Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

Microenvironment and Immunology

Cancer Research

Tie2 Expression on Macrophages Is Required for Blood Vessel Reconstruction and Tumor Relapse after Chemotherapy

Lin Chen[1,2], Jie Li[1,2], Fei Wang[1], Chengliang Dai[2], Fan Wu[2], Xiaoman Liu[2], Taotao Li[3], Rainer Glauben[4], Yi Zhang[1], Guangjun Nie[5], Yulong He[3], and Zhihai Qin[1,2]

Abstract

Tumor relapse after chemotherapy is a major hurdle for successful cancer therapy. Chemotherapeutic drugs select for resistant tumor cells and reshape tumor microenvironment, including the blood supply system. Using animal models, we observed on macrophages in tumor tissue a close correlation between upregulated Tie2 expression and tumor relapse upon chemotherapy. Conditional deletion of Tie2 expression in macrophages significantly prohibited blood supply and regrowth of tumors. $Tie2^+$ macrophages were derived from tumor-infiltrating $Tie2^-CD11b^+$ cells and hypoxia-induced Tie2 expression on these cells. Mechanistically, expression of Tie2 prevented macrophages from apoptosis in stress conditions via the AKT-dependent signaling pathway. Together, these results demonstrate that Tie2 expression by macrophages is necessary and sufficient to promote the reconstruction of blood vessels after chemotherapy, shedding new light on developing novel strategies to inhibit tumor relapse. *Cancer Res; 76(23); 6828–38. ©2016 AACR.*

Introduction

The molecular mechanisms leading to tumor relapse after treatment with cytotoxic drugs are still not completely understood (1). Many studies focused on the analysis of chemoresistant, either intrinsically or acquired, characteristics of tumor cells during treatment (2). However, solid tumors are complex organs with a reactive stroma accompanying cancer cells. There is increasing evidence that the outcome of chemotherapy is also dependent on non–tumor cell factors. It is now widely accepted that tumor-specific immune responses can determine the efficacy of anticancer therapies with conventional cytotoxic drugs (3). In another example of chemotherapy for prostate cancer, cytotoxic drugs caused fibroblasts to pump out growth factors into the tumor microenvironment, and this process attenuated the effects of cytotoxic drugs by promoting tumor cell survival and disease progression (4). Other components, such as extracellular matrix and blood vessel system, would also influence the outcome of chemotherapy (3, 5, 6).

Vascular reconstruction is a key step for wound healing, which is also involved in tumor relapse after chemotherapy (7, 8). Anticancer therapies cause vascular damage, hypoxia, and tumor necrosis, which together induce the recruitment of myeloid cells, proliferation of endothelial cells, and migration of pericytes (9, 10). These cells help reconstruct the vascular system to remove tissue debris and bring oxygen and nutrients for tissue repair. However, the role of myeloid cells for vascular reconstruction after chemotherapy and the underlying molecular mechanism is still not clear.

Angiopoietin–Tie2 pathways play essential roles in angiogenesis (11). Tie2 gene knockout mice are embryonic lethal due to vascular dysplasia (12). Tie2, which is a receptor tyrosine kinase, together with Tie1, mediates various functions of a group of angiopoietin ligands such as angiopoietin1/2 (Ang1/Ang2; refs. 9, 11). Tie2 is broadly expressed on vascular endothelial cells, and Angiopoietin–Tie2 pathways are involved in the proliferation, migration, and stabilization of endothelial cells (13). For example, Ang1-mediated signals are important for stabilization of the vascular endothelium. During the process of tissue remodeling, Ang2 is expressed by the endothelial cells of actively remodeling vessels and helps promote the dissociation of pericytes from preexisting vessels and increases vascular permeability, which facilitates the infiltration of proteases, cytokines, and angiogenic myeloid cells (13). Notably, targeting Ang2 is now under clinical trials in various cancers (14, 15).

Except for endothelial cells, Tie2 is also expressed by a subpopulation of macrophages. Tie2-expressing macrophages (TEM) are known to promote angiogenesis (16). Initially, TEMs are

[1]The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, Henan, China. [2]Key Laboratory of Protein and Peptide Pharmaceuticals, Institute of Biophysics, Chinese Academy of Sciences, Beijing, China. [3]Laboratory of Vascular and Cancer Biology, Cyrus Tang Hematology Center, Collaborative Innovation Center of Hematology, Jiangsu Key Laboratory of Preventive and Translational Medicine for Geriatric Diseases Thrombosis and Hemostasis Key Lab of the Ministry of Health, Jiangsu Institute of Hematology, the First Affiliated Hospital, Soochow University, Suzhou, China. [4]Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Medical Department I, Berlin, Germany. [5]National Centre for Nanoscience and Technology of China, Beijing, China.

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

L. Chen, J. Li, and F. Wang contributed equally to this work.

Corresponding Authors: Zhihai Qin, The First Affiliated Hospital of Zhengzhou University, Zhengzhou University, Zhengzhou, Henan 450052, China. Phone: 86-10-64888435; Fax: 86-10-64888570; E-mail: zhihai@ibp.ac.cn; and Yulong He, Soochow University, Suzhou, China. Phone: 86-512-65884937; Fax: 86-512-65880929; E-mail: heyulong@suda.edu.cn

doi: 10.1158/0008-5472.CAN-16-1114

Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

described to play a promoting role in unperturbed tumor growth and embryonic development. These myeloid cells migrate toward and adhere to sprouting blood vessels and are essential for vascular anastomosis and formation of functional vessel system (16, 17). Although TEMs play an important role in the angiogenesis in many tumor models, the proportion of TEMs varies in different types of tumors. Interestingly, Tie2-positive macrophages have been reported to accumulate in tumor microenvironment after chemotherapy, antiangiogenic treatment, radiotherapy, or in injured muscle tissue of ischemic limb (18, 19), suggesting that TEMs are involved in vascular reconstruction. However, the exact molecular mechanism for that is still not known.

Several studies indicate that Tie2 is important for the function of TEMs, for example, as a chemotaxis receptor or adhesion receptor to endothelial cells (19–21). Many studies trying to figure out the role of Tie2 on macrophages were based on the stimulation of angiopoietins (20–22). Mazzieri and colleagues demonstrated that Ang2 blockage did not inhibit recruitment of TEMs but impeded their upregulation of Tie2 and ability to restore angiogenesis in tumors (11). However, besides Tie2, integrin is also a functional receptor for angiopoietins both in endothelial cells and in myeloid cells (23–25). Scholz and colleagues reported that Ang2 promotes myeloid cell infiltration and adhesion to endothelial cells in an integrin-dependent manner (26). Therefore, it is necessary to directly deplete Tie2 to verify its role on macrophages.

In this study, we employed the Cre-loxP system to delete the *Tie2* gene on macrophages *in vivo*. Our results uncover an important role of Tie2 on the viability of macrophages in stressed tumor microenvironment. Tie2 deletion on macrophages impaired the vascular reconstruction during tumor relapse after chemotherapy.

Materials and Methods

Mice

The generation of Tie2$^{flox/flox}$ mice was described before (27). LyzCre; Tie2$^{-/flox}$ (M^{Tie2-}) and LyzCre;Tie2$^{+/flox}$ (Con) mice were generated by breeding LyzCre$^{+/+}$;Tie2$^{+/-}$mice with Tie2$^{flox/flox}$ mice. C57BL/6 mice were purchased from Vital River. All mice were bred under specific pathogen-free conditions in the animal facilities at the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China) and Soochow University (Suzhou, China). All animal studies were performed with sex- and age-matched mice after being approved by the Institutional Laboratory Animal Care and Use Committee.

Cell lines

MCA205 is a methylcholanthrene-induced fibrosarcoma cell line of C57BL/6 origin (28) and was originally provided by Dr. Steve Rosenberg in 2000. The macrophage cell line RAW264.7 was a kind gift from Dr. Shengdian Wang (Institute of Biophysics) in 2010. All cells were cultured in DMEM supplemented with 10% (vol/vol) FBS and 100 U/mL penicillin/streptomycin. Authentication of cell lines was assured by short tandem repeat (STR) profiling, regular morphology checks, and growth curve analysis.

Plasmid and transfection

The expression plasmid encoding Tie2-GFP plasmid was kindly provided by Dr. Naoki Mochizuki (National Cardiovascular Center Research Institute, Osaka, Japan; ref. 29). RAW264.7 cells were transfected with Tie2-GFP or mock plasmid using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction and changed to complete medium after 3 hours. The next day, selective medium containing 750 μg/mL G418 was added and the cells were cultured for 3 weeks. At that time, cells were analyzed and sorted by FACS. GFP-positive cells were either continued in mass culture or picked individually as clones and grown in G418 (200 μg/mL) for 3 additional weeks. Three Tie2-GFP clones (Tie2-1, -2, and 3) and 2 mock clones (Mock-1 and -2) were used to further analysis in the present study.

