic drug was given daily for 18 days prior to the analysis shown. A, VEGF receptor-selective inhibitors suppress tumor angiogenesis (CD31 expression) and (B) block metronomic cyclophosphamide induction of granzyme B in U251 tumors grown in SCID mice, as compared with untreated (UT) controls, determined 6 days after the third cyclophosphamide injection (day 18). C, VEGF receptor-selective inhibitors block metronomic cyclophosphamide-induced immune recruitment in U251 tumors, as determined by qPCR analysis of NK cell markers NKp46 and NK1.1, macrophage marker CD68, and dendritic cell markers CD74 and CD209. qPCR data were normalized to untreated tumors, whose relative RNA level was set to 1. Bars, mean \pm SE for $n = 5-6$ tumors/group.

suppressed these immune factors to basal or below basal levels. Axitinib also suppressed the induction by metronomic cyclophosphamide of human tumor cell-expressed MICB (Fig. 3C), which is an activating ligand for the receptor NKG2D found on NK and other immune cells (26). This finding suggests that axitinib might further inhibit metronomic cyclophosphamide-induced tumor cell-targeted antitumor immunity by blocking the expression and subsequent presentation of activation signals by cyclophosphamide-damaged tumor cells. Finally, axitinib shifted the balance of tumor-associated macrophages from antitumor M1 [marked by inducible nitric oxide synthase (iNOS)] to protumor M2 macrophages (marked by arginase-1; Fig. 3D). Thus, the inhibitory effects of axitinib on metronomic cyclophosphamide-induced tumor regression likely result from interference with the innate immune response at multiple levels.

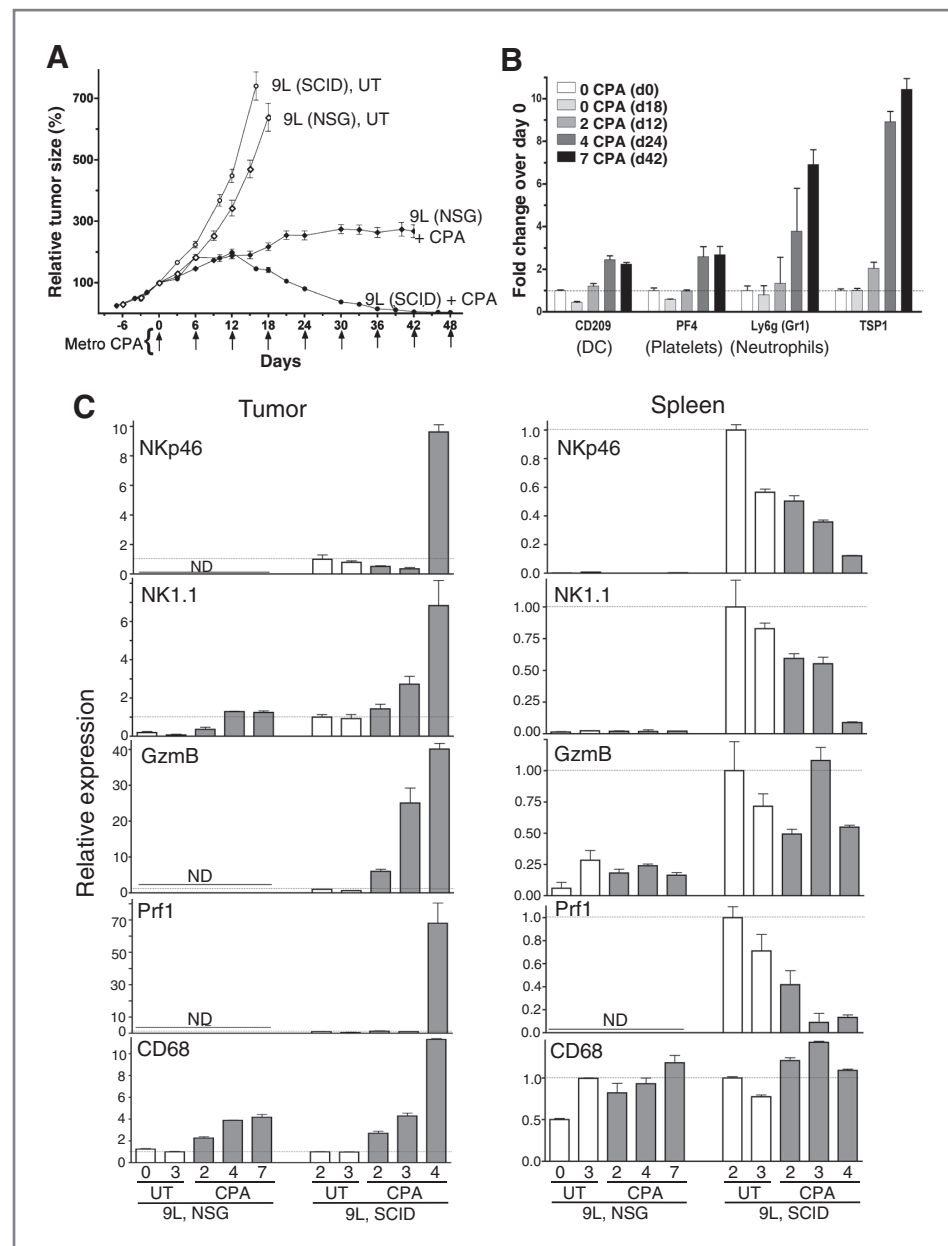
Next, we used 2 other antiangiogenic tyrosine kinase inhibitors, cediranib (AZD2171; ref. 27) and AG-028262 (28), to investigate the importance of VEGF receptor signaling for metronomic cyclophosphamide-induced antitumor immunity. These 2 chemicals exhibit selectivity for VEGF receptor inhibition comparable with or greater than that of axitinib (27, 28). In U251 tumors grown in SCID mice, cediranib and

