

Expansion of myeloid immune suppressor Gr⁺CD11b⁺ cells in tumor-bearing host directly promotes tumor angiogenesis

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Summary

We demonstrate a novel tumor-promoting role of myeloid immune suppressor Gr⁺CD11b⁺ cells, which are evident in cancer patients and tumor-bearing animals. These cells constitute approximately 5% of total cells in tumors. Tumors coinjected with Gr⁺CD11b⁺ cells exhibited increased vascular density, vascular maturation, and decreased necrosis. These immune cells produce high levels of MMP9. Deletion of MMP9 in these cells completely abolishes their tumor-promoting ability. Gr⁺CD11b⁺ cells were also found to directly incorporate into tumor endothelium. Consistent with this observation, Gr⁺CD11b⁺ cells acquire endothelial cell (EC) properties in tumor microenvironment and proangiogenic culture conditions. Our data provide evidence that Gr⁺CD11b⁺ cells of immune origin induced by tumors directly contribute to tumor growth and vascularization by producing MMP9 and differentiating into ECs.

Introduction

Tumors must escape from host immune surveillance in order to survive and grow. The development, progression, and metastasis of tumors also depend on tumor blood vessels. A number of recent findings demonstrate that angioblasts, the progenitors for endothelial cells contributing to embryonic vascular development, also contribute to tumor vascular formation (Asahara et al., 1997; Lyden et al., 2001; Rafii et al., 2002). These progenitor cells were further identified as VEGFR2-positive bone marrow (BM)-derived cells. Transplantation of wild-type BM to *Id* mutant mice (Lyden et al., 1999) or placental growth factor null mice (Carmeliet et al., 2001; Hattori et al., 2002) restored their ability to form tumor blood vessels and rescued tumor growth in these mutant mice. These results demonstrate that vasculogenesis is sufficient to restore tumor vascular formation ability in these genetic models. However, contradicting data (De Palma et al., 2003) showed the absence of transplanted BM cells in tumor endothelium. Of interest, the authors discovered that BM contribution to tumor vessels relied not on the endothelial progenitor

cells (EPCs), but rather on a hematopoietic cell population (i.e., CD45- and CD11b-positive, but CD31-negative) that homed specifically to the tumors and contributed indirectly to neovascularization. Recently, a report showed the absence of BM-derived EC precursors in the enhanced tumor growth in β 3-integrin null mice, which they instead related to circulating blood cells such as macrophages (Taverna et al., 2004). All of these studies show the intense research and debate on this subject. Clearly, how host hematopoietic cells contribute to tumor growth, especially tumor angiogenesis, needs to be further elucidated.

The host contribution to tumor progression is reflected by the roles of tumor infiltrating cells, which orchestrate the tumor microenvironment, and participate in the neoplastic process by fostering proliferation, survival, and metastasis (Coussens and Werb, 2002). These infiltrating cells promote tumor angiogenesis by producing angiogenic factors and matrix-degrading enzymes such as matrix metalloproteinase 9 (MMP9), a critical regulator of tumor angiogenesis, vasculogenesis, and metastasis (Fridman et al., 2003; Hamano et al., 2003; Hiratsuka et al., 2002).

SIGNIFICANCE

Escape from immune surveillance and promotion of tumor angiogenesis are essential for tumors to grow and progress. These two aspects are governed by the immune system and the angiogenic network of the host. In this report, an expansion of myeloid immune suppressor Gr⁺CD11b⁺ cells was found to directly promote tumor growth through the mechanisms of tumor angiogenesis and vasculogenesis. The proangiogenic role of myeloid immune suppressor cells induced by tumors might constitute a critical mechanism by which tumors subvert their host. Interventions aimed at eliminating these cells may improve antitumor immune response and concurrently inhibit tumor angiogenesis.

Studies have shown that MMP9 functions as an angiogenic switch during tumorigenesis by releasing vascular endothelial growth factor (VEGF) from the matrix (Bergers et al., 2000). In addition, there has been an increasing recognition of strong adaptive responses of cells in response to environmental stimuli (Fernandez Pujol et al., 2001; Harraz et al., 2001; Schmeisser et al., 2001). ECs and hematopoietic cells share the same progenitors, hemangioblasts. The monocyte/macrophage/dendritic cell (DC) system is closely related to ECs, and it has been speculated that these cells might differentiate into ECs under certain conditions. Understanding the role of hematopoietic cells in the tumor microenvironment is of immense interest.

Accumulating evidence since the 1980s has demonstrated that myeloid immune suppressor Gr+CD11b+ cells, of myeloid macrophage/DC lineage, are significantly increased in spleens and BM of animals bearing large tumors and in conditions associated with impaired immune reactivity (Bronte et al., 2000; Gabrilovich et al., 1999; Kusmartsev and Gabrilovich, 2002; Melani et al., 2003; Serafini et al., 2004). These cells are also found in the peripheral blood of cancer patients, including lung, breast, and head and neck cancer (Almand et al., 2001; Young and Lathers, 1999). Gr+CD11b+ cells express CD11b, a specific marker for myeloid cells of the macrophage lineage, and a marker for granulocytes, Gr-1. Gr+CD11b+ cells are known to be immune suppressive (Almand et al., 2001; Serafini et al., 2004; Young and Lathers, 1999). They express abnormally low levels of MHC class II molecules and low/undetectable levels of costimulatory molecules. They are unable to process and present antigens, and therefore do not induce effective antitumor responses (Bronte et al., 2000; Gabrilovich et al., 1996; Kusmartsev and Gabrilovich, 2002). Despite the data defining the immune-suppressive effect of these cells, it is unclear whether Gr+CD11b+ cells play other roles in tumor growth. Here we show these myeloid immune suppressor cells actively contribute to tumor angiogenesis by producing MMP9, and can differentiate into ECs. Our data demonstrate a novel mechanism used by tumors to exploit the host and thus promote tumor development and growth.

Results

Gr+CD11b+ cells infiltrated into tumors and were dramatically increased in tumor-bearing hosts

The possibility that Gr+CD11b+ cells may contribute to tumor angiogenesis led us to examine whether these cells infiltrate into tumors. Single cell suspensions from tumor tissues were labeled with fluorescence-conjugated antibodies, and analyzed by flow cytometry. Gr+CD11b+ cells comprised on average 5.64% of total tumor cells (Figures 1A and 1B) in a colorectal cancer (MC26) model, and 5.41% in a Lewis lung carcinoma (3LL) model (Supplemental Figure S1 at <http://www.cancer-cell.org/cgi/content/full/6/4/409/DC1/>). It was previously reported that the number of Gr+CD11b+ cells was greatly increased in the spleens of mice with large tumors. This was assessed in our two experimental models: Balb/c mice were inoculated s.c. with 5×10^5 MC26 cells, and C57BL/6 mice with 5×10^5 3LL cells. Single cell suspensions from spleens of normal mice and mice with tumors equal or larger than 1.5 cm in diameter were made, and the presence of Gr+CD11b+ cells was evaluated by flow cytometry. Gr+CD11b+ cells made up over 40% of the cell population in spleens of MC26-tumor-bearing mice

30–35 days after tumor inoculation, but only 3% in the spleens of normal mice (Figures 1C and 1D). In the 3LL (which grew significantly quicker) model, Gr+CD11b+ cells were also significantly increased to over 12% in spleens of tumor-bearing mice 21–28 days after tumor inoculation compared to 0.9% in normal spleens (Supplemental Figure S1 at <http://www.cancer-cell.org/cgi/content/full/6/4/409/DC1/>). In addition, we observed that the production of Gr+CD11b+ cells and the tumor infiltration of these cells increased as tumor size increased and with the duration of tumor growth (Figures 1E–1G). These data suggest that tumors affect hematopoiesis of the tumor hosts, manifested by the increase of Gr+CD11b+ cells in both spleens and tumors.

To further characterize the composition of Gr+CD11b+ cells, these cells were sorted from spleens of normal and tumor-bearing mice using FACS, then spun onto glass slides, stained by H&E, and examined by a hematology pathologist in a blinded fashion. Each subset of cells was counted, and the percentage was determined (Figures 1H–1J). We found that there was a significant increase in the whole spectrum of myeloid lineage cells, including myeloblasts, promyelocytes, myelocytes, metamyelocytes, bands, and neutrophils, ranging from less differentiated to terminally differentiated myeloid cells, in the tumor-bearing mice compared to that of normal controls ($p < 0.05$) (Figure 1J). These data reveal that tumors are associated with an increased fraction of myeloid cells.

Tumor growth was increased when tumor cells were coinjected with Gr+CD11b+ cells

It is well described that Gr+CD11b+ cells are associated with immune suppression in tumor-bearing hosts, but it is unclear whether these cells affect tumor growth directly. To examine this, we sorted Gr+CD11b+ cells from the spleens of mice bearing large MC26 tumors using FACS (Figure 2A). We achieved approximately 91% purity of Gr+CD11b+ cells, with the rest composed mostly of dead cells and debris. MC26 at 5×10^5 were coinjected subcutaneously (s.c.) with Gr+CD11b+ cells (0.5×10^5), sorted from tumor-bearing mice or normal mice, into BALB/c mice. Tumor size was measured by calipers. We found that tumor volume and growth rate are significantly higher when coinjected with tumor-derived Gr+CD11b+ cells than tumor cells alone or tumor cells coinjected with Gr+CD11b+ cells from normal mice ($p < 0.05$) (Figure 2B). However, this difference diminished approximately 28 days after tumor inoculation for the MC26 model (Figure 2B) and 21 days for the 3LL model (Figure 5A). This is likely due to the significant production of Gr+CD11b+ cells of the hosts bearing large tumors later in the experiments (Figures 1E–1G), which predominate the Gr+CD11b+ cells we coinjected. These data support the hypothesis that Gr+CD11b+ cells directly promote tumor growth.

