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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 $\alpha_L\beta_2$ (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, RM1 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 $\alpha_L^{-/-}$ 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 $\beta_2$ 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, endothelial cells fail to form tubes in the presence of conditioned medium collected from TNF-$\alpha$-stimulated PMNs (5), 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 $\alpha_L^{-/-}$ 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 $\beta_2$ 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.

Materials and Methods

**Materials**

Mouse VEGF165 and keratinocyte-derived cytokine (KC) were from Biosource International (Camarillo, CA); heparin 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-$\alpha$-smooth muscle actin antibody (A2; Millipore, Temecula, CA); rabbit anti-smooth muscle actin antibody (A2; Millipore, Temecula, CA); rabbit anti-smooth muscle actin antibody (A2; Millipore, Temecula, CA).
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. Cytochrome oxidase and pentoxifylline were from Sigma-Aldrich.

**Mice**

The α5/−/− mice were generated as described previously (16), and α5/−/− 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° 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, SMAs 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, fibronectin, and Ly-6G– or MOMA-2-positive tissue 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 Plg binding to α5 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, 6, and 6 d after Matrigel-IC 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 i.v. with injection of 10° 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 α5/−/−→WT and WT→α5/−/− mice using WT and α5/−/− 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 α5 and α5:1 domains**

Glutathione S-transferase (GST)-fused α5, and α5:1 domains were purified with glutathione chromatography. Real-time protein-protein interactions were analyzed using a Biacore 3000 instrument (Biacore). Plg was immobilized on CM5 biosensor chips by amine coupling. Experiments were analyzed using a Biacore 3000 instrument (Biacore AB). Plg was purified 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, 6, and 6 d after Matrigel-IC implantation. The Matrigel plugs were collected 8 d after injection, sectioned, and stained with anti-CD31 to examine vascular formation. The inhibitors of protein synthesis (cycloheximide-10 μg/ml) or PMNs were incubated 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 wiping the inside with a cotton swab to remove nonmigrated cells. The migrated cells were quantified using the Cyquant Cytokinesis Plate (Molecular Probes, Eugene, OR) according to the manufacturer’s instructions.

**Regulation of VEGF by PMNs**

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 nM Tris-Cl buffer (pH 7.4) containing 0.14 mM NaCl, 0.1% BSA, 1 mM CaCl2, and 1 mM MgCl2. The cells were washed three times, and 50 μl of the cell suspension (1×10° 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 Pm-specific fluorescent substrate H-D-Val-Leu-Lys-7-amido-4-methylcoumarin (2 mM) mixture was added, and Plg formation was monitored over 45 min at 37˚C at α5α5 = 370 nm and α5α5 = 470 nm using a fluorescence plate reader (SpectraMax GeminiXS; Molecular Devices).

**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 (cytocheximide-10 μg/ml) or PMN degranulation (pentoxifylline, 3, 3-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, and from WT and α5/−/− mouse CD117+ bone marrow progenitor cells with Trizol reagent 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 on an iCycler (Bio-Rad iCycler V2.0 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 of 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 × 10^6 of WT MAECs were added to each well in 250 μl of chosen PMN-conditioned DMEM:F-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 TNF-α-stimulated PMNs before its addition to MAECs. Live time-lapse photography was performed for 12 h, using 5-min intervals on a Leica DMIRB 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 α5β1− mouse using the Sigma-Plot software program (SPSS); p < 0.05 was considered to be statistically significant.

Results

Integrin α5β2 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, α5β2 and α1β2, in angiogenesis. These two integrins share the same β2-subunit, and their α-subunits are 49% identical (19). Angiogenesis was analyzed in the α5β2−/−, α1β2−/−, 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 α1β2−/− and WT mice. In contrast, angiogenesis was significantly impaired in the α5β2−/− 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 α5β2−/− compared with tumors in the α1β2−/− 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 the α5β2−/− mice compared with the α1β2−/− and WT mice: 6200 μm² ± 1000 versus 12800 ± 2000 in α1β2−/− 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 α5β2−/− mice, melanomas grown in these mice were 70–80% smaller (p < 0.01, n = 7 per group) than those derived in the α1β2−/− and WT mice: 40 ± 10 mg in α5β2−/− versus 202 ± 42 mg in WT and 178 ± 34 mg in α1β2−/− mice. In addition, the average weight of RM1 prostate tumors recovered from the α5β2−/− mice was ~40–50% lower than from the α1β2−/− and WT mice (p < 0.05, n = 8 per group): 295 ± 30 mg in α5β2−/− versus 497 ± 80 mg in WT and 570 ± 100 mg in α1β2−/− 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 αL−/− mice, whereas the Matrigel plugs from α5β2−/− 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 αM−/− mice was reduced by ~75% compared with the implants from the αL−/− 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).