Tumor transplantation and chemotherapy

Exponentially growing tumor cells were harvested, washed, and injected subcutaneously into the abdomen region of mice. Tumor size was assessed using Vernier calipers 2 to 3 times per week after tumors reached a palpable size. Tumor volume was determined using the $(L \times W \times W)/2$ formula with L as the longest diameter and W as the diameter at the position perpendicular to L.

For chemotherapy of tumors, a single intratumoral injection of doxorubicin (0.7 mg/mL, 100 μL/mouse, Sangon Biotech) was given on days 11 to 12 after tumor inoculation. Intraperitoneal injection of cyclophosphamide (10 mg/mL, 200 μL/mouse, Sigma) was given on day 14 after tumor inoculation. Tumor size was assessed in the following days 2 to 3 times per week.

FACS analysis

Single-cell suspensions prepared from peripheral blood, spleen, and tumor tissue were stained with the following directly labeled mouse-specific mAbs: phycoerythrin (PE)-labeled anti-Tie2 (eBioscience), fluorescein isothiocyanate (FITC)- or Percp/Cy5.5–labeled anti-CD11b (BD Biosciences), PE- or allophycocyanin (APC)-labeled anti-Ly6C (BD Biosciences), APC-labeled anti-Ly6G (BD Biosciences), and APC-labeled anti-F4/80 (BD Biosciences). Dead cells were excluded by 7-aminoactinomycin D (7-AAD) staining. Cells were collected on a FACSCalibur (BD Biosciences) and analyzed by FlowJo software (TreeStar).

Histology and immunostaining

Preparation of cryostat or paraffin tissue sections was done as described previously (30). After routine processing, 7-μm-thick tumor tissue sections were stained with hematoxylin and eosin (H&E) for histologic analysis.

Frozen tumor tissue sections were incubated with anti-Tie2 (R&D Systems), anti-CD11b, anti-CD31, anti-NG2 antibodies (all from Abcam, Cambridge Science Park), and species-matched Alexa dye–labeled or horseradish peroxidase (HRP)-conjugated secondary antibodies. Sections were counterstained with 4, 6′-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) and evaluated under the microscope (DP71, OLYMPUS) for bright-field and fluorescence microscopy or under Olympus FV1000 confocal microscope for fluorescence microscopy. For HRP-conjugated secondary antibody staining and H&E staining, whole slide images were taken under Leica SCN400 Slide Scanner (Leica Microsystems).

Western blot analysis

Cell extracts were analyzed by Western blotting as described (31). The primary antibodies used were anti-Tie2 (R&D Systems), anti-phospho-AKT, anti-AKT, anti-phospho-ERK, and anti-ERK

Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

(Cell Signaling), as well as anti-β-actin (Cell Signaling). HRP-conjugated rabbit anti-goat or goat anti-rabbit IgG were used as secondary antibodies.

Isolation and adoptive transfer of CD11b$^+$Ly6C$^+$ cells

To study the fate of adoptively transferred CD11b$^+$Ly6C$^+$ cells, CD11b$^+$Ly6C$^+$ cells were FACS sorted from the spleen of tumor-bearing EGFP mice by FACSAria II (BD Biosciences). A total of 5×10^6 isolated CD11b$^+$Ly6C$^+$ cells were injected into doxorubicin-treated tumor-bearing C57BL/6 mice via the tail vein.

To study the role of Tie2, CD11b$^+$Ly6C$^+$ cells were isolated from the spleens of tumor-bearing Con and M^{Tie2-} mice by FACS. After labeling with carboxyfluorescein succinimidyl ester (CFSE), 1×10^7 isolated CD11b$^+$Ly6C$^+$ cells were injected into doxorubicin-treated tumor-bearing C57BL/6 mice via the tail vein.

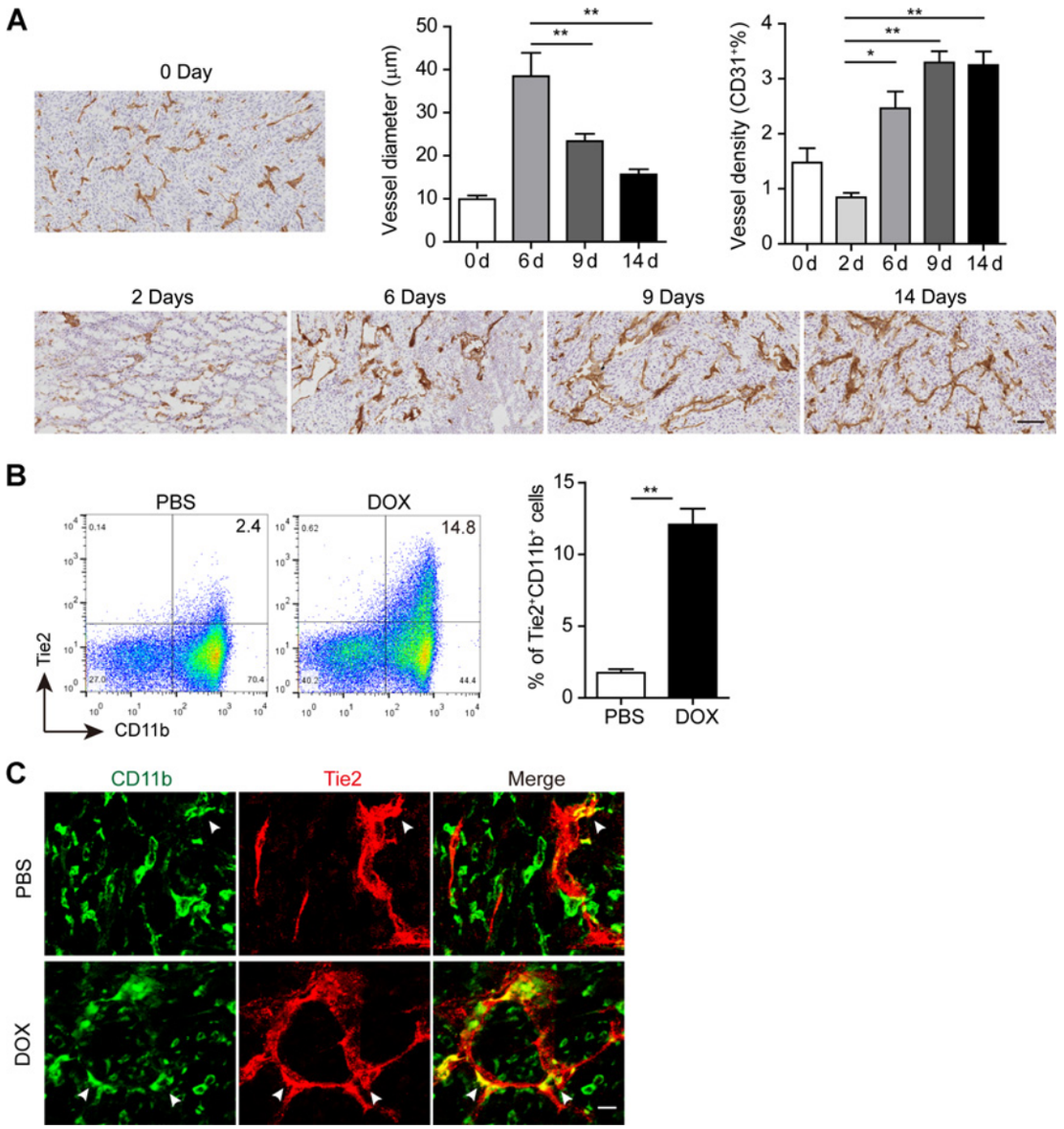

Figure 1.

Accumulation of TEM in tumor microenvironment after chemotherapy. **A,** Vigorous vessel reconstruction was found during tumor regrowth after chemotherapy. Transplanted MCA205 tumors were intratumorally injected with doxorubicin (DOX) or PBS as control (0 day). At 2, 6, 9, and 14 days after drug treatment, tumors were stained for CD31 (brown). Scale bars, 100 μm. Statistical analysis of the vessel diameter and density is shown. **, $P < 0.01$. **B,** Tie2$^+$ macrophages accumulated in tumor microenvironment after chemotherapy. The proportion of Tie2$^+$CD11b$^+$ cells was evaluated by FACS using single-cell suspensions prepared from tumors that received doxorubicin ($n = 4$) or PBS ($n = 4$) treatment 9 days before. Dead cells were excluded by 7-AAD staining. Statistical analysis is shown. **, $P < 0.01$. Representative results of 1 of 4 experiments are shown. **C,** TEMs were closely associated with blood vessels during tumor relapse. Nine days after treatment, tumor sections were stained for CD11b and Tie2 and analyzed via confocal microscopy. Six serial images were taken from tumor sections where the focus (z-dimension) was moved by 2 μm between images and images of 3D reconstruction were shown. TEMs (arrowhead) were present at vascular junctions. Scale bar, 10 μm.