AG-028262 strongly inhibited tumor angiogenesis, as expected (Fig. 4A); however, they also blocked metronomic cyclophosphamide-induced NK cell activity (Fig. 4B) and the recruitment of all 3 classes of innate immune cells (Fig. 4C). Moreover, by the sixth day of treatment, both drugs blocked U251 regression induced by metronomic cyclophosphamide, resulting in tumor growth stasis that continued until the study was terminated on treatment day 18, whereas over that same time period, tumors treated with metronomic cyclophosphamide alone regressed a further 50% in volume. Thus, interference with metronomic chemotherapy-induced antitumor immunity and tumor regression are general responses to VEGF receptor inhibitors.

Metronomic cyclophosphamide-induced tumor regression requires innate immune cells

To test whether the tumor-infiltrating innate immune cells contribute functionally to tumor regression, we investigated the effects of metronomic cyclophosphamide treatment on 9L gliosarcomas grown in NOD-SCID-IL2R γ -null (NSG) mice, which, unlike SCID mice, are NK cell deficient and have dysfunctional macrophages and dendritic cells due to loss of the important immunostimulatory IL-2R γ receptor (13). In

Figure 5. Antitumor activity of metronomic cyclophosphamide (CPA) in NSG mice (adaptive and innate immunocompromised). **A**, 9L tumor growth profiles in NSG and SCID mice, with tumors implanted 14 days prior to the first cyclophosphamide treatment ($n = 2$ tumors/group). **B**, qPCR of the indicated factors in 9L tumors \pm metronomic cyclophosphamide treatment and isolated on day 0 or 6 days after the second, fourth, or seventh treatment cycle (days 12–42, as marked). Bars, mean \pm SE for $n = 5$ –6 tumors/group. **C**, qPCR of the indicated factors in 9L tumors (left) and spleens (right) \pm metronomic cyclophosphamide treatment isolated from NSG or SCID mice on day 0 or 6 days after the second, fourth, or seventh treatment cycle. For each comparison, qPCR data were normalized to the first untreated (UT) SCID tumor or spleen group, whose relative RNA level was set to 1. The absence of NKp46, granzyme B (GzmB), and perforin (Prf1) reflects the NK cell deficiency of NSG mice. See Supplementary Materials for C_T values determined by qPCR. White bars, untreated tumor and spleen samples; shaded bars, treated samples. Bars, mean \pm SD for tumor ($n = 4$ –6) and spleen ($n = 2$ –3) pools. DC, dendritic cell; ND, not detectable.



NSG mice, metronomic cyclophosphamide-induced 9L tumor growth delay led to growth stasis after several treatment cycles but no regression (Fig. 5A). While macrophages, dendritic cells, and markers for other innate immune cell types such as neutrophils and platelets are present in the tumors and are increased by metronomic cyclophosphamide (Fig. 5B and C), NK cells (NKp46) and their cytotoxic effectors, granzyme B and perforin, were undetectable (Fig. 5C). Thus, the large differential antitumor response between NSG mice (tumor growth stasis) and SCID mice (full tumor regression; Fig. 5A) can be attributed to the diminished innate immune response to metronomic cyclophosphamide in NSG mice (Fig. 5C, left: NSG vs. SCID). NK cell factors (NKp46, NK1.1, GzmB, and Prf1) were also deficient in spleen in both untreated and cyclophos-

