

# Integrin $\alpha_4\beta_1$ Promotes Monocyte Trafficking and Angiogenesis in Tumors

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## Abstract

**Monocytes and macrophages extensively colonize solid tumors, where they are thought to promote tumor angiogenesis. Here, we show that integrin  $\alpha_4\beta_1$  (VLA4) promotes the invasion of tumors by myeloid cells and subsequent neovascularization. Antagonists of integrin  $\alpha_4\beta_1$ , but not of other integrins, blocked the adhesion of monocytes to endothelium *in vitro* and *in vivo* as well as their extravasation into tumor tissue. These antagonists prevented monocyte stimulation of angiogenesis *in vivo*, macrophage colonization of tumors, and tumor angiogenesis. These studies indicate the usefulness of antagonists of integrin  $\alpha_4\beta_1$  in suppressing macrophage colonization of tumors and subsequent tumor angiogenesis. These studies further indicate that suppression of myeloid cell homing to tumors could be a useful supplementary approach to suppress tumor angiogenesis and growth.** (Cancer Res 2006; 66(4): 2146-52)

## Introduction

The growth of new blood vessels, or neovascularization, stimulates the healing of injured tissues but also promotes tumor growth and inflammatory disease (1–3). Tumor angiogenesis is regulated by both endothelial and nonendothelial cells present within the tumor compartment. Vascular and lymphatic endothelial cells directly participate in sprouting angiogenesis (1–5). In contrast, stromal fibroblasts express angiogenic growth factors and stimulate angiogenesis (6). Additionally, bone marrow–derived cells infiltrate tumors and may directly participate in neovascularization (7–13), giving rise to ~15% of the neovasculature (12).

Another bone marrow–derived cell type, the CD14<sup>+</sup> or CD11b<sup>+</sup> myeloid lineage cell, is also found extensively in tumors and other neovascular or repairing tissues (14–16). Macrophages express growth factors that stimulate angiogenesis and lymphangiogenesis (15–18). These cells also may transdifferentiate into endothelial cells and thereby promote angiogenesis (7, 14). Thus, myeloid cells may directly and indirectly participate in angiogenesis (7–18). Several studies have shown recently that macrophages play important roles in wound healing, inflammation, and cancer. Indeed, monocytes and macrophages have been shown recently to promote angiogenesis and lymphangiogenesis in healing wounds and tumors, and up to 5% of the cells in a tumor may be macrophages (15–18). As myeloid cells are circulating cells, understanding how they invade tumors and

manipulating this mechanism could provide new means to inhibit tumor angiogenesis.

Although these important bone marrow–derived cells are present within tumors, little is known about the mechanisms by which these cells traffic into and out of the tumor microenvironment. Immune cell trafficking *in vivo* is regulated by several members of the integrin, immunoglobulin superfamily and selectin adhesion molecule families (19–24). Because myeloid cells express functional integrins  $\alpha_2\beta_1$ ,  $\alpha_4\beta_1$ ,  $\alpha_5\beta_1$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ ,  $\alpha_M\beta_2$ , and  $\alpha_X\beta_2$  as well as other adhesion proteins, it has been unclear how these cells might enter the tumor microenvironment from the circulation. Our studies on the roles of integrins and their ligands revealed important functions for a key integrin in trafficking of monocytes to tumor blood vessels. In the studies presented here, we show that fibronectin receptor integrin  $\alpha_4\beta_1$  (VLA4) selectively promotes the homing of monocytes to tumors and that this integrin is essential for the participation of myeloid cells in angiogenesis and tumor growth. Our studies also suggest that suppression of monocyte/macrophage homing to tumors could be a useful supplementary approach to suppress tumor angiogenesis and growth.

## Materials and Methods

**Myeloid cell isolation and culture.** Peripheral blood mononuclear cells were purified by Percoll gradient centrifugation from pooled human buffy coats obtained from the San Diego Blood Bank (pooled from 4–5 donors). Myeloid cells were isolated from mononuclear cell populations by positive selection for CD14 or CD11b by two rounds of magnetic bead immune selection according to the manufacturer's directions (Miltenyi Biotech, Auburn, CA). All studies with human cells were conducted with approval of the Human Subjects Institutional Review Board of the University of California at San Diego. All studies were done two to four times.

**Fluorescence-activated cell sorting analysis.** Marker analysis was done by fluorescence-activated cell sorting (FACS) analysis three to four times using purified monocytes from separate fresh, pooled human mononuclear cells. FACS analysis was done at the University of California at San Diego Cancer Center Shared Resource. APC-conjugated mouse anti-human CD11b and CD14 antibodies were from Miltenyi Biotech. PE-conjugated mouse anti-human  $\alpha_4\beta_1$  (9F10) and  $\alpha_5\beta_1$  (IIA1) were from Becton Dickinson (San Jose, CA). Anti- $\beta_2$  (CLB-LFA 1/1) was from eBiosciences (San Diego, CA). FITC- or PE-conjugated mouse anti-human antibodies against  $\alpha_v\beta_3$  (LM609) and  $\alpha_v\beta_5$  (PIF6) were from Chemicon International (Temecula, CA).

