Dual Role of the Leukocyte Integrin $\alpha_M\beta_2$ in Angiogenesis

Dmitry A. Soloviev, Stanley L. Hazen, Dorota Szpak, Kamila M. Bledzka, Christie M. Ballantyne, Edward F. Plow and Elzbieta Pluskota

_J Immunol_ 2014; 193:4712-4721; Prepublished online 26 September 2014;
doi: 10.4049/jimmunol.1400202

http://www.jimmunol.org/content/193/9/4712

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2014/09/26/jimmunol.1400202.DCSupplemental

**References**

This article cites 46 articles, 20 of which you can access for free at:
http://www.jimmunol.org/content/193/9/4712.full#ref-list-1

**Subscription**

Information about subscribing to _The Journal of Immunology_ is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Dual Role of the Leukocyte Integrin \(\alpha_{M}\beta_2\) in Angiogenesis

Dmitry A. Soloviev,* Stanley L. Hazen,† Dorota Szpak,* Kamila M. Bledzka,* Christie M. Ballantyne,‡ Edward F. Plow,* and Elzbieta Pluskota*

Polymorphonuclear neutrophils (PMNs) and macrophages are crucial contributors to neovascularization, serving as a source of chemokines, growth factors, and proteases. \(\alpha_{M}\beta_2\) (CD11b/CD18) and \(\alpha_\beta_2\) (CD11a/CD18) are expressed prominently and have been implicated in various responses of these cell types. Thus, we investigated the role of these \(\beta_2\) integrins in angiogenesis. Angiogenesis was analyzed in wild-type (WT), \(\alpha_L\)-knockout (\(\alpha_{M}^{-/-}\)), and \(\alpha_L\)-deficient (\(\alpha_{L}^{-/-}\)) mice using B16F10 melanoma, RMI prostate cancer, and Matrigel implants. In all models, vascular area was decreased by 50–70% in \(\alpha_{M}^{-/-}\) mice, resulting in stunted tumor growth as compared with WT mice. In contrast, \(\alpha_L\) deficiency did not impair angiogenesis and tumor growth. The neovessels in \(\alpha_{M}^{-/-}\) mice were leaky and immature because they lacked smooth muscle cell and pericytes. Defective angiogenesis in the \(\alpha_{M}^{-/-}\) mice was associated with attenuated PMN and macrophage recruitment into tumors. In contrast to WT or the \(\alpha_2^{-/-}\) leukocytes, the \(\alpha_{M}^{-/-}\) myeloid cells showed impaired plasmin (Plm)-dependent extracellular matrix invasion, resulting from 50–75% decrease in plasminogen (Plg) binding and pericellular Plm activity. Surface plasmon resonance verified direct interaction of the \(\alpha_M\)-domain, the major ligand binding site in the \(\alpha_2\) integrins, with Plg. However, the \(\alpha_L\)-domain failed to bind Plg. In addition, endothelial cells failed to form tubes in the presence of conditioned medium collected from TNF-\(\alpha\)-stimulated PMNs derived from the \(\alpha_{M}^{-/-}\) mice because of severely impaired degranulation and secretion of VEGF. Thus, \(\alpha_{M}\beta_2\) plays a dual role in angiogenesis, supporting not only Plm-dependent recruitment of myeloid cells to angiogenic niches, but also secretion of VEGF by these cells. The Journal of Immunology, 2014, 193: 4712–4721.

B one marrow–derived myeloid cells, particularly polymorphonuclear neutrophils (PMNs) and macrophages, are key regulators of tumor progression and metastasis. One of the major tumor promoting functions of these cells is their facilitation of angiogenesis (reviewed in Refs. 1, 2). PMNs and macrophages contribute to angiogenesis via a variety of well-established mechanisms. One example is their capacity to produce and secrete a variety of proangiogenic factors such as vascular endothelial growth factor A (VEGF-A), fibroblast growth factor (FGF), IL-8, IL-10, CXCL1/GRO, and COX-2 (3, 4). In addition, PMNs and macrophages are a rich source of numerous proteases, including neutrophil elastase, cathepsin G, and several metalloproteinases, which are crucial for extracellular matrix (ECM) degradation and remodeling and regulate the bioavailability of various proangiogenic stimuli (5), all requisite events in angiogenesis (reviewed in Refs. 4, 6–8). In addition, PMNs and macrophages secrete urokinase-type plasminogen activator (uPA), which converts plasminogen (Plg) to plasmin (Plm). There are diverse Plg receptors on leukocyte surface (reviewed in Ref. 9), and bound Plm facilitates leukocyte migration/invasion by directly degrading ECM, and encourages leukocyte recruitment in a variety of in vivo models of inflammation (10–12).

\(\alpha_{M}\beta_2\) (CD11b/CD18) and \(\alpha_2\) (CD11a/CD18), the two most broadly studied members of the \(\beta_2\) integrin subfamily, are particularly enriched in PMNs and macrophages, where they regulate diverse cell functions, including migration, adhesion, the respiratory burst, and cytokine production (13). In addition, we have previously demonstrated that \(\alpha_M\beta_2\) enhances uPA-dependent Plg activation on the PMN surface (14, 15), which has the potential to influence their recruitment to inflammatory or angiogenic sites in vivo. Based on these observations, we hypothesized that \(\alpha_M\beta_2\) and \(\alpha_2\beta_2\), as key regulators of leukocyte functions, might be implicated in angiogenesis. In this study, we used \(\alpha_{M}^{-/-}\) and \(\alpha_2^{-/-}\) mice and three distinct in vivo angiogenesis models to show that \(\alpha_{M}\beta_2\), but not \(\alpha_2\beta_2\), is a critical contributor to angiogenesis. This function of \(\alpha_{M}\beta_2\) is mediated by two distinct mechanisms: 1) support of Plm-dependent PMN and macrophage recruitment to angiogenic niches; and 2) enhancement of leukocyte production and secretion of the primary angiogenic growth factor, VEGF-A.

Materials and Methods

Materials

Mouse VEGF165 and keratinocyte-derived cytokine (KC) were from Biosource International (Camarillo, CA); hepatarin was from Sigma-Aldrich (St. Louis, MO); biotin-conjugated anti-mouse CD31 mAb was from BD Pharmingen (San Jose, CA); rabbit anti-smooth muscle actin (SMA; Abcam, Cambridge, MA); rabbit anti-neutrophil/gliad gent (NG2; Millipore, Temecula, CA); rabbit anti-mouse laminin (Serotec, Oxford,
UK), goat anti-Fibrin II (Accurate Chemical, Westbury, NY), purified, or FITC-labeled rat anti-Ly6G, clone 1A8, specific for mouse PMNs were from BD Pharmingen (San Jose, CA); anti-mouse macrophage–monocyte mAb (MOMA-2) was from Chemicon (Temecula, CA); and rat LEAF TM purified anti-mouse α5 integrin (clone M1/70) was from BioLegend (San Diego, CA). Ghu-Plg was isolated from normal human plasma by affinity chromatography on lysine-sepharose followed by gel filtration. Growth factor–reduced Matrigel matrix was from BD Biosciences (San Diego, CA). Murine recombinant TNF-α was from R&D Systems. Cycloheximide and pentoxifylline were from Sigma-Aldrich.