phamide-treated NSG spleens as compared with SCID mice (Fig. 5C, right: NSG vs. SCID), as expected. Progressive depletion of NK cells from SCID mouse splenic reservoirs with continued metronomic cyclophosphamide treatment was also apparent (Fig. 5C, right). The tumor growth stasis seen in NSG mice likely reflects residual antitumor immune cell responses (Fig. 5B and C) and the intrinsic cytotoxicity of cyclophosphamide toward tumor cells and tumor endothelial cells. The delayed increase in the NK cell markers NKp46 and perforin in cyclophosphamide-treated SCID mouse 9L tumors (Fig. 5C) is consistent with the delayed onset of tumor regression (Fig. 5A), further implicating NK cell function in this response to metronomic cyclophosphamide. Finally, a strong increase in TSP1 expression was seen in the metronomic cyclophosphamide-

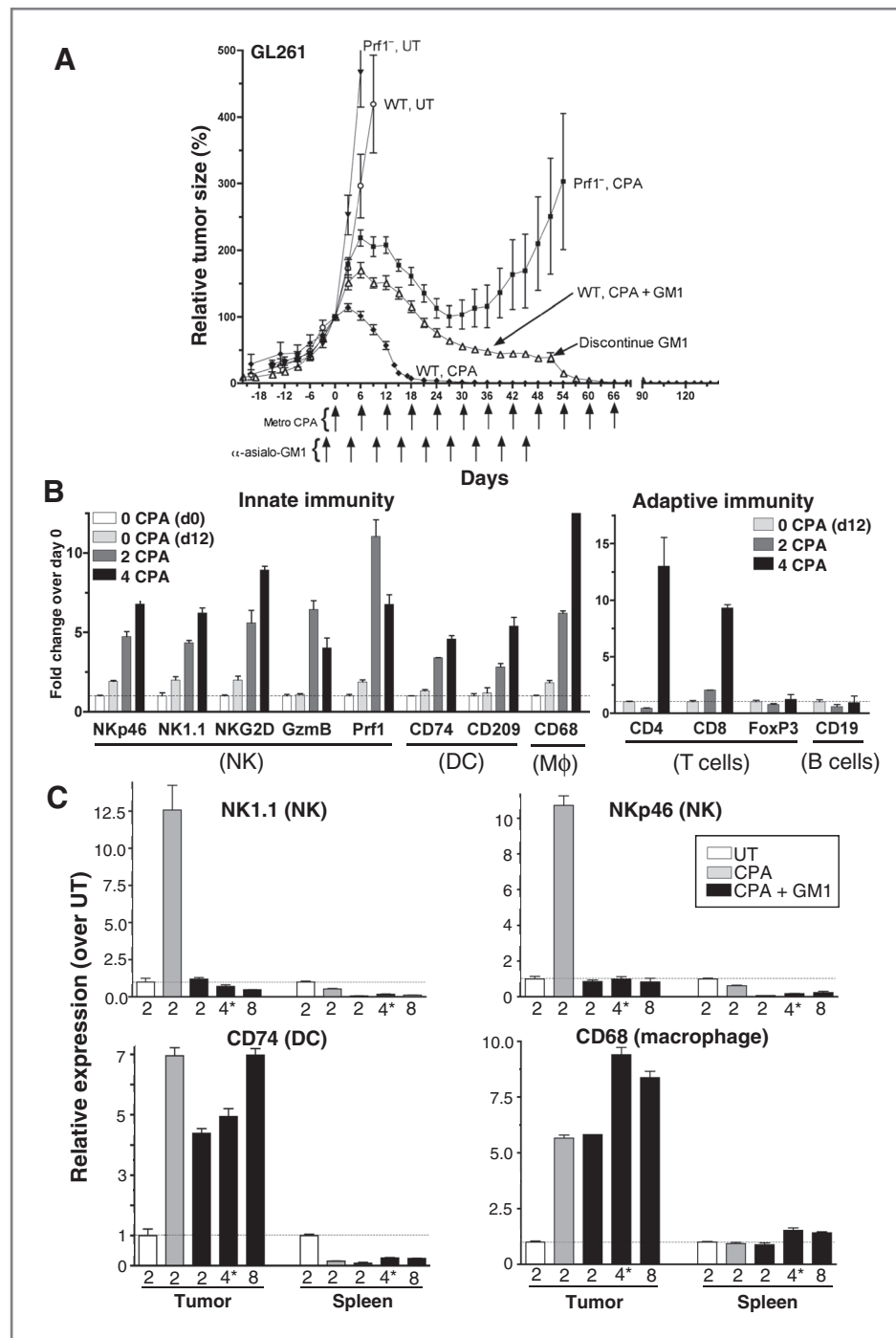


Figure 6. Response of GL261 tumors to metronomic cyclophosphamide (CPA) in C57BL/6 wild-type (WT) and perforin-knockout mice (Prf1^{-/-}) and impact of NK cell depletion. **A**, tumor growth profiles ($n = 12$ tumors/group) showing metronomic cyclophosphamide-induced regression in wild-type (WT) mice; regression is delayed and incomplete in Prf1^{-/-} mice and also following NK cell depletion in WT mice (anti-asialo-GM1 antibody given every 6 days beginning 3 days prior to the first cyclophosphamide injection; arrows). Anti-asialo-GM1 was discontinued on day 51; cyclophosphamide was terminated on days 60 and 54 in WT and Prf1^{-/-} mice, respectively, and on day 66 in the CPA + GM1 group. Tumors were inoculated 28 days prior to the first cyclophosphamide treatment (day 0). **B**, qPCR analysis of mouse (host cell) innate and adaptive immune factors in untreated GL261 tumors collected on days 0 and/or 12, and in tumors 6 days after either 2 or 4 cyclophosphamide treatment cycles, as marked. M ϕ , macrophage; DC, dendritic cell. Genes assayed include those shown in earlier figures, as well as markers for helper (CD4) and cytotoxic effector (CD8) T cells and Treg cells (FoxP3) and B cells (CD19). The strong increase in CD4- and CD8-marked T cells was delayed when compared with the innate immune cells and the onset of tumor regression. Data were normalized to the first untreated (UT) tumor group, whose relative RNA level was set to 1. Bars, mean \pm SE for $n = 5$ –6 tumors/group. **C**, qPCR analysis showing depletion of NK cells by anti-asialo-GM1; tumors and spleens