Perivascular M2 Macrophages Stimulate Tumor Relapse after Chemotherapy

Russell Hughes1, Bin-Zhi Qian2, Charlotte Rowan1, Munitta Muthana1, Ioanna Kekilkoglou3, Oakley C. Olson3, Simon Tazzyman1, Sarah Danson1, Christina Addison3, Mark Clemons3, Ana Maria Gonzalez-Angulo6, Johanna A. Joyce4, Michele De Palma3, Jeffrey W. Pollard2,7, and Claire E. Lewis1

Abstract

Tumor relapse after chemotherapy-induced regression is a major clinical problem, because it often involves inoperable metastatic disease. Tumor-associated macrophages (TAM) are known to limit the cytotoxic effects of chemotherapy in preclinical models of cancer. Here, we report that an alternatively activated (M2) subpopulation of TAMs (MRC1+TIE2+CXCR4+) accumulate around blood vessels in tumors after chemotherapy, where they promote tumor revascularization and relapse, in part, via VEGF-A release. A similar perivascular, M2-related TAM subset was present in human breast carcinomas and bone metastases after chemotherapy. Although a small proportion of M2 TAMs were also present in hypoxic tumor areas, when we genetically ablated their ability to respond to hypoxia via hypoxia-inducible factors 1 and 2, tumor relapse was unaffected. TAMs were the predominant cells expressing immunoreactive CXCR4 in chemotherapy-treated mouse tumors, with the highest levels expressed by MRC1+ TAMs clustering around the tumor vasculature. Furthermore, the primary CXCR4 ligand, CXCL12, was upregulated in these perivascular sites after chemotherapy, where it was selectively chemotactic for MRC1+ TAMs. Interestingly, HMOX-1, a marker of oxidative stress, was also upregulated in perivascular areas after chemotherapy. This enzyme generates carbon monoxide from the breakdown of heme, a gas known to upregulate CXCL12. Finally, pharmacologic blockade of CXCR4 selectively reduced M2-related TAMs after chemotherapy, especially those in direct contact with blood vessels, thereby reducing tumor revascularization and regrowth. Our studies rationalize a strategy to leverage chemotherapeutic efficacy by selectively targeting this perivascular, relapse-promoting M2-related TAM cell population. Cancer Res; 75(17); 1–13. ©2015 AACR.

Introduction

The regrowth of tumors after treatment with cytotoxic agents poses a major threat to survival in cancer patients, particularly those with inoperable primary and/or metastatic tumors as they often rely heavily on chemotherapy to slow tumor growth and reduce its burden. The development of resistance results in poor survival rates for patients with, for example, inoperable pancreatic or lung cancer, who often survive for less than 12 months after diagnosis (1, 2). New therapeutic strategies are, therefore, urgently needed to delay or prevent tumor regrowth after early cycles of chemotherapy as these would extend life. Malignant tumors contain various CD11b+ myeloid cells, including granulocytes, myeloid-derived suppressor cells (MDSC), and tumor-associated macrophages (TAM; ref. 3). The latter are recruited as monocytes from the peripheral blood, which are themselves derived from progenitor cells in the bone marrow (4, 5). After entry into tumors, monocytes differentiate into macrophages (6) and promote tumor progression by stimulating tumor invasion, neovascularization and metastasis, and suppressing antitumor immunity (5, 7). TAMs express a broad spectrum of activation states between the two extreme forms of “classical” (M1) and “alternative” (M2) activation, with a tendency toward the latter (8). M2-skewed TAMs are characterized by their upregulation of various receptors, including the mannose receptor C-type lectin [MRC1/CD206] and the angiopoietin receptor, TIE2 (9). Indeed, TAMs expressing high levels of these two receptors have been shown to play an essential role in promoting angiogenesis in untreated mouse tumors (10).

A variety of anticancer therapies have been shown to stimulate the recruitment of CD11b+ myeloid cells by mouse tumors (11, 12). For example, TAMs accumulate in mouse tumors after chemotherapy (13–15), ionizing radiation (16–18), and the vascular disrupting agent combretastatin-A4-P (CA-4-P; ref. 19).

1Department of Oncology, University of Sheffield Medical School, Sheffield, United Kingdom. 2MRC Centre for Reproductive Health, Queen’s Medical Research Institute, University of Edinburgh, Edinburgh, Scotland, United Kingdom. 3Swiss Institute for Experimental Cancer Research (ISREC), School of Life Sciences, Ecole Polytechnique Fédérale de Lausanne (EPFL), Lausanne, Switzerland. 4Cancer Biology and Genetics Program, Memorial Sloan Kettering Cancer Center, New York, New York. 5Breast Medical Oncology, The University of Texas MD Anderson Cancer Center, Houston, Texas. 6Department of Developmental and Molecular Biology, Albert Einstein College of Medicine, New York, New York.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

R. Hughes and B.-Z. Qian share first authorship of this article.

J.W. Pollard and C.E. Lewis share senior authorship of this article.