Neovasculature in α5β2−/− mice is immature

The presence of smooth muscle cells and pericytes within vasculature 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 αL−/− mice 30–37% of total CD31+ blood vessels stained for SMA, whereas only 15% of blood vessels formed in the αM−/− mice expressed this marker (p < 0.02, n = 8; Fig. 2B). Containing for CD31 and NG2 revealed reduced pericytes interacting with blood vessels in prostate tumors in the αM−/− mice as compared with WT and αL−/− mice (25 ± 8 μm versus 50–55 ± 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 the αM−/− mice (2.7 ± 0.3 μm) compared with WT and αL−/− mice (5.8–6.1 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 the αM−/− 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 the αM−/− mice compared with WT and αL−/− 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 the αM−/− mice also showed reduced maturity and leakiness (data not shown). In addition, the permeability of preexisting blood vessels in dorsal skin of the αM−/− 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 the αM−/− and WT mice, indicating that preexisting vasculature in the αM−/− mice was normal (Supplemental Fig. 1).

Impaired PMN and macrophage recruitment to angiogenic sites in αM−/− 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 αM−/− mice (CD11b+Gr-1+) 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 αL−/− 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 $\alpha_M$β2 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 ($\alpha_M^{-/-}$→WT) resulted in reduced RM1 tumor growth and angiogenesis (tumor weight: $p = 0.0169$; vascular area: $p = 0.028$ for $\alpha_M^{-/-}$→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 = 0.012 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<sup>−/−</sup> 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<sup>−/−</sup> deficiency did not affect BMT engraftment efficiency (Supplemental Table I). In addition, flow cytometry of circulating total leukocytes with anti-mouse αMβ2 mAb confirmed its absence in the αM<sup>−/−</sup>→WT chimeras and its persistence 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. Neovasculature 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 mouse (n = 4 mice/group). Data are expressed as mean ± SEM (n = 60) and are representative of three independent experiments including four mice per group. (F) 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.
αMβ2 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 αM−/− leukocytes to invade tumors could be caused by impaired Plg activation on the leukocyte surface. Because αMβ2 and αLβ2 are the most abundant β2-integrins on PMNs and macrophages, we used a modified Boyden chamber system to elucidate the role of

FIGURE 3. Reduced leukocyte infiltration of tumors in the αM−/− mice. (A) Prostate tumor sections from WT and αL−/− mice were double stained with FITC-labeled rat anti-CD11b (M1/70) mAb (green) and rat anti-Ly6G (Gr-1; clone 1A8-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 αM−/− mice. Data are means ± SEM (n = 8 mice per group) and are representative of three independent experiments. Scale bars, 50 μm. *p < 0.05.

FIGURE 4. Defective hematopoietic cells contribute to reduced tumor growth and angiogenesis in the αM−/− mice. (A) Average weight and (B) CD31-positive vascular area in RM1 prostate tumors in mice undergoing BMT with WT or αM−/− 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 αM−/− donor marrow. Data represent mean ± SEM (n = 5 mice per group). (E and F) Intravenous administration of blocking mAb (M1/70) to αM inhibits KC-dependent angiogenesis in Matrigel plug model in WT mice. M1/70 and normal rat IgG2b (3.5 mg/kg) 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 μm. (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 PMN 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 $\alpha_L^{-/-}$ 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
of Alexa488-conjugated soluble Plg to αM<sup>−/−</sup> peritoneal PMNs and macrophages as compared with WT and αL<sup>−/−</sup> leukocytes (Fig. 5C).