**Mice**

The αɛ−/− mice were generated as described previously (16), and αɛ−/− mice were purchased from the Jackson Laboratory. Both strains were backcrossed for 12 generations into a C57BL/6J background. The study was conducted under protocols approved by the IACUC of the Cleveland Clinic.

**Angiogenesis models in vivo**

Eight- to twelve-week-old mice were injected s.c. with 10^6 murine B16F10 melanoma or RM1 prostate cancer cells. Tumors were collected 8–14 d after injection and were weighed, photographed, and processed for immunohistochemical staining. Endothelial cells (ECs) were identified using a biotinylated mouse CD31 mAb, SAs with anti-SMA Ab, pericytes with anti-NG2 Ab, fibrin with anti-Fibrin II Ab, basement membrane with anti-laminin Ab, PMNs with rat anti-Ly6G (clone 1A8), and monocytes–macrophages with MOMA-2 mAbs. Stained sections were analyzed using fluorescent imaging microscopy (Leica, Wetzlar, Germany) and ImagePro Plus Capture and Analysis software (Media Cybernetics). CD31, fibrin, and Ly6G- or MOMA2-positive area was quantified in 5–10 independent fields. The average area per field was determined from duplicate measurements of each of the fields analyzed. Matrigel plug assay was performed as described (17). Mice were injected with 500 μl growth factor-reduced Matrigel mixed at 4°C with heparin (26 U/ml) alone or with KC (500 ng/ml) or VEGF 165 (100 ng/ml; R&D Systems). Matrigel plugs were harvested 8 d after injection and snap-frozen, and 8-μm sections were processed for immunohistochemical analyses as described above. In αɛ−/− integrin blocking experiments, WT mice were injected intravenously with rat LEAF anti-mouse α5 integrin (clone M1/70; BioLegend) or isotype-matched normal rat IgG (3.5 mg/kg), 4 h before and then 2, 4, and 6 d after Matrigel-KC implantation. The Matrigel plugs were collected 8 d after injection, sectioned, and stained with anti-CD31 to examine vascular formation.

**Bone marrow transplantation**

Two-month-old recipient mice were lethally X-irradiated with a total dose of 9 Gy and reconstituted with i.v. injection of 10^7 bone marrow (BM) cells isolated from the femurs of donor mice. Mice were used 6–8 wk after BM transplantation (BMT). Engraftment efficiency was examined 6 wk after BMT in chimeric αɛ−/−→WT and WT→αɛ−/− mice using WT and αɛ−/− mice, which did not undergo BMT as controls did. Single-cell suspensions from spleens and thymuses from these mice were prepared, and percentages of individual leukocyte subsets were measured with flow cytometry using FITC-labeled Abs to cell-specific markers (Ly-6G for PMN, F4/80 for macrophages, CD19 for B lymphocytes, CD3 for T lymphocytes) and FITC-labeled isotype-matched Abs as controls.

**Plg binding to αɛ and αLI domains**

Glutathione S-transferase (GST)-fused αɛL and αLI domains were purified with glutathione chromatography. Real-time protein–protein interactions were analyzed using a Biacore 3000 instrument (Biacore AB). Plg was immobilized on CM5 biosensor chips by amine coupling. Experiments were performed at 22°C in 10 mM HEPES buffer (pH 7.4) containing 150 mM NaCl and 0.005% surfactant P20 (flow rate, 25 μl/min). Surface plasmon resonance (SPR) sensograms were obtained by injecting various concentrations of GST-tagged αɛL or αLI domain over immobilized Plg and reference flow cells. Surfaces were regenerated with 30-s pulses of 10 mM NaOH. Association–disassociation curves were determined after the subtraction of the reference surface values and buffer binding at six selected concentrations. Sensorgrams were analyzed using BIAevaluation software (version 4.01; GE Healthcare).

**PMN and macrophage isolation**

Mouse PMNs for use in mouse aortic endothelial cell (MAEC) tube formation and Plg activation assays (see below) were isolated from blood drawn from hearts of anesthetized animals into sterile acid-citrate-dextrose (1:7 volume 145 mM sodium citrate, pH 4.6, and 2% dextrose). Blood was transferred to 1.25% dextran T500 solution (1:9) to sediment erythrocytes for 30 min at room temperature (14, 18). Leukocyte-rich supernatants were washed with PBS once, and PMNs were isolated by magnetic positive selection using mouse anti-Ly6G MicroBead Isolation Kit (Miltenyi Biotech, Auburn, CA) according to the manufacturer’s instructions. Eluted cells were 98% granulocytes, of which more than 96% were neutrophils and 1–2% were eosinophils. Contaminating lymphocytes and monocytes were less than 2% as determined by Wright staining. PMN viability was >98% as determined by trypan blue staining. The PMN yield was usually ∼4×10^6 per mouse, and blood pooled from 10–15 mice was used. For Plg activation assays, lymphocytes and monocytes were collected from buffy coats and washed twice with the HBSS buffer. Leukocytes were obtained from blood pooled from 7–10 mice.

For matrix invasion and Plg binding assays, macrophages and PMNs were isolated from peritoneal lavages. PMNs at 6 h and macrophages at 72 h after i.p. thioglycolate injection, when their recruitment was at the highest levels for each cell type (10). PMNs constitute 92% and macrophages constitute 90% of all cells in the 6- and 72-h peritoneal lavages, respectively. PMNs and macrophages harvested from lavages are referred to as “peritoneal PMN” or “peritoneal macrophages” in the manuscript to distinguish them from blood cells.

**Matrix invasion assays ex vivo**

Prechilled tissue culture inserts with porous (8-μm pore size) polyester membrane (Costar, Corning, NY) were coated with 50 μg/ml Matrigel (0.5 mg/ml) overnight at 4°C. Next a matrix was rehydrated with 600 μl DMEM F-12 for 1 h. Peritoneal PMNs or macrophages were suspended in serum-free DMEM F-12 medium (Life Technologies, Carlsbad, CA), and added to matrix-coated inserts (1×10^6/ml), which were placed in a 24-well plate containing serum-free DMEM F-12 supplement with or without 100 ng/ml KC. Plg (90 μg/ml) was added to appropriate controls, and cells were incubated for 18 h at 37°C. Assays were stopped by removing the inserts and washing the inside of a cotton swab to remove nonmigrated cells. The migrated cells were quantified using the Cyquant Cytoskeleton Proliferation Kit (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

**Plg activation on the leukocyte surface**

Peripheral blood PMNs or lymphocyte–monocyte mixtures were incubated with KC (100 ng/ml) for 1 h at 37°C in the presence of 25 nM single-chain uPA (sc-uPA) in 10 mM Tris-Cl buffer (pH 7.4) containing 0.14 mM NaCl, 0.1% BSA, 1 mM CaCl_2 and 1 mM MgCl_2. The cells were washed three times, and 50 μl of the cell suspension (1×10^6 cells/well) were added to each well of the microtiter plates. Peritoneal macrophages, which had not been incubated with KC or sc-uPA, were also added to the plates. Next, 100 μl of a Glu-Plg (1 μM) and Plg-specific fluorescent substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (2 mM) mixture was added, and Plm formation was monitored over 45 min at 37°C at αε = 370 nm and αɛ = 470 nm using a fluorescence plate reader (SpectraMax GeminiXS; Molecular Devices).