RT-PCR and real-time quantitative PCR

Total RNA was extracted using TRIzol (Tiangen), and cDNA was synthesized using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen). Quantitative real-time PCR (qPCR) was performed using SYBR Green (TransGen). Quantification was performed by comparing the C_t values of each sample with a standard curve and normalizing to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Values were expressed as fold induction in comparison with untreated controls. The following primers were used in this study: mTie2-For GCCGCGGACTGACTACGAGC, mTie2-Rev GGAGGAGGGAGTCCGATAGACGC; mGAPDH-For TGGCCTTCCGTGTTCCTAC, mGAPDH-Rev GAGTTGCTGTTGAAGTCGCA.

Hypoxia induction

CD11b$^+$Ly6C$^+$ cells were isolated from the spleen of tumor-bearing C57BL/6 mice. Isolated cells were maintained under standard tissue culture conditions in addition with 10% MCA205 tumor supernatant, under normoxic condition of 95% air, 5% CO_2 at 37°C or hypoxic condition with 1.5% O_2, 4.5% CO_2, 94% N_2 (Thermo Forma cell incubator; Forma Scientific). Cells were collected for mRNA and protein analysis after being cultured under normoxia or hypoxia for indicated time.

Isolated CD11b$^+$Ly6C$^+$ cells were also treated with 200 μmol/L cobalt chlorides ($CoCl_2$) in a 5% CO_2, 95% air humidified atmosphere at 37°C for various time periods and then collected for mRNA and protein analysis.

Quantitative and statistical analyses

The vessel diameter and CD31-positive area were quantified by Image Pro Plus software (Media Cybernetics). All data are expressed as mean ± SEM. Differences between 2 groups were compared using a 2-tailed unpaired Student *t* test or 2-way ANOVA (GraphPad Prism). All experiments were repeated 3 to 4 times. *P* values less than 0.05 were considered statistically significant.

Results

Accumulation of TEMs in tumor microenvironment after chemotherapy

Like in humans, most of the mouse tumors relapse after chemotherapy even in immunocompetent mice (32). In this study, fibrosarcoma cells (MCA205; 5×10^5 per mouse) were subcutaneously transplanted on the back of C57BL/6 mice and treated with doxorubicin (70 μg/mouse). The growth of tumor was significantly inhibited during the first week, followed by a rapid relapse (Supplementary Fig. S1).

Analyzing tumor sections by CD31 staining after chemotherapy, we observed that tumor relapse was associated with vigorous vessel reconstruction within 14 days. Furthermore, the vessel reconstruction process seemed to be well-organized: the vessel diameters were gradually decreased, whereas the vessel density was dramatically increased (Fig. 1A). We then examined tumor blood vessels at day 9 after chemotherapy when the vessel reconstruction stayed in an intermediate state. We found that blood vessels at this stage in the tumor were much less covered by pericytes, which can be used to monitor the maturation of blood vessels (Supplementary Fig. S2; ref. 33). Interestingly, FACS analysis showed that the proportion of CD11b$^+$Tie2$^+$ cells among the myeloid cells was dramatically increased compared with the untreated group (Fig. 1B). These CD11b$^+$Tie2$^+$ cells did not express Ly6G (a typical marker for neutrophils) and showed typical monocytic light scattering features in peripheral blood, which are consistent with previously reported characteristics of TEM (Supplementary Fig. S3; ref. 16). Furthermore, immunofluorescent double staining showed that the accumulated Tie2$^+$CD11b$^+$ macrophages were intimately juxtaposed with blood vessels, especially at the vascular branching points (Fig. 1C). These results suggest that active vessel reconstruction during tumor relapse after chemotherapy is closely associated with the accumulation and attachment of TEM around the tumor blood vessels.

Tie2 deletion in myeloid cells impaired blood vessel reconstruction and tumor relapse

To investigate the effect of Tie2 expression on macrophages after chemotherapy, we generated mice with *Tie2* gene knockout in macrophages by the Cre-loxP system, as described before (27). Briefly, Tie2 deletion in the myeloid lineage, mainly neutrophils and monocytes/macrophages, was achieved by crossing the transgenic line with the lysozyme M promoter–driven Cre recombinase (Lyz-Cre) with *Tie2^{Flox}* mice (34, 35). Supplementary Figure S4A shows the schematic illustration of mouse mating strategy. Mice with the myeloid cell–specific deletion of *Tie2* (LyzCre;Tie2$^{-/flox}$) will be termed M^{Tie2-} mice, whereas littermate control mice (LyzCre;Tie2$^{+/flox}$) will be termed as control mice (Con; Supplementary Fig. S4A). The modified alleles were identified by PCR amplification (Supplementary Fig. S4B). Furthermore, PCR amplification confirmed that *Tie2* genes were efficiently deleted in peritoneal as well as tumor-infiltrating macrophages (Supplementary Fig. S4B).

MCA205 tumor cells were subcutaneously transplanted into Con or M^{Tie2-} mice and treated with doxorubicin. The conditional knockout of Tie2 in macrophages remarkably impaired the regrowth of MCA205 after treatment with doxorubicin (Fig. 2A). The tumor isolated from Con mice was full of blood vessels, whereas the tumor from M^{Tie2-} mice appeared pale and lacked blood perfusion (Fig. 2A). Analysis by CD31 staining showed much less and also discontinued formation of blood vessels, especially around necrotic area, in the tumor of M^{Tie2-} mice (Fig. 2B). FITC-dextran perfusion experiments confirmed that the functional vessels in the M^{Tie2-} mice were dramatically decreased, and most of the perfused vessels were concentrated on the peripheral area. Mammalian cells require oxygen and nutrients for their survival; therefore, they are distributed within 100 to 200 μm from blood vessels (36). Here, we defined an "irrigated area" as the area within 100 μm from the nearest FITC-dextran perfused blood vessels. We calculated the "irrigated area" in tumor tissues by the procedure shown in Fig. 2C. The statistic results revealed that the proportion of irrigated tumor area was significantly smaller in the tumor from M^{Tie2-} mice than that of Con mice after chemotherapy (Fig. 2C). H&E staining displayed higher percentage of necrotic area in tumors from M^{Tie2-} mice (Fig. 2D). To study whether this effect is drug-dependent, we established another chemotherapeutic relapse mouse model using cyclophosphamide. Similarly, the tumor relapse of MCA205 after cyclophosphamide treatment was also inhibited in M^{Tie2-} mice (Fig. 2E). Taken together, we conclude that Tie2 deletion in macrophages inhibited tumor relapse after chemotherapy.

Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

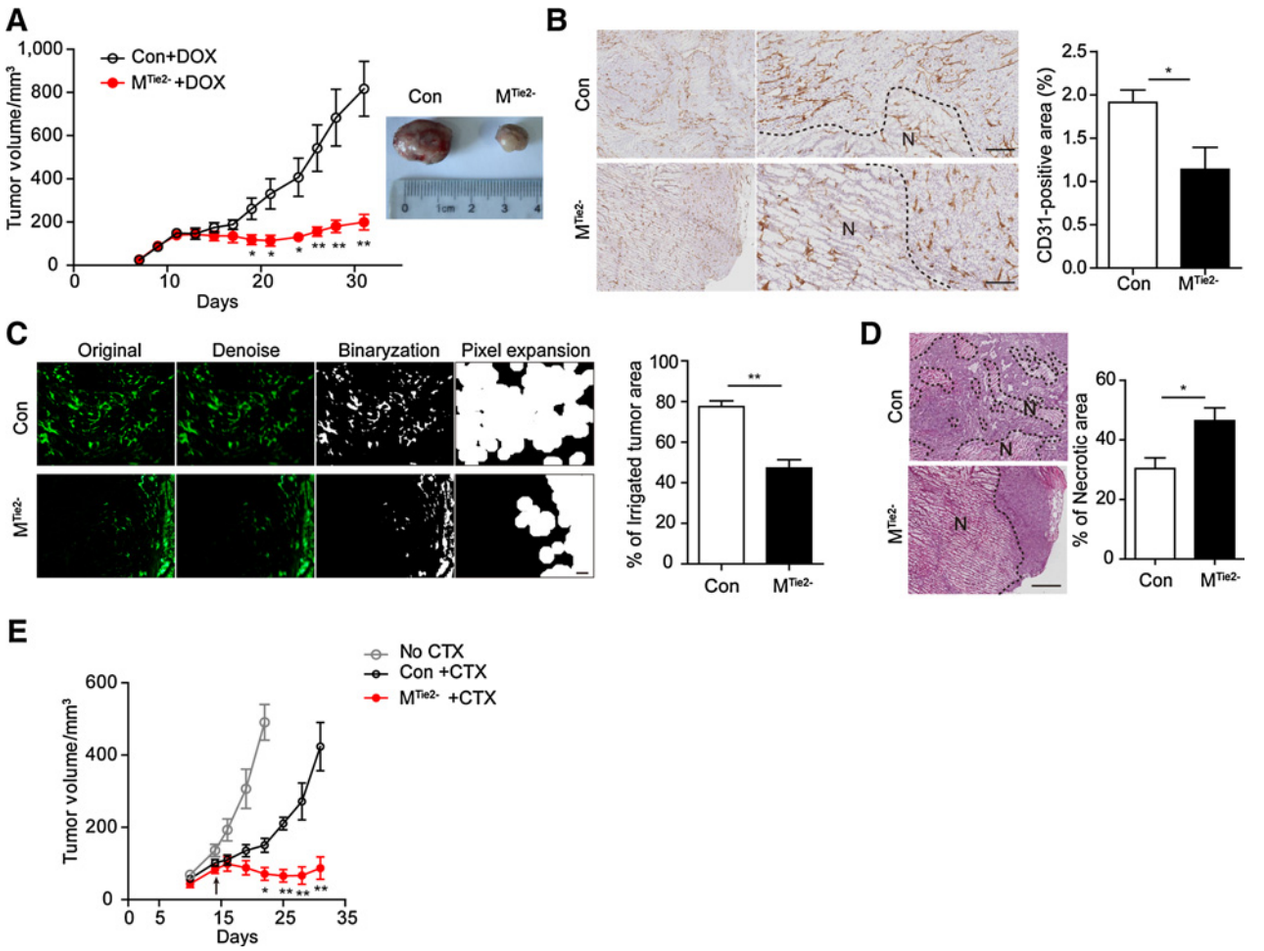

Figure 2.