To determine whether Plg interacts directly with the αM<sup>−</sup>- and αL<sup>−</sup>-domain, the major site of ligand binding in β<sub>2</sub> integrins (13), we performed SPR experiments. GST-tagged αM<sup>−</sup>-domain interacted with Plg immobilized on biosensor chips in a concentration-dependent manner, whereas the GST-tagged αL<sup>−</sup>-domain did not bind Plg (Fig. 5D). GST alone also did not interact with Plg. From the progress curves of the Plg:αM<sup>−</sup>-domain interaction, we estimated a K<sub>d</sub> = 1.76 ± 0.9 x 10<sup>−7</sup>. 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, αL<sup>−/−</sup>, and αM<sup>−/−</sup> 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 αM<sup>−</sup>-/PMNs showed a 50% reduction in Plm generation as compared with WT and αL<sup>−/−</sup>PMNs (p < 0.03, n = 5; Fig. 5E). In contrast, Plg activation was similar on WT, αM<sup>−/−</sup>, and αL<sup>−/−</sup> lymphocytes (Fig. 5E). As with PMNs, peritoneal αM<sup>−</sup>-deficient macrophages also exhibited severely reduced (by 75%) Plg activation compared with the αL<sup>−/−</sup> and WT macrophages (p < 0.05, n = 5; Fig. 5F). In control experiments, we examined α<sub>L</sub> expression on αM<sup>−</sup>-deficient PMN, αM levels on αL-null, and control WT PMNs (both peritoneal and circulating) by flow cytometry. Neither α<sub>L</sub> nor αM deficiency altered expression of its counterpart β<sub>2</sub>-integrin on PMNs (data not shown), confirming that Plg recognition and activation is αMβ<sub>2</sub>-specific. In addition, αMβ<sub>2</sub> functions as a Plg receptor, and this function is critical in Plm-dependent leukocyte recruitment to angiogenic sites.

**αMβ<sub>2</sub> regulates secretion of VEGF-A by PMNs**

Our data demonstrate a critical role of αMβ<sub>2</sub> in leukocyte recruitment to angiogenic sites. However, we considered a possibility that αMβ<sub>2</sub> might also regulate other proangiogenic leukocyte functions such as production and secretion of angiogenic stimulators. The CD11b<sup>+</sup>/Gr-1<sup>+</sup> 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 PMNs of any mouse strains tested, suggesting that αM<sup>−/−</sup>-stimulated PMNs (3 x 10<sup>6</sup> cells/well) were incubated in 24-well TC plates in the absence or presence of TNF-α (20 ng/ml) for 2 h at 37°C. Cycloheximide (10 µg/ml) or pentoxifylline (300 µM) were added 60 min before addition of TNF-α. VEGF concentration was measured in supernatants using mouse VEGF Quantikine ELISA Kit. Data are means ± SEM and are representative of three independent experiments.

**FIGURE 6.** αMβ<sub>2</sub> supports angiogenesis via regulation of VEGF secretion by PMNs. (A) Peripheral blood WT, αM<sup>−/−</sup>, or αL<sup>−/−</sup> PMNs (3 x 10<sup>6</sup> cells/well) were incubated in 24-well TC plates in the absence or presence of TNF-α (20 ng/ml) for 2 h at 37°C. Cycloheximide (10 µg/ml) or pentoxifylline (300 µM) were added 60 min before addition of TNF-α. VEGF concentration was measured in supernatants using mouse VEGF Quantikine ELISA Kit. Data are means ± SEM of triplicate samples and are representative of three independent experiments. (B) Bright field microscopy of tube formation by WT MAECs in the presence of conditioned media collected from WT, αM<sup>−/−</sup>, or αL<sup>−/−</sup> peripheral blood PMNs stimulated with TNFα (upper panels). Inhibitors of VEGF: neutralizing anti-VEGF mAb, isotype matched rat IgG<sub>2a</sub> (100 µg/ml), and recombinant mouse sFLT-1 (100 ng/ml) were preincubated for 60 min before addition of TNF-α. The images were taken after 6 h incubation in 37°C, 5% CO<sub>2</sub>. Scale bars, 75 µm. (C) Quantification of tube formation. The number of closed tubes was counted in 20 different fields of each treatment and plotted as mean ± SEM and are representative of two independent experiments (Fig. 6A). Taken together, these data suggest that αMβ<sub>2</sub> integrin does not regulate VEGF synthesis, but rather its secretion via control of PMN degranulation, which is consistent with prior data implicating αMβ<sub>2</sub> in degranulation of human PMNs ex vivo (25).
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 αL−/− 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 CD117+ resting PMNs from each mouse strain (Supplemental Fig. 2). We failed to detect mRNA for VEGF in PMNs, which were almost as low as in supernatants of TNF-α-stimulated αL−/− and αM−/− 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 anti-VEGF mAb (clone 2G11-2A05), its isotype control rat IgG₂α, 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 was able to migrate, they would likely to have PMN cells, but approximately 10% of these mice were able to migrate via VEGF-A de novo synthesis; however, it enhances PMN degranulation by 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 αM−/− and αL−/− 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 αM-I-domain directly interacts with Plg, but the αL-I-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 αMβ2 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 αM-I- and αL-I-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 neoangiogenesis 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 responsible for angiogenic switch in some tumors (5). Consistent with the finding of decreased Plm activity and the capacity of αMβ2 to bind and activate MMP-9 (43), we observed reduced MMP-9 activity in tumor extracts from the αM−/− mice (data not shown). As a key source of proteases and proangiogenic factors, PMNs and macrophages are essential for angiogenesis. Angiogenesis is severely blunted in neutropenic mice (5, 29, 44, 45) or in mice in which macrophages have been eliminated (2). Robust PMN and macrophage recruitment is observed into ischemic tissues including tumors (46), and in many tumors recruitment of these cells correlates with poor host survival (reviewed in Ref. 2). With such evidence, the virtual absence of PMNs and macrophages in the angiogenic tissue of the αM−/− mice provides a mechanism to account for profound reduction in neovascularization and tumor growth in these animals. In addition to defective recruitment, the αM−/− PMNs also exhibited severely attenuated secretion of VEGF-A because of impaired degranulation, and supernatants collected from these cells did not support EC tube formation in in vitro assays. 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 neovascularization 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+/Ly6G−