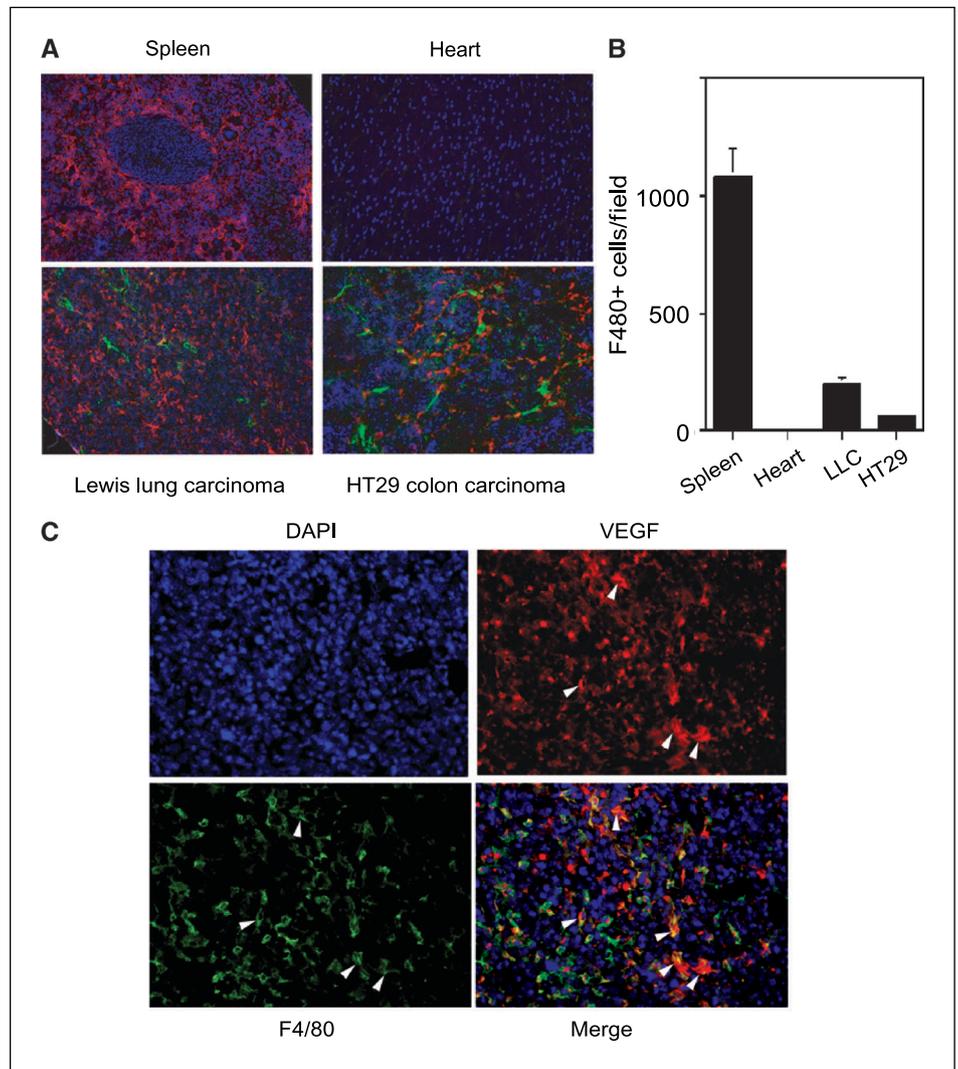
**Immunohistochemistry.** Cryosections of HT29 colon carcinoma xenograft tumors, Lewis lung carcinoma tumors, murine spleen, murine heart, and Matrigel samples were fixed in ice-cold acetone for 2 minutes, air dried, and incubated in 5% bovine serum albumin (BSA) in PBS and then in primary antibodies (5  $\mu$ g/mL) for 2 hours at room temperature. Negative control slides were incubated in block buffer during this step. Five normal tissue replicates and 10 replicate tumors were examined in each study. Slides were washed four times for 5 to 10 minutes in PBS, incubated in secondary antibody for 1 hour at room temperature, washed, and stained with 4',6-diamidino-2-phenylindole (DAPI). Anti-mouse CD31 was from PharMingen (San Jose, CA). Anti-F4/80 was from Serotec (Raleigh, NC).

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

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**Figure 1.** Macrophages populate tumors and express VEGF-A. **A**, cryosections of murine spleen and heart as well as cryosections of murine Lewis lung carcinoma tumors and HT29 colon carcinoma tumors grown in nude mice were immunostained to detect expression of the macrophage antigen F4/80 (red) and the endothelial cell antigen CD31 (green). Nuclei were immunostained with DAPI (blue). **B**, quantification of the average number of F4/80<sup>+</sup> macrophages per ×200 microscopic field in spleen, heart, Lewis lung carcinoma (LLC), and HT29 colon carcinoma cryosections. **C**, cryosections of HT29 colon carcinoma xenograft tumors were immunostained to detect expression of the macrophage antigen F4/80 (green) and the endothelial cell mitogen VEGF-A (red). Nuclei were immunostained with DAPI (blue). Fifty-nine percent of F4/80<sup>+</sup> cells in the tumors are positive for VEGF expression. Arrowheads, macrophages expressing VEGF-A.



Alexa 488 and Alexa 568 secondary antibodies were from Invitrogen (Carlsbad, CA). Immunofluorescence was quantified in 5 to 10 independent fields per tumor or normal tissue replicate. The average number of immunofluorescent cells per field per tissue was determined by averaging the replicate measurements for each of the 5 or 10 replicate samples. Negative control staining for secondary antibody alone was done on serial sections of each tissue sample. Two blinded observers independently viewed slides and quantification was done by counting of observable fluorescent structures in 5 to 10 microscopic fields (×200) under conditions predetermined to exhibit negligible background fluorescence.

**Adhesion assays.** Adhesion assays were done for 30 minutes on 48-well plates coated with 5 μg/mL recombinant H120 CS-1 fibronectin (from Martin J. Humphries, University of Birmingham, Birmingham, United Kingdom), plasma fibronectin, vitronectin, or recombinant soluble VCAM (R&D Systems, Minneapolis, MN) as described (25). Assays were done in the presence of adhesion medium, 25 μg/mL function-blocking anti-α<sub>4</sub>β<sub>1</sub> (HP2/1; a gift from Biogen-Idec, Cambridge, MA), anti-α<sub>5</sub>β<sub>1</sub> (JBS5; Chemicon International), anti-α<sub>v</sub>β<sub>3</sub> (PIF6; from Dr. David Cheresh, Scripps Research Institute, La Jolla, CA), or anti-α<sub>v</sub>β<sub>3</sub> (LM609; from Dr. David Cheresh), anti-β<sub>1</sub> (P4C10), or anti-β<sub>7</sub>. The number of cells adhering per field was quantified in five fields per treatment condition. All assays were done with triplicate samples and each assay was done three or more times using purified monocytes from separate fresh, pooled human mononuclear cells.