Tie2 deletion in myeloid cells impaired the reconstruction of blood vessels and tumor relapse after chemotherapy. **A,** Tie2 deletion in macrophages impaired tumor relapse after chemotherapy. MCA205 tumor cells were subcutaneously transplanted into Con or M^{Tie2-} mice and intratumorally injected with doxorubicin (DOX; 70 μg/mouse) on day 12. Tumor growth of MCA205 in Con ($n = 7$) and M^{Tie2-} mice ($n = 7$) was shown. Representative photo of tumors obtained at the endpoint of the experiment is shown in the right panel. **B,** Tie2 deletion in macrophages impaired vessel reconstruction around tumor necrotic area. Tumor sections from doxorubicin-treated Con ($n = 4$) and M^{Tie2-} mice ($n = 4$) were obtained 14 days after doxorubicin treatment and stained for CD31 (brown). Enlarged representative blood vessels around the necrotic area are also shown. The dotted line shows the necrotic area (N). Scale bar, 200 μm. Quantitative analysis of CD31-positive area is shown. *, $P < 0.05$. **C,** Fourteen days after doxorubicin treatment, blood vessels of tumors from Con and M^{Tie2-} mice were perfused and visualized by FITC-dextran (green). Panels show the steps how "irrigated tumor area" are calculated. Statistical analysis of the percentage of irrigated tumor area is also shown. Con, $n = 4$; M^{Tie2-}, $n = 6$, 3.64 mm^2 /tumor are analyzed. Scale bar, 200 μm. **D,** Fourteen days after doxorubicin treatment, tumor sections from Con and M^{Tie2-} mice were stained with H&E. The dotted line shows the necrotic area (N). Statistical analysis of the percentage of necrotic area is shown. Con, $n = 4$; M^{Tie2-}, $n = 6$. Scale bar, 735 μm. **E,** Inhibited tumor relapse in M^{Tie2-} mice after treatment with cyclophosphamide (CTX). Con ($n = 6$) and M^{Tie2-} ($n = 5$) mice were subcutaneously transplanted with MCA205, and CTX was given intraperitoneally at 14 days after transplantation (arrow). Tumor growth of MCA205 in Con mice ($n = 4$) without CTX treatment was also shown as control. *, $P < 0.05$; **, $P < 0.01$.

Hypoxia induced Tie2 expression on $CD11b^+Ly6C^+$ cells

In addition, the origin of TEMs in tumors after chemotherapy was investigated. In tumor-bearing mice, $CD11b^+Ly6C^+$ cells represent a heterogeneous population of cells with great plasticity (37). These $CD11b^+Ly6C^+$ cells abundantly infiltrated into tumors after chemotherapy, which peaked at day 3 and gradually returned to the normal level at day 9 (Supplementary Fig. S5). Thus, we wondered whether chemotherapy-induced infiltrated $CD11b^+Ly6C^+$ cells could be the precursors for local TEMs.

Hypoxia is one of the most important conditions in the tumor microenvironment that causes tumor necrosis (36). Indeed, dramatically increased hypoxia-inducible factor-1α (HIF1α)-positive area was found in doxorubicin-treated tumor (Supplementary Fig. S6). Thus, the role of hypoxia in the induction of Tie2 expression on infiltrated $CD11b^+Ly6C^+$ cells was investigated. Treatment with $CoCl_2$, a chemical inducer of HIF1α (38), was efficient to upregulate the level of Tie2 mRNA in isolated $CD11b^+Ly6C^+$ cells (Fig. 3A). Western blot analysis also showed that both hypoxia (1.5% O_2) and $CoCl_2$ were able to induce Tie2 expression in $CD11b^+Ly6C^+$ cells from Con mice but not M^{Tie2-} mice as expected (Fig. 3B). The direct effect of cytotoxic drugs on the induction of Tie2 expression on $CD11b^+Ly6C^+$ cells was also investigated; however, we did not find upregulated Tie2 expression at indicated concentrations and culture time of doxorubicin (Supplementary Fig. S7). These results indicate that hypoxia is an

Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

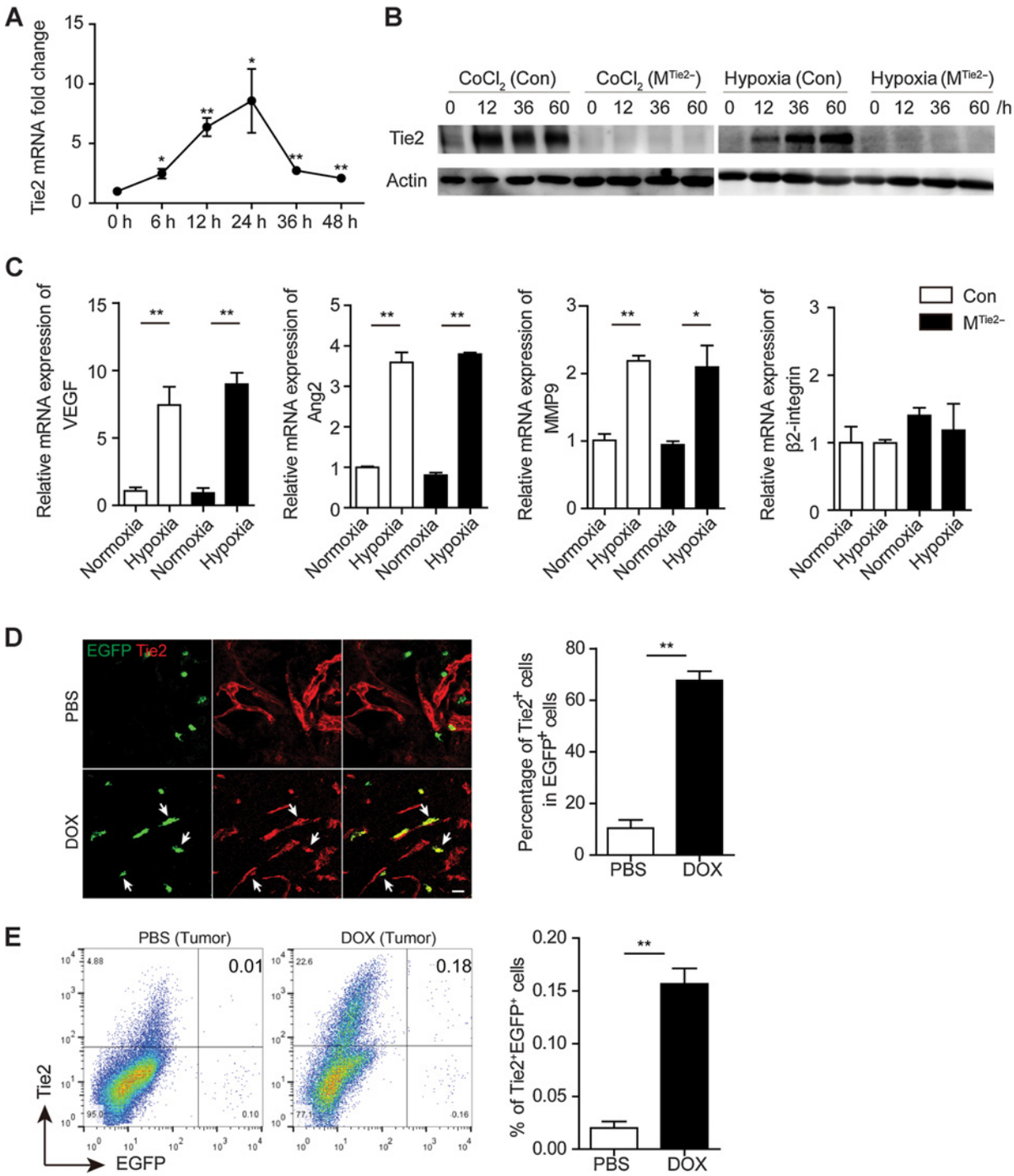

Figure 3.