Increased tumor angiogenesis, vascular maturation, and decreased tumor cell apoptosis and tumor necrosis were observed in tumors coinjected with Gr+CD11b+ cells

The MC26 tumors were harvested 21 days after tumor inoculation. We observed more visible blood vessels on the surface of the tumors that received a coinjection of MC26 and tumor-derived Gr+CD11b+ cells (Figure 3B) than MC26 cells alone (Figure 3A). It is known that tumor necrosis results when tumor

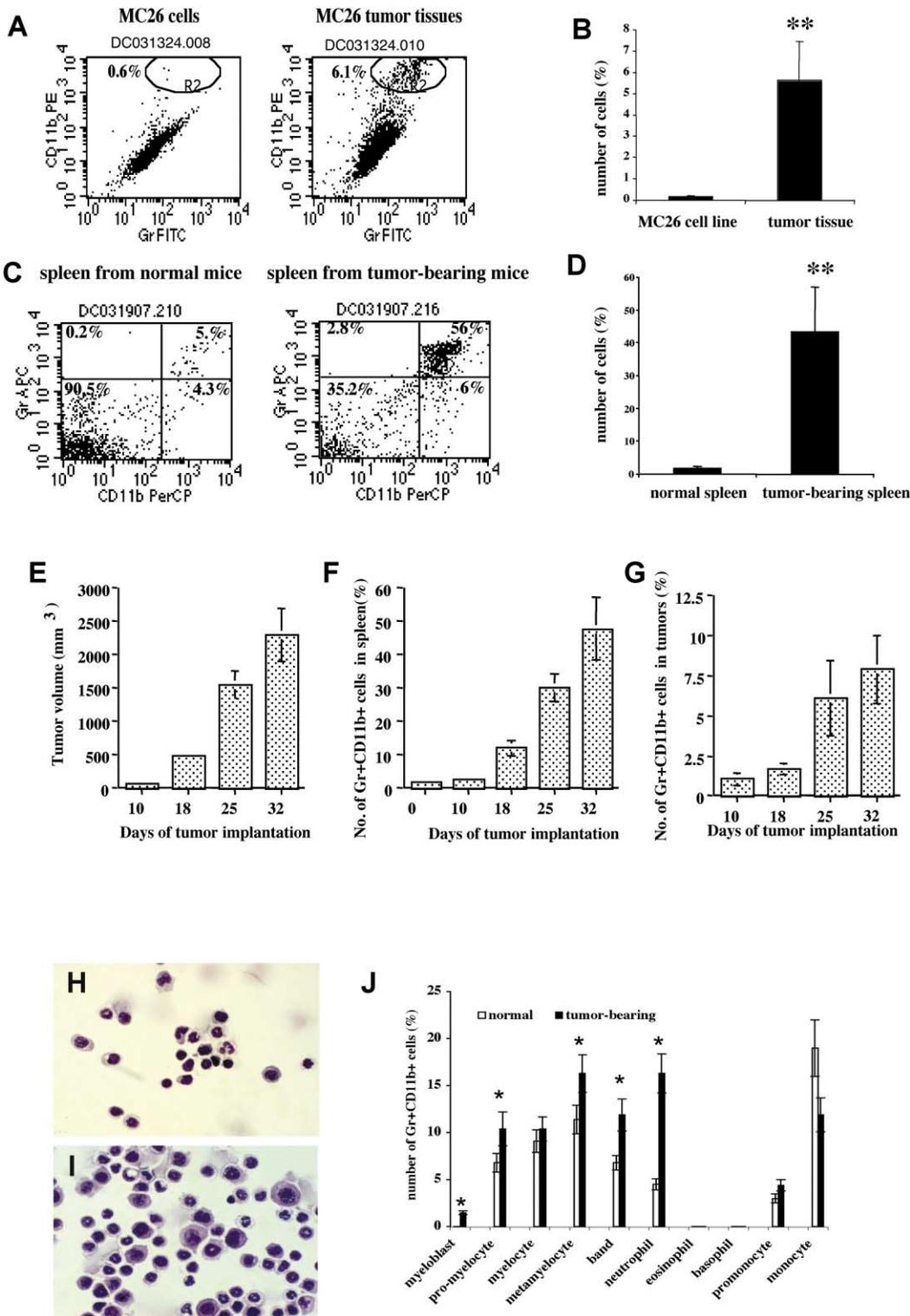


Figure 1. Gr+CD11b+ cells infiltrated into tumor tissues and were significantly increased in spleens of tumor-bearing mice

A and B: Flow cytometry analysis to assess the infiltrating Gr+CD11b+ cells in MC26 tumors 35 days after tumor inoculation (**A**), as quantified in **B**. ** indicates $p < 0.01$.

C and D: Gr+CD11b+ cells in spleens of normal and MC26 tumor-bearing mice 35 days after tumor inoculation by flow cytometry analysis (**C**), as quantified in **D**. ** indicates $p < 0.01$.

E–G: 5×10^5 MC26 cells were injected s.c. in Balb/c mice. Tumor growth was shown (**E**). The percentage of Gr+CD11b+ cells in spleens (**F**) and tumor tissues (**G**) of tumor-bearing mice was analyzed by flow cytometry. $n = 5$ mice per group.

H–J: Sorted Gr+CD11b+ cells from the spleens of normal mice (**H**) and MC26 tumor-bearing mice (**I**) were stained with H&E. Cell compositions of the Gr+CD11b+ populations were quantified (**J**) (4 or more mice per group). *, $p < 0.05$.

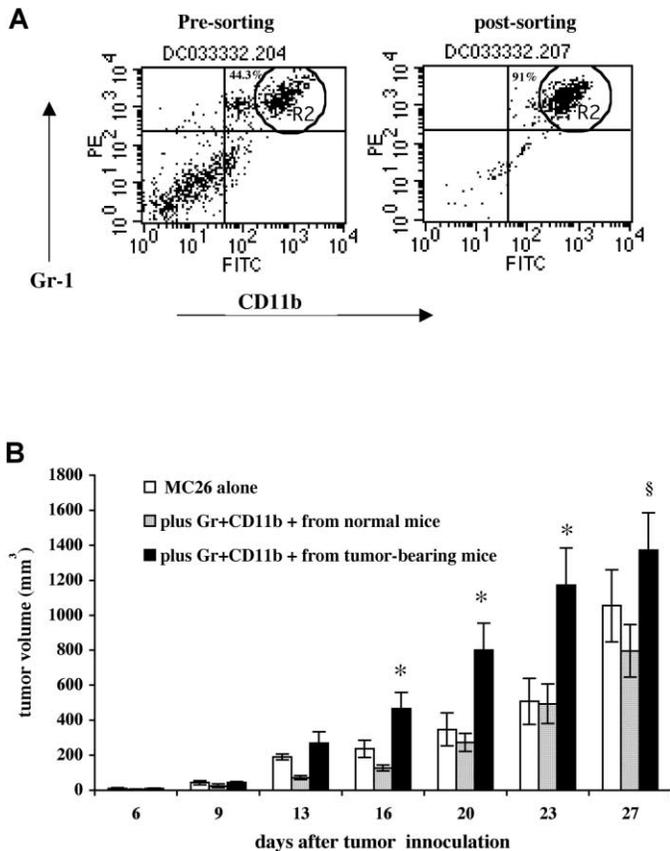


Figure 2. Tumor growth of MC26 was significantly increased when coinjected with tumor derived Gr+CD11b+ cells

A: Single-cell sorting of Gr+CD11b+ cells from spleens of MC26 tumor-bearing mice 35 days after tumor inoculation using CD11b-FITC and Gr-1-PE antibodies. Flow cytometry analysis of Gr+CD11b+ cells before and after sorting was shown.

B: Isograft models of tumor growth with Gr+CD11b+ cells. 5×10^5 MC26 cells and sorted 0.5×10^5 Gr+CD11b+ cells from tumor mice were injected s.c. in Balb/c mice. MC26 cells alone and Gr+CD11b+ cells from normal mice were used as controls. Results were presented as the mean \pm SEM (5–8 mice per group). *, $p < 0.05$ and §, $p > 0.05$.

growth outstrips its blood supply. Therefore, we examined tumor necrosis using GdDTPA contrast agent-enhanced magnetic resonance imaging (MRI). We found that tumors were significantly less necrotic when coinjected with Gr+CD11b+ cells (Figure 3D), compared to tumor cells alone ($p < 0.05$) (Figure 3C). The mean fractional necrosis was 15% for MC26 coinjected with Gr+CD11b+ cells, with 31% for MC26 cells alone (Figure 3E). Histological analysis for hypoxia in tumor sections showed large numbers of hypoxic cells in the center of the tumors without coinjection (Figure 3F), but many fewer hypoxic cells in the tumors coinjected with Gr+CD11b+ cells (Figure 3G).