Additionally, 25,000 5-(and-6)-[4-chloromethyl(benzoyl)amino] tetramethylrhodamine (CMTMR; Invitrogen)-labeled human CD11b<sup>+</sup> and CD14<sup>+</sup>

monocytes per well were plated on human umbilical vascular endothelial cell monolayers for 30 minutes at 37°C in the presence of 25 μg/mL anti-α<sub>4</sub>β<sub>1</sub> (HP2/1), anti-α<sub>v</sub>β<sub>3</sub> (PIF6), anti-β<sub>1</sub> (P4C10), or anti-β<sub>7</sub> (PharMingen). All assays were done in HEPES-buffered HBSS containing 3% BSA, 1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MgCl<sub>2</sub>, and 0.1 mmol/L MnCl<sub>2</sub>. The number of cells adhering per field was quantified in five fields per treatment condition. All assays were done with triplicate samples and each assay was done three or more times using purified monocytes from separate fresh, pooled human mononuclear cells.

**Matrigel studies.** Nude mice were injected s.c. with 400 μL growth factor-depleted Matrigel supplemented with saline, 400 ng/mL vascular endothelial growth factor-A (VEGF-A), 1 × 10<sup>6</sup> CMTMR-labeled human CD11b<sup>+</sup> or CD14<sup>+</sup> monocytes isolated as described above, or 1 × 10<sup>6</sup> CMTMR-labeled human cells and VEGF-A together (*n* = 10 for each group). After 5 days, mice were injected with FITC-labeled *Bandeiraea simplicifolia* lectin, and 15 minutes later, mice were sacrificed and Matrigel plugs were excised, cryopreserved, and sectioned. Vessels were quantified in the center (inner 2 mm) and edge (outer 2 mm) of the Matrigel sections in 5 to 10 microscopic fields per slide for each individual replicate Matrigel plug. The average number of vessels per treatment condition per group was determined by counting blood vessels as independent lectin-positive structures within the Matrigel. In other studies, nude mice were injected s.c. with 400 μL growth factor-depleted Matrigel supplemented with saline, 400 ng/mL VEGF-A, and 1 × 10<sup>6</sup> unlabeled human CD14<sup>+</sup> cells (*n* = 10). After 5 days, mice were sacrificed and Matrigel plugs were excised,

cryopreserved, and sectioned. Sections (5  $\mu\text{m}$ ) were immunostained with anti-CD31 and anti-LYVE to detect blood vessels and lymphatic vessels, respectively. Experiments were done thrice using purified monocytes from separate fresh, pooled human mononuclear cells.

**Tumor studies.** CMTMR-labeled human monocytes ( $2 \times 10^6$ ) were incubated in medium, 50  $\mu\text{g}$  anti- $\alpha_v\beta_3$  (LM609), or 50  $\mu\text{g}$  anti-human  $\alpha_4\beta_1$  (HP2/1) in 100  $\mu\text{L}$  saline on ice for 30 minutes before injecting into nude mice bearing 0.5  $\text{cm}^3$  s.c. Lewis lung carcinomas. Animals were sacrificed 1 hour later. In additional studies,  $2 \times 10^6$  CMTMR-labeled human monocytes were injected into nude mice bearing 0.5  $\text{cm}^3$  HT29 tumors. Mice were treated for 5 days with saline, 200  $\mu\text{g}$  anti-human  $\alpha_v\beta_3$ , or 200  $\mu\text{g}$  anti-human  $\alpha_4\beta_1$  (HP2/1) every other day ( $n = 10$ ). After 5 days, tumors were excised and immunostained to detect CD31. In other studies, nude mice bearing 0.5  $\text{cm}^3$  Lewis lung carcinoma tumors ( $n = 12$ ) were treated for 5 days with 100  $\mu\text{L}$  saline, 200  $\mu\text{g}$  anti-murine  $\alpha_{M\beta_2}$ , or anti-200  $\mu\text{g}$  anti-murine  $\alpha_4\beta_1$  (PS/2) every other day ( $n = 10-12$ ). Alternatively, mice with 0.1  $\text{cm}$  HT29 colon carcinoma tumors were treated for 4 weeks with saline, 200  $\mu\text{g}$  anti- $\alpha_{M\beta_2}$ , or anti-200  $\mu\text{g}$  PS/2 every other day ( $n = 10$ ). In these two studies, tumors and their surrounding connective tissue were excised and cryopreserved and sections were immunostained with anti-F4/80 and anti-CD31 to detect macrophages. Each study was done two to three times using freshly isolated monocytes from pooled human buffy coats.