<table>
<thead>
<tr>
<th>Treatment</th>
<th>WT</th>
<th>αM−/−</th>
<th>αL−/−</th>
</tr>
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<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>
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Values are expressed relative to GAPDH mRNA levels. PMNs were incubated in the presence 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.
PMNs known to support vascular maturation via elevated levels of proangiogenic factors VEGF and MMP-9 (22).

Taken together, our studies demonstrate that integrin αMβ2 promotes angiogenesis in vivo via a dual mechanism: first, as a P1g receptor, αMβ2 supports Plm-dependent recruitment of myeloid cells to angiogenic niches; second, αMβ2 enhances VEGF-A secretion by PMN degranulation. Based on our findings, selective antagonists of αMβ2 can be considered as a new target to inhibit tumor angiogenesis.

Acknowledgments

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Disclosures

The authors have no financial conflicts of interest.

References


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

<table>
<thead>
<tr>
<th>Mice</th>
<th>Antibody or total cell #</th>
<th>Spleen (% positive cells)</th>
<th>Thymus (% positive cells)</th>
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<tr>
<td>Ly6G</td>
<td>8.60 ± 1.5</td>
<td>1.10 ± 0.3</td>
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<td>CD19</td>
<td>22.16 ± 4.2</td>
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<td>F4/80</td>
<td>20.91 ± 1.09</td>
<td>1.42 ± 0.2</td>
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<td>CD3</td>
<td>94.90 ± 6.5</td>
<td>90.3 ± 8.5</td>
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<td>Total cell #</td>
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<tr>
<td>Ly6G</td>
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<td>17.5 ± 1.93</td>
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<td>F4/80</td>
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<td>Total cell #</td>
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<td><strong>WT mice no BMT</strong></td>
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<td>Ly6G</td>
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<td><strong>αM&lt;sup&gt;−/−&lt;/sup&gt; mice no BMT</strong></td>
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<tr>
<td>Ly6G</td>
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<td>CD19</td>
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<td>F4/80</td>
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<td>1.70 ± 0.4</td>
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<td>CD3</td>
<td>92.20 ± 9.1</td>
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<td>Total cell #</td>
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<td>216 ± 38 x 10&lt;sup&gt;6&lt;/sup&gt;</td>
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Bone marrow transplant was performed from WT to αM<sup>−/−</sup> mice (WT → αM<sup>−/−</sup>) and from αM<sup>−/−</sup> 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<sup>−/−</sup> 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 µl 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 56ºC 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 (3×10^6 cells/well) were incubated in 24-well TC plates in the absence or presence of TNFα (20ng/ml) for 2h at 37ºC. Cycloheximide (10µg/ml) or pentoxifylline (300 µM) were added 60 min before addition of TNFα. 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.