To further investigate whether increased tumor vascularity could explain the increased tumor growth rate, we stained tumor samples with an anti-CD31 antibody (Figures 4A–4D). Blood vessels were counted in more than 10 randomly selected high power fields from 4 independent MC26 tumors (Figure 4E) in a blinded evaluation. A significantly higher blood vessel density was observed in tumors derived from MC26 coinjected with

Gr+CD11b+ cells derived from tumor mice than those derived from normal mice ($p < 0.05$) (Figure 4E), with an average of 25.92 ± 2.4 and 17.56 ± 2.2 ($p < 0.05$) for the MC26 tumor model. Similar results were observed in the 3LL tumor model, with an average of 120 ± 15.4 and 77 ± 8.1 vessels ($p < 0.05$) for the 3LL coinjected with Gr+CD11b+ cells of tumor mice and normal mice, respectively (Supplemental Figure S2 at <http://www.cancer.org/cgi/content/full/6/4/409/DC1/>). Considering the difference in tumor volume between the two groups, the difference of total blood vessels is actually more pronounced. Very interestingly, we found many viable islands consisting of tumor cells, CD31-positive cells, and blood vessels within large necrotic regions in tumors derived from a coinjection of tumor cells with tumor derived Gr+CD11b+ cells (Figure 4D and Supplemental Figure S2D). CD31-positive cells were abundant and grouped around tumor blood vessels. Some of these cells were seen within the tumor endothelium (Figure 4D), which was greatly reduced in tumors derived from coinjection with normal Gr+CD11b+ cells (Figure 4C, Supplemental Figure S2). In addition, tumor sections were costained with antibodies against CD31 and α SMC (α smooth muscle actin). The percentage of α SMC and CD31 double-positive vessels from the total of CD31+ vessels was used as an index for vessel maturation/stability. We found significantly more α SMC-positive vessels in MC26 tumors coinjected with tumor-derived Gr+CD11b+ cells than controls (Figures 4F–4H) ($p < 0.05$); this increase correlated with a significant reduction in apoptotic cells in tumors with coinjection (Figures 4I–4K) ($p < 0.05$). The data is in agreement with a recent publication that stromal MMP9 regulates the vascular architecture by promoting pericyte recruitment (Chantraine et al., 2004). Collectively, our data indicate that coinjection of tumor cells with tumor-derived Gr+CD11b+ cells is associated with increased tumor angiogenesis, vascular maturation, and decreased tumor apoptosis and necrosis.

Gr+CD11b+ cells contributed to tumor angiogenesis through the production of MMP9

We observed increased tumor angiogenesis, reduced tumor necrosis, and enhanced tumor growth from the coinjection of MC26 cells with tumor-derived Gr+CD11b+ cells. We next investigated the mechanisms involved in this process. Previous studies demonstrate that tumor-infiltrating neutrophils, macrophages, and mast cells produce MMP9 and contribute to skin carcinogenesis (Coussens et al., 2000). MMP9 is also known to promote tumor angiogenesis by regulating the bioavailability of VEGF (Bergers et al., 2000; Rodriguez-Manzaneque et al., 2001). To test the role of MMP9 in Gr+CD11b+ cells, we sorted Gr+CD11b+ cells from spleens of MMP9 null and wt tumor-bearing mice, respectively. These cells were then coinjected with syngeneic 3LL tumor cells into wt C57Bl/6 mice, the genetic background of the MMP9 null mice used in this study. Tumor size was then measured. Tumors from 3LL cells coinjected with MMP9 null Gr+CD11b+ cells grew significantly slower than those derived from coinjection with Gr+CD11b+ cells from a wt background ($p < 0.001$) (Figure 5A). In fact, it appeared that MMP9 null Gr+CD11b+ cells suppressed tumor growth compared to the control 3LL alone ($p = 0.021$). This difference diminished at later time points, likely due to the large production of Gr+CD11b+ cells from the hosts. These tumors also have decreased tumor blood vessel counts ($p < 0.05$) (Figure 5B).

To confirm the results, Gr+CD11b+ cells from normal and

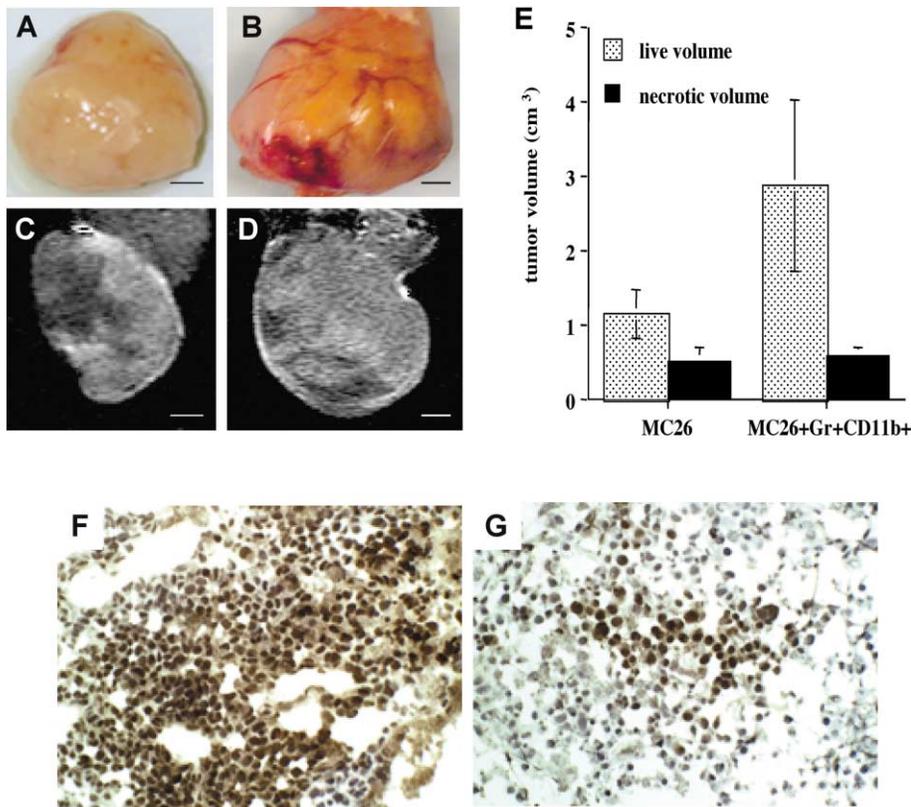


Figure 3. Increased tumor blood vessels and decreased tumor necrosis in MC26 tumors co-injected with Gr+CD11b+ cells when compared to MC26 cells alone

A and B: More blood vessels on the surface of tumors derived from MC26 cells co-injected with tumor derived Gr+CD11b+ cells (**B**) than with MC26 alone (**A**) 21 days after the tumor inoculation. Scale bar: 0.25 mm.

C–E: MR imaging of tumors derived from MC26 alone (**C**) or MC26 cells co-injected with tumor derived Gr+CD11b+ cells (**D**), as quantification of live and dead tumor volume (**E**). $p < 0.05$, $n = 3$ pairs.

F and G: Immunohistochemistry of pimonidazole adducts for hypoxia cells (brown color) in tumor sections from MC26 alone (**F**) and MC26 co-injected with Gr+CD11b+ cells (**G**).

MC26 tumor-bearing mice were isolated, and cell lysates were analyzed by gelatin zymography for MMP activity. It is very clear that Gr+CD11b+ cells derived from tumor-bearing mice produced high levels of pro-MMP9 and MMP9 compared with cells derived from normal mice. In contrast, there was no significant difference in MMP2 levels between the two groups (Figure 5C). In addition, in situ hybridization of the tumor sections showed significantly more MMP9-positive cells in 3LL tumors co-injected with tumor-derived Gr+CD11b+ cells than the tumor cell alone (Figures 5D and 5E). MMP9-positive cells were visible throughout the tumors as well as around and in the vascular walls of tumors co-injected with Gr+CD11b+ cells (Figure 5E), which is similar to the immunohistochemical staining of CD31 in tumor sections (Figure 4D). Collectively, these data demonstrate that production of MMP9 by Gr+CD11b+ cells contributes to tumor angiogenesis and tumor growth.

MMP9 derived from Gr+CD11b+ cells regulated VEGF bioavailability in tumors and sKitL (soluble Kit-ligand) release in BM

Our data demonstrated an essential role of MMP9 of Gr+CD11b+ cells in tumor promotion. We next studied the molecular mechanisms. MMP9 has been shown to function as an angiogenic switch and release VEGF from extracellular matrix (Bergers et al., 2000); therefore, we investigated the association of VEGFR2/VEGF using an antibody that only recognizes the complex. We found that there were significantly more VEGFR2/VEGF-positive vessels in the MC26 tumors co-injected with tumor-derived Gr+CD11b+ cells than in controls (Figures 6A and 6B). Immunohistochemical analysis of tumor sections showed

no difference of VEGF levels between the two groups, and no difference of VEGF production was found in the culture media of normal and tumor-derived Gr+CD11b+ cells (data not shown). These results suggest that the difference of VEGFR2 activation is likely due to the bioavailability of VEGF. Therefore, we evaluated VEGF release in tumor tissues. We found that there was significantly more VEGF released in MC26 tumor co-injected with Gr+CD11b+ from tumor-bearing mice than from normal mice (Figure 6C) ($p < 0.01$). Addition of GM6001, a MMP inhibitor, significantly reduced VEGF release from the tumor tissues compared to the untreated group (Figure 6C) ($p < 0.01$). After examination the angiogenic function of VEGF released from the tumors, we found MC26 tumors co-injected with Gr+CD11b+ cells from tumor-bearing mice showed stronger effects in inducing EC migration than those co-injected with Gr+CD11b+ cells from nontumor mice ($p < 0.05$). Addition of either a VEGF inhibitor or MMP inhibitor blocked EC migration induced by the tumor tissues ($p < 0.01$) (Figure 6D). Collectively, these data suggest that Gr+CD11b+ cells produce MMP9 that regulates bioavailability of VEGF and promotes tumor angiogenesis.