**Regulation of VEGF by PMNs**

Peripheral blood WT, αɛ−/− or αLI−/− PMNs were incubated in 24-well tissue culture plates (Costar, 3×10^5 cells/well) in 250 μl DMEM F-12 medium in the absence or presence of TNF-α (20 ng/ml) for 2 h at 37°C. The inhibitors of protein synthesis (cycloheximide-10 μg/ml) or PMN degranulation (pentoxifylline, 3.7-dimethyl-1-[5-oxohexyl]-xantine, 300 μM) were added to PMNs 60 min before the addition of TNF-α. Supernatants were collected and centrifuged at 1500 rpm in a Beckman GS-6 centrifuge for 10 min, and VEGF was measured using mouse VEGF Quantikine ELISA Kit (R&D Systems). In parallel, lactoferrin concentrations were measured in PMN supernatants using mouse Lactoferrin LTF/ELISA Kit (Cusabio).

**Quantitative real-time PCR**

Total RNA was isolated from peripheral blood mouse PMNs, either resting or stimulated with TNF-α (20 ng/ml) for 2 h at 37°C. The inhibitors of protein synthesis (cycloheximide-10 μg/ml) or PMN degranulation (pentoxifylline, 3.7-dimethyl-1-[5-oxohexyl]-xantine, 300 μM) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and random hexamers, according to the manufacturer’s protocol. Total RNA (4 μg) was reverse transcribed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen) and random hexamers, according to the manufacturer’s protocol. Real-time quantitative PCR of the CDNA template was performed with an iCycler (Bio-Rad). VEGF and GAPDH primers were from SABiosciences (Frederick, MD). The PCR contained 150 ng of CDNA, 10 μM forward and reverse primers, and 12.5 μl 2× RT2 Real Time SYBR Green PCR Master mix (SABiosciences) in a total volume of 25 μl. All
PCR were performed in triplicate. Results were calculated as expression of the target gene relative to expression of the reference gene (GAPDH).

MAE tube formation assay

Twenty-four-well tissue culture plates were coated with 250 µl Growth Factor-Reduced Matrigel (BD Biosciences, San Diego, CA) and incubated at 37°C for 30 min. When the Matrigel solidified, 1.25 × 10⁶ of WT MAECs were added to each well in 250 µl of chosen PMN-conditioned DMEMF-12 medium obtained as described in Regulation of VEGF by PMNs and supplemented with 10% FBS and 90 µg/ml heparin. Inhibitors of VEGF, neutralizing LEAF rat anti-mouse VEGF mAb (BioLegend), isotype matched rat IgG₂a (100 µg/ml; BioLegend), and recombinant mouse soluble VEGF receptor-1 (sFLT-1; 100 ng/ml; R&D Systems) were preincubated for 60 min with conditioned media of WT TNFα-stimulated PMNs before its addition to MAECs. Live time-lapse photography was performed for 12 h, using 5-min intervals on a Leica DMIRM Inverted Microscope equipped with a Roper Scientific CoolSNAP HQ Cooled CCD camera, a temperature controller, and a CO₂ incubation chamber. Snapshots were taken using MetaMorph Software. Tube formation was analyzed and quantified using ImageJ software program 1.34.

Statistical analysis

Data are expressed as mean ± SEM. To determine significance, a one-way ANOVA test was performed to compare angiogenic responses between the three mouse genotypes, and a two-tailed Student t test was performed for comparisons between WT and the αMβ2 mice using the Sigma-Plot software program (SPSS); p < 0.05 was considered to be statistically significant.

Results

Integrin αMβ2 is critical in angiogenesis in vivo

Leukocytes, particularly PMNs and macrophages, are important supporters of angiogenesis as a source of proteases and angiogenic factors (reviewed in Refs. 1, 2). The β2 integrins are crucial in the regulation of a variety of leukocyte responses, including adhesion, migration, and cytokine production (reviewed in Ref. 13). Accordingly, we examined the role of two prominent members of the β2 integrin subfamily, αMβ2 and αβ2, in angiogenesis. These two integrins share the same β2 subunit, and their α-subunits are 49% identical (19). Angiogenesis was analyzed in the αMβ2−/−, αβ2−/−, and control WT mice using two tumor models, murine B16F10 melanoma, and RM1 prostate cancer. These tumors are highly vascularized, and their growth is heavily dependent on an angiogenic response (20, 21). Staining of tumor sections for endothelial cells with CD31 (green fluorescence) revealed normal, well-developed, thick vasculature in tumors grown in the αMβ2−/− and WT mice. In contrast, angiogenesis was significantly impaired in αMβ2−/− mice as only a few short and thin vessel-like structures were observed in melanomas and prostate tumors from these mice (Fig. 1A). Blood vessel area of the melanoma sections was attenuated by ~70% in the αMβ2−/− compared with tumors in the αMβ2−/− and WT mice (p < 0.01, n = 8 per group; Fig. 1B, upper panel). In addition, significantly reduced vascular area was observed in RM1 prostate tumors grown in αMβ2−/− mice compared with αMβ2−/− and WT mice: 6200 µm² ± 1000 versus 12800 ± 2000 in αMβ2−/− and 12000 ± 2150 µm² in WT mice (p < 0.01, n = 8 per group; Fig. 1B, lower panel). Consistent with the blunted angiogenic response in the αMβ2−/− mice, melanomas grown in these mice were 70–80% smaller (p < 0.01, n = 7 per group) than those derived in the αMβ2−/− and WT mice: 40 ± 10 mg in αMβ2−/− versus 202 ± 42 mg in WT and 178 ± 34 mg in αMβ2−/− mice. In addition, the average weight of RM1 prostate tumors recovered from the αMβ2−/− mice was ~40–50% lower than from the αMβ2−/− and WT mice (p < 0.05, n = 8 per group): 295 ± 30 mg in αMβ2−/− versus 497 ± 80 mg in WT and 570 ± 100 mg in αMβ2−/− mice (Fig. 1C, 1D).

