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The Role of IL-1β in the Early Tumor Cell–Induced Angiogenic Response

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In this study, we assessed the involvement of IL-1β in early angiogenic responses induced by malignant cells using Matrigel plugs supplemented with B16 melanoma cells. We found that during the angiogenic response, IL-1β and vascular endothelial growth factor (VEGF) interact in a newly described autoinduction circuit, in which each of these cytokines induces the other. The IL-1β and VEGF circuit acts through interactions between bone marrow–derived VEGF receptor 1^+/IL-$1R1^+$ immature myeloid cells and tissue endothelial cells. Myeloid cells produce IL-1β and additional proinflammatory cytokines, which subsequently activate endothelial cells to produce VEGF and other proangiogenic factors and provide the inflammatory microenvironment for angiogenesis and tumor progression. These mechanisms were also observed in a nontumor early angiogenic response elicited in Matrigel plugs by either rIL-1β or recombinant VEGF. We have shown that IL-1β inhibition stably reduces tumor growth by limiting inflammation and inducing the maturation of immature myeloid cells into M1 macrophages. In sharp contrast, only transient inhibition of tumor growth was observed after VEGF neutralization, followed by tumor recurrence mediated by rebound angiogenesis. This occurs via the reprogramming of VEGF receptor 1^+/IL-$1R1^+$ cells to express hypoxia inducible factor-1α, VEGF, and other angiogenic factors, thereby directly supporting proliferation of endothelial cells and blood vessel formation in a paracrine manner. We suggest using IL-1β inhibition as an effective antitumor therapy and are currently optimizing the conditions for its application in the clinic. *The Journal of Immunology*, **2013, 190: 3500–3509.**

Progressively growing tumors emerge from a unique microenvironment derived from the interactions between tumor cells and the host that support tumor proliferation, invasiveness, and immune escape (reviewed in Refs. 1–6). Myeloid cells, such as myeloid-derived suppressor cells (MDSCs) and other hemopoietic precursors, neutrophils, and monocytes/macrophages, are recruited to the tumor microenvironment by factors released by the malignant cells, and they are subsequently educated in situ to acquire a proinvasive and proangiogenic as well as immunosuppressive phenotype (reviewed in Refs. 1, 7–14).

The angiogenic switch in tumors occurs after disruption of the local, delicate balance of pro- and antiangiogenic factors, which causes changes in the malignant cells, such as switching on secretion of vascular endothelial growth factor (VEGF), as well as changes in the tumor microenvironment (reviewed in Refs. 5, 15–20). Inflammation usually accompanies pathological angiogenesis, whereas it is not observed during a physiological angiogenic response. It has been suggested that inflammatory mediators act in parallel pathways to direct angiogenic factors during tumor-induced angiogenesis. However, Grunewald et al. (21) demonstrated the requirement for inflammation in the angiogenic response, even when VEGF is overexpressed in tissues. The proximity of myeloid cells to endothelial cells (ECs) lining blood vessels suggests a paracrine role for myeloid cells in the angiogenic response by mechanisms not yet completely elucidated. Most studies assessed mechanisms of tumor-mediated angiogenesis in established tumors, whereas interactions between cancer cells and their microenvironment in the angiogenic switch were not examined, due to difficulties in recovering and evaluating small or even nonapparent tumors at early stages of tumor invasiveness.

The necessity for microenvironment-derived IL-1β for tumor-mediated angiogenesis and the ability of the IL-1 inhibitor, the IL-1 receptor antagonist (IL-1Ra), to reduce the angiogenic response and to attenuate tumor progression was demonstrated by us (22–24) and by others (25–28). However, the mechanisms through which IL-1β induces the angiogenic switch and activates tumor progression have not yet been characterized.

VEGF-A has been identified as a predominant regulator of tumor angiogenesis (reviewed in Refs. 5, 15–20). In cancer, in response to hypoxia and/or inflammatory signals, VEGF is upregulated and

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Abbreviations used in this article: bFGF, basic fibroblast growth factor; BM, bone marrow; EC, endothelial cell; IL-1Ra, IL-1 receptor antagonist; iMC, immature myeloid cell; KO, knockout; MDSC, myeloid-derived suppressor cell; MHC II, MHC class II; MVD, microvessel density; PDGF, platelet-derived growth factor; rVEGF, recombinant vascular endothelial growth factor; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor; vWF, von Willebrand factor; WT, wild-type.