MMP9 is also known to release sKitL in BM, permitting the transfer of hematopoietic stem cells from the quiescent to proliferative niche (Heissig et al., 2002). Therefore, we examined the effects of host MMP9 on Gr+CD11b+ cell production and tumor infiltration. Spleens, BM, and tumors were harvested from both normal and 3LL-bearing wt and MMP9 null mice, and the presence of Gr+CD11b+ cells was analyzed. We found that deletion of MMP9 in hosts significantly inhibited the production of Gr+CD11b+ cells in spleens (Figure 6E, $p < 0.01$) and BM (Figure 6F, $p < 0.05$). There was also a significant reduction

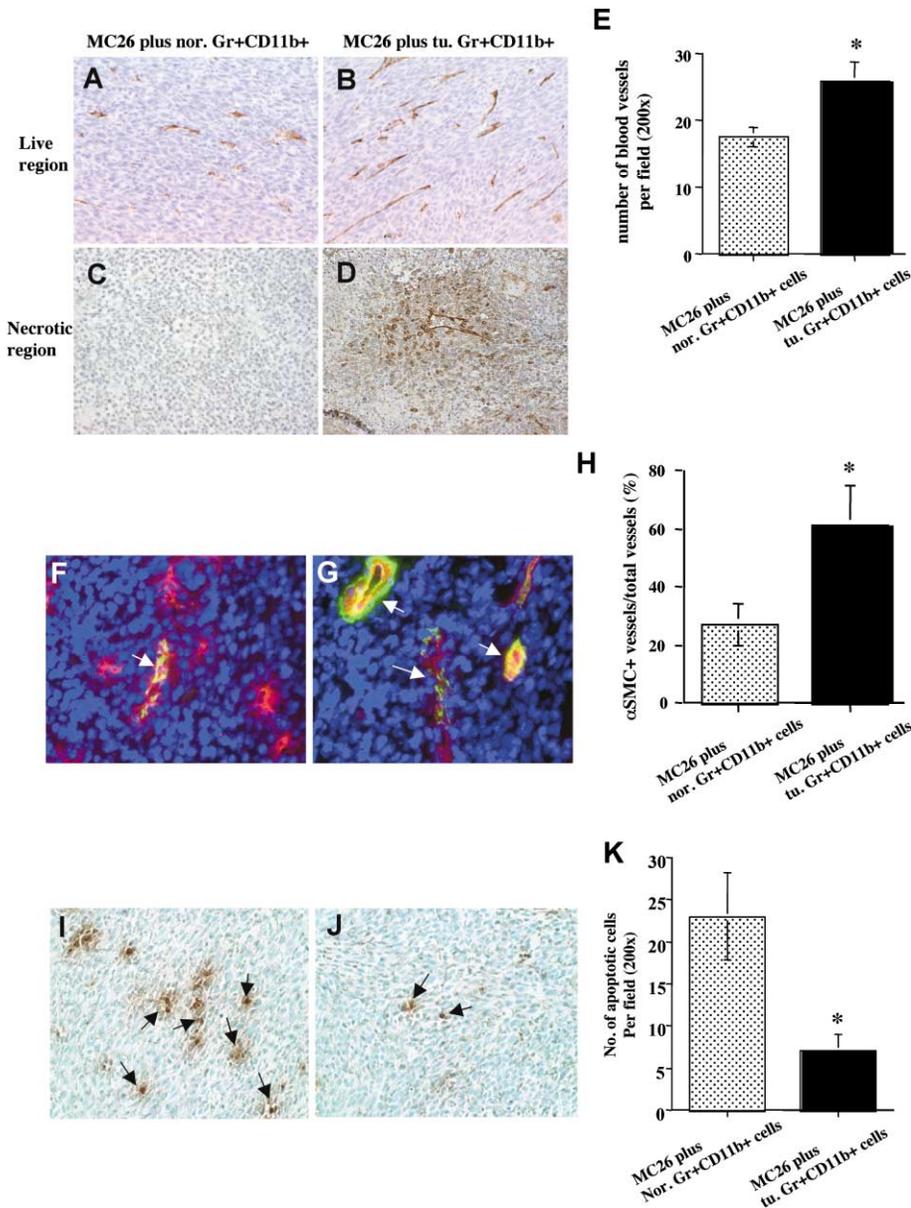


Figure 4. Increased tumor blood vessel density and vascular stability and decreased apoptosis from MC26 tumors co-injected with tumor derived Gr+CD11b+ cells

A–D: Tumor sections were stained with an anti-CD31 antibody (brown color). Blood vessels in live (**A** and **B**) and necrotic regions (**C** and **D**) of tumors derived from MC26 co-injected with Gr+CD11b+ cells derived from normal mice (**A** and **C**) and tumor-bearing mice (**B** and **D**) 21 days after the tumor inoculation are shown.

E: Tumor blood vessels were counted in 10 randomly selected fields from MC26 tumors 21 days after the tumor inoculation. *, $p < 0.05$.

F–H: Immunofluorescent analysis of α SMC (green) and CD31 (red) double-positive vessels, as indicated by arrows in tumor sections from MC26 co-injected with Gr+CD11b+ cells of normal mice (**F**) and tumor-bearing mice (**G**). DAPI staining (blue) for nuclei. The percentage of α SMC and CD31 double-positive vessels from the total of CD31-positive vessels was quantified (**H**). * indicates $p < 0.05$

I–K: TUNEL assay for apoptotic cells as indicated by arrows in tumor sections of MC26 co-injected with Gr+CD11b+ cells of normal (**I**) and tumor-bearing mice (**J**). Quantification of apoptotic cells in these tumor sections (**K**). *, $p < 0.01$.

of Gr+CD11b+ infiltrating in tumors from MMP9 null mice than from wt mice (Figure 6G, $p < 0.01$). Finally, we investigated sKitL release from BM stromal cells cocultured with Gr+CD11b+ cells. Both BM stromal cells and Gr+CD11b+ cells expressed sKitL. Addition of Gr+CD11b+ cells from MC26 tumor-bearing mice significantly increased the release of sKitL from BM stromal cells, compared to Gr+CD11b+ cells derived from normal mice ($p < 0.01$). This could be blocked with a MMP inhibitor GM6001 (Figure 6H, $p < 0.01$). Together, our data show that host MMP9 regulates Gr+CD11b+ cells production and tumor infiltration. MMP9 from Gr+CD11b+ cells regulates the release of sKitL in BM and potentially affects production and mobilization of endothelial and hematopoietic stem cells in BM.

Gr+CD11b+ cells directly incorporated into tumor endothelium

To further investigate the contribution of Gr+CD11b+ cells to tumor growth in vivo, we sorted Gr+CD11b+ cells from the

spleens of MC26 tumor-bearing mice and labeled them with a vital dye 5-CFDA, and co-injected with MC26 cells into Balb/c mice. Frozen tumor sections collected 21 days after tumor injection were examined under a fluorescence microscope. Strikingly, we found Gr+CD11b+ cells lining the endothelial wall of tumor blood vessels (Figure 7A). Some of them showed the long thin shape of ECs (Figures 7A and 7B). Some labeled Gr+CD11b+ cells still retained a rounded shape, and clustered around tumor blood vessels in a manner similar to what we have observed in CD31 staining (Figure 4D). Immunostaining of tumor sections with an anti-CD31 antibody confirmed that the fluorescently labeled cells were indeed tumor endothelium (Figure 7B). To further verify this observation, we used Rosa-26 mice, a transgenic line that ubiquitously expresses β -galactosidase. Sorted Gr+CD11b+ cells from 3LL tumor-bearing Rosa-26 mice were co-injected with 3LL cells into C57BL/6 mice. Tumor samples were analyzed for β -gal expression. We ob-