from mice in **A** were collected 6 days after 2 cyclophosphamide cycles (i.e., day 12) without anti-asialo-GM1 (UT) or 6 days after the second, 3 days (*) after the fourth, and 6 days after the eighth cyclophosphamide cycle with anti-asialo-GM1 treatment. Anti-asialo-GM1 depleted NK cells from tumors and spleens and blocked their recruitment into metronomic cyclophosphamide-treated tumors. The partial regression seen with anti-asialo-GM1 in **A** may result from the unimpeded tumor recruitment of dendritic cells and macrophages, seen in **C** (bottom). qPCR data were normalized to untreated (UT), whose relative RNA level was set to 1. Bars, mean \pm SE for $n = 5$ –6 tumors/group. See also Supplementary Fig. S3.

treated NSG mouse 9L tumors (Fig. 5B), despite the lack of tumor regression. Thus, TSP1 and its antiangiogenic activity are not sufficient to drive tumor regression, which may help explain why TSP1 production is not a reliable marker for clinical response to metronomic chemotherapy (29).

Metronomic cyclophosphamide-activated antitumor immunity and tumor regression in an immunocompetent, syngeneic mouse model

To ascertain whether the strong innate immune responses to metronomic cyclophosphamide treatment are limited to animals with deficiencies in T- and B-adaptive immune cells, the impact of metronomic cyclophosphamide was investigated in immunocompetent C57BL/6 mice bearing the syngeneic glioma GL261. These studies additionally enabled us to determine whether metronomic cyclophosphamide activates a T- or B-cell adaptive immune response and whether regulatory suppressor T cells (Treg) are also recruited to the tumors and might interfere with antitumor immunity (30). Strikingly, metronomic cyclophosphamide fully regressed all GL261 tumors (Supplementary Table S1), with no tumor regrowth seen at day 140, that is, 80 days after halting metronomic cyclophosphamide (Fig. 6A). Large increases in tumor-associated NK cells, dendritic cells, and macrophages occurred at the onset of tumor regression and continued for at least 4 metronomic cyclophosphamide cycles (Fig. 6B, left). We also observed delayed recruitment of CD4⁺ helper T cells and CD8⁺ cytotoxic T-effector cells to the regressing tumors, but no increase in Tregs (marked by FoxP3; Fig. 6B, right). The latter finding is consistent with the reported selectivity of metronomic cyclophosphamide for killing Tregs but not cytotoxic antitumor T-effector cells (30, 31).