Corresponding Author: Claire E. Lewis, University of Sheffield Medical School, Beech Hill Road, Sheffield, Yorkshire S10 2RX, United Kingdom. Phone/Fax: 44-114-271-2903; E-mail: claire.lewis@sheffield.ac.uk
doi: 10.1158/0008-5472.CAN-14-3587

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Importantly, the mononuclear phagocyte growth factor CSF1 (13) and chemokines CCL2 (14) and CXCL12 (16, 19) are increased in tumors after such anticancer therapies, and can trigger monocyte recruitment (4, 5). These cells then reduce the efficacy of chemotherapy by limiting vascular permeability via their expression of MMP9 (14), promoting resistance to therapy-induced death via their expression of cathepsin serine proteases (15) and by suppressing the recruitment/activation of cytotoxic T cells (12, 13).

Considerable evidence has emerged recently for M2-activated macrophages playing an important role in repair and remodeling after tissue injury. For example, they are prominent in diseased tissues in spinal cord injury, myocardial infarction, and various forms of renal disease. (20). Furthermore, TAMs with similar phenotypes have been implicated in tumor relapse after therapies like irradiation and CA-4-P (17, 19). Although MRC1⁺ TAMs are increased in MMTV-PyMT mammary tumors after doxorubicin treatment (14), the role of M2 TAMs in tumor relapse after chemotherapy has not been defined.

Our studies show that MRC1⁺ TAMs are elevated in mouse tumors after treatment with various chemotherapeutic agents. Moreover, this TAM subset was further defined as MRC1⁺ TIE2⁺CXCR4⁺VEGFA⁺ and shown to accumulate preferentially in vascularized, CXCL12-rich regions of tumors after chemotherapy. Blockade of CXCR4 signaling prevented this close association with the tumor vasculature after chemotherapy, resulting in a marked delay in subsequent tumor revascularization and relapse. These findings suggest that selective targeting of vessel-associated, M2-skewed TAMs after chemotherapy could increase the relapse-free survival of cancer patients.

Materials and Methods

Mouse studies

To investigate the mechanisms regulating tumor relapse after chemotherapy, we primarily used the Lewis lung carcinoma model (21) and the MMTV-PyMT model of breast cancer (15). These syngeneic tumor models respond to chemotherapy, with an initial phase of tumor growth inhibition, followed by a distinct regrowth phase. Transgenic tumor models were also obtained from either chemotherapy alone (paclitaxel, doxorubicin/cyclophosphamide) or with paclitaxel (10 mg/kg) 2 hours before sacrifice to label hypoxia and proliferating cells, respectively.

4T1/paclitaxel studies

Female Balb/c mice (>8 weeks) were orthotopically implanted with 1 x 10⁶ 4T1 murine mammary adenocarcinoma cells. Tumors were grown for 10 days before mice were injected i.p. with either cremophore or cremophore + paclitaxel (10 mg/kg) every 5 days for a total of three doses, the mice were sacrificed and tissues harvested 4 days after the last treatment. Orthotopic MMTV-PyMT/doxorubicin studies were performed as previously described (15). Mice bearing implanted tumors were injected i.v. with a single dose of doxorubicin (5 mg/kg) when at 250 mm³ and sacrificed 7 days after therapy.

Monocyte/macrophage-specific ablation of Vegfa studies

Female Tg(Ifitm-Cre-Mer)1meri.Vegfa(loxP) mice (>8 weeks), shown to specifically lack the expression of VEGFA in the mouse cyto/macrophage lineage after treatment with tamoxifen (23), or Cre-negative litter mates (ages 8 weeks +) were orthotopically implanted with the syngeneic MMTV-PyMT cell line F246-6. Vegfa floxed mice were the kind gift of Dr. Napoleone Ferrara, Genentech. Mice with established tumors were treated with a single i.v. injection of either vehicle (PBS) or doxorubicin (5 mg/kg) and 24 hours later with tamoxifen via their food (3 mg/20 g bodyweight/day). Treatment with tamoxifen was then maintained throughout tumor regrowth. Tumor growth was monitored by caliper measurements. In the LLC1/TAM adoptive transfer studies, C57BL/6 mice implanted with LLCs and treated with cyclophosphamide as outlined above, 48 hours after the last dose of cyclophosphamide, donor mice were sacrificed, their tumors removed, enzymatically dissociated, and MRC1⁺ and MRC1⁻ TAMs were purified by flow cytometry. Recipient mice were randomized into three groups and received either 50 x 10⁶ MRC1⁺ TAMs, 50 x 10⁶ MRC1⁻ TAMs, or saline via a 25 μL intratumoral injection. After injection of these cells or saline, tumor regrowth was then monitored.