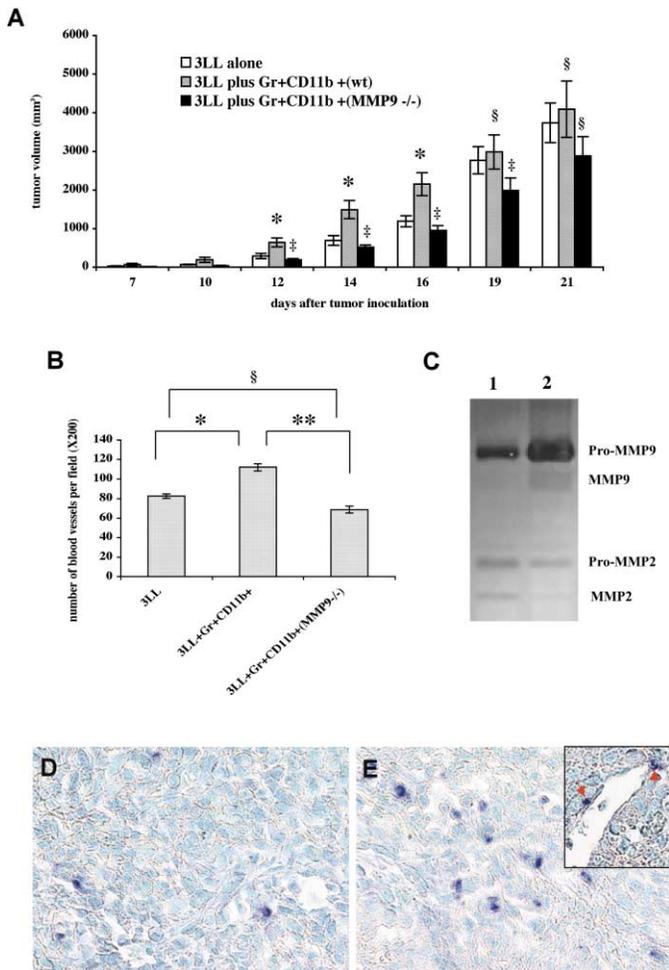


Figure 5. Gr+CD11b+ cells produced MMP9 that contributed to tumor angiogenesis

A: 5×10^5 3LL cells and 0.5×10^5 Gr+CD11b+ cells with MMP9 null background were injected s.c. into C57Bl/6 mice. 3LL cells alone and Gr+CD11b+ cells from wt tumor-bearing mice were used as controls. Results were presented as the mean \pm SEM (8–12 mice per group). †, $p < 0.001$, §, $p > 0.05$, compared to 3LL plus wt Gr+CD11b+ cells. *, $p < 0.05$, compared to the 3LL cells alone.

B: CD31-positive blood vessels were counted in ten randomly selected fields from 4 independent 3LL tumors 14 days after the tumor inoculation. Results were presented as the mean \pm SEM. * and **, $p < 0.05$; §, $p > 0.05$.

C: Gelatin zymography of sorted Gr+CD11b+ cells from the spleens of normal mice (lane 1) and tumor-bearing mice (lane 2) was shown.

D–E: MMP9 in situ hybridization showed more MMP9-positive cells in 14-day tumor tissues derived from 3LL co-injected with tumor derived Gr+CD11b+ cells (**E**) than 3LL alone (**D**). MMP9-positive cells were seen lining in the tumor endothelium as indicated by arrows (inset in **E**).

served “blue” (β -gal-positive) tumor endothelium of large (Figure 7C) and small vessels (Figure 7D) derived from coinjection of tumor cells with Gr+CD11b+ of Rosa-26 mice. A costaining with a VE-Cadherin antibody confirmed that the β -gal-positive cells were indeed endothelium (Figure 7C). These data demonstrate that Gr+CD11b+ cells have the potential to differentiate and incorporate into tumor endothelium.

EPCs have been identified as VEGFR2-positive, and $4.39 \pm 0.56\%$ of Gr+CD11b+ cells also express VEGFR2; there-

fore, we sought to determine the impact of VEGFR2-positive Gr+CD11b+ population on tumor growth and tumor angiogenesis. Because the percentage of VEGFR2-positive Gr+CD11b+ cells is very small, we therefore sorted Gr+CD11b+/VEGFR2– cells and coinjected them with MC26 cells into Balb/c mice. We did not observe a statistically significant decrease in tumor growth from coinjection with Gr+CD11b+/VEGFR2– cells compared to coinjection with the whole Gr+CD11b+ population, which includes VEGFR2+ cells (Figure 7E). In addition, we found a small number of vital dye-labeled Gr+CD11b+/VEGFR2– cells also incorporated into the tumor endothelium when coinjected with MC26 cells (data not shown). Collectively, these data suggest that Gr+CD11b+VEGFR2+ cells likely give rise to the cells incorporated into tumor endothelium; however, VEGFR2– cells also have the potential under tumor conditions.

Finally, we examined the expression of endothelium markers on Gr+CD11b+ cells of tumor mice. We observed $4.39 \pm 0.56\%$, $6.71 \pm 1.02\%$, and $4.02 \pm 0.66\%$ of the cells expressing VEGFR2, VE-Cadherin, and both markers, respectively, indicating that Gr+CD11b+ cells are closely related to ECs. In addition, Gr+CD11b+ cells from tumor-bearing mice had a significantly increased number of VEGFR2+, VE-Cadherin+, and VEGFR2+/VE-Cadherin+ Gr+CD11b+ cells, compared with non-tumor-bearing mice ($p < 0.01$) (Figure 7F).

The expression of endothelial markers is dramatically increased in Gr+CD11b+ cells residing in tumor tissues, and Gr+CD11b+ cells acquired EC properties in proangiogenic culture conditions

In recent years, there has been an increased recognition of strong adaptive responses of non-tumor cells in response to environmental stimuli. We hypothesized that Gr+CD11b+ cells might have a plasticity in the tumor microenvironment. To examine this, we compared the endothelial marker expression of Gr+CD11b+ cells residing in tumor tissues with those from spleens of tumor-bearing mice. Very interestingly, the percentage of Gr+CD11b+ cells expressing both VE-Cadherin and VEGFR2 was dramatically increased in cells from tumor tissues when compared to those from spleens ($p < 0.01$) (Figure 8A), suggesting that the tumor microenvironment may induce differentiation of Gr+CD11b+ cells toward ECs. Growth factors influence cell differentiation, and VEGF, bFGF, and IGF are important angiogenic factors. We therefore cultured Gr+CD11b+ cells on fibronectin-coated plates in the presence of VEGF, bFGF, and IGF for a week in attempt to mimic the tumor microenvironment. Under this condition, Gr+CD11b+ cells take up low-density lipoprotein (DiI-LDL) within a few hours of incubation, a property of ECs (Figure 8B). Gr+CD11b+ cells attached to the dishes and displayed a cobblestone-like morphology (Figure 8C). The cultured Gr+CD11b+ cells express VEGFR2 and VE-Cadherin cell surface markers (Figure 8C). Very interestingly, sorted VEGFR2-negative Gr+CD11b+ cells express VEGFR2 and VE-Cadherin after one week in culture (Figure 8C). Further analysis by flow cytometry confirmed that sorted VEGFR2-negative Gr+CD11b+ cells express VEGFR2, VE-Cadherin, and both markers after one week in culture, with no statistical difference found when compared with sorted total Gr+CD11b+ population under the same conditions (Figure 8D). These results are in agreement with our early observation that Gr+CD11b+/VEGFR2– cells also gave rise to tumor endothelium and pro-

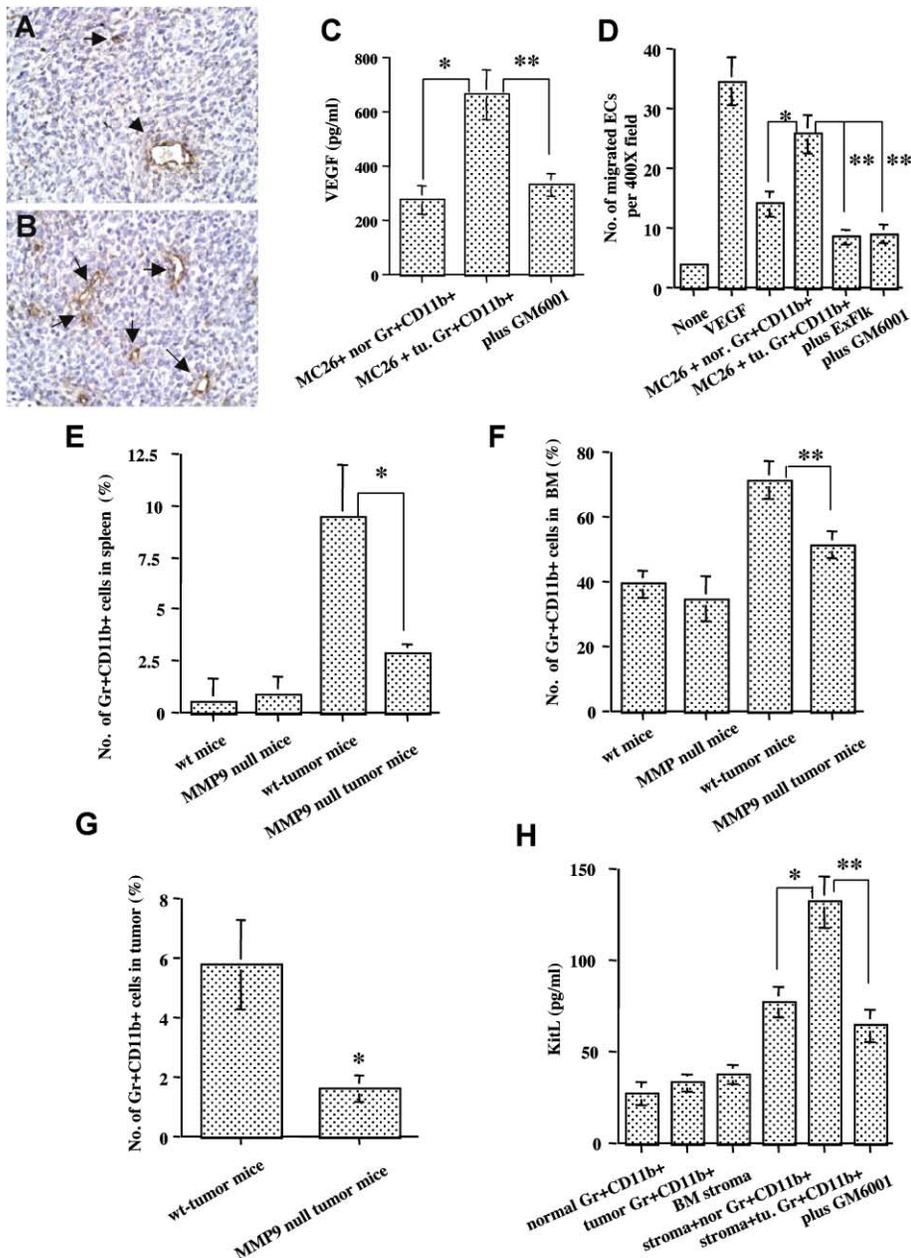


Figure 6. MMP9 produced in tumor-derived Gr+CD11b+ cells released in tumors promoting angiogenesis; in BM, it released sKitL contributing to Gr+CD11b+ cell production