Tumor-infiltrating CD11b$^+$Ly6C$^+$ cells were essential precursors for Tie2 macrophages. **A** and **B,** Hypoxia-induced Tie2 expression in CD11b$^+$Ly6C$^+$ cells. **A,** Tie2 expression in FACS-sorted CD11b$^+$Ly6C$^+$ cells from the spleen of tumor-bearing Con mice were induced by 200 μmol/L $CoCl_2$ for indicated time and detected by real-time PCR. *, $P < 0.05$; **, $P < 0.01$. **B,** Protein levels of Tie2 in CD11b$^+$Ly6C$^+$ cells from Con mice or M^{Tie2-} mice under 200 μmol/L $CoCl_2$ or hypoxia (1.5% O_2) added conditions were detected by Western blotting. Cells were cultured additionally with tumor supernatant. $CoCl_2$ or hypoxia was applied at indicated time points before mRNA/protein extraction. **C,** Hypoxia-induced proangiogenic gene expression in CD11b$^+$Ly6C$^+$ cells. CD11b$^+$Ly6C$^+$ cells from Con mice or M^{Tie2-} mice were cultured for 12 hours under hypoxia condition. mRNA level of indicated genes was detected by real-time PCR. *, $P < 0.05$; **, $P < 0.01$. **D,** Tie2 and EGFP double staining shows that infiltrated EGFP$^+$ CD11b$^+$Ly6C$^+$ cells became Tie2-positive in doxorubicin (DOX)-treated tumor. CD11b$^+$Ly6C$^+$ cells were isolated from EGFP mice and adoptively transferred to control ($n = 3$) or doxorubicin-treated ($n = 3$) tumor-bearing C57BL/6 mice. Tumor sections were stained for EGFP and Tie2 at 9 days after adoptive transfer and analyzed via fluorescence microscopy. Scale bar, 20 μm. The proportion of Tie2$^+$ cells in EGFP$^+$ cells in each group is shown on the right. **, $P < 0.01$. **E,** Proportion of Tie2-expressing EGFP$^+$ cells was evaluated by FACS using single-cell suspensions prepared from control ($n = 3$) or doxorubicin-treated ($n = 3$) tumor tissues. Dead cells were excluded by 7-AAD staining. Statistical analysis is shown on the right. **, $P < 0.01$.

Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

important factor for infiltrated $CD11b^+Ly6C^+$ cells to become Tie2-positive macrophages in the tumor microenvironment.

Besides *Tie2*, hypoxia also upregulated the expression of several proangiogenic factors, such as *VEGF*, *Ang2*, and *MMP9*, in $CD11b^+Ly6C^+$ cells. However, *Tie2* deficiency did not impair the upregulation of these genes (Fig. 3C).

Next, we investigated the condition that induced Tie2 expression on macrophages *in vivo*. $CD11b^+Ly6C^+$ cells, which were isolated from the spleen of tumor-bearing Rosa26-EGFP C57BL/6 mice (EGFP mice), were adoptively transferred into doxorubicin-treated tumor-bearing or control mice. Higher proportion of infiltrated $EGFP^+$ cells demonstrated Tie2-positive staining as well as perivascular location in the doxorubicin-treated mice (Fig. 3D and E). However, even in the doxorubicin-treated mice, $EGFP^+$ cells can only become Tie2-positive in the tumor microenvironment but not in the spleen and blood (Supplementary Fig. S8).

Tie2 maintains the viability of macrophages in stressed condition via AKT pathway

To clarify the role of Tie2 expression on macrophages, RAW264.7 cells, a macrophage cell line, were transfected with an expression plasmid encoding GFP (Mock-2) or a Tie2–GFP fusion protein (Tie2-3; ref. 29). As shown by immunofluorescence, Tie2 expression was detected in macrophages expressing Tie2–GFP fusion protein (Supplementary Fig. S9).

Ang2, a key ligand of Tie2 during vascular remolding, was used as the activator of Tie2 in the following experiments. Under normal culture conditions, no difference in cell proliferation was observed after RAW264.7 macrophages were transfected with Tie2 (Fig. 4A). Unexpectedly, we found that Tie2 expression can sustain the viability of macrophages under serum-starving condition. Importantly, Tie2 expression did not protect RAW264.7 from doxorubicin-induced cell death (Fig. 4A). Further analysis showed that serum starving induced less apoptosis in TEMs, as reflected by the Annexin V/PI double staining (Fig. 4B). Similar results were obtained when the other mock-transfected (Mock-1) and Tie2-transfected clones (Tie2-1 and Tie2-2) were used (Supplementary Fig. S10).

The signaling pathways involved in Ang2-mediated Tie2 activation were analyzed. Both AKT and ERK signaling pathways could be activated via Tie2 and they play important roles in the survival of eukaryotic cells in stressed conditions (39, 40). Here, we found that Ang2 stimulation of Tie2 induced phosphorylation of AKT but not ERK (Fig. 4C). In parallel, treatment with LY294002, which inhibits PI3K-dependent AKT phosphorylation, dramatically decreased the viability of TEMs under serum-starving condition. Additional treatment with U0126, an inhibitor of ERK phosphorylation, did not influence the viability of the stressed TEMs (Fig. 4D). These results suggest that Tie2 promotes the viability of $CD11b^+Ly6C^+$ cells through the AKT signaling pathway in stressed condition. Furthermore, in primary Tie2-positive macrophages, derived from $CD11b^+Ly6C^+$ cells induced by hypoxia, we also confirmed that Tie2 expression plays a critical role in sustaining their viability under serum-starving condition (Fig. 4E).

To confirm whether Tie2 is also important for the viability of tumor-infiltrated myeloid cells *in vivo*, isolated $CD11b^+Ly6C^+$ cells from either Con mice or M^{Tie2-} were labeled with CFSE and then adoptively transferred to doxorubicin-treated tumor-bearing mice. As shown in Fig. 4F, after chemotherapy, the number of tumor-infiltrated $CFSE^+$ cells from M^{Tie2-} mice is sharply decreased within 9 days. Remarkably, in the spleen, *Tie2* gene depletion did not influence either the proliferating activity or the number of $CFSE^+$ cells after adoptive transfer (Supplementary Fig. S11). These results indicate that Tie2 provides survival advantages to macrophages under stress conditions, such as tumor microenvironment after chemotherapy.

$CD11b^+Ly6C^+$ cell depletion eliminated the effect of Tie2 expression on macrophages

To identify the source for TEM attached to the blood vessels, we employed all-*trans* retinoic acid (ATRA) to eliminate $CD11b^+Ly6C^+$ cells in tumor-bearing mice (41). Tumor-bearing M^{Tie2-} and Con mice were administrated with ATRA and doxorubicin as shown by a schematic illustration (Fig. 5A). Indeed, ATRA was able to effectively reduce $CD11b^+Ly6C^+$ cells both in spleen and in tumor (Fig. 5B and C). As shown in Fig. 5D, in the group without ATRA treatment, Con mice gained significantly larger tumor volume than M^{Tie2-} mice from day 8 after doxorubicin treatment. However, in the ATRA-treated group, no significant difference in tumor volumes between Con mice and M^{Tie2-} mice was observed until 22 days after doxorubicin treatment (Fig. 5D). This suggests that $CD11b^+Ly6C^+$ cells represent the source population of TEM and are responsible for the different relapse velocity between Con mice and M^{Tie2-} mice after chemotherapy.

Discussion

By employing a macrophage-specific Tie2-knockout mouse model, we show here that Tie2 expression on tumor-infiltrating macrophages is crucial for the repair of the blood vessel system in the tumor and, consequently, tumor relapse after chemotherapy. We further demonstrated that hypoxia microenvironment induces Tie2 expression in $CD11b^+Ly6C^+$ cells. Tie2 expression in turn promoted the viability of macrophages in stressed conditions via the AKT-dependent signaling pathway. This finding may be important for developing novel strategies to overcome the resistance of some clinical chemotherapeutic applications.