Human breast carcinomas and metastatic bone lesions

Four patients with newly diagnosed breast cancer underwent neoadjuvant chemotherapy as previously described (24). Residual tumor tissues were collected at the time of surgery (which took place 21–28 days after the last dose of paclitaxel). Biopsies of bone metastatic lesions were also obtained from five advanced breast cancer patients. All biopsies were obtained under informed consent following procedures approved by the Ottawa Health Sciences Research Ethics Board. Samples were collected as previously described (25). All biopsies were taken within 6 to 15 days of treatment with either chemotherapy alone (paclitaxel, doxetaxel, or 5-fluorouracil/doxorubicin/cyclophosphamide) or without pamidronate.

Immunofluorescence and IHC studies

Frozen tumor sections were blocked with 1% BSA and 5% goat serum for 30 minutes and incubated with various primary antibodies (Supplementary Table S1), for 1 hour at room temperature. Alexa fluor-conjugated anti-rabbit or anti-rat secondary antibodies were used with unconjugated primary antibodies. For BrdUrd staining, DNA in frozen tissue sections was denatured with 2N HCl for 15 minutes before immunostaining, as described above. Nuclei in all tumor sections were counterstained with DAPI. Formalin-fixed, paraffin-embedded tissues were rehydrated, peroxidase blocked, then antigen retrieved, serum blocked, and incubated with primary antibodies for 1 to 2 hours.
Primary antibodies were detected with appropriate ABC or Polymer detection kits followed by chromagen staining with DAB.

Distribution of MRC1<sup>+</sup> TAMs relative to blood vessels in human primary breast tumors

The number of MRC1<sup>+</sup> TAMs present within a 250-μm radius of a given blood vessel was counted in six randomly selected areas per tumor section. Then, within this 250 μm radius, the distance of each MRC1<sup>+</sup> TAM was measured to the nearest vessel and the number of MRC1<sup>+</sup> TAMs within or beyond 150 μm from that vessel was expressed as % MRC1<sup>+</sup> TAMs within the region being analyzed. These were defined as "perivascular" or "avascular" MRC1<sup>+</sup> TAM subsets, respectively.

Tumor dissociation/flow-cytometric studies

Tumors were enzymatically dissociated and labeled with antibodies as described previously (9). All antibody incubations were performed for 1 hour at 4°C. Cell staining analyses and cell sorting experiments were performed using BD LSRII and BD FACSaria, respectively.

VEGFA ELISA studies

CD45<sup>−</sup>CD11b<sup>+</sup>Ly6G<sup>−</sup>F4/80<sup>+</sup> TAMs were isolated from three pooled, dissociated control, and cyclophosphamide-treated LLC1 tumors. One hundred thousand sorted cells were seeded in 100 μL medium of medium and cultured overnight. Conditioned medium was examined for VEGFA release using a mouse VEGFA Quantikine Sandwich ELISA (R&D Systems).

Chemotaxis assay

MRC1<sup>−/Hi</sup> (CD11b<sup>+</sup>Ly6G<sup>−</sup>F4/80<sup>+</sup> MRC1<sup>Hi</sup>) and MRC1<sup>−/Lo</sup> (CD11b<sup>+</sup>Ly6G<sup>−</sup>F4/80<sup>+</sup> MRC1<sup>Lo</sup>) TAMs were FACSorted from dissociated cyclophosphamide-treated LLC1 tumors and seeded into Transwell inserts (4 μm pores, VWR International, 50 × 10<sup>3</sup> cell per well. Lower chambers contained either medium alone, medium supplemented with 10 nmol/L BSA or 10 nmol/L recombinant murine CXCL12 (100 ng/mL). After an incubation of 6 hours at 37°C, the chambers were disassembled and the membranes stained with crystal violet. The upper surface of each Transwell insert was scraped to remove non-migrated cells before quantification.

Quantification of spontaneous LLC1 lung metastases

Sections (4 μm thick) were cut from formalin-fixed, paraffin-embedded lungs and stained with haematoxylin and eosin. These stained sections were imaged using the Aperio slide scanner (Leica Biosystems), and the number of metastases per section and total metastatic area quantified using ImageScope analysis software (Leica Biosystems).

Statistical analysis

All data represent mean values ± SEM. P values of less than 0.05 were considered to be significant. All statistical comparisons were made using the nonparametric Mann–Whitney U test (paired or unpaired as appropriate).

Results

M2-skewed (MRC1<sup>+</sup> TIE2<sup>Hi</sup>/VEGFA<sup>−</sup>) TAMs are abundant in mouse tumors after chemotherapy and promote their relapse

Three i.p. injections of the cytotoxic agent, cyclophosphamide, resulted in the complete cessation of LLC1 tumor growth, followed by regrowth beginning 7 days after treatment stops (Fig. 1A, left). Forty-eight hours after the last cyclophosphamide injection (day 6), LLC1s contained significantly (P < 0.05) shorter blood vessels and more hypoxia than size-matched controls (Supplementary Fig. S1A). Consistent with earlier observations for MMTV-PyMT implants treated with paclitaxel (13), TAMs were enriched among those leukocytes present in LLC1s 48 hours after cyclophosphamide (Supplementary Fig. S1B). In addition, there was a significant (P < 0.05) increase in the overall number of F4/80<sup>+</sup> TAMs in cyclophosphamide-treated LLCs compared with size-matched controls. This was also seen in paclitaxel-treated, orthotopic 4T1 tumors, and doxorubicin-treated orthotopic MMTV-PyMT implants (Supplementary Fig. S1C).