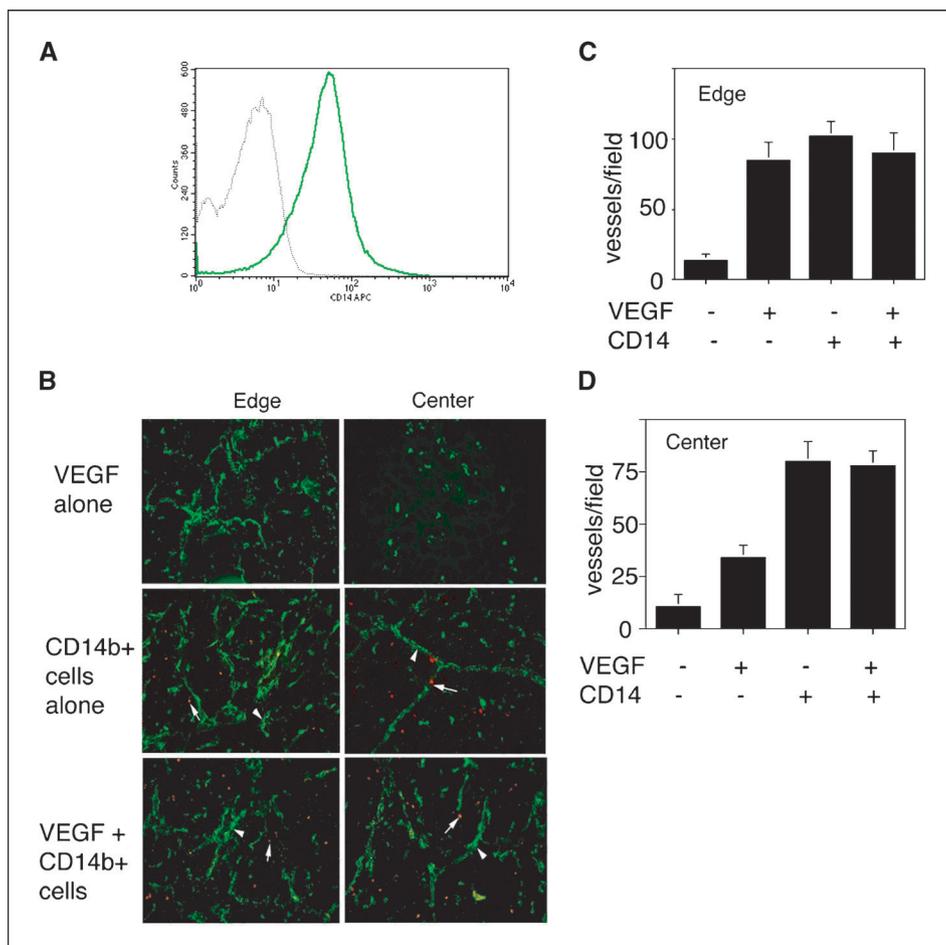
**Intravital microscopy.** Human monocytes were labeled with CMTMR in culture medium for 15 minutes on ice and washed according to the manufacturer's directions. Labeled cells ( $1 \times 10^6$ ) were i.v. injected into mice with N202 syngeneic green fluorescent protein-expressing tumor spheroids grown on transplanted mammary fat pad under transparent dorsal skinfold chambers. Cells were resuspended in saline, 100  $\mu\text{g}$  anti-human  $\alpha_4\beta_1$  antibodies (HP2/1) in saline, or 100  $\mu\text{g}$  anti-human  $\alpha_v\beta_5$  antibodies (PIF6) in saline for an initial serum concentration of 50  $\mu\text{g}/\text{mL}$

( $n = 2$ ). Animals were sedated (15-20 minutes), while *in vivo* fluorescence microscopy was done using a Mikron Instrument microscope (Mikron Instruments, Oakland, NJ) equipped with epi-illuminator and video-triggered stroboscopic illumination from a xenon arc (MV-7600, EG&G, Salem, MA). A silicon-intensified target camera (SIT68, Dage-MTI, Michigan City, IN) is attached to the microscope. A Hamamatsu image processor (Argus 20) with firmware version 2.50 (Hamamatsu Photonic System, Bridgewater, NJ) was used for image enhancement and to capture images to a computer. A Zeiss Achroplan  $\times 20/0.5$  W objective 10/0.22 was used to capture images.

**Animal studies.** All experiments on animals were done with the prior approval of the University of California at San Diego Institutional Animal Care and Use Committee and conformed to the national guidelines and regulations on ethical animal research.

## Results

**Tumor are infiltrated by VEGF-expressing macrophages.** To study the mechanisms by which bone marrow-derived myeloid cells traffic to the tumor microenvironment, we first examined normal and tumor tissues for the presence of bone marrow-derived myeloid cells, such as macrophages. Although normal mouse spleen has a high F4/80<sup>+</sup> macrophage content (average,  $1,075 \pm 121$  macrophages per field), other normal tissues, such as mouse heart or skin, are devoid of these cells (Fig. 1A and B). In contrast to most normal tissues, tumors have a high macrophage content; an average of  $199 \pm 33$  F4/80<sup>+</sup> macrophages per field can be observed in Lewis lung carcinoma tumors and an average of  $61 \pm 1$  F4/80<sup>+</sup> macrophages per field can be observed in HT29



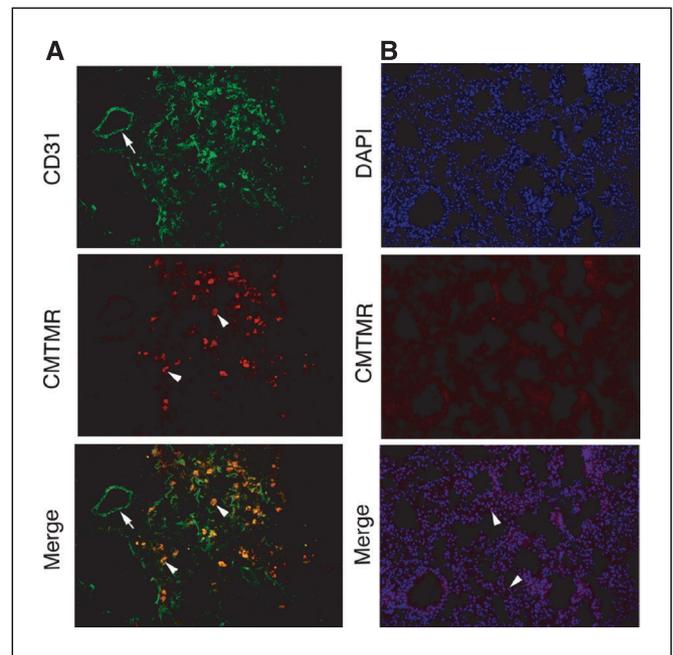
**Figure 2.** CD14<sup>+</sup> monocytes stimulate angiogenesis. **A**, monocytes purified by immune selection for CD14 expression were analyzed by FACS for expression levels of CD14 (green line). Gray FACS profile represents binding of fluorescent nonspecific IgG. **B**, representative micrographs of microvessels at the edge and center of growth factor-depleted Matrigel containing 400 ng/mL VEGF-A, 1 million CMTMR-labeled CD14<sup>+</sup> cells (red, arrows), or 400 ng/mL VEGF-A + 1 million CD11b<sup>+</sup> cells. Microvessels were identified by FITC-lectin reactivity (green, arrowheads). **C**, quantification of lectin-positive microvessels at the edge of Matrigel containing VEGF-A ( $P < 0.03$ ), CD14<sup>+</sup> cells ( $P < 0.001$ ), or VEGF-A + CD14<sup>+</sup> cells ( $P < 0.0002$ ). **D**, quantification of lectin-positive microvessels at the center of Matrigel containing VEGF-A, CD14<sup>+</sup> cells ( $P < 0.0002$ ), or VEGF + CD14<sup>+</sup> cells ( $P < 0.0004$ ).