secreted by tumor cells or induced in cells of the microenvironment. The biological effects of the members of the VEGF family are mediated by two tyrosine kinase receptors, VEGF receptor (VEGFR) 1 and VEGFR2. VEGFR2 is mainly expressed in ECs and its signaling is involved in migration and proliferation of ECs. In contrast, signaling through VEGFR1 in ECs does not induce their activation and rather acts as a VEGF decoy receptor. VEGFR1 is expressed on a variety of myeloid cells, such as monocytes/macrophages and immature myeloid cells (iMCs), where its signaling is essential for cell migration to sites of inflammation and tumors (reviewed in Refs. 29, 30). Myelomonocytic VEGFR1-positive cells were shown to be especially important in tumor invasiveness and in creating the metastatic niche of invasive tumors (11, 12, 29, 31–33). Signaling through VEGFR1 in myeloid cells was shown to be involved in their migration to tumor sites, contributing significantly to angiogenesis and tumor progression.

In the current study, we assessed the role of IL-1β in the early angiogenic response occurring in plugs of basement membrane-like matrix (Matrigel, BD Biosciences, San Jose, CA) containing tumor cells, emphasizing the interactions between IL-1β and VEGF, the major proinflammatory and angiogenic factors, respectively. We showed that IL-1β and VEGF induce each other and act in a differential, but complementary, manner in the induction of angiogenesis in Matrigel plugs supplemented by tumor cells. This cross talk between IL-1β and VEGF occurs as a result of in situ interactions between infiltrating myeloid cells of bone marrow (BM) origin and tissue-resident ECs. The intricacies of the involvement of IL-1β in the early, tumor-induced, angiogenic response described in this study suggest the feasibility of using IL-1 neutralization approaches in anticancer therapy.

Materials and Methods

Mice

IL-1Ra and IL-1β knockout (KO) mice were generated as described (34). GFP transgenic mice (C57BL/6-Tg [UBC-GFP] 30Scha/J) and IL-1R1 KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Wild-type (WT) C57BL/6 mice were obtained from Harlan. Animal studies were approved by the Animal Care Committee of Ben-Gurion University.

Cell lines

B16-F10 and B16-mCherry melanoma cell lines were cultured under standard conditions. B16-mCherry melanoma cells were used to discriminate between malignant cells and inflammatory cells. Both cell lines have similar growth patterns in mice. Cell lines were routinely tested for mycoplasma and for their ability to generate characteristic tumors in recipient mice.

Tumor models

For tumor-invasiveness studies, 2×10^5 B16-F10 tumor cells were injected into the footpad of mice. Neutralizing Abs against IL-1β (AF-401-NA) or VEGF (AF-493-NA) (R&D Systems, Minneapolis, MN) (10 μg/mouse, i.p.) were injected once a week after tumor challenge. Tumor development was measured with a caliper every other day, and the experiments were terminated when tumor size reached a diameter of 8 mm.

For the Matrigel plug assay, 0.5 ml Matrigel (BD Biosciences) was mixed with 2×10^5 B16 cells and injected s.c. into mice. In some experiments, neutralizing Abs against IL-1β (1 μg/plug) or VEGF (1 μg/plug) were added to the Matrigel mixture and incubated at 4°C for 2 h before injection into mice. At different time intervals, Matrigel plugs were removed for further examination.

BM transplantation experiments

WT mice were irradiated with a lethal dose of γ-Cobalt (130 Gy γ-Cobalt per mouse), and 24 h later, mice were injected i.v. with GFP-tagged BM-derived cells (2.5×10^6/mouse in 0.2 ml PBS). After 2 mo, these mice were injected with Matrigel (BD Biosciences) supplemented with tumor cells.

Recovering cells from Matrigel plugs

Matrigel plugs were obtained from mice and further digested using a mixture of enzymes (1 g/100 ml collagenase type IV, 20,000 units/100 ml DNase type IV, 1 mg/ml hyaluronidase type V [Sigma-Aldrich, Rehovot, Israel]) at 37°C for 1 h, as described (35). Recovered cells were analyzed and used for further experiments. MACS separation columns (Miltenyi Biotec, Bergisch-Gladbach, Germany) were used for isolation of specific infiltrating cells using anti-mouse CD11b, anti-mouse VEGFR1, or anti-mouse VEGFR2 (all from eBioscience, San Diego, CA) (35). Supernatants from digested Matrigel plugs were collected and stored at 20°C for subsequent evaluation. In all experiments in which Matrigel-infiltrating cells were characterized or isolated for further experiments, we used B16-mCherry cells.

The aortic ring sprouting assay

Aortas were removed from 6-wk-