Cyclophosphamide treatment of LLC1s resulted in a significant (P < 0.05) increase in the number of F4/80<sup>+</sup> TAMs expressing the M2-marker, MRC1, relative to size-matched controls. In contrast, there was a significant (P < 0.05) drop in F4/80<sup>+</sup>/MRC1<sup>+</sup> TAMs after cyclophosphamide (Fig. 1A, right). Also, TAMs from cyclophosphamide-treated LLC1s expressed higher surface MRC1 than those from size-matched controls (Fig. 1B). Consistent with previous findings in untreated mouse tumors (9, 10), MRC1<sup>Hi</sup> TAMs in cyclophosphamide-treated LLC1s coexpressed elevated TIE2 (Fig. 1C and D). A similar increase in MRC1<sup>+</sup> TAMs was also seen in orthotopic 4T1 and MMTV-PyMT implants after paclitaxel and doxorubicin, respectively (Supplementary Fig. S1D). In all three models, the vast majority of MRC1<sup>+</sup> cells F4/80<sup>+</sup> TAMs, and the small number of F4/80<sup>+</sup> MRC1<sup>−</sup> (presumably dendritic) cells were not significantly increased after treatment with cyclophosphamide, paclitaxel, or doxorubicin (Supplementary Fig. S2A).

BrdUrd uptake was negligible in MRC1<sup>+</sup> TAMs in both size-matched control and cyclophosphamide-treated LLC1 tumors, indicating their nonproliferative status (Supplementary Fig. S2B). As chemotherapy-induced changes in circulating hematopoietic stem and progenitor cells (HSPCs) could, in theory, contribute to the increase of TAM numbers after therapy, we also performed dual immunofluorescence labeling for HSPCs markers, c-Kit and Sca1. Although c-Kit<sup>−</sup>Sca1<sup>−</sup> cells were detected in the spleens of LLC1 tumor-bearing mice (positive control for the staining), no such cells were detected in either control or cyclophosphamide-treated LLC1s (Supplementary Fig. S2C). These data indicate that the chemotherapy-induced increase in TAMs is most likely to be due to increased monocyte recruitment rather than the proliferation of existing TAMs or their differentiation from recruited HSPCs.

We then isolated F4/80<sup>+</sup>MRC1<sup>−/Hi</sup> and F4/80<sup>+</sup>MRC1<sup>−/Lo</sup> TAMs from cyclophosphamide-treated LLC1 tumors by FACS and injected them into cyclophosphamide-treated LLC1 tumors in littermates (Fig. 2A). MRC1<sup>−/Hi</sup> TAMs, but not MRC1<sup>−/Lo</sup> TAMs from treated tumors or TAMs from vehicle-treated tumors, accelerated tumor regrowth after cyclophosphamide. This was accompanied by a significant (P < 0.05) increase in the number of MRC1<sup>+</sup> TAMs, CD31<sup>+</sup> blood vessels, and a moderate increase in BrdUrd<sup>+</sup> (proliferating) cells in cyclophosphamide-treated LLC1s receiving MRC1<sup>−/Hi</sup> TAMs. Very few (< 1%) of CD45<sup>+</sup> leukocytes contained immunodetectable BrdUrd (Fig. 2B–E).

MRC1<sup>+</sup> TAMs are proangiogenic in untreated tumors and express the important proangiogenic mediator VEGFA (9, 26). Moreover, myeloid-specific deletion of VEGFA is known to have profound effects on the vascularization and progression of untreated mouse tumors (27). We, therefore, investigated the role of VEGFA derived from MRC1<sup>+</sup> TAMs in tumor-relapse after...
chemotherapy. As reported previously in mouse tumors (25), VEGFA was expressed by both TAMs and other (F4/80+/C0) cells in control LLC1s (Fig. 3A and Supplementary Fig. S3A). However, within 48 hours of the last dose of cyclophosphamide, VEGFA was expressed almost exclusively by MRC1+ TAMs (Supplementary Fig. S3B). As the expression of VEGFA was closely associated with MRC1+ TAMs, and because the latter were more abundant in cyclophosphamide-treated LLCs (Fig. 3B and C), we postulated that the macrophage population as a whole found within cyclophosphamide-treated tumors might be capable of producing higher levels of VEGFA. Indeed, the release of VEGFA was found to be significantly greater for TAMs sampled from cyclophosphamide-treated LLCs (Fig. 3D).