Impaired angiogenesis in the αMβ2−/− mice was corroborated using Matrigel as a third angiogenic model. Mice were injected with Matrigel alone or with Matrigel supplemented with VEGF or KC (keratinocyte-derived factor) to stimulate angiogenesis. CD31 staining of Matrigel plugs containing VEGF or KC revealed well-formed vasculature in the implants in WT and αMβ2−/− mice, whereas the Matrigel plugs from αMβ2−/− mice showed no distinct vascular formations, although a few CD31-positive ECs were discerned within the plugs (Fig. 1E). Regardless of the angiogenic factor used, blood vessel area in the Matrigel implants in the αMβ2−/− mice was reduced by ~75% compared with the implants from the αMβ2−/− and WT animals (p < 0.01, n = 8 per group; Fig. 1F). In control Matrigel plugs without proangiogenic cytokines, no blood vessels were detected in any of the three mouse genotypes tested (data not shown).

Neovascularization in αMβ2−/− mice is immature

The presence of smooth muscle cells and pericytes within vascular structures is a key indicator of its maturity because they stabilize blood vessels. We double-stained melanoma and prostate tumor sections with Abs to CD31 and to SMA (Fig. 2A, top panel) or to NG2 chondroitin sulfate proteoglycan, a marker of pericytes (Fig. 2A bottom panel). In prostate tumors grown in WT and αMβ2−/− mice 30–37% of total CD31+ blood vessels stained for SMA, whereas only 15% of blood vessels formed in αMβ2−/− mice expressed this marker (p < 0.02, n = 8; Fig. 2B). Costaining for CD31 and NG2 revealed reduced pericytes interacting with blood vessels in prostate tumors in αMβ2−/− mice as compared with WT and αMβ2−/− mice (25 ± 8 µm versus 30–55 ± 10 µm; p < 0.05, n = 60; 4 mice per group; Fig. 2D, top panel, and 2E). Immunostaining for laminin showed a 50% reduction in basement membrane thickness of blood vessels in αMβ2−/− mice (2.7 ± 0.3 µm) compared with WT and αMβ2−/− mice (5.8–6.1 ± 1.3 µm; p < 0.03, n = 60; 4 mice per group; Fig. 2D, lower panel, and 2F). With decreased maturation and laminin deposition, we considered that vasculature in αMβ2−/− mice might be leaky. Plasma leakage measured as an area that is positive for plasma-derived fibrin was enhanced by 2.5-fold in tumors grown in αMβ2−/− mice compared with WT and αMβ2−/− mice (13.8 ± 1.8% versus 5.2 ± 1.1% and 3.8 ± 0.7%, respectively; p < 0.03, n = 20; 4 mice per group; Fig. 2G, 2H). The vasculature in melanoma tumors grown in αMβ2−/− mice also showed reduced maturity and leakiness (data not shown). In addition, the permeability of preexisting blood vessels in dorsal skin of αMβ2−/− and WT mice was examined using Evans blue dye injected i.v. Baseline permeability upon injection of control PBS and VEGF-A–induced vascular permeability were similar in αMβ2−/− and WT mice, indicating that preexisting vasculature in αMβ2−/− mice was normal (Supplemental Fig. 1).

Impaired PMN and macrophage recruitment to angiogenic sites in αMβ2−/− mice

The CD11b+Gr-1+ myeloid cells consisting primarily of PMNs are crucial enhancers of angiogenesis, and they contribute to angiogenic switch in many tumors (4, 5, 22). Tumor growth and angiogenesis were impaired in the αMβ2−/− (CD11b−/−) mice; therefore, we considered the possibility that this integrin regulates recruitment of these cells to growing tumors. First, we examined infiltration of CD11b+Gr-1+ cells in prostate and melanoma tumors in WT and αMβ2−/− mice by double staining with anti-CD11b (green) and anti-Ly6G (Gr-1) mAbs (red). As shown in Fig. 3A, 70–80% of CD11b+ cells were also positive for Gr-1, and numerous CD11b−Gr-1+ cells were detected in prostate tumors in...
WT and $\alpha_L^{-/-}$ mice, with no differences in their recruitment observed in the two mouse strains. Similar results were obtained with melanoma tumors (data not shown). An assessment of CD11b$^+$/Gr-1$^+$ cell recruitment to tumors in $\alpha_M^{-/-}$ (CD11b$^-$) mice was not feasible because of the absence of the $\alpha_M$ integrin subunit on these cells; therefore, we stained tumor sections only with PMN-specific anti-Ly6G (Gr-1) mAb. Indeed, PMN infiltration into both melanoma and prostate tumors was significantly reduced in the $\alpha_M^{-/-}$ mice compared with WT and $\alpha_L^{-/-}$ littermates (Fig. 3B). Quantification of Ly6G-positive areas in tumor sections verified these observations: 900 ± 210 $\mu$m$^2$ in $\alpha_M^{-/-}$ versus 3600 ± 1120 $\mu$m$^2$ in WT and 3950 ± 350 $\mu$m$^2$ in $\alpha_L^{-/-}$ mice in melanomas ($p < 0.02$, $n = 8$) and 1200 ± 265 $\mu$m$^2$ in $\alpha_M^{-/-}$ versus 6020 ± 840 $\mu$m$^2$ in WT and 7135 ± 1780 $\mu$m$^2$ in $\alpha_L^{-/-}$ mice in prostate tumors ($p < 0.01$, $n = 8$ per group; Fig. 3C). Next, tumor sections were stained with MOMA-2. Macrophage infiltration into both tumors was decreased by 50–60% in the $\alpha_M^{-/-}$ mice as compared with WT and $\alpha_L^{-/-}$ littermates (Fig. 3D, 3E). In melanomas grown in the $\alpha_M^{-/-}$ mice, macrophage-positive area was 1820 ± 160 $\mu$m$^2$ compared with 5610 ± 985 $\mu$m$^2$ in WT and 5240 ± 450 $\mu$m$^2$ in $\alpha_L^{-/-}$ mice, whereas in prostate tumors it was 3600 ± 750 $\mu$m$^2$ in $\alpha_M^{-/-}$ mice versus 7500 ± 1100 $\mu$m$^2$ in WT and 7280 ± 1300 $\mu$m$^2$ in $\alpha_L^{-/-}$ littermates ($p < 0.05$, $n = 8$ per group; Fig. 3E). In addition, staining of Matrigel implant sections with anti-PMN and MOMA-2 Abs revealed robust VEGF- and KC-dependent leukocyte infiltration into the centers of the implants in WT and $\alpha_L^{-/-}$ mice, whereas it was inhibited in the $\alpha_M^{-/-}$ mice (data not shown).