Our results provide a novel mechanism whereby tumor stromal responses contribute to chemotherapeutic resistance. Extensive studies focus on intrinsic or acquired genetic alterations of tumor cells that render chemotherapy ineffective (42). Fifteen years ago, we showed that *IFNγR* deficiency on stromal cells succumb the effectiveness of cyclophosphamide (8). Recently, increasing evidences suggest that stromal cells in the tumor environment help tumor evade killing by chemotherapeutic drugs. Actually, chemotherapy often imposes a stress on stromal cells or even the host as a whole. Different responses of stromal cells to chemotherapy will influence the final efficacy (43). From a pathophysiological point of view, chemotherapeutic drugs induce injury, or "wounds," in the tumor tissue. Logically, tissue repair responses, such as inflammation, will be elicited by the injury, which subsequently promotes tumor regrowth (44). Here, the results showed that $Tie2^+$ macrophages invaded massively and distributed in the necrotic area after chemotherapy. These cells favor prosperous revascularization and subsequently a tumor relapse. Hughes and colleagues (45) also showed that a subset of $MRC1^+TIE2^{Hi}CXCR4^{Hi}$ macrophages accumulated around blood vessels in tumors after chemotherapy, where they promote tumor revascularization and relapse, partly via VEGF-A release. The invasiveness of $Tie2^+$ macrophages in necrotic area following

Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

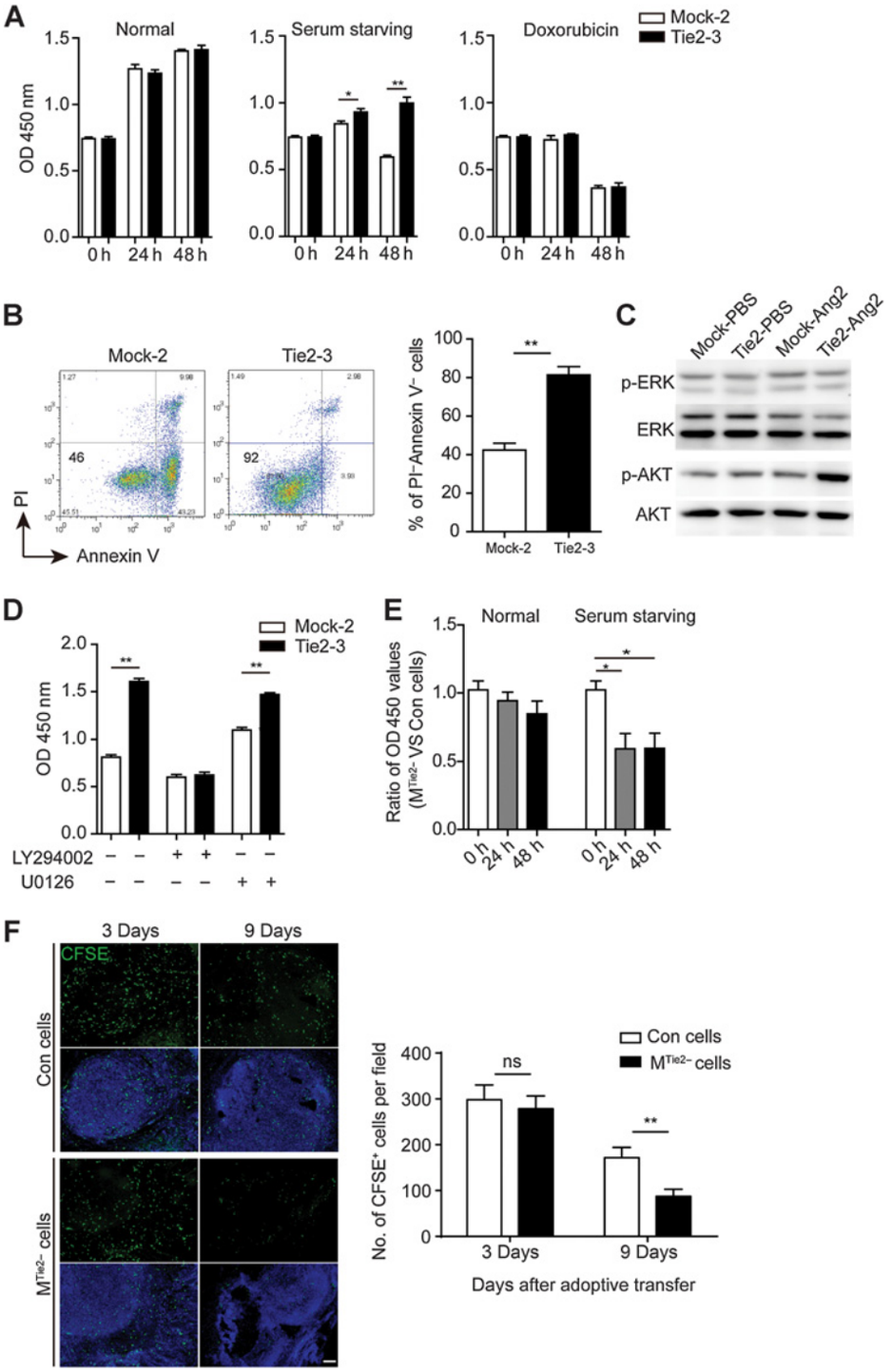

Figure 4.
Tie2 sustains the viability of macrophages in stressed condition via AKT pathway. **A,** Tie2 sustained the viability of macrophages in serum-starving conditions. The mock (Mock-2)- or Tie2-transfected RAW264.7 cells (Tie2-3) were cultured with 10% FBS (normal), 1% FBS (serum-starving), or doxorubicin (5 μg/mL) for indicated time. Cell viability was determined by CCK-8 assay. Shown are representative results of 1 of 3 similar experiments. **B,** Tie2 rescues serum deprivation-induced apoptosis in macrophages. Macrophages were cultured under normal or serum-starving conditions for 48 hours, and then cell apoptosis was detected through propidium iodide (PI) and Annexin V double staining and FACS analysis. Experiments were performed in the presence of Ang2 (200 ng/mL; **A** and **B**). *, $P < 0.05$; **, $P < 0.01$. **C,** Mock (Mock-2)- or Tie2-transfected RAW264.7 cells (Tie2-3) were stimulated with 200 ng/mL Ang2 or PBS as control for 12 hours under serum-starving conditions. Activation of AKT and ERK signaling pathways was analyzed by Western blotting. **D,** Tie2 promoted the viability of myeloid cells via AKT pathway. Macrophages were cultured under serum-starving conditions for 48 hours with either AKT inhibitor LY294002 or ERK inhibitor U0126. Cell viability was determined by CCK-8 assay. **E,** CD11b^{+}Ly6C^{+} cells isolated from spleens of Con and M^{Tie2-} tumor-bearing mice were cultured under hypoxia (1.5% O_2) conditions to induce Tie2 expression and then were cultured under normal or serum-starving conditions for 48 hours. Cell viability was determined by CCK-8 assay. Experiments were performed in the presence of Ang2 (200 ng/mL; **D** and **E**). *, $P < 0.05$; **, $P < 0.01$. **F,** CD11b^{+}Ly6C^{+} cells isolated from Con andM^{Tie2-} tumor–bearing mice were labeled with CFSE and then adoptively transferred to doxorubicin-treated recipient tumor-bearing C57BL/6 mice. At 3 and 9 days after the adoptive transfer, CFSE^{+} cells in tumors were analyzed by immunofluorescence. Scale bar, 200 μm. Statistical analysis is shown in the right. **, $P < 0.01$.

chemotherapy may represent a kind of tissue repair response in the tumor, which provides a potential target to overcome chemotherapeutic resistance in the clinic.

The mechanism of how the blood supply system is reconstructed after chemotherapy might be quite different from that of the original tumor angiogenesis. Except for causing massive tumor cell death, chemotherapy impairs the vascular system through killing endothelial cells, inducing structure disorders, and raising vascular ruptures in the tumor (46). However, the basic ingredients that constitute vascular system are still left after chemotherapy (47). Therefore, vascular formation after chemotherapy should be a kind of "fixing" process of a broken