xenograft tumors grown in mice (Fig. 1A and B). Fifty-nine percent of these macrophages express VEGF-A as shown in Fig. 1C, consistent with previous observations that myeloid cells express proangiogenic growth factors, such as IL-8, VEGF-A, and VEGF-C (15, 16).

**Monocytes stimulate angiogenesis *in vivo*.** To test the potential of myeloid cells to induce angiogenesis, human monocytes, which are characterized by expression of markers, such as CD14, CD11b, or CD68, were purified by immune selection from human peripheral blood cells by magnetic bead immunoselection for either CD14 (Fig. 2) or CD11b (Supplementary Fig. S1) expression. The purity of the CD14<sup>+</sup> cells was confirmed by flow cytometric immunostaining for expression of the monocytic marker CD14, as 90% of the purified cell population expressed CD14 (Fig. 2A). CD14<sup>+</sup> monocytes were labeled with the fluorescent cell tracking dye CMTMR (Fig. 2B, arrows) and embedded in growth factor–depleted Matrigel supplemented with or without added VEGF-A. In the presence of VEGF-A alone, new blood vessel growth was observed only in the outer 2 mm (edges) of the Matrigel but not in the inner 2 mm (center) of the Matrigel. In contrast, in the absence of added growth factor, CD14<sup>+</sup> myeloid cells potently stimulated angiogenesis at the edges and the center of the Matrigel plug, indicating that CD14<sup>+</sup> cells can stimulate angiogenesis (Fig. 2B–D). The combination of VEGF-A and CD14<sup>+</sup> monocytes enhanced angiogenesis throughout the Matrigel plug (Fig. 2B–D). These studies indicate that CD14<sup>+</sup> monocytes are able to induce angiogenesis directly. Similarly, when monocytes were purified by immune selection for CD11b expression, they stimulated angiogenesis in the absence of added growth factors and enhanced VEGF-A-induced angiogenesis (Supplementary Fig. S1). Together, these studies confirm that myeloid cells directly stimulate angiogenesis *in vivo* and suggest that tumor-associated macrophages could stimulate tumor angiogenesis.

**Monocytes promote lymphangiogenesis *in vivo*.** In addition to vascular angiogenesis, macrophages may also promote lymphangiogenesis, the outgrowth of lymphatic vessels (15). Lymphangiogenesis has been shown to also occur during tumor growth and may serve to promote tumor metastasis through the lymphatic system (4, 5). To determine whether purified monocytes directly promote lymphangiogenesis *in vivo*, CD14<sup>+</sup> monocytes were implanted in Matrigel in the presence or absence of VEGF-A. Monocytes alone stimulated an outgrowth of LYVE-1<sup>+</sup> vessels at the edge of the Matrigel, as did VEGF-A alone (Supplementary Fig. S2). No lymphatic vessels were observed in the center of the Matrigel. Together, these studies indicate that myeloid cells stimulate both angiogenesis and lymphangiogenesis *in vivo*, suggesting that tumor-associated macrophage stimulation of tumor angiogenesis and lymphangiogenesis may thereby help promote tumor growth and spread.

To determine whether CD14<sup>+</sup> monocytes expressed angiogenic growth factors *in vivo*, monocytes were embedded in growth factor–depleted Matrigel and injected into mice. After 5 days, Matrigel plugs were cryopreserved and cryosections were immunostained with anti-VEGF-A antibodies. We found that  $94 \pm 13\%$  of the embedded monocytes expressed VEGF-A, suggesting that monocytes likely stimulate angiogenesis by expressing angiogenic growth factors (Supplementary Fig. S3). Indeed, recent studies of healing ocular wounds showed that macrophages express VEGF-A and VEGF-C, thereby stimulating ocular angiogenesis and lymphangiogenesis (15).

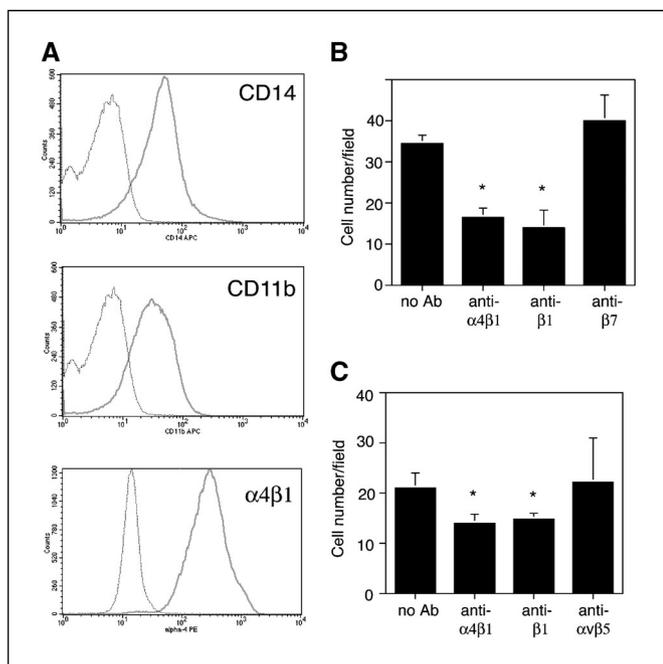


**Figure 3.** Human myeloid cells home to tumors. A, cryosections of Lewis lung carcinomas from mice injected with CMTMR-labeled human CD14<sup>+</sup> monocytes (red, arrowheads) were immunostained to detect blood vessels with anti-CD31 antibodies (green, arrows). B, cryosections of lungs from the above mice injected with CMTMR-labeled human CD14<sup>+</sup> monocytes (red, arrowheads) were stained to detect nuclei (blue).