**BMT and suppression with blocking Abs confirm the importance of $\alpha_M\beta_2$ in angiogenesis**

Next, we sought to confirm that reduced tumor growth and angiogenesis in the $\alpha_M^{-/-}$ mice are due to impaired functions of bone marrow-derived cells, including leukocytes, and are not caused by defective vascular cells. Therefore, we performed BMT experiments and examined growth and angiogenesis in RM1 tumors. Transplantation of $\alpha_M^{-/-}$ BM into WT hosts ($\alpha_M^{-/-} \rightarrow$ WT) resulted in reduced RM1 tumor growth and angiogenesis (tumor weight: $p = 0.0169$; vascular area: $p = 0.028$ for $\alpha_M^{-/-} \rightarrow$ WT versus WT→WT; $n = 5$; Fig. 4A, 4B). Alternatively, transplantation of WT...
BM into the αM<sup>-/-</sup> hosts (WT→αM<sup>-/-</sup>) restored growth and angiogenesis of prostate tumors to those of control WT mice receiving WT bone marrow (RM1 weight: p = 0.0183; vascular area: p = 0.0112) for WT→αM<sup>-/-</sup> versus αM<sup>-/-</sup>→WT; n = 5; Fig. 4A, 4B). These results suggest that blunted tumor growth and angiogenesis in the αM<sup>-/-</sup> mice is a consequence of altered bone marrow-derived cell functions. In addition, image analysis of RM1 tumor sections stained with PMN-specific anti-Ly6G and anti-monocyte–macrophage MOMA-2 Abs revealed impaired recruitment of these cells to tumors grown in WT recipients receiving αM<sup>-/-</sup> BM as compared with control WT→WT mice, indicating a crucial role of αMβ2 in this process (p = 0.0184 for PMNs [Ly6G] and p = 0.0167 for MOMA-2; n = 5; Fig. 4C, 4D). In contrast, transplantation of WT BM to αM<sup>-/-</sup> recipients restored not only angiogenesis, but also PMN and macrophage migration into tumors (PMNs: p = 0.021; MOMA-2: p = 0.0454 for WT→αM<sup>-/-</sup> versus αM<sup>-/-</sup>→WT; n = 5; Fig. 4C, 4D). To exclude the possibility that αM deficiency might impair recovery of the immune system upon BMT, we assessed engraftment efficiency 6 wk after BMT in chimeric αM<sup>-/-</sup>→WT and WT→αM<sup>-/-</sup> mice using WT and αM<sup>-/-</sup> mice not undergoing BMT as controls. We have collected spleens and thymuses from these mice, prepared single-cell suspensions, and measured percentages of PMN, macrophage, B cells, and T cells by flow cytometry using FITC-labeled Abs to cell specific markers (Ly-6G, F4/80, CD19, CD3, respectively) and FITC-labeled isotype-matched Abs as controls. We found that the content of individual leukocyte subsets was similar in respective organs in both chimeric mouse lines as well as in control mice (no BMT), indicating that αM deficiency did not affect BMT engraftment efficiency (Supplemental Table I). In addition, flow cytometry of circulating total leukocytes with anti-mouse αM mAb confirmed its absence in the αM<sup>-/-</sup>→WT chimeras and its presence in the WT→αM<sup>-/-</sup> mice (data not shown).

To confirm the importance of the αM integrin in angiogenesis, WT mice were injected with a rat anti-αM blocking Ab (clone M1/70) that has been shown to inhibit neointima formation in rabbits specifically (23). These neutralizing Abs bind to the ligand-binding site within the αM subunit and inhibit its interactions with ligands thereby mimicking the gene ablation. Matrigel implants were harvested after 8 d, and the Ab was injected before Matrigel injection and then every 2 d. Vasculature was analyzed in Matrigel sections stained with anti-CD31 mAb (Fig. 4E). The anti-αM Ab reduced KC-induced angiogenesis in Matrigel implants by ~60–80% as compared with noninjected mice, whereas isotype-matched normal rat IgG<sub>2b</sub> had no effect indicating specificity (p < 0.05 mice injected with anti-αM versus not injected; n = 4 mice per group; Fig. 4E, 4F). These experiments verify the importance of αMβ2 on myeloid cells in tumor-induced angiogenesis via regulation of leukocyte recruitment to sites of neovascularization.

**FIGURE 2.** Neovascularure in prostate tumors in αM<sup>-/-</sup> mice is immature and leaky. Immunohistochemistry and image analyses of RM1 prostate tumors implanted into WT, αM<sup>-/-</sup>, and αL<sup>-/-</sup> mice. (A) Costaining for SMA (green) and CD31 (red; top panel) and for NG2 (green) and CD31 (red; bottom panel) in RM1 prostate tumors. Nuclei are stained with DAPI. Scale bars, 50 μm. (B and C) Quantification of the data presented in (A), top and bottom panels, respectively. Data are expressed as mean ± SEM and are representative of three independent experiments (n = 8 mice/group). (D) CD31-stained (top panel) and laminin-stained (bottom panel) blood vessels in tumors grown in WT, αM<sup>-/-</sup>, and αL<sup>-/-</sup> mice. Scale bars, 50 μm (top panel) and 25 μm (lower panel). (E) Vessel diameter was measured in 60 tumor vessels cut perpendicular to their longitudinal axis in 8-μm-thick sections, stained for CD31 from each host (WT, RM1 prostate tumors. Nuclei are stained with DAPI. Scale bars, 50 and laminin-stained (bottom panel) blood vessels in tumors grown in WT, αM<sup>-/-</sup>, and αL<sup>-/-</sup> mice. Scale bars, 50 μm (top panel) and 25 μm (lower panel). (F) Thickness of laminin-positive basement membrane in blood vessels formed in WT, αM<sup>-/-</sup>, and αL<sup>-/-</sup> mice. Data are mean ± SEM (n = 60) and are representative of three independent experiments including four mice per group. (G) Representative photograph of fibrin content (brown) in tumors grown in WT, αM<sup>-/-</sup>, and αL<sup>-/-</sup> mice. Tumor sections were stained with anti-fibrin Ab. Data are means ± SEM (n = 8) and are representative of three independent experiments including eight mice per group.
\( \alpha_M \beta_2 \) facilitates leukocyte recruitment to angiogenic sites via interaction with Plg and enhancement of Plg activation

As Plm-dependent ECM proteolysis greatly facilitates leukocyte invasion (10–12), we hypothesized that the inability of \( \alpha_M^{-/-} \) leukocytes to invade tumors could be caused by impaired Plg activation on the leukocyte surface. Because \( \alpha_M \beta_2 \) and \( \alpha_L \beta_2 \) are the most abundant \( \beta_2 \)-integrins on PMNs and macrophages, we used a modified Boyden chamber system to elucidate the role of

\[ \text{FIGURE 3.} \text{ Reduced leukocyte infiltration of tumors in the } \alpha_M^{-/-} \text{ mice. (A) Prostate tumor sections from WT and } \alpha_L^{-/-} \text{ mice were double stained with FITC-labeled rat anti-CD11b (M1/70) mAb (green) and rat anti-Ly6G (Gr-1; clone IA8-red). Numerous CD11b\(^{+}/\)Gr-1\(^{+}\) cells are present on merged images (yellow-orange) in both mouse strains. Representative images of the melanoma and prostate tumor sections stained with PMN-specific anti-Ly6G (B) and the monocyte–macrophage-specific MOMA-2 (D) Abs. Image analysis shows reduced Ly6G-positive (C) and MOMA-2-positive (E) area in melanoma and prostate tumors in the \( \alpha_M^{-/-} \) mice. Data are means±SEM (n=8 mice per group) and are representative of three independent experiments. Scale bars, 50 \( \mu \text{m.} \) *\( p < 0.05. \)