A and B: Immunohistochemistry of VEGF/VEGFR2 complex as indicated by arrows in tumor sections of MC26 cojected with Gr+CD11b+ cells from normal mice (**A**) and tumor-bearing mice (**B**). **C:** VEGF release in culture media measured by ELISA. MC26 tumor tissues were harvested 2 weeks after inoculation, cut into small pieces (0.5 mm size), and cultured in serum-free conditions in a 24-well plate for 24 hr, with each well containing 15 pieces of small tumor tissues. The results were presented as the mean \pm SEM of 3 independent experiments. * and **, $p < 0.01$.

D: Transwell filters with HUVECs were placed into a 24-well plate, which contains 15 pieces of tumors in each well. A VEGF inhibitor (ExFlk at 1 μ g/ml) and an MMP inhibitor (GM6001 at 20 μ M) were included. Recombinant VEGF at 20 ng/ml was used as a control. Migrated HUVECs were counted 5 hr after incubation. *, $p < 0.05$; **, $p < 0.01$.

E-G: Flow cytometry analysis of Gr+CD11b+ cells in spleens (**E**), BM (**F**), and tumor tissues (**G**) of C57BL/6 and MMP9 null mice with or without 3LL tumors 28 days after tumor implantation. $n = 5$ mice per group. *, $p < 0.01$; **, $p < 0.05$.

H: Confluent BM stromal cells were incubated with Gr+CD11b+ cells. GM6001 at 20 μ M was included in the coculture with tumor-derived Gr+CD11b+ cells. sKitL was measured in the culture media 24 hr after incubation by ELISA. * and **, $p < 0.01$.

moted tumor growth as total Gr+CD11b+ cells did (Figure 7E). Together, our data demonstrate that Gr+CD11b+ cells have a certain plasticity and acquire EC properties in the tumor microenvironment in vivo and angiogenic conditions in vitro in a dynamic manner.

To assess the possible role of MMP9 in Gr+CD11b+ differentiation into ECs, we sorted those cells from MMP9 null and wt mice bearing 3LL tumors and cultured them under the same proangiogenic conditions. MMP9 null Gr+CD11b+ cells displayed endothelial cell morphology and expressed both VEGFR2 and VE-Cadherin after one week in culture, with no difference found when compared to wt Gr+CD11b+ cells (Figure 8C). This result suggests that MMP9 function does not affect Gr+CD11b+ cell differentiation to ECs.

Discussion

BM-derived cells play important roles in tumor vascular development (De Palma et al., 2003; Luttun et al., 2002; Lyden et al., 2001; Reyes et al., 2002). There are two major themes of research in this area. First, tumor vascular formation involves endothelial progenitors through vasculogenesis (Asahara et al., 1997; Carmeliet et al., 2001; Luttun et al., 2002; Lyden et al., 2001; Reyes et al., 2002). Second, host inflammatory cells, including mast cell and macrophages, infiltrate tumor tissues, alter the microenvironment, and promote tumor angiogenesis (Coussens et al., 2000; Kamate et al., 2002). Our data demonstrate that myeloid immune suppressor Gr+CD11b+ cells directly contribute to tumor vascular development through

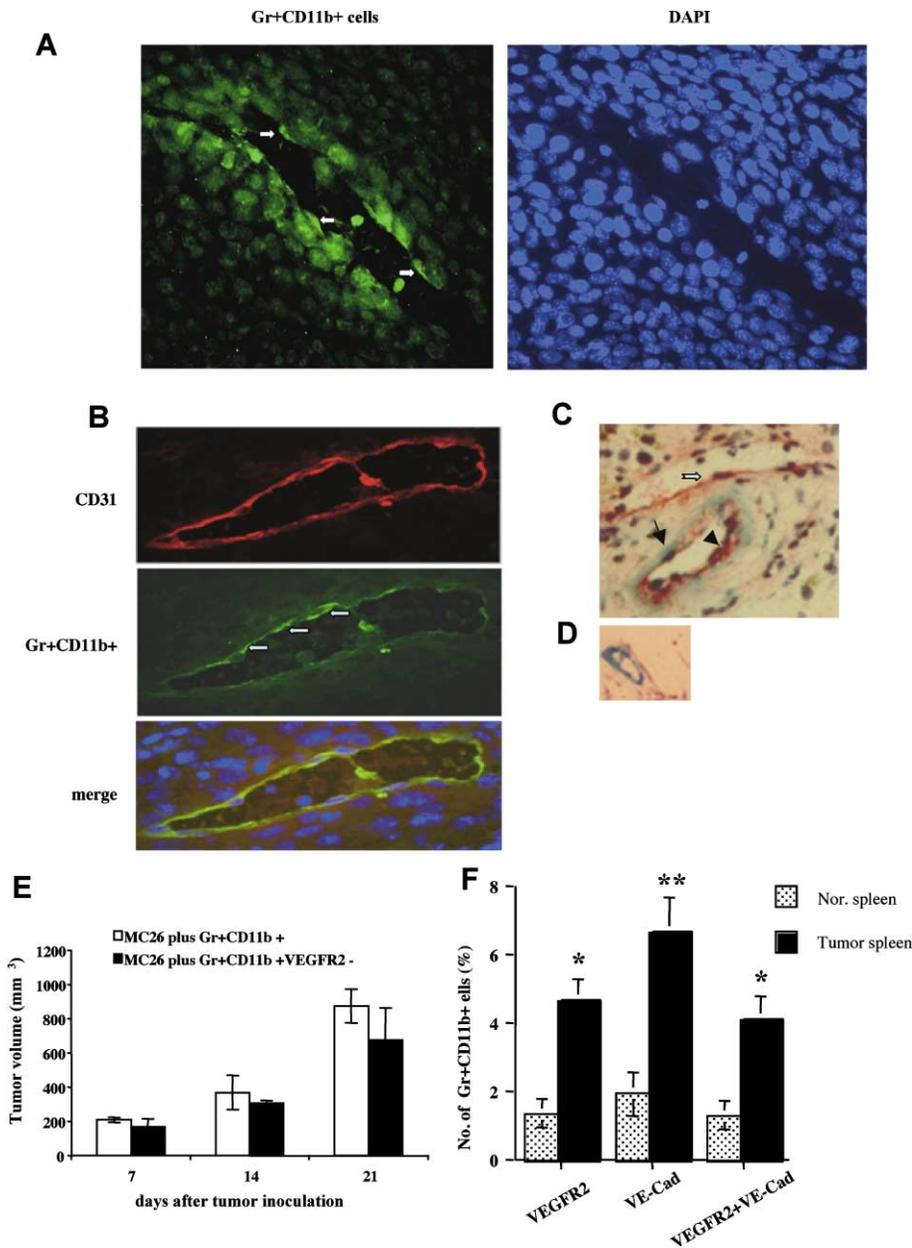


Figure 7. Gr+CD11b+ cells directly incorporated into tumor endothelium

A: 0.5×10^5 5-CFDA-labeled Gr+CD11b+ cells and 5×10^5 MC26 cells were coinjected s.c. in Balb/c mice. Tumors were harvested 21 days after implantation and frozen sections were examined under fluorescence microscopy. CFDA-labeled Gr+CD11b+ cells were observed to line the tumor endothelium (arrow). DAPI staining for nuclei.

B: Colocalization of Gr+CD11b cells with tumor endothelium. Tumor sections were stained with a CD31 antibody: CD31-positive endothelial cells (upper panel) and CFDA-labeled Gr+CD11b+ cells (arrow, middle panel) were colocalized in tumor endothelium (lower panel).

C: β -gal staining of 3LL tumor tissues derived from coinjection of tumor cells with Gr+CD11b+ cells of tumor-bearing Rosa-26 mice. The section was costained with an anti-VE-Cadherin antibody (red). β -gal-positive cells were colocalized with VE-Cadherin-positive tumor endothelium (solid arrow). Open arrow indicates a surrounding host derived vessel.

D: A small β -gal-positive vessel is shown.

E: 5×10^5 MC26 cells plus 0.5×10^5 Gr+CD11b+/VEGFR2- cells was injected s.c. in Balb/c mice. MC26 cells plus whole Gr+CD11b+ cells were used as controls. Tumor volume was measured. Results are presented as the mean \pm SEM (3–4 mice per group) ($p = 0.02$).

F: Quantification of Gr+CD11b+ cells expressing VEGFR2, VE-Cadherin, and both markers in the spleens of MC26 tumor-bearing mice and normal mice by flow cytometry ($n = 4$ pairs). **, $p < 0.01$, *, $p < 0.05$.

two major mechanisms: producing MMP9 and differentiating into ECs.