Although macrophages are found within tumors (Fig. 1), it is not clear how myeloid cells enter the tumor microenvironment. To determine how bone marrow–derived myeloid precursor cells traffic to the tumor microenvironment, we injected mice bearing Lewis lung carcinoma tumors with CMTMR-labeled human CD14<sup>+</sup> monocytes. Fluorescently labeled CD14<sup>+</sup> monocytes homed to Lewis lung carcinoma tumors and extravasated into the tumor tissue (Fig. 3A). Few CD14<sup>+</sup> cells were found within other organs, including lungs (Fig. 3B).

**Integrin  $\alpha_4\beta_1$  is expressed by monocytes.** To determine how monocytes arrest in peripheral vasculature, we examined the expression and function of cell adhesion molecules in this process. Monocytes purified for CD14 expression are also CD14, CD11b, and integrin  $\alpha_4\beta_1$  positive (Fig. 4A). Monocytes purified by immune selection for CD11b (integrin  $\alpha_M\beta_2$ ) are both CD11b<sup>+</sup> (90%) and CD14<sup>+</sup> (90%) and express significant levels of integrins  $\alpha_4\beta_1$  (100%),  $\alpha_5\beta_1$  (100%),  $\alpha_v\beta_3$  (99%), and  $\alpha_v\beta_5$  (99%; Supplementary Fig. S4). Myeloid cells thus express several integrins, some of which have been shown to play roles in lymphocyte or progenitor cell homing (20, 23).

**Integrin  $\alpha_4\beta_1$  mediates myeloid cell adhesion to endothelium *in vitro*.** To determine whether monocyte integrins are functional, we examined the ability of these cells to attach to and migrate on various extracellular matrix proteins. CD11b<sup>+</sup> monocytes adhered to and migrated on CS-1 fibronectin, plasma fibronectin, vitronectin, and collagen. Adhesion to vitronectin was mediated by integrins  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$  (Supplementary Fig. S4), as function-blocking antibodies to these integrins, but not to other integrins, prevented cell attachment to this matrix proteins. Adhesion to plasma fibronectin was mediated primarily by  $\alpha_5\beta_1$  and secondarily by  $\alpha_v\beta_3$ , as function-blocking antibodies to  $\alpha_5\beta_1$  and  $\alpha_v\beta_3$ , but not to other integrins, prevented cell



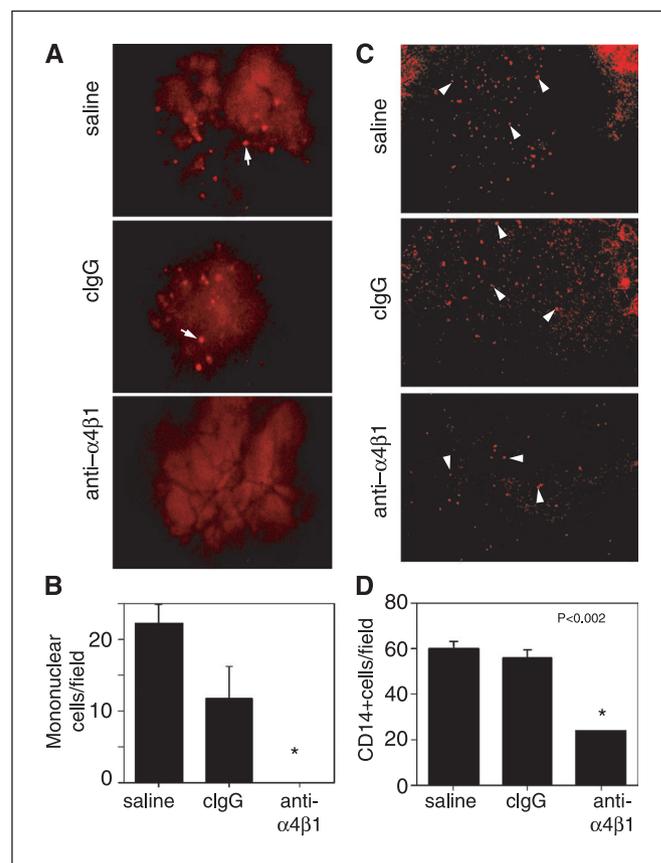
**Figure 4.** Integrin  $\alpha_4\beta_1$  mediates adhesion of human monocytes to endothelium. **A**, FACS profiles for CD11b, CD14, and  $\alpha_4\beta_1$  integrins on monocytes purified by immune selection for CD14 expression. Gray FACS profile represents binding of fluorescent nonspecific IgG. **B**, CD14<sup>+</sup> monocyte adhesion to recombinant CS-1 fibronectin in the presence of medium, anti-integrin  $\alpha_4\beta_1$  ( $P < 0.05$ ), anti-integrin  $\beta_1$  ( $P < 0.05$ ), or anti-integrin  $\beta_7$ . **C**, CD14<sup>+</sup> monocyte adhesion to human endothelial cell monolayers in the presence of medium, anti-integrin  $\alpha_4\beta_1$  ( $P < 0.05$ ), anti-integrin  $\beta_1$  ( $P < 0.05$ ), or anti-integrin  $\alpha_v\beta_5$ . \*,  $P < 0.05$ , statistically significant.