\[ \text{FIGURE 4.} \text{ Defective hematopoietic cells contribute to reduced tumor growth and angiogenesis in the } \alpha_M^{-/-} \text{ mice. (A) Average weight and (B) CD31-positive vascular area in RM1 prostate tumors in mice undergoing BMT with WT or } \alpha_M^{-/-} \text{ donor marrow. Data represent mean±SEM (n=5 mice per group). (C) Ly6G-positive and monocyte–macrophage-positive (D) area in RM1 tumors in mice undergoing BMT with WT or } \alpha_M^{-/-} \text{ donor marrow. Data represent mean±SEM (n=5 mice per group). (E and F) Intravenous administration of blocking mAb (M1/70) to } \alpha_M \text{ inhibits KC-dependent angiogenesis in Matrigel plug model in WT mice. } \text{M1/70 and normal rat IgG}_{2b} (3.5 mg/kg) \text{ were injected before Matrigel injection and on days 2, 4, and 6. Matrigel implants were harvested on day 8, sectioned, and stained with anti-CD31 mAb. (E) Representative images of Matrigel sections stained with anti-CD31 (brown). Scale bars, 50 \( \mu \text{m.} \) (F) Quantification of the CD31-positive area in Matrigel implants. Data are means±SEM (n=4) and are representative of two independent experiments. *\( p < 0.05. \)\]
\( \alpha_M \beta_2 \) and \( \alpha_L \beta_2 \) in mouse peritoneal PMNs and macrophage migration through a Matrigel matrix barrier (ECM extracted from Engelbreth-Holm-Swarm mouse sarcoma) in response to KC (Fig. 5A, 5B). In the absence of KC, there was minimal migration of leukocytes into the lower chambers in the presence or absence of added Plg. Although KC-induced migration of PMNs and macrophages was significantly impeded by Matrigel, this barrier effect was overcome by addition of Plg to the \( \alpha_M^{-/-} \) and WT leukocytes (Fig. 5A, 5B). In contrast, addition of Plg to the \( \alpha_M^{-/-} \) cells failed to improve their migration through the Matrigel barrier, suggesting that the capability of these cells to bind and activate Plg was limited (\( p = 0.014 \alpha_M^{-/-} \) versus WT PMNs and \( p = 0.005 \alpha_M^{-/-} \) versus WT macrophages; \( n = 3 \); Fig. 5A, 5B). Indeed, flow cytometry showed a 50–60% reduction in binding.

\[ \text{FIGURE 5.} \ \alpha_M \beta_2 \text{ directly interacts with Plg, enhances its activation, and facilitates Plm-dependent leukocyte recruitment. KC-directed transmigration of peritoneal PMNs (A) and macrophages (B) through Matrigel-coated inserts is Plg-dependent and is impaired in } \alpha_M^{-/-} \text{ leukocytes. The data are means } \pm \text{ SEM of triplicate samples and are representative of two independent experiments including three mice per group. (C) Reduced binding of soluble Alexa488-labeled Plg to peritoneal } \alpha_M^{-/-} \text{ PMNs and macrophages. The cells were incubated with increasing concentrations of Plg as indicated for 30 min at 37°C. After two washings, Plg binding to cell surface was analyzed using a FACSCalibur flow cytometer and CellQuest software. The cells incubated without Plg were set as negative controls. The data are means } \pm \text{ SEM of triplicate samples (} n = 3 \text{ mice per group) and are representative of two independent experiments. (D) Plg was immobilized on the CMS5 sensor chip surfaces (500 RU). Sensorgrams obtained for a concentration series of GST-\alpha_M-I (left panel) and GST-\alpha_I-I domain (right panel). (E and F) Reduced Plg activation on the surface of peripheral blood } \alpha_M^{-/-} \text{ PMNs and peritoneal macrophages. The results are means } \pm \text{ SEM of triplicate samples (} n = 5 \text{ mice per group) and are representative of two independent experiments. * } p < 0.05. \]
of Alexa488-conjugated soluble Plg to α_{M}^{−/−} peritoneal PMNs and macrophages as compared with WT and α_{L}^{−/−} leukocytes (Fig. 5C).

To determine whether Plg interacts directly with the α_{M}^{−} and α_{L}^{−} domain, the major site of ligand binding in β_{2} integrins (13), we performed SPR experiments. GST-tagged α_{M}^{−} domain interacted with Plg immobilized on biosensor chips in a concentration-dependent manner, whereas the GST-tagged α_{L}^{−} domain did not bind Plg (Fig. 5D). GST alone also did not interact with Plg. From the progress curves of the Plg:α_{M}^{−} domain interaction, we estimated a K_{D} = 1.76 ± 0.9 × 10^{-7}. This value was derived by fitting the kinetic data to a 1:1 global Langmuir model, and the stoichiometry observed at ligand saturation was 1:1.

Next, we compared Plg activation on the surface of WT, α_{L}^{−/−}, and α_{M}^{−/−} leukocytes using a fluorogenic plasmin-specific peptide substrate. Peripheral blood PMNs and lymphocytes were stimulated with KC and pretreated with sc-uPA to activate the integrin and enable sc-uPA binding to leukocyte surface, respectively. No plasmin activity was detected in the absence of leukocytes. The α_{M}^{−/−} PMNs showed a 50% reduction in Plm generation as compared with WT and α_{L}^{−/−} PMNs (p < 0.03, n = 5; Fig. 5E). In contrast, Plg activation was similar on WT, α_{M}^{−/−}, and α_{L}^{−/−} lymphocytes (Fig. 5E). As with PMNs, peritoneal α_{M}^{−} deficient macrophages also exhibited severely reduced (by 75%) Plg activation compared with the α_{M}^{−/−} and WT macrophages (p < 0.05, n = 5; Fig. 5F). In control experiments, we examined α_{L} expression on α_{M}^{−}-deficient PMN, α_{M} levels on α_{L}-null, and control WT PMNs (both peritoneal and circulating) by flow cytometry. Neither α_{L} nor α_{M} deficiency altered expression of its counterpart β_{2}-integrin on PMNs (data not shown), confirming that Plg recognition and activation is α_{M}β_{2}-specific. In addition, α_{M}β_{2} functions as a Plg receptor, and this function is critical in Plm-dependent leukocyte recruitment to angiogenic sites.

α_{M}β_{2} regulates secretion of VEGF-A by PMNs

Our data demonstrate a critical role of α_{M}β_{2} in leukocyte recruitment to angiogenic sites. However, we considered a possibility that α_{M}β_{2} might also regulate other proangiogenic leukocyte functions such as production and secretion of angiogenic stimulators. The CD11b/Gr-1^{+} cells, which primarily constitute PMNs, are of particular interest because they secrete high levels of MMP-9 and VEGF, leading to “angiogenic switch” in many tumors and to a failure of anti-VEGF therapies (5, 22). Therefore, we compared the VEGF-A content of supernatants released from peripheral blood PMN of WT and α_{M}^{−/−} and α_{L}^{−/−} mice. In the absence of TNF-α stimulation, VEGF-A levels were low and similar in PMNs of all three genotypes. Notably, upon stimulation with TNF-α, VEGF-A content was substantially lower (by ∼60%) in the α_{M}^{−/−} PMN-conditioned medium compared with medium harvested from WT or α_{L}^{−/−} PMNs (p < 0.05, n = 6; Fig. 6A).