The importance of Gr+CD11b+ cells in tumor growth is not only that they are significantly increased in tumor-bearing hosts, but also that these cells constitute about 5% of the total cells in tumor tissues. We hypothesize that Gr+CD11b+ cells may not only be immune-suppressive but may also contribute to tumor growth directly. We found that there are two mechanisms by which Gr+CD11b+ cells promote tumor growth and enhance tumor angiogenesis. First, Gr+CD11b+ cells produce high levels of MMP9, an important mediator in angiogenesis (Bergers et al., 2000; Coussens et al., 2000; Heissig et al., 2002). Stromal MMP9 was shown to regulate vascular stability by recruiting pericytes (Chantrain et al., 2004). Host contribution of MMP9 from tumor-infiltrating cells was shown to play a critical role in

tumor development (Coussens et al., 2000; Huang et al., 2002). We observed that MMP9, but not MMP2, is overproduced in Gr+CD11b+ cells by zymography, and this production was essential for their ability to promote tumor angiogenesis. These data were confirmed by microarray analysis with the Affymetrix U74AV₂ array, which revealed a 5.4-fold increase in MMP9 mRNA but no difference in 21 other MMPs (data not shown). We found that MMP9 from Gr+CD11b+ cells of tumor-bearing hosts regulates bioavailability of VEGF in tumors and promotes tumor angiogenesis and vascular stability, as well as release of sKitL in BM, potentially regulating the production and mobilization of Gr+CD11b+ cells. Selective deletion of MMP9 in Gr+CD11b+ cells eliminated their ability to promote tumor growth, and, in fact, led to inhibition of tumor formation. This implies and is in agreement with the proposal that tumor angio-

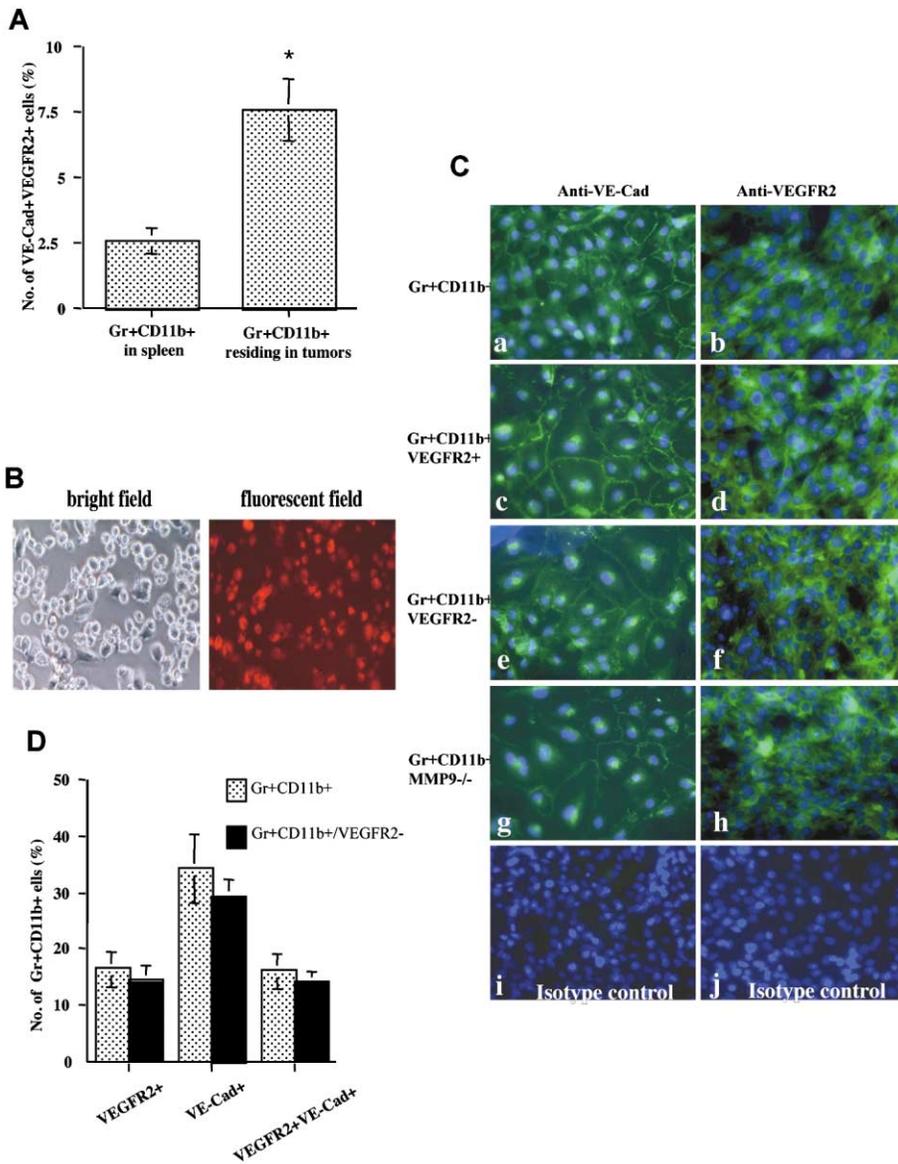


Figure 8. Tumor microenvironment and angiogenic factors induced Gr+CD11b+ cell differentiation toward ECs

A: The expression of VEGFR2 and VE-Cadherin in Gr+CD11b+ cells from MC26 tumor tissues and spleens of tumor-bearing mice was analyzed by flow cytometry. Results are presented as the mean \pm SEM from three experiments. * $p < 0.01$.

B: Gr+CD11b+ cells took up LDL. Cultured Gr+CD11b+ cells were incubated with fluorescent dye-labeled LDL for 2 hr. The micro images were taken under a microscope.

C: Gr+CD11b+ cells differentiated into ECs in vitro. Total Gr+CD11b+ cells (**a** and **b**), Gr+CD11b+/VEGFR2+ cells (**c** and **d**) and Gr+CD11b+/VEGFR2- (**e** and **f**) were sorted from the spleens of MC26 tumor-bearing mice, and MMP9 null Gr+CD11b+ cells (**g** and **h**) were from the spleens of 3LL tumor-bearing MMP9 null mice. The cells were cultured in the presence of bFGF, VEGF, and IGF for a week. Cells were stained with antibodies against VE-Cadherin (**a**, **c**, **e**, and **g**), VEGFR2 (**b**, **d**, **f**, and **h**), and isotype controls (**i** and **j**). The slides were costained with DAPI, and analyzed by an Olympus fluorescence microscope.

D: Sorted total Gr+CD11b+ cells and Gr+CD11b+/VEGFR2- cells were cultured for a week. The expression of VEGFR2 and VE-Cadherin on these cells was analyzed by flow cytometry.

genesis can be targeted by transplantation of genetically modified hematopoietic cells (De Palma et al., 2003).

The second mechanism is our finding that Gr+CD11b+ cells directly incorporate into tumor endothelium in vivo. VEGFR2+ Gr+CD11b+ cells are likely giving rise to tumor endothelium. However, cultured VEGFR2- Gr+CD11b+ cells acquire the expression of EC markers in the proangiogenic conditions in vitro and incorporate into tumor endothelium in vivo, which suggests that the participation of these cells in tumor progression is not static but rather dynamic. In agreement with this observation, Gr+CD11b+ cells exhibited significantly increased expression of several EC markers in the tumor microenvironment in vivo and proangiogenic conditions in vitro, indicating that these cells have significant plasticity. Our observation is supported by several previous reports: immature DCs, in a transient stage of differentiation in the myeloid lineage, can form cord-like structures in matrigel (Schmeisser et al., 2001). When cultured in the presence of angiogenic factors, including VEGF,

these cells develop into endothelial-like cells. They have increased expression of ECs markers (Fernandez Pujol et al., 2001). Although there are contradicting reports regarding developmental plasticity of adult progenitor cells (Joshi and Enver, 2002; Wagers et al., 2002), our results support that certain levels of plasticity exist in the tumor microenvironment. Tumors thus can affect the differentiation of Gr+CD11b+ cells not only systemically, but also locally.

Studies from cancer patients showed Gr+CD11b+ cells were composed of immature macrophages, immature DC, and immature myeloid cells at early stages of differentiation as well as a small percentage (<2%) of hematopoietic progenitor cells (Almand et al., 2001). In characterizing Gr+CD11b+ cells, we found that they contain cell subsets expressing c-kit ($4.82 \pm 0.51\%$), CD34 ($7.1 \pm 0.95\%$), and Sca-1 ($18 \pm 3.3\%$), markers often found in progenitor cells. But Gr+CD11b+ cells express both Gr+ and CD11b, unlike conventional myeloid progenitor cells, and are therefore much more differentiated than classical

progenitors. The fact that these cells express markers for progenitor cells indicates that they are likely immature myeloid cells in an intermediate stage of differentiation. In addition, these cells express ECs markers, indicating their close relationship with ECs. The Gr+CD11b+ cells are different from BM-derived EPCs that are described as CD11b-negative (Hattori et al., 2001). Therefore, Gr+CD11b+ cells may represent a new population of cells that have the potential to differentiate into ECs with particular relevance to tumor angiogenesis.