attachment to fibronectin (Supplementary Fig. S4). Adhesion to the CS-1 domain of cellular fibronectin was mediated only by integrin  $\alpha_4\beta_1$  (Supplementary Fig. S4). Because integrin  $\alpha_4\beta_1$  is also a receptor for the immunoglobulin superfamily molecule VCAM that is expressed by activated endothelium (20), we also examined the ability of monocytes to attach to plates coated with recombinant soluble VCAM. Antagonists of  $\alpha_4\beta_1$  blocked monocyte attachment to VCAM, indicating that integrin  $\alpha_4\beta_1$  is a functionally active receptor for both CS-1 fibronectin and VCAM (Supplementary Fig. S4). To determine whether CD11b<sup>+</sup> monocytes can attach to proliferating vascular endothelium that has been stimulated by angiogenic growth factors, we plated fluorescently labeled monocytes onto endothelial monolayers *in vitro*. Monocytes bound strongly to endothelium and antibody antagonists of  $\alpha_4\beta_1$  as well as recombinant soluble VCAM blocked cell attachment to endothelial monolayers (Supplementary Fig. S4). In contrast, antibody antagonists of other integrins, including  $\beta_2$ ,  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$ , or  $\alpha_5\beta_1$ , did not inhibit attachment to endothelium (Supplementary Fig. S4). Similarly, CD14<sup>+</sup> cells adhered to CS-1 fibronectin (Fig. 4B) and endothelium (Fig. 4C) *in vitro* in an  $\alpha_4\beta_1$ -dependent manner. Thus,  $\alpha_4\beta_1$  mediates the attachment of monocytes to both CS-1 fibronectin and vascular endothelium.

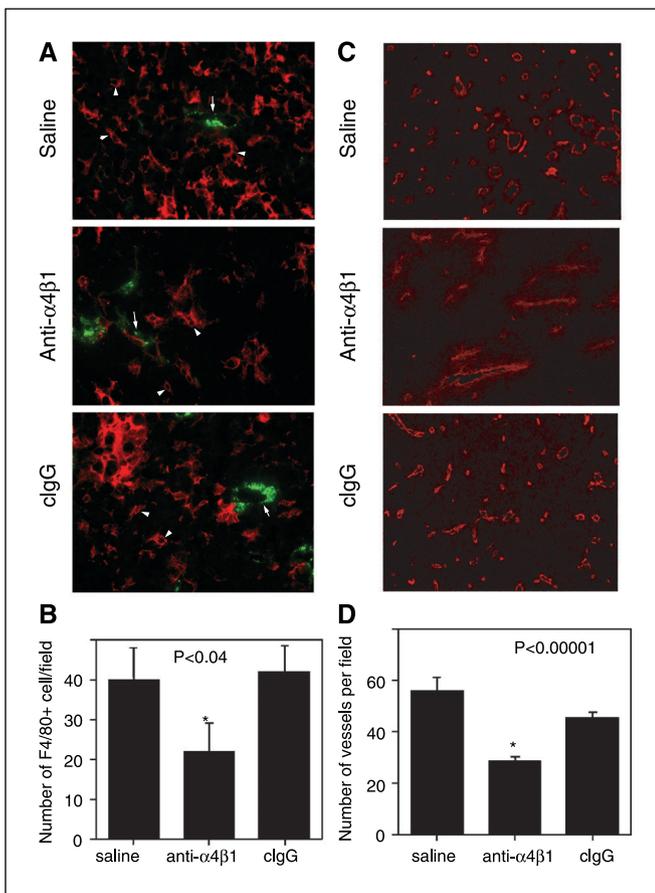
**Integrin  $\alpha_4\beta_1$  mediates myeloid cell adhesion to endothelium *in vivo*.** To examine the role of integrins in the homing of monocytes to tumors, we injected fluorescently labeled human monocytes into mice bearing human breast carcinomas under cover glasses in the presence of antibody antagonists of human

integrins. In this model, only integrins expressed on the injected human cells could be affected by the antagonists of human integrins. Within 15 minutes, monocytes had homed to the tumor vasculature and had adhered *in vivo*. In the presence of integrin  $\alpha_4\beta_1$  antagonists, however, *in vivo* adhesion was blocked (Fig. 5A and B). To examine the role of integrins in the homing of monocytes to lung carcinomas, we injected fluorescently labeled human monocytes purified by immune selection for expression of CD14 into nude mice with Lewis lung carcinomas in the presence of antibody antagonists of human integrins. We found that inhibitors of integrin  $\alpha_4\beta_1$ , but not of integrin  $\alpha_v\beta_3$  (or other integrins; data not shown), blocked cell homing to the tumor vasculature (Fig. 5C and D). These studies indicate that integrin  $\alpha_4\beta_1$  expressed by the injected circulating monocytes regulates homing of human monocytes to tumor neovasculature *in vivo*.

**Integrin  $\alpha_4\beta_1$  promotes tumor invasion by macrophages and subsequent angiogenesis.** To determine whether  $\alpha_4\beta_1$  promotes invasion of murine tumors by murine macrophages *in vivo*, we treated animals bearing Lewis lung carcinoma tumors with antagonists of integrin  $\alpha_4\beta_1$  or isotype-matched control



**Figure 5.** Integrin  $\alpha_4\beta_1$  promotes monocyte homing to tumors. **A**, intravital images at  $\times 200$  of tumors in animals injected with CMTMR-labeled human mononuclear cells (red, arrowheads) in the presence of saline, anti- $\alpha_4\beta_1$ , or isotype-matched control anti-integrin antibody (anti- $\alpha_v\beta_3$ ). **B**, average number  $\pm$  SE of CMTMR-labeled mononuclear cells per  $\times 200$  field for saline, anti- $\alpha_v\beta_3$  (clgG), and anti- $\alpha_4\beta_1$  treated animals observed by intravital imaging. \*,  $P < 0.002$ , statistically significant. **C**, cryosections of lung carcinomas from mice injected with CMTMR-labeled human CD14<sup>+</sup> monocytes (red, arrowheads) and treated with saline, anti-human  $\alpha_v\beta_3$  (clgG), or anti-human  $\alpha_4\beta_1$  antibodies. **D**, average number  $\pm$  SE of CMTMR-labeled CD14<sup>+</sup> monocytes per  $\times 200$  field for saline, anti- $\alpha_v\beta_3$ , and anti- $\alpha_4\beta_1$  treated animals. \*,  $P < 0.002$ , statistically significant.