VEGF is stored in specific granules in human PMNs (24). To determine whether α_{M}β_{2} regulates de novo synthesis of VEGF its secretion, or both, we used cycloheximide, an inhibitor of protein synthesis, and pentoxifylline, an inhibitor of PMN degranulation. Cycloheximide did not affect VEGF content in supernatants of TNF-α-stimulated PMNs of any mouse strains tested, suggesting that TNF-α did not stimulate de novo VEGF synthesis (Fig. 6A).

This interpretation was corroborated by quantitative real-time polymerase chain reaction assays, which revealed that VEGF mRNA levels were similar not only in resting and TNF-α-treated PMNs, but also in WT, α_{M}^{−/−}, and α_{L}^{−/−} PMNs (Table I). In contrast, pentoxifylline inhibited secretion of VEGF from PMNs and reduced its concentration by 80–85% in supernatants from WT and α_{L}^{−/−} PMNs and by additional 20% from α_{M}^{−/−} PMNs.
Table I. Comparison of VEGF-A mRNA content in WT, αM/−/−, and αL/−/− peripheral blood PMNs

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WT</th>
<th>αM/−/−</th>
<th>αL/−/−</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.22</td>
<td>0.19</td>
<td>0.2</td>
</tr>
<tr>
<td>TNF-α (20 ng/ml)</td>
<td>0.24</td>
<td>0.20</td>
<td>0.24</td>
</tr>
</tbody>
</table>

Values are expressed relative to GAPDH mRNA levels. PMNs were incubated in the presence of or absence of TNF-α (20 ng/ml) for 2h at 37°C. Total RNA was isolated using Trizol reagent, and RT-PCR has been performed as described in Materials and Methods.

To corroborate this conclusion, we measured the concentration of lactoferrin, a marker of PMN-specific granules, in PMN supernatants. The relative changes of lactoferrin and VEGF in the PMN-conditioned media were highly similar. Importantly, the αM/−/− PMNs, but not the αL/−/− PMNs, showed severely impaired release of lactoferrin into the supernatants of TNF-α-stimulated αM/−/− PMNs, which were almost as low as in supernatants of resting PMNs from each mouse strain (Supplemental Fig. 2). We also sought to examine VEGF production and secretion in CD11b+ progenitor cells isolated from bone marrow of WT, αM/−/−, and αL/−/− mice. However, we failed to detect mRNA for VEGF in these immature cells.

Next, we analyzed the capacity of WT MAECs to form tubes in the presence of the conditioned media derived from WT, αM/−/−, or αL/−/− TNF-α-stimulated PMNs. MAECs in the presence of media collected from WT or αL/−/− PMNs formed well-organized tubelike networks. In contrast, tubes formed by MAECs in the presence of the αM/−/− PMN-conditioned media were incomplete (p < 0.05, n = 20; Fig. 6B, 6C). In control samples, TNF-α alone did not support tube formation by ECs. To confirm that tube formation by MAECs in the presence of WT or αL/−/− PMN supernatants is VEGF-dependent, we added neutralizing rat antimouse VEGF mAb (clone 2G11-2A05), its isotype control rat IgG2a, or sFLT-1. The effectiveness of these VEGF inhibitors has been previously established (26, 27). These inhibitors of VEGF almost completely inhibited (by 75–80%) tube formation by MAEC (p < 0.05, n = 20). In contrast, the isotype control Ab did not have any effect (Fig. 6B, bottom panel, and 6C). Although other proangiogenic factors are likely to be present in PMN supernatants, VEGF appears to be the key stimulator of this process in our experimental system. Finally, supplementation of αM/−/− PMN conditioned medium with recombinant mouse VEGF to the same concentration as in medium from WT PMNs (520 pg/ml) enhanced the numbers of closed tubes by 2.5-fold and almost completely restored MAEC tube formation (Fig. 6B, top panel). αMβ2 does not directly regulate VEGF-A de novo synthesis; however, it enhances VEGF-A secretion from PMN intracellular stores via its enhancement of PMN degranulation.

Discussion

The goal of this study was to examine involvement of the two major leukocyte β2 integrins in angiogenesis: αMβ2 and αLβ2. Using the αMβ2 and αLβ2-deficient mice, we demonstrate that αMβ2 promotes angiogenesis in model melanoma and prostate tumors, as well as in Matrigel implants, whereas the αLβ2 integrin does not. Blood vessel formation and tumor growth were impaired in αM/−/− mice as compared with the αL/−/− or WT mice. Impaired angiogenesis in αM/−/− mice was due to dramatic reduction in recruitment of PMNs and macrophages into the tumors and Matrigel implants. Furthermore, we showed that Plg binding and activation on the surface of αM/−/− PMNs and macrophages and their Plm-dependent invasion through Matrigel were significantly attenuated as compared with the αL/−/− and WT cells. These data were consistent with the SPR sensorgrams showing that recombinant αMβ2-domain directly interacts with Plg, but the αLβ2-domain does not. These findings are in agreement with prior studies showing that αMβ2 recognizes urokinase (uPA) and Plg enhancing their reciprocal activation on PMN surface (14, 15, 28). To our knowledge, this is the first report implicating αMβ2 in angiogenesis and demonstrating its intimate interplay with Plg in vivo. Although, the β2-deficient mice showed slowed angiogenesis in healing wounds (29), none of the individual β2 integrin family members was shown to contribute to this process. The implication of αMβ2 in angiogenesis and Plg binding and activation is highly specific as both αLβ2 did not show any impairment in these responses. This distinction may be explained, at least in part, by the relatively low sequence identity between the ligand binding αMβ2- and αLβ2-domains resulting in the αMβ2 promiscuity for many structurally unrelated ligands, whereas αLβ2 shows a limited ligand repertoire with little overlap in ligand recognition with αMβ2 (19).

Regarding the ligand repertoire, the two β2 integrins αMβ2 and αLβ2 are more similar to αMβ2 than to αLβ2, and it would be interesting to examine their roles in angiogenesis. The critical role of αMβ2-dependent Plg activation in PMN and macrophage recruitment to angiogenic niches is in accord with previous studies demonstrating the importance of cell-bound plasmin in leukocyte recruitment in a variety of in vivo models of inflammation (10–12) and with a crucial role of the Plg system in angiogenesis (30–36). Pericellular proteolysis is critical for initiation of angiogenesis as evidenced by suppression of neovascularization in mice deficient in various proteases (31, 37–39) and by administration of a variety of protease inhibitors (40, 41). Among the proteases implicated in angiogenesis, in addition to plasmin, are its activators and metalloproteinases (reviewed in Ref. 4, 8). Plasmin is one of pro-MMP-9 activators (42) and PMN-derived MMP-9 is a mechanism to account for profound reduction in neovascularization and tumor growth in these animals. In addition to defective recruitment, the αMβ2/−/− mice have impaired secretion of VEGF-A because of impaired degranulation, and supernatants collected from these cells did not support EC tube formation in vitro. As enhanced VEGF is the hallmark and a key contributor to CD11b/Gr-1−/−-dependent (mostly PMNs) resistance of many tumors to VEGF targeting anticancer therapies, we have focused our investigations on the regulation of this pivotal cytokine. We cannot exclude that other proangiogenic factors, particularly those stored in PMN granules, might also be regulated by αMβ2 via its influence on degranulation.