MDSCs (CD11b+Gr1+) in tumor-bearing mice are also reported to express MRC1 raising the possibility that some VEGFA-expressing MRC1+ cells in cyclophosphamide-treated tumors might have been MDSCs (28). However, we found that both granulocytic (CD45+CD11b+Ly6G+) and monocytic (CD45+CD11b+Ly6G–Ly6C+) MDSC subsets were depleted in cyclophosphamide-treated tumors, and that the expression of MRC1 on monocytic MDSCs was significantly (P < 0.05) lower than that on TAMs (Supplementary Fig. S3C). Taken together, these data suggest that MDSCs are unlikely to represent a significant proportion of the MRC1Hi cells in our LLC1 tumors.

We previously showed that a subset of TAMs accumulates in hypoxic areas of untreated mouse and human tumors (29), where they respond to hypoxia by upregulating hypoxia-inducible factors (HIF) 1 and 2 and a wide array of HIF-dependent M2 genes (30). As we detected some MRC1+ TAMs in the hypoxic regions of cyclophosphamide-treated LLC1 tumors (Supplementary Fig. S4A), albeit at much lower numbers than in the well-vascularized, normoxic (PIMO+/C0) regions (Fig. 4A), we investigated the possibility that hypoxia-regulated transcriptional programming
(via HIFs 1 and 2) might contribute to the relapse-promoting functions of TAMs. LLC1s were implanted into LysMCre/+ HIF1fl/fl or LysMcre/+ HIF2fl/fl myeloid-specific knockout mice and treated with vehicle or cyclophosphamide. HIF knockout was seen to have no effect on the number of MRC1+ TAMs in either the hypoxic or PIMO+/C0 areas in cyclophosphamide-treated LLC1s, or on tumor relapse (Supplementary Fig. S4B and S4C). IHC staining demonstrated a reduction of >90% in TAMs expressing HIF1α or 2α in control LLC1s, confirming high efficiency of LysCre-targeted HIF allele ablation in our mice (Supplementary Fig. S4D). These data suggest that hypoxia and HIF1/2-regulated transcriptional programming do not regulate the relapse-promoting functions of MRC1+ TAMs.

MRC1+ TAMs accumulate in well-vascularized areas of tumors after chemotherapy in both mouse and human tumors and promote tumor relapse: role of VEGFA

Previous studies have shown that MRC1+ TAMs can reside close to blood vessels in untreated mouse tumors (10). Furthermore, the aforementioned HIF knockout study demonstrated an increase in MRC1+ TAMs in PIMO- vascularized areas (PIMO- VA) in cyclophosphamide-treated tumors (Supplementary Fig. S4B). We, therefore, conducted a more detailed immunofluorescence staining analysis of the distribution of these cells in control and cyclophosphamide-treated LLC1s. This showed that, although MRC1+ TAMs were evenly distributed between PIMO- VA and PIMO+ hypoxic areas in size-matched control tumors,
there was a significant ($P < 0.05$) increase in the number of $F4/80^{+}\text{MRC1}^{+}$ TAMs in the former in cyclophosphamide-treated LLC1s (Fig. 4A). As cyclophosphamide treatment also resulted in reduced tumor vascularity (Supplementary Fig. S1A), we normalized MRC1$^{+}$ TAM numbers to CD31$^{+}$ VA of control and cyclophosphamide-treated LLC1s. Interestingly, this showed that the increase in MRC1$^{+}$ TAMs in PIMO$^{-}$ VA is independent of changes in the vasculature (Fig. 4A, far right).

Further immunofluorescent analysis of 4T1 and PyMT-MMTV tumors demonstrated a similar increase in the number of vessel-associated MRC1$^{+}$ TAMs after treatment with paclitaxel and doxorubicin, respectively (Fig. 4B). Furthermore, because the majority of MRC1$^{+}$ TAMs coexpressed VEGFA in both control and cyclophosphamide-treated LLCs, this meant that there was also a significant increase in VEGFA$^{+}$ TAMs in perivascular areas (Fig. 4C, top). Interestingly, there was also
as I g n i fi cant (P < 0.05) increase in the number of MRC1⁺ VE G FA⁺ TAMs in direct contact with the abluminal surface of the tumor vasculature in cyclophosphamide-treated tumors (Fig. 4C, bottom). However, the preferential accumulation of MRC1⁺ TAMs in PIMO⁺ VA regions of chemotherapy-treated tumors was no longer present after relapse, suggesting that the accumulation of MRC1⁺ TAMs was an acute, transient response to therapy (Supplementary Fig. S5A).

MRC1⁺ TAMs were also seen to preferentially localize in perivascular areas (i.e., within 150 μm of blood vessels) in human breast carcinomas 21 to 28 days after three cycles of neoadjuvant paclitaxel (Fig. 4D, left and middle), and in breast cancer metastases in the bone after chemotherapy (with or without bisphosphonates; Fig. 4D, right).