**Figure 6.** Integrin  $\alpha_4\beta_1$  promotes macrophage invasion of murine tumors. *A*, cryosections of Lewis lung carcinoma tumors from animals treated with saline, anti- $\alpha_M\beta_2$  (*cIgG*), or anti- $\alpha_4\beta_1$  antibodies were immunostained with anti-CD31 (*green*) or anti-F4/80 (*red*). Magnification,  $\times 400$ . *B*, average number of F4/80<sup>+</sup> macrophages in animals with Lewis lung carcinoma tumors that were treated with saline, anti- $\alpha_M\beta_2$  (*cIgG*), or anti- $\alpha_4\beta_1$ . \*,  $P < 0.04$ , statistically significant. *C*, cryosections of Lewis lung carcinoma tumors from (*A*) were immunostained with anti-CD31 (*red*) to identify blood vessels. Magnification,  $\times 200$ . *D*, average number of CD31<sup>+</sup> blood vessels in animals with Lewis lung carcinoma tumors that were treated with saline, anti- $\alpha_M\beta_2$  (*cIgG*), or anti- $\alpha_4\beta_1$ . \*,  $P < 0.00001$ , statistically significant.

anti-integrin antibodies. Treatment with integrin  $\alpha_4\beta_1$  antagonists, but not other antagonists, significantly suppressed the number of F4/80<sup>+</sup> macrophages within tumors (Fig. 6*A* and *B*). These antagonists also reduced blood vessel density within treated tumors (Fig. 6*C* and *D*) and partially suppressed tumor growth (Supplementary Fig. S5). Additionally, antagonists of integrin  $\alpha_4\beta_1$  suppressed the number of tumor-associated macrophages in a mouse model of colon carcinoma growth (data not shown). These studies therefore indicate that integrin  $\alpha_4\beta_1$  regulates monocyte/macrophage invasion of tumors and subsequent neovascularization.

## Discussion

**Significance of integrin  $\alpha_4\beta_1$ -mediated monocyte homing to tumors.** Our studies show that integrin  $\alpha_4\beta_1$  promotes the homing of myeloid cells/macrophages to tumor tissue. This work identifies a key mechanism regulating myeloid cell homing to tumor tissues. Once localized in a tumor, myeloid cells/macrophages can promote tumor growth and spread by expressing

growth factors that stimulate angiogenesis and lymphangiogenesis (1–5, 14). Our work shows that blocking macrophage homing to tumors may suppress macrophage-induced tumor angiogenesis.

Integrin  $\alpha_4\beta_1$ -VCAM interactions promote important cell adhesion events *in vivo*. This integrin promotes fusion of the chorion with the allantois and fusion of endocardium with myocardium during early embryonic development (26, 27). Integrin  $\alpha_4\beta_1$  and fibronectin or VCAM interactions also regulate T-cell and natural killer cell trafficking (19, 20, 28) as well as circulating cell homing to the bone marrow (21–23). The studies presented here indicate a key role for this pair of molecules in the homing of monocytes/macrophages to tumors.

Our results suggest that other integrins do not play a large role in monocyte homing to tumor neovasculature. Because myeloid cells express functional integrins  $\alpha_2\beta_1$ ,  $\alpha_4\beta_1$ ,  $\alpha_5\beta_1$ ,  $\alpha_V\beta_3$ ,  $\alpha_V\beta_5$ ,  $\alpha_M\beta_2$ , and  $\alpha_X\beta_2$  as well as other adhesion proteins, it has been unclear how these cells enter the tumor microenvironment from the circulation. Our studies clearly show that integrin  $\alpha_4\beta_1$  plays the major role in homing to tumors. Although CD11b (integrin  $\alpha_M\beta_2$ ) has been shown previously to promote trafficking of myeloid cells into inflamed tissues (29), our studies suggest that this integrin does not regulate trafficking to tumors.

In studies of myeloid cell homing to tumors, we observed that these cells traveled throughout the vessels of the tumor but only attached within the tumor vessels at the periphery of the tumor. Analyses of cell homing showed the role of integrin  $\alpha_4\beta_1$  in homing of myeloid cells to tumors. Immunohistochemistry of cryosections shows myeloid cells distributed extensively in tumor tissue. Thus, our studies indicate that myeloid cells home to tumors and extravasate into tumor tissue.

We found that inhibition of integrin  $\alpha_4\beta_1$  *in vivo* blocks trafficking of myeloid cells while inhibiting overall tumor angiogenesis in the Lewis lung carcinoma model by  $\sim 50\%$  and tumor growth by  $\sim 25\%$ . These results suggest that macrophages contribute to tumor angiogenesis and that blocking their homing may help in suppressing tumor angiogenesis. Although it is generally held that tumor angiogenesis results from tumor cell and stromal cell secretion of proangiogenic growth factors, our studies and those of others suggest that macrophages play an important supportive role in this process. For example, recent studies have shown an important role for myeloid cells in promoting angiogenesis in response to wounds and ischemia (15). Thus, antagonists of macrophage-mediated angiogenesis promise to be useful in combination with inhibitors of sprouting angiogenesis to thoroughly suppress tumor angiogenesis.

Our studies show that integrin  $\alpha_4\beta_1$  promotes myeloid cell homing to tumors and that inhibitors of this integrin can suppress both monocyte/macrophage accumulation and angiogenesis in tumors. Our studies suggest that suppression of monocyte/macrophage homing to tumors may be a useful supplementary approach to suppress macrophage-mediated tumor angiogenesis and growth.

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