The Gr+CD11b+ cells, as immature myeloid cells, have the ability to migrate through tumor hosts, as do other immune cells. It may be that tumors take advantage of this property of immune cells and recruit them to the tumor site. These immune cells produce high levels of MMP9, and also directly differentiate and incorporate into the vascular endothelium, promoting tumor vascularization and tumor progression. The fact that these cells are overproduced, exist as immature myeloid cells in the tumor-bearing hosts, and can be reprogrammed is very important in understanding tumor-host interactions.

We have shown that VEGF, a potent angiogenic factor abundantly expressed in tumors, regulates the production and immune suppression of Gr+CD11b+ cells in vivo. In this report, we identify a novel function of these cells in promoting tumor angiogenesis. It is conceivable that the tumor microenvironment and VEGF modulate the production and differentiation of Gr+CD11b+ cells, which then exhibit different functions, including host immune suppression and promotion of tumor angiogenesis. There are preclinical and clinical trials using anti-VEGF antibody and all-trans-retinoic acid to induce the differentiation of these cells in an attempt to improve host antitumor response (Gabrilovich et al., 1999; Kusmartsev et al., 2003) (D. Gabrilovich, personal communication). Based on our findings, we suggest that cancer vaccine strategies directed toward enhancing the antitumor immune response should take these cells into account together with their proangiogenic properties of MMP9 production and plasticity. Therapeutic approaches directed at this aspect of the host-tumor interface may have synergistic immune and antiangiogenic effectiveness.

Experimental procedures

Cell lines and mice

MC26 colorectal cancer cell line, and Lewis lung carcinoma cell line (3LL), were maintained per standard cell culture techniques.

Eight- to ten-week-old female Balb/c or C57Bl/6 mice and Rosa-26 mice were purchased from Harlan Inc. (Indianapolis, IN) and Jackson Laboratory (Bar Harbor, ME). MMP9 knockout mice (Vu et al., 1998) were backcrossed into a C57Bl/6 background to $n > 10$. The animals were housed in pathogen-free units at Vanderbilt University Medical Center, in compliance with IACUC regulations.

Flow cytometry analysis

Single cell suspensions were made from spleens and BM of normal and tumor-bearing mice. For tumor tissues, we followed a published protocol (Ljung et al., 1989). These cells were labeled with fluorescence-conjugated antibodies (BD Pharmingen) and isotype-matched IgG controls. The cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Single-cell sorting

Splenocytes from normal or tumor-bearing mice were stained with fluorescence-labeled antibodies and sorted with a FACStarPlus flow cytometer (Becton Dickinson, Franklin Lakes, NJ). Gr+CD11b+ cells and the subpopu-

lations were collected for tumor growth, MMP9 zymography, cytospin, and in vitro differentiation experiments.

Cytospin and HE staining

Sorted Gr+/CD11b+ cells were spun onto glycine-coated slides (Cytospin 3, Shandon, PA). The cells were fixed in 4% paraformaldehyde and stained with HE.

Isograft models of tumor biology

MC26 or 3LL cells (5×10^5 cells), combined with Gr+CD11b+ cells (0.5×10^5), were injected subcutaneously (s.c.) into the right flank of Balb/c mice or C57Bl/6 mice, respectively. The size of tumors was determined by direct measurement of tumor dimensions at 2–3 day intervals using calipers. The equation volume = length \times (width)² \times 0.5 was used to calculate tumor volume.

Magnetic resonance imaging

Anesthetized tumor-bearing mice were examined with a 3.0 Tesla MR scanner (GE Medical Systems). Imaging included coronal 1 mm slice thickness with 0.1 mm gap conventional gradient echo T1-weighted images before and after injection of 0.05 ml of GdDTPA (Magnevist). The total tumor volume was assessed in each MR examination by ROI-based volumetry. The tumor area was traced on the coronal T1-weighted image. To determine areas of little or no contrast enhancement (i.e., necrotic areas), coregistered precontrast images were subtracted from postcontrast images; the pixels of the generated difference image were counted to determine the area within the tumor that did not enhance with contrast.

Immunohistochemistry

Tumor sections were incubated with an anti-CD31 antibody (Pharmingen) or anti-VEGF/VEGFR2 complex antibody (Brekken et al., 2000), respectively. A biotinylated 2nd antibody was applied, followed by incubation with streptavidin-conjugated HRP. Peroxidase activity was localized with diaminobenzidine (Vector Laboratories).

For CD31 and α SMC double staining, frozen sections were blocked with M.O.M. solution (Vector Lab), incubated with antibodies to CD31 and to α SMA (DAKO) overnight (4°C), and visualized by using Texas Red-conjugated goat anti-rat and Cy2-conjugated goat anti-mouse 2nd antibodies (Jackson Immuno Research Laboratories).

For β -galactosidase staining, tumor tissues from Rosa-26 mice were stained with a X-gal solution, followed by costaining with an anti-VE-Cadherin antibody and an AP-conjugated 2nd antibody (Vector Lab).

For apoptosis, frozen tumor sections were stained with ApopTag Plus Peroxidase in situ Apoptosis Detection Kit (Intergen). For hypoxia, one hour before sacrifice, mice were i.p. injected with Hypoxyprobe-1 at 60 mg/kg (Chemicon International, Inc). Tumor tissues were processed and frozen sections were incubated with Hypoxyprobe-1-MAb1 according to the manufacturer's recommendation.

Immunoassay for cytokines and chemokines

Conditioned media and tissue lysates were analyzed for VEGF, TNF α , and sKitL expression by ELISA using commercial kits (R&D Systems).

In vitro cell migration assay

Human umbilical vein ECs (HUVECs) were seeded into the top chamber of Transwell filters (VWR Scientific) coated with fibronectin. The filters were placed in a 24-well plate that contains 15 small pieces of tumor tissues (0.5 mm size) per well in serum-free media. Recombinant soluble VEGFR2 (ExFlk) (Lin et al., 1998) at 1 μ g/ml or MMP inhibitor GM6001 at 20 μ M was added into the bottom chamber. Migrated ECs were counted (8 fields/filter) 5 hr after incubation.

BM stromal cell culture

BM cells from Balb/c mice were seeded onto fibronectin coated 24-well plates for 48 hr, and floating cells were removed by washing with PBS. Gr+CD11b+ cells were seeded into either fresh wells or on top of the stromal cells in serum-free media. GM6001 at 20 μ M was used. Conditioned media was collected 24 hr after the coculture for analysis of sKitL levels.

In situ hybridization of MMP9

An 835 bp fragment of the 3'UTR (Witty et al., 1995) was labeled with digoxigenin according to manufacturer's instructions (Roche). Tumor sections were microwaved in 10 mM citrate (pH 6.0), treated with 5 µg/ml proteinase K, and incubated with the labeled probe for 16 hr at 50°C, followed by incubation with an AP-labeled anti-DIG antibody and incubation with the BCIP/NBT. The slides were counterstained with Contrast Green (Kirkegaard and Perry).

Zymography of MMP9

Cells were lysed in buffer containing 500 mM Tris-HCl (pH 7.4), 200 mM NaCl, 10 mM CaCl₂, and 1% Triton X-100. Aliquots of 20 µg proteins were analyzed by gelatin zymography on SDS-PAGE with substrate (1 mg/ml of gelatin). After electrophoresis, gels were washed in buffer (50 mM Tris-HCl [pH 7.5] with 100 mM NaCl and 2.5% Triton X-100) followed by incubation with 50 mM Tris-HCl (pH 7.5) and 10 mM CaCl₂ for 20 hr at 37°C, and then stained with 0.25% Coomassie Brilliant Blue R-250.

In vitro differentiation of Gr+CD11b+ cells

Bright Gr+CD11b+ cells and a subpopulation of cells were sorted from spleens of tumor-bearing mice by FACS, seeded on fibronectin-coated tissue culture slides (Nalgene, Rochester, NY), and cultured at 37°C. The media consists of 70% endothelial growth media (Clonetics Inc, Walkersville, MD) and 30% NIH3T3 culture media plus 20 ng/ml VEGF (NCI), 2.5 ng/ml IGF (R&D Systems Inc, Minneapolis, MN), and 2 ng/ml bFGF (NCI). The cells were processed for immunofluorescent staining with anti-VEGFR2, anti-VE-Cadherin antibodies (Santa Cruz), and appropriate Alexis-488 conjugated secondary antibodies (Molecular Probes, Eugene, OR), with nuclei stained with DAPI. The cells were examined under an Olympus IX70 microscope.

For LDL uptake assay, Gr+CD11b+ cells were cultured on fibronectin-coated wells for a week. The assay was carried out in the serum-free EBM medium (Clonetics) in the presence of Dil-acLDL (Molecular Probes) at 10 µg/ml for 2–3 hr, then washed and examined.

Gr+CD11b+ cell labeling

Gr+CD11b+ cells were labeled with 5-CFDA SE (Molecular Probes) for long-term tracking of cells in vivo. A cell suspension containing labeled 0.5×10^5 Gr+CD11b+ cells, and 5×10^5 MC26 cells were coinjected s.c. into the right flank of Balb/c mice. Tumors were removed 21 days after the inoculation and cryopreserved in OCT (Sakura Finetek Inc.). Frozen sections were mounted on glass slides and stained with DAPI and CD31 antibody, and examined.

Statistical analysis

The REML-based mixed effect model and ANOVA were used to analyze the differences of tumor growth. The rest data were analyzed by the Student's t test. All data were expressed as means \pm SEM, and differences were considered statistically significant when the p value < 0.05.

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