Immuno fluorescence staining of VEGFA in orthotopic MMTV-PyMT tumors excised at day 6 indicated that only MRC1⁺, not MRC1⁻/C⁻, TAMs expressed detectable levels of VEGFA in vehicle and doxorubicin-injected Cre⁻/C⁻ tumors. Indeed, >98% of vessel-associated MRC1⁺ TAMs were VEGFA⁺ in both groups of tumors (Fig. 5A and B). So, we investigated the role of VEGFA expression by these MRC1⁺ TAMs in tumor relapse using the Csf1r⁻Mer-iCre-Mer inducible cre-recombinase/estrogen receptor fusion protein...
model for the tamoxifen-induced ablation of VEGFA selectively in monocytes/macrophages (23).

Female Csf1r-Mer-iCre-Mer FVB/n mice were orthotopically implanted with syngeneic MMTV-PyMT tumors and administered tamoxifen 24 hours after a single injection of either vehicle or doxorubicin. In addition, tamoxifen was given continuously thereafter to ensure VEGFA knockout until mice were sacrificed at the end of the experiment (day 14), with the same treatment given to control Cre-recombinase negative mice (Fig. 5C). As the implanted PyMT cells in these tumors did not carry the Csf1r-driven Cre recombinase and expression of Csf1r is largely confined to TAMs in such tumors, the knockdown of VEGFA was restricted to the macrophage lineage, a significant source of VEGFA in the PyMT model (as in cyclophosphamide-treated LLC1s; ref. 31). Tamoxifen-induced ablation of this TAM-derived VEGFA (Fig. 5C) caused a significant (P < 0.05) delay in the growth of the tumors. 

Figure 5.
Effect of TAM-derived VEGFA on relapse of orthotopic MMTV-PyMT tumors after doxorubicin (DOX) treatment. A, representative immunostaining for VEGFA in MRC1+ TAMs in vascularized (CD31+) areas of doxorubicin-treated, Cre+, MMTV-PyMT tumors. This was not seen for MRC1+ TAMs anywhere in the same tumors (B). C, female Tg(Csf1r-Mer-iCre-Mer)1jwp;Vegfafl/fl mice bearing MMTV-PyMT tumors were administered tamoxifen for 24 hours to delete VEGFA expression in TAMs (right panels) after a single injection of either vehicle alone or doxorubicin. D and E, growth of tumors treated with vehicle alone in Cre+ and Cre− mice (n = 3–6/group; D) and regrowth of tumors in Cre− and Cre+ mice after treatment with doxorubicin (n = 3–4/group; E). F, CD31 staining of vessels in tumors in Cre− or Cre+ mice given doxorubicin. Bars, 50 μm. *, P < 0.05 with respect to tumors at the same time point in the respective Cre− group.
of vehicle-treated MMTV-PyMT tumors (Fig. 5D). Furthermore, relapse of doxorubicin-treated tumors lacking VEGFA\(^+\) TAMs (Cre\(^+\)) was significantly (\(P < 0.05\)) slower than that of doxorubicin-treated tumors in VEGFA-expressing (Cre\(^-\)) mice (Fig. 5E). This was accompanied by a significant (\(P < 0.05\)) decrease in vessel area in these tumors (Fig. 5F).

Pharmacologic blockade of CXCR4 prevents perivascular accumulation of MRC1\(^+\) VEGFA\(^+\) TAMs after chemotherapy and delays tumor relapse. The majority of F4/80\(^+\) MRC1\(^+\) TAMs expressed the CXCL12 receptor, CXCR4 in both control and cyclophosphamide-treated LLC1s (Fig. 6A) and 98\% of all CXCR4-expressing cells were TAMs.
in cyclophosphamide-treated LLC1 tumors, compared with 78% in control LLCs (where other cell types were also CXCR4+; Fig. 6B). As reported previously (32, 33), LLC1 cells do not express CXCR4 (Fig. 6A and B). Moreover, less than 1% of CD31+ blood vessels expressed CXCR4 in cyclophosphamide-treated tumors (Fig. 6C). MRC1Hi TAMs in both control and cyclophosphamide-treated tumors expressed elevated levels of CXCR4 compared with the MRC1Lo TAMs (Fig. 6D).

These changes in CXCR4+ cells after cyclophosphamide were accompanied by a marked increase in tumor levels of its ligand, CXCL12, on day 6 (Fig. 6D, far right). These CXCL12+ cells were CD31+ (Fig. 6E, left) and most likely tumor cells and/or fibroblasts (34). As a hypoxia-inducible gene (35), it was not surprising to find CXCL12 more highly expressed in hypoxic than normoxic areas of control LLC1 tumors. However, after cyclophosphamide, tumor levels of HIF1 and -2 were markedly reduced in all areas of tumors (Supplementary Fig. S6A, i and ii), but CXCL12 was abundant in both hypoxic and normoxic, vascularized areas (Supplementary Fig. S6B). This suggested CXCL12 upregulation by factors other than hypoxia in such tumors.