Published OnlineFirst October 10, 2016; DOI: 10.1158/0008-5472.CAN-16-1114

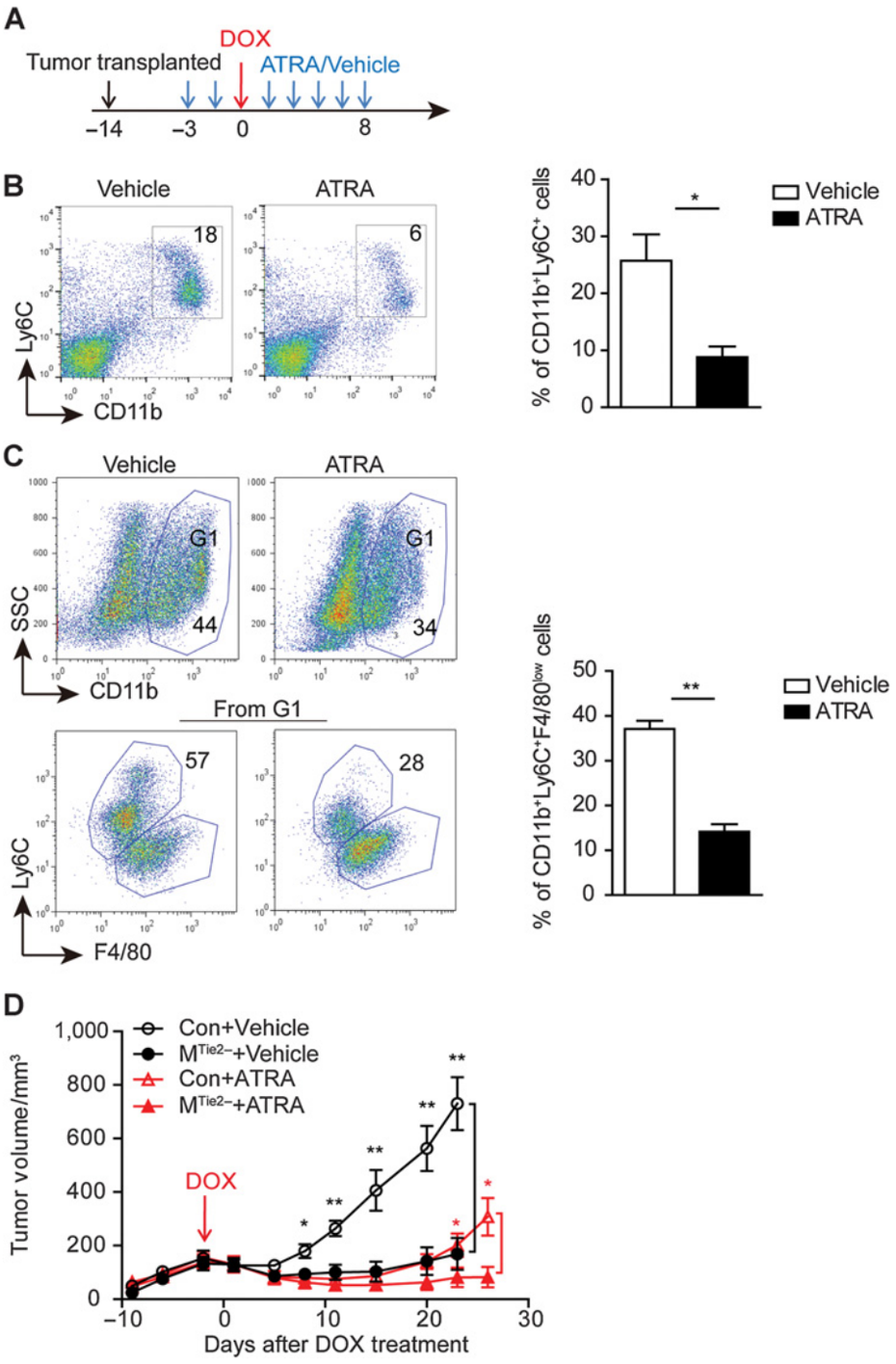

Figure 5.
$CD11b^+Ly6C^+$ cell depletion delayed the tumor recovery from chemotherapy. **A,** Schematic illustration of doxorubicin (DOX) plus ATRA treatment. **B** and **C,** $CD11b^+Ly6C^+$ cell depletion efficacy of ATRA was analyzed by FACS. Four days after doxorubicin treatment, the proportions of $CD11b^+Ly6C^+$ cells in spleens (**B**) and $CD11b^+Ly6C^+F4/80^{low}$ cells in tumors (**C**) were analyzed. Dead cells were excluded by 7-AAD staining. Statistical analysis is shown on the right. $n = 3$ (ATRA group); $n = 3$ (vehicle group). *, $P < 0.05$; **, $P < 0.01$. **D,** Treatment with ATRA delayed the tumor relapse after chemotherapy. Con and M^{Tie2-} mice were subcutaneously transplanted with MCA205 and treated with doxorubicin and ATRA as described in **A**. Tumor growth was monitored. $n = 5$ (ATRA groups); $n = 4$ (vehicle groups). *, $P < 0.05$; **, $P < 0.01$.

vascular system. In fact, a huge amount of vascular fragment was observed in tumor necrosis after chemotherapy. In the following observation along with tumor relapse, the number of vascular fragment was reduced, whereas long and well-networked vascular structure appeared gradually. We perceived this process as that, unless engulfed by macrophages, some useful vascular fragments or single endothelial cells probably were reused for neovascularization, which we called vascular repair. As for the tumor, the obvious benefits of this vascular reconstruction are immediate restoration of blood supply and a quick initiation of tumor recurrence. Predictably, fixing of the remnant vascular segments produces a chaotic and complex vascular system, as observed in tumor after chemotherapy. Interestingly, Liu and colleagues recently demonstrated in zebrafish that macrophages generated mechanical traction forces to pull the endothelial ends and facilitate their ligation, thus mediating the repair of the

cerebrovascular rupture (48). It might be a similar scenario during vascular reconstruction after chemotherapy (Supplementary Fig. S12).

An interesting question is that what is the driving factor for the expression of Tie2 on macrophages after chemotherapy? The mechanism we proved here is that hypoxia induced Tie2 expression on macrophages *in vitro*. Similarly, Murdoch and colleagues also found that hypoxia upregulated the cell surface expression of Tie2 on human monocytes and macrophages *in vitro* (21). Besides, it was shown here that doxorubicin induced higher level of hypoxia in tumor microenvironment (Supplementary Fig. S6), which is consistent with the results of Cao and colleagues (49). We have found that higher percentage of transferred cells became Tie2-positive in doxorubicin-treated tumor than in normal tumor *in vivo* (Fig. 3D). However, whether it is hypoxia that induced Tie2 expression on macrophages *in vivo* still needs further investigation. The cell debris or exosomes derived from necrotic tumor cells may also play a role in this process.

Our investigations suggest that combining chemotherapy with treatment targeting TEMs may delay the recurrence after chemotherapy. Indeed, we have found that the percentage of CD14$^+$Tie2$^+$ cells in peripheral blood increased significantly after chemotherapy in patients with lung cancer with progressive disease. These results suggest that TEMs may also play a vital role in the tumor relapse after chemotherapy in patients. Although there are no drugs specifically against TEMs, clinical trials already combine anti-Ang2 antibody with chemotherapeutic drugs for the cure of patients with cancer (15). Our research provides new clues for the understanding of their possible synergistic effect. Besides inhibiting the function of endothelial cells, anti-Ang2 treatment might also impede the survival of TEMs accumulated in the tumor after chemotherapy, which subsequently impairs vascular reconstruction and tumor recurrence. In addition, depletion of myeloid cells has also been reported to enhance the efficacy of chemotherapy for solid tumors (50), possibly due to blocking the origin of TEMs in the tumor microenvironment. In conclusion, our findings provide a new target for overcoming chemotherapeutic resistance of cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: L. Chen, J. Li, Z. Qin
Development of methodology: L. Chen, J. Li
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): L. Chen, J. Li, C. Dai, T. Li, Y. Zhang, Y. He
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): L. Chen, J. Li, Y. Zhang, Z. Qin
Writing, review, and/or revision of the manuscript: L. Chen, J. Li, C. Dai, R. Glauben, G. Nie, Z. Qin
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Wang, C. Dai, F. Wu, X. Liu

Acknowledgments

We would like to thank Dr. Naoki Mochizuki for generously providing us with Tie2-GFP plasmid; Qingyang Hu for programming for irrigated area quantification; Zhenzhen Wu for intracardially perfusion, Junying Jia and Zhenwei Yang for outstanding assistance with flow cytometry and real-time PCR; Qingcui Li, Ruirui Wang, and Yuan Wang for technical support; and Chen Ni and Yuqiao Sheng for helpful comments.

Grant Support

This work was supported by the Ministry of Science and Technology of China (2012CB917103, 2016YFC1302305 to Z. Qin) and the National Natural Science Foundation of China (91229203, 81630068, 31670881 to Z. Qin). Y. He was supported by the Priority Academic Program Development of Jiangsu Higher Education Institutions.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received April 27, 2016; revised August 18, 2016; accepted September 14, 2016; published OnlineFirst October 10, 2016.

References

1. Chabner BA, Longo DL. Cancer chemotherapy and biotherapy: principles and practice. Philadelphia: Lippincott Williams & Wilkins; 2011.
2. Longley DB, Johnston PG. Molecular mechanisms of drug resistance. J Pathol 2005;205:275–92.
3. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. Annu Rev Immunol 2013;31:51–72.
4. Sun Y, Campisi J, Higano C, Beer TM, Porter P, Coleman I, et al. Treatment-induced damage to the tumor microenvironment promotes prostate cancer therapy resistance through WNT16B. Nat Med 2012;18:1359–68.
5. Schrader J, Gordon-Walker TT, Aucott RL, van Deemter M, Quaas A, Walsh S, et al. Matrix stiffness modulates proliferation, chemotherapeutic response, and dormancy in hepatocellular carcinoma cells. Hepatology 2011;53:1192–205.
6. Loeffler M, Kruger JA, Niethammer AG, Reisfeld RA. Targeting tumor-associated fibroblasts improves cancer chemotherapy by increasing intratumoral drug uptake. J Clin Invest 2006;116:1955–62.
7. Hudis CA. Clinical implications of antiangiogenic therapies. Oncology (Williston Park) 2005;19:26–31.
8. Ibe S, Qin Z, Schuler T, Preiss S, Blankenstein T. Tumor rejection by disturbing tumor stroma cell interactions. J Exp Med 2001;194:1549–59.
9. De Palma M, Lewis CE. Macrophage regulation of tumor responses to anticancer therapies. Cancer Cell 2013;23:277–86.
10. Shaked Y, Henke E, Roodhart JM, Mancuso P, Langenberg MH, Colleoni M, et al. Rapid chemotherapy-induced acute endothelial progenitor cell mobilization: implications for antiangiogenic drugs as chemosensitizing agents. Cancer Cell 2008;14:263–73.
11. Mazzieri R, Pucci F, Moi D, Zonari E, Ranghetti A, Berti A, et al. Targeting the ANG2/TIE2 axis inhibits tumor growth and metastasis by impairing angiogenesis and disabling rebounds of proangiogenic myeloid cells. Cancer Cell 2011;19:512–26.
12. Dumont DJ, Gradwohl G, Fong GH, Puri MC, Gertsenstein M, Auerbach A, et al. Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. Genes Dev 1994;8:1897–909.
13. Huang H, Bhat A, Woodnutt G, Lappe R. Targeting the ANGPT-TIE2 pathway in malignancy. Nat Rev Cancer 2010;10:575–85.
14. Pfizer. Safety and PK study of CVX-060 in patients with advanced solid tumors. In: ClinicalTrials.gov [Internet]. Bethesda, MD: National Library of Medicine (US). 2011- [cited 2014 Sep 1]. Available from: http://clinicaltrials.gov/ct2/show/NCT00879684?term=ang2&rank=7. NLM Identifier: NCT00879684.
15. Pfizer. PF-04856884 (CVX-060) in combination with axitinib in patients with previously treated metastatic renal cell carcinoma. In: ClinicalTrials.gov [Internet]. Bethesda, MD: National Library of Medicine (US). 2014- [cited 2014 Sep 1]. Available from: http://clinicaltrials.gov/ct2/show/NCT01441414?term=ang2&rank=3. NLM Identifier: NCT01441414.
16. De Palma M, Venneri MA, Galli R, Sergi Sergi L, Politi LS, Sampaolesi M, et al. Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. Cancer Cell 2005;8:211–26.