Causal role for innate immune cells in tumor regression

The functional consequence of NK cell recruitment was probed by NK cell depletion using anti-asialo-GM1 antibody (32), which resulted in delayed and substantially incomplete GL261 tumor regression (Fig. 6A; Supplementary Table S1). Tumor cell recruitment of NK cells was fully blocked by asialo-GM1 antibody over multiple cyclophosphamide treatment cycles, and splenic NK cell levels were also suppressed (Fig. 6C, top). Metronomic cyclophosphamide recruitment of dendritic cells and macrophages was unaffected by NK cell depletion (Fig. 6C, bottom) and may contribute to the partial tumor regression observed. Importantly, NK cell recruitment and complete tumor regression were both restored following termination of asialo-GM1 antibody treatment on day 51 (Fig. 6A). Thus, while other immune cells likely contribute to metronomic cyclophosphamide-induced tumor regression, NK cells are required both for the early onset and for the completeness of tumor regression. Metronomic cyclophosphamide-induced GL261 regression was also delayed and even less complete in C57BL/6 mice deficient in perforin (Fig. 6A; Supplementary Table S1). Perforin deficiency severely decreases not only NK cell but also NKT cell and cytotoxic T-cell cytolytic activity (14). The further diminished antitumor response in the perforin-knockout mice suggests that adaptive immune lymphocytes contribute to tumor regression. Markers for host adaptive

immune CD8⁺ T lymphocytes and innate immune NK cells, macrophages, and dendritic cells were all strongly induced in GL261 tumors by metronomic cyclophosphamide treatment of the perforin-knockout mice (Supplementary Fig. S4A). Thus, despite intact immune cell mobilization, the impairment of lymphocyte cytolytic function in the absence of perforin is sufficient to seriously impair metronomic cyclophosphamide-induced tumor regression. Thus, the host immune system is essential for metronomic cyclophosphamide-induced tumor regression.

MTD cyclophosphamide induces a weak, transient innate immune response

U251 tumors grown in SCID mice initially responded to MTD cyclophosphamide treatment; however, the response was short-lived and was followed by resumption of rapid growth during the drug-free recovery period between treatment cycles (Fig. 7A). An initial, modest innate immune response to MTD cyclophosphamide dissipated during the recovery period, whereas the response to metronomic cyclophosphamide was sustained and became maximal by day 18, as judged by the increased expression of the cytotoxic effector granzyme B (Fig. 7B, top) and other immune cell markers (Fig. 7C). MTD cyclophosphamide treatment did increase overall granzyme B exposure by approximately 40%; however, the increase following metronomic cyclophosphamide treatment was more than 10-fold higher (Fig. 7B, bottom).

Discussion

The present findings identify innate immune cell recruitment, and not TSP1-mediated antiangiogenesis, as a major, functionally important mechanism for the dramatic regression of brain tumor xenografts treated with cyclophosphamide on a 6-day repeating, metronomic schedule. This immune response involves a strong NK cell component that contributes functionally to tumor regression. These findings are based on studies in 3 brain tumor models, including a syngeneic, fully immunocompetent mouse model, indicating that it is not due to immune dysregulation as a result of the lack of an adaptive immune system. In contrast, MTD cyclophosphamide did not induce immune recruitment leading to tumor regression, indicating that the scheduling of chemotherapy is a critical requirement for this innate immune response. Finally, several VEGF receptor-selective antiangiogenesis drugs were shown to block innate immune recruitment, implicating VEGF receptors in the mechanism whereby metronomic cyclophosphamide activates innate immunity. The latter finding is particularly important given the widespread efforts to develop effective ways to combine VEGF receptor inhibitors with traditional cytotoxic anticancer drugs (6).

Metronomic cyclophosphamide-induced tumor infiltration by macrophages, dendritic cells, and NK cells was established using both structural and functional markers for each immune cell type. In the case of NK cells, strong induction of several functional markers was observed, notably Nkp46, perforin, and granzymes A, B, and C. Moreover, functionality was *de facto* established by GM1 antibody