Cyclophosphamide is known to induce oxidative stress (36), a cellular response known to regulate the expression of CXCL12 (37), so we investigated the expression of a well-defined marker for oxidative stress, heme oxygenase-1 (HMOX-1) in control versus cyclophosphamide-treated tumors. Interestingly, this was found to be upregulated in perivascular, PIMO+/CXCL12-rich areas of tumors after cyclophosphamide treatment but not in control tumors (Supplementary Fig. S6C, i and ii). Both MRC1+ TAMs and MRC1+ cells expressed HMOX-1 in these vascularized areas (Supplementary Fig. S6C, iii).

We then investigated whether CXCL12 might recruit and/or retain CXCR4+ MRC1+ TAMs in LLC1 tumors. First, we showed in an in vitro chemotaxis assay that CXCL12 is selectively chemotactic for MRC1+Hi TAMs isolated from LLC1 tumors (Fig. 6E, right). Then we administered cyclophosphamide to LLC1-bearing mice alone or in combination with the CXCR4 antagonist, plerixafor (AMD3100; Fig. 7A). This was feasible as TAMs were the predominant cell type expressing CXCR4 after cyclophosphamide (Fig. 6B). CXCR4 blockade significantly inhibited cyclophosphamide-induced accumulation of F4/80+ MRC1+ TAMs in the PIMO+ VA (while the numbers in PIMO- areas of LLC1 tumors were unchanged, as was F4/80+ MRC1+ TAMs in either of these areas) and delayed tumor relapse (Fig. 7A and B; Supplementary S5B). At day 10 (when tumors had relapsed in the cyclophosphamide alone group), a similar distribution of MRC1+ TAMs was seen compared with day 6 (the start of the relapse period; Fig. 7B and C). In addition, tumors administered cyclophosphamide + AMD3100 contained significantly (P < 0.05) shorter CD31+ blood vessels, higher levels of hypoxia, and fewer BrdUrd+ (proliferating) cells than tumors exposed to cyclophosphamide with the vehicle for AMD3100 (PBS; Fig. 7D; Supplementary Fig. S5C).

We then examined the effect of CXCR4 blockade on the distribution of MRC1+ TAMs across PIMO+ VAs in cyclophosphamide-treated tumors. Interestingly, this was found to significantly (P < 0.05) reduce the number of MRC1+ TAMs in direct contact with the abluminal surface of CD31+ blood vessels (standardized by CD31+ vessel area as this differed between cyclophosphamide + PBS and cyclophosphamide + AMD3100 groups), and increase them elsewhere in the PIMO+ VAs (Fig. 7E). These data suggest that CXCR4 regulates the direct association of alternatively activated TAMs with blood vessels in cyclophosphamide-treated LLC1s.

A recent study demonstrated a marked rebound in the growth of pulmonary metastases after treatment with an antibody to CCL2 (38). This was the result of increased mobilization of monocytes from the bone marrow after CCL2 inhibition, and increased blood vessel formation and cancer cell proliferation in the lungs. We, therefore, investigated the possibility of a similar rebound effect after CXCR4 inhibition using AMD3100 as this could disrupt the bone marrow niche. When we extended the length of the AMD3100 experiment, primary LLC1 tumors relapsed eventually, but not at an accelerated rate, and mice receiving cyclophosphamide + AMD3100 showed increased survival compared with those in the cyclophosphamide alone group (or controls). Importantly, there was also no rebound in pulmonary metastases (Supplementary Fig. S8).

**Discussion**

Our studies show that M2-skewed TAMs (MRC1+TIE2+CXCR4+VEGFA+) selectively accumulate in vascularized, well-oxygenated areas of LLC1 tumors after treatment with cyclophosphamide. A similar increase in such vessel-associated, M2-skewed TAMs was also seen in orthotopic 4T1 and MMTV-PyMT implants after treatment with paclitaxel and doxorubicin, respectively, and in human breast carcinomas after neoadjuvant treatment with paclitaxel. This perivascular accumulation was found to be CXCR4-dependent, especially the increased, direct contact of this TAM subset with the abluminal surface of blood vessels in chemotherapy-treated tumors. When this was disrupted using a CXCR4 inhibitor, tumor revascularization and regrowth after chemotherapy were markedly impaired. We also show, using an inducible, macrophage-specific, gene knockdown model that VEGFA expressed by such MRC1+ TAMs mediates, in part, their ability to promote tumor relapse after therapy. Consistent with this, genetic deletion of HIF signaling in TAMs in hypoxic tumor areas had no effect on this rescue.