17. De Palma M, Venneri MA, Roca C, Naldini L. Targeting exogenous genes to tumor angiogenesis by transplantation of genetically modified hematopoietic stem cells. Nat Med 2003;9:789–95.
18. Kioi M, Vogel H, Schultz G, Hoffman RM, Harsh GR, Brown JM. Inhibition of vasculogenesis, but not angiogenesis, prevents the recurrence of glioblastoma after irradiation in mice. J Clin Invest 2010;120:694–705.
19. Patel AS, Smith A, Nucera S, Biziato D, Saha P, Attia RQ, et al. TIE2-expressing monocytes/macrophages regulate revascularization of the ischemic limb. EMBO Mol Med 2013;5:858–69.
20. Coffelt SB, Tal AO, Scholz A, De Palma M, Patel S, Urbich C, et al. Angiopoietin-2 regulates gene expression in TIE2-expressing monocytes and augments their inherent proangiogenic functions. Cancer Res 2010;70:5270–80.
21. Murdoch C, Tazzyman S, Webster S, Lewis CE. Expression of Tie-2 by human monocytes and their responses to angiopoietin-2. J Immunol 2007;178:7405–11.
22. Coffelt SB, Chen YY, Muthana M, Welford AF, Tal AO, Scholz A, et al. Angiopoietin 2 stimulates TIE2-expressing monocytes to suppress T cell activation and to promote regulatory T cell expansion. J Immunol 2011;186:4183–90.
23. Thomas M, Felcht M, Kruse K, Kretschmer S, Deppermann C, Biesdorf A, et al. Angiopoietin-2 stimulation of endothelial cells induces alphavbeta3 integrin internalization and degradation. J Biol Chem 2010;285:23842–9.
24. Bezuidenhout L, Zilla P, Davies N. Association of Ang-2 with integrin beta 2 controls Ang-2/PDGF-BB-dependent upregulation of human peripheral blood monocyte fibrinolysis. Inflammation 2009;32:393–401.
25. Felcht M, Luck R, Schering A, Seidel P, Srivastava K, Hu J, et al. Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. J Clin Invest 2012;122:1991–2005.
26. Scholz A, Lang V, Henschler R, Czabanka M, Vajkoczy P, Chavakis E, et al. Angiopoietin-2 promotes myeloid cell infiltration in a beta(2)-integrin-dependent manner. Blood 2011;118:5050–9.
27. Shen B, Shang Z, Wang B, Zhang L, Zhou F, Li T, et al. Genetic dissection of tie pathway in mouse lymphatic maturation and valve development. Arterioscler Thromb Vasc Biol 2014;34:1221–30.
28. Qin Z, Schwartzkopff J, Pradera F, Kammertoens T, Seliger B, Pircher H, et al. A critical requirement of interferon gamma-mediated angiostasis for tumor rejection by CD8+ T cells. Cancer Res 2003;63:4095–100.
29. Fukuhara S, Sako K, Minami T, Noda K, Kim HZ, Kodama T, et al. Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. Nat Cell Biol 2008;10:513–26.
30. Zhang J, Chen L, Xiao M, Wang C, Qin Z. FSP1+ fibroblasts promote skin carcinogenesis by maintaining MCP-1-mediated macrophage infiltration and chronic inflammation. Am J Pathol 2011;178:382–90.
31. Chen L, Li J, Zhang J, Dai C, Liu X, Wang J, et al. S100A4 promotes liver fibrosis via activation of hepatic stellate cells. J Hepatol 2015;62:156–64.
32. Tredan O, Galmarini CM, Patel K, Tannock IF. Drug resistance and the solid tumor microenvironment. J Natl Cancer Inst 2007;99:1441–54.
33. Armulik A, Abramsson A, Betsholtz C. Endothelial/pericyte interactions. Circ Res 2005;97:512–23.
34. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I. Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transgenic Res 1999;8:265–77.
35. Fantin A, Vieira JM, Gestri G, Denti L, Schwarz Q, Prykhozhij S, et al. Tissue macrophages act as cellular chaperones for vascular anastomosis downstream of VEGF-mediated endothelial tip cell induction. Blood 2010;116:829–40.
36. Carmeliet P, Jain RK. Angiogenesis in cancer and other diseases. Nature 2000;407:249–57.
37. Talmadge JE, Gabrilovich DI. History of myeloid-derived suppressor cells. Nat Rev Cancer 2013;13:739–52.
38. Piret JP, Mottet D, Raes M, Michiels C. CoCl2, a chemical inducer of hypoxia-inducible factor-1, and hypoxia reduce apoptotic cell death in hepatoma cell line HepG2. Ann N Y Acad Sci 2002;973:443–7.
39. Kim JY, Kim YJ, Lee S, Park JH. The critical role of ERK in death resistance and invasiveness of hypoxia-selected glioblastoma cells. BMC Cancer 2009;9:27.
40. Hou M, Cui J, Liu J, Liu F, Jiang R, Liu K, et al. Angiopoietin-like 4 confers resistance to hypoxia/serum deprivation-induced apoptosis through PI3K/Akt and ERK1/2 signaling pathways in mesenchymal stem cells. PLoS One 2014;9:e85808.
41. Kusmartsev S, Cheng F, Yu B, Nefedova Y, Sotomayor E, Lush R, et al. All-trans-retinoic acid eliminates immature myeloid cells from tumor-bearing mice and improves the effect of vaccination. Cancer Res 2003;63:4441–9.
42. Vidal S, Rodriguez-Bravo V, Galsky M, Cordon-Cardo C, Domingo-Domenech J. Targeting cancer stem cells to suppress acquired chemotherapy resistance. Oncogene 2014;33:4451–63.
43. Daenen LG, Houthuijzen JM, Cirkel GA, Roodhart JM, Shaked Y, Voest EE. Treatment-induced host-mediated mechanisms reducing the efficacy of antitumor therapies. Oncogene 2014;33:1341–7.
44. Schafer M, Werner S. Cancer as an overhealing wound: an old hypothesis revisited. Nat Rev Mol Cell Biol 2008;9:628–38.
45. Hughes R, Qian B-Z, Rowan C, Muthana M, Keklikoglou I, Olson OC, et al. Perivascular M2 macrophages stimulate tumor relapse after chemotherapy. Cancer Res 2015;75:3479–91.
46. Cooney MM, van Heeckeren W, Bhakta S, Ortiz J, Remick SC. Drug Insight: vascular disrupting agents and angiogenesis[mdash]novel approaches for drug delivery. Nat Clin Prac Oncol 2006;3:682–92.
47. Tonnesen MG, Feng X, Clark RA. Angiogenesis in wound healing. J Investig Dermatol Symp Proc 2000;5:40–6.
48. Liu C, Wu C, Yang Q, Gao J, Li L, Yang D, et al. Macrophages mediate the repair of brain vascular rupture through direct physical adhesion and mechanical traction. Immunity 2016;44:1162–76.
49. Cao Y, Eble JM, Moon E, Yuan H, Weitzel DH, Landon CD, et al. Tumor cells upregulate normoxic HIF-1alpha in response to doxorubicin. Cancer Res 2013;73:6230–42.
50. De Palma M, Lewis CE. Cancer: macrophages limit chemotherapy. Nature 2011;472:303–4.

Cancer Research
The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

AACR American Association for Cancer Research

Tie2 Expression on Macrophages Is Required for Blood Vessel Reconstruction and Tumor Relapse after Chemotherapy

Lin Chen, Jie Li, Fei Wang, et al.

Cancer Res 2016;76:6828-6838. Published OnlineFirst October 10, 2016.