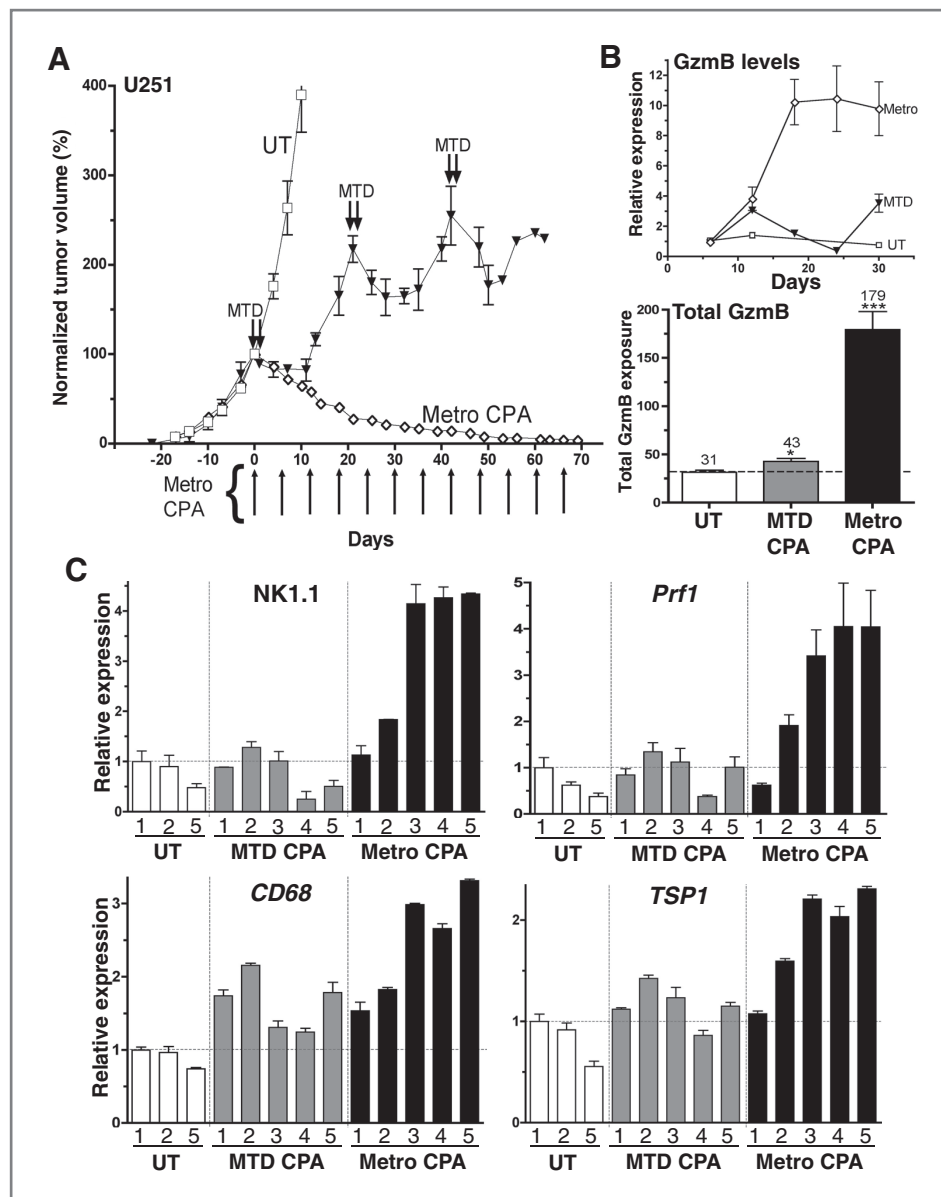


Figure 7. Response of U251 tumors to metronomic versus MTD cyclophosphamide. **A**, growth curves for U251 tumor in SCID mice administered either metronomic or MTD cyclophosphamide ($n = 12$ tumors/group). Untreated (UT) and metronomic cyclophosphamide (CPA)-treated U251 curves are the same as in Fig. 1A. **B**, U251 tumors isolated at the indicated times after initiating cyclophosphamide treatment as in **A** were analyzed for granzyme B expression (top) and total (integrated) granzyme B RNA levels from day 0 through day 30, which increased 1.4-fold (from 31 to 43 arbitrary units; MTD cyclophosphamide) versus 5.8-fold (from 31 to 179 arbitrary units; metronomic cyclophosphamide; bottom). *, $P < 0.05$; ***, $P < 0.0001$ versus UT tumors. **C**, qPCR analysis of marker genes in U251 tumors collected after the indicated number of 6-day schedule intervals (1 = day 6 after first cyclophosphamide treatment, 5 = day 30). Data were normalized to the first untreated tumor group, whose relative RNA level was set to 1. Bars, mean \pm SE for $n = 5$ –6 tumors/group.

depletion, which seriously compromised metronomic cyclophosphamide-induced tumor ablation, with tumor regression resuming shortly after antibody depletion was terminated. Functional markers for the tumor-recruited macrophages included the macrophage cytolytic effectors lysozyme 1 and 2, which showed large increases in the treated tumors, and in the case of dendritic cells, DC-SIGN (CD209), which is important for dendritic cell function and antigen presentation. The increases in DC-SIGN exceeded those of the dendritic cell structural markers CD207 (Langerin) and CD74, suggesting an increase in dendritic cell activity in addition to recruitment in response to metronomic cyclophosphamide treatment. Markers for other innate immune cells, such as platelets and neutrophils, were also increased; however, further studies will be required to fully characterize

their involvement in metronomic cyclophosphamide-activated antitumor immunity and tumor regression.

Conceivably, other cytotoxic chemotherapeutic drugs may also induce a sustained antitumor innate immune response when given on a metronomic schedule (1). Metronomic chemotherapies may thus complement strategies to counter immune evasion by tumor cells, such as *ex vivo* immune cell augmentation, host immune ablation, and adoptive immunotherapy (33). In other studies, low-dose chemotherapy can modulate various antitumor immune responses. Examples include the ability of vinblastine to induce dendritic cell maturation (34) and that of cyclophosphamide to suppress Tregs (30, 31). Several daily, low-dose metronomic regimens have been shown to enhance antitumor immunity by selectively killing Treg suppressor cells and thereby restoring

antitumor lymphocyte effector function; however, an increase in tumor NK cells was not established (31, 35, 36). In contrast, recruitment of immune cell infiltrates to the tumor microenvironment was shown here and is likely to be critical to the major tumor regression responses that we observed. Moreover, our finding that large increases in tumor-associated NK cells occur not only in immunocompetent C57BL/6 mice but also in immunodeficient SCID mice, which are devoid of Tregs, indicates that the NK cell response described here is both novel and mechanistically distinct from the relief of immunosuppression by Tregs reported previously using daily low-dose metronomic schedules (31, 35, 36).

Cyclophosphamide given on a 6-day metronomic schedule may also potentiate antitumor adaptive immunity, as suggested by the increase in CD8⁺ T cells through downregulation of iNOS (37). Although iNOS regulation was not explored in the present study, we did observe a temporal delay in the recruitment of adaptive immune cytotoxic CD8⁺ T cells to the regressing GL261 tumors. Perforin knockout, which greatly impairs both innate and adaptive lymphocyte effector function (14), had a greater impact on tumor regression than antibody depletion of NK cells alone (Fig. 6A), suggesting a role for adaptive CD8⁺ T cells in the antitumor response to metronomic cyclophosphamide.

The tumor regression responses reported here were robust and were validated in 3 brain tumor xenograft models. Future studies will be required to extend these findings to other tumor models, including orthotopic brain tumor models. Importantly, many orthotopic sites have endogenous innate immune cell populations, including the liver, which contains Kupffer and NK cells (38), and the brain, where microglia and NK cells can infiltrate tumors (39). Although the blood–brain barrier often impedes chemotherapeutic drug access to brain tumors, 4-hydroxy-CPA, the active metabolite of cyclophosphamide, is membrane permeable and can cross the blood–brain barrier (40). Furthermore, brain tumors may be leaky, disrupting surrounding extracellular matrix and the blood–brain barrier itself (41).

Innate immune cell recruitment is shown to be a target for the inhibitory effects of several VEGF receptor–selective inhibitors on metronomic cyclophosphamide–induced tumor regression. This finding has important implications for treatments combining chemotherapy, in particular metronomic chemotherapy, with VEGF receptor inhibitory antiangiogenic drugs. While VEGF receptor signaling inhibitors can improve responses to some metronomic therapies (e.g., see ref. 29), those studies typically use daily low-dose metronomic drug treatment, which may be less effective at eliciting an antitumor immune response than the 6-day repeating metronomic cyclophosphamide schedule used here (J.C. Doloff and D.J. Waxman; unpublished data). Furthermore, the suppression of innate immune cell recruitment by VEGF receptor inhibitors suggests that these drugs decrease immune surveillance, which could help explain the increases in metastatic incidence and progression recently linked to this class of antiangiogenic agents (42). Supporting this hypothesis, metastatic infiltration of tumor cells in NOD/SCID mice is increased by NK cell–depleting antibody, and metastasis is even more severe when tumor

cells are grown in additionally immunocompromised NSG mice (43). Furthermore, numerous GL261 tumors implanted in perforin knockout C57BL/6 mice were not restricted to the subcutaneous space and infiltrated into the intraperitoneal activity.

VEGF receptors, which are targeted by the 3 antiangiogenic drugs used here, have been implicated in dendritic cell differentiation and are important for dendritic cell–endothelial cell cross-talk, transdifferentiation, and tumor-associated macrophage infiltration (44). Endothelial cell VEGF signaling is also important for chemokine expression and secretion in proinflammatory responses (17), suggesting an additional mechanism whereby the inhibition of VEGF signaling could block innate immune cell recruitment. Indeed, in our models, the VEGF receptor inhibitor axitinib blocked induction of host (mouse) chemokines IL-12 β and CXCL14 by metronomic cyclophosphamide treatment. IL-12 is expressed and secreted by activated dendritic cells, neutrophils, and macrophages and can activate antitumor NK cells and T cells (45), whereas CXCL14 stimulates activated NK cell migration (46). We observed that splenic NK cell reservoirs were decreased over time in metronomic cyclophosphamide–treated SCID mice, perhaps reflecting net immune cell migration out from the spleen and into the treated tumors. A corresponding decrease in splenic NK cell factors was not seen in immunocompetent C57BL/6 mice, which had higher basal levels of NK cells, both in spleen and in untreated tumors (Supplementary Fig. S4B).

The suppression of the innate immune response by VEGF receptor inhibitors reported here is likely due to the inhibition of VEGF signaling, rather than a secondary response to the associated loss of blood vessels required for immune cell trafficking into the tumor compartment, insofar as antiangiogenic agents that decrease tumor vascularity without inhibiting VEGF signaling do not block metronomic cyclophosphamide–stimulated antitumor innate immunity (J.C. Doloff and D.J. Waxman; unpublished data). Thus, inhibition of antitumor innate immunity is not a characteristic of antiangiogenesis *per se*. Antiangiogenic agents that target tumor endothelial cells without inhibition of VEGF receptor include the tubulin-targeting cytotoxic agent Oxi4053 and the cell-cycle inhibitor TPN-470, both of which can enhance antitumor activity when combined with metronomic chemotherapy (3, 47). In addition, TPN-470 inhibits both tumor metastases and primary tumor growth (48).

The empirical observation that a 6-day metronomic cyclophosphamide schedule is optimal with regard to antitumor activity (3) could reflect the life span of host immune cells such as platelets, which are first-line immune responders to tissue inflammation and damage and have a life span of 5 to 10 days (49). While other metronomic chemotherapy schedules, including daily, low-dose regimens, show antitumor activity (1), we suggest that an intermittent metronomic schedule, such as the every-6-day bolus cyclophosphamide regimen used here, may be optimal with respect to activation of innate immunity: sufficiently frequent to repeatedly induce tumor cytotoxicity and inflammation and activate cytokine/chemokine attractants leading to an innate immune response, whereas at the same time sufficiently infrequent to minimize

the killing of immune cells recruited to the tumor. While the metronomic dose of cyclophosphamide used here is higher than metronomic dosages used in patients with cancer, where daily metronomic dosing is most often used (1), our metronomic schedule (140 mg/kg cyclophosphamide, every 6 days) is, in fact, slightly lower in total drug exposure than that of the low-dose, daily metronomic cyclophosphamide regimen used in mouse models by others (25 mg/kg, daily, which corresponds to a total dose of 150 mg/kg every 6 days) to model metronomic dosing in the clinic (50). The exact dosing and metronomic timing requirements for an effective antitumor immune response can be expected to vary with the chemotherapeutic drug and are likely to benefit from efforts at optimization.

The precise nature of the cytotoxic damage, stress response, and cytokine and chemokine signals required for metronomic cyclophosphamide to elicit an antitumor immune response are unknown. The ability of metronomic cyclophosphamide to induce such a response is likely to vary between tumors, insofar as the same metronomic cyclophosphamide dose and schedule used in the present study did not elicit regression (51) or an innate immune response (J.C. Doloff and D.J. Waxman; unpublished data) in the case of PC-3 prostate tumor xenografts. Given the absence of immune cell involvement in the PC-3 model, it is not surprising that the combination of metronomic cyclophosphamide with axitinib was more effective, rather than less effective against PC-3 tumors than metronomic cyclophosphamide alone (51). Conceivably, an innate immune response leading to tumor regression may be achieved with PC-3 and other tumors by appropriate choice of drug, dose, and metronomic schedule. Indeed, tumors derived from immortalized fibroblasts are regressed via an apoptosis-independent mechanism involving macrophages when cyclo-

phosphamide is given at 170 mg/kg on a 5-day repeating schedule (52), and regression of mammary MX-1, ovarian SK-OV-3, and neuroblastoma SK-NAS tumor xenografts is achieved when paclitaxel is given on various metronomic schedules (53). Further studies to investigate the involvement of NK and other immune cells in these tumor regression responses would be of interest.

Chemotherapeutic drugs elicit a variety of distinct types of DNA damage and activate different cellular stress responses, some of which are known to activate NK cells (54), which could be one mechanism whereby metronomic cyclophosphamide activates an innate immune response. In U251 tumors grown in SCID mice, we found that metronomic cyclophosphamide induced tumor cell expression of MICB, a ligand for the innate immune cell activating receptor NKG2D. Axitinib blocked this increase in MICB expression, further implicating tumor cell-specific expression of MICB in targeting of NK and other immune cells to the drug-treated tumors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interests were disclosed.

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VEGF Receptor Inhibitors Block the Ability of Metronomically Dosed Cyclophosphamide to Activate Innate Immunity–Induced Tumor Regression

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