The mobilization and accumulation in tumors, of such BMDCs as myelomonocytic cells, MDSCs, endothelial progenitor cells (EPC) and macrophages after various forms of anti-cancer therapy are now well established (13–19, 39, 40). However, our data show that M2-skewed TAMs represent a significant proportion of such BMDCs in tumors after chemotherapy, and is not accompanied by TAM proliferation or increased numbers of stem/progenitor cells. These data suggest that increased recruitment of circulating monocytes precedes the accumulation of such M2 TAMs in tumors after chemotherapy. It remains to be seen whether monocytes are already M2-skewed uponarrival in treated tumors and/or activated by factors produced in the perivascular niche. Of note, the recruitment, activation, and perivascular retention of these cells does not appear to be just an acute response to chemotherapy-induced tumor damage, as increased numbers of perivascular M2 TAMs persisted throughout the relapse phase.

Our observations suggest that the accumulation of MRC1+ VEGFA+ TAMs on and around the tumor vasculature plays an important part in tumor revascularization and relapse after chemotherapy. Furthermore, this was accompanied by a marked change in the pattern of CXCL12 expression in tumors. Although CXCL12 was mainly confined to hypoxic areas of control tumors, it was upregulated in vascularized, well-oxygenated areas after chemotherapy.
chemotherapy. This correlated with an increase in the expression of the enzyme, HMOX-1, a marker of oxidative stress, in such perivascular areas. Cytotoxic agents such as cyclophosphamide induce oxidative stress in tumors (41), which activates cellular expression of HMOX-1. This, in turn, generates carbon monoxide from the breakdown of heme, a gas known to upregulate both CXCL12 and VEGFA in neighboring cells (37). Interestingly, HMOX-1 also regulates expression of MRC1 by macrophages (42). So, chemotherapy induction of this stress pathway in perivascular areas could both retain TAMs via local CXCL12 induction, and upregulate their expression of VEGFA and MRC1.

 Interestingly, the CXCL12-induced retention of myeloid cells around the vasculature is also essential to the process of neovascularization in nonmalignant tissues (43).

Other factors may also contribute to the retention of MRC1+ TAMs. Our observation that MRC1+ TAMs expressed elevated TIE2 suggests that ANGPT2 expressed by the tumor endothelium (44) might also be involved. Indeed, ANGPT2 has an established role in retaining vascular modulatory myeloid cells in proximity to the vasculature in progressing mouse tumors (26).

In untreated mouse tumors, perivascular M2-skewed TAMs have an established vascular modulatory role and facilitate tumor growth and progression (26). Our observation that chemotherapy induces the accumulation of MRC1+VEGFA+ TAMs around tumor blood vessels strongly infers a proangiogenic role for these cells. This is supported by our finding that pharmacologic inhibition of their chemotherapy-induced accumulation resulted in reduced subsequent tumor revascularization. Furthermore, in doxorubicin-treated, MMTV-PyMT tumor implants VEGFA was found to be expressed predominantly by MRC1+ TAMs, consistent with our previous finding that TAMs...
are a major source of VEGFA in MMTV-PyMT tumors (31). The selective ablation of VEGFA in MRC1+ TAMs resulted in delayed tumor relapse, post-therapy. However, PyMT tumors still relapsed, albeit at a reduced rate, in the absence of TAM-derived VEGFA, indicating that other TAM-derived factors may also contribute to tumor relapse and/or the possible induction of resistance mechanisms in tumors to VEGFA knockout. The presence of the latter has been demonstrated in tumors after anti-VEGFA therapy and shown to include increased expression of such alternative proangiogenic mediators as FGF2 (45), ANGPT2 (46), or PIGF (47) and the recruitment of tumor-promoting, CD11b+ Gr1+ myeloid cells (48).

Recently, the Condeelis group has used intravital imaging to characterize a distinct subset of perivascular TAMs in untreated mouse tumors that make contact with endothelial cells and tumor cells expressing high levels of VEGFA (a protein that enhances their motility), and directly stimulate tumor cell intravasation (49). These cell trios have been termed "tumor microenvironments of metastasis" (TMEM). It is possible that some of the perivascular MRC1+ M2-skewed TAMs accumulating around tumor blood vessels after chemotherapy form TMEM and promote metastasis as well as relapse, a dangerous combination in patients with inoperable tumors.

The effect of CXCR4 blockade on tumor relapse after chemotherapy in our study suggests that CXCR4 inhibitors might be successfully combined with chemotherapy. This combination could extend relapse-free survival in patients with inoperable tumors, although our data suggests that multiple rounds of the inhibitor would have to be administered to maintain a suppressive effect on relapse. Such sustained use of a CXCR4 antagonist after chemotherapy could conceivably disrupt the marrow niche. However, this is unlikely to lead to clinical problems as a recent, first-in-human clinical trial has shown that repeated daily injections of the CXCR4 antagonist, LY2510924 over a number of consecutive, 28-day cycles was well tolerated in advanced cancer patients (50).

References
Macrophages and Tumor Relapse


Perivascular M2 Macrophages Stimulate Tumor Relapse after Chemotherapy

Russell Hughes, Bin-Zhi Qian, Charlotte Rowan, et al.

_Cancer Res_ Published OnlineFirst August 12, 2015.

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Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-3587

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