This important issue is open for further investigation. Even if αM/−/− PMNs had been able to migrate, they would likely to have failed to promote angiogenesis because of an inability to supply the requisite amounts of the major proangiogenic stimulus, VEGF. The neovascularure in growing tumors, but not preexisting blood vessels, in αM/−/− mice showed immature and leaky phenotype. This might be caused by insufficient infiltration of CD11b/Gr-1−/−/Ly6G−...
PMNs known to support vascular maturation via elevated levels of proangiogenic factors VEGF and MMP-9 (22).

Taken together, our studies demonstrate that integrin α3β1 promotes angiogenesis in vivo via a dual mechanism: first, as a Plg receptor, α3β1 supports Plm-dependent recruitment of myeloid cells to angiogenic niches; second, α3β1 enhances VEGF-A secretion by PMN degranulation. Based on our findings, selective antagonists of α3β1 can be considered as a new target to inhibit tumor angiogenesis.

Acknowledgments

We thank Dr. Tatiana Byzova for expertise and advice regarding the in vivo angiogenesis models.

Disclosures

The authors have no financial conflicts of interest.

References

Supplemental Table 1. BMT engraftment efficiency in WT → αM⁻/⁻ and αM⁻/⁻ → WT mice.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Antibody or total cell #</th>
<th>Spleen (% positive cells)</th>
<th>Thymus (% positive cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT → αM⁻/⁻</td>
<td>Ly6G</td>
<td>8.60 ± 1.5</td>
<td>1.10 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>CD19</td>
<td>22.16 ± 4.2</td>
<td>1.04 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>F4/80</td>
<td>20.91 ± 1.09</td>
<td>1.42 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CD3</td>
<td>94.90 ± 6.5</td>
<td>90.3 ± 8.5</td>
</tr>
<tr>
<td></td>
<td>Total cell #</td>
<td>170.1 ± 3.6 x 10⁶</td>
<td>180 ± 45 x 10⁶</td>
</tr>
<tr>
<td>αM⁻/⁻ → WT</td>
<td>Ly6G</td>
<td>7.48 ± 2.04</td>
<td>1.60 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>CD19</td>
<td>17.5 ± 1.93</td>
<td>1.61 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>F4/80</td>
<td>25.1 ± 4.1</td>
<td>1.73 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>CD3</td>
<td>95.6 ± 12.1</td>
<td>95.0 ± 7.4</td>
</tr>
<tr>
<td></td>
<td>Total cell #</td>
<td>208.5 ± 44 x 10⁶</td>
<td>190 ± 20 x 10⁶</td>
</tr>
<tr>
<td>WT mice no BMT</td>
<td>Ly6G</td>
<td>6.4 ± 1.2</td>
<td>1.23 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>CD19</td>
<td>18.06 ± 5.1</td>
<td>1.40 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>F4/80</td>
<td>19.2 ± 3.8</td>
<td>1.37 ± 0.31</td>
</tr>
<tr>
<td></td>
<td>CD3</td>
<td>92.16 ± 10.8</td>
<td>93.9 ± 6.8</td>
</tr>
<tr>
<td></td>
<td>Total cell #</td>
<td>197 ± 30 x 10⁶</td>
<td>224 ± 35 x 10⁶</td>
</tr>
<tr>
<td>αM⁻/⁻ mice no BMT</td>
<td>Ly6G</td>
<td>7.50 ± 1.4</td>
<td>1.52 ± 0.21</td>
</tr>
<tr>
<td></td>
<td>CD19</td>
<td>25.01 ± 6.2</td>
<td>1.14 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>F4/80</td>
<td>22.02 ± 2.8</td>
<td>1.70 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>CD3</td>
<td>92.20 ± 9.1</td>
<td>95.49 ± 9.3</td>
</tr>
<tr>
<td></td>
<td>Total cell #</td>
<td>190 ± 25 x 10⁶</td>
<td>216 ± 38 x 10⁶</td>
</tr>
</tbody>
</table>

Bone marrow transplant was performed from WT to αM⁻/⁻ mice (WT → αM⁻/⁻) and from αM⁻/⁻ to WT mice as described in Materials and Methods. Mice were sacrificed 6 weeks after BMT, single cell suspensions were prepared from spleen and thymus and stained with FITC-labeled mAbs to the markers of PMNs (Ly6G), B cells (CD19), macrophages (F4/80) and T cells (CD3). The data are expressed as % of positive cells for each marker as compared to the respective FITC-labeled isotype matched control antibodies. WT and αM⁻/⁻ mice without BMT served as controls. The experiment was performed twice with 5 mice per group (n=10).
Supplemental Figure. 1 Preexisting vasculature is not leaky in the $\alpha_M^{-/-}$ mice.

A. Representative photographs of Evans blue leakage from dorsal skin vasculature of WT (left panels) and $\alpha_M^{-/-}$ (right panels) mice 30 min upon application of PBS (upper panels) or VEGF-A (lower panels). B. Quantification of dorsal skin vasculature permeability. The data are expressed as mean ± SEM and are representative of two independent experiments with 4 mice per group, $P>0.05$ WT vs $\alpha_M^{-/-}$ for both VEGF and PBS-injected groups (n=7). Five minutes after Evans blue injection intravenously into anesthetized mice 10 $\mu$l of PBS or VEGF (100 ng; R7D systems) was injected intradermally at adjacent location in the flanks of the mice. After 30 min skin samples of similar size were removed, weighted, photographed and Evans blue was extracted with 1 ml of formamide overnight at 56°C with constant shaking. The amount of extracted dye was measured spectrophotometrically at 610 nm.

Supplemental Figure. 2 Impaired degranulation of the $\alpha_M^{-/-}$ peripheral blood PMNs. Peripheral blood WT, $\alpha_M^{-/-}$ or $\alpha_L^{-/-}$ PMNs (3x10^6 cells/well) were incubated in 24-well TC plates in the absence or presence of TNF$\alpha$ (20ng/ml) for 2h at 37°C. Cycloheximide (10$\mu$g/ml) or pentoxifylline (300 $\mu$M) were added 60 min before addition of TNF$\alpha$. VEGF concentration was measured in supernatants using mouse Lactoferrin LTF/LF Elisa Kit (Cusabio). Data are means ± SEM of triplicate samples and are representative of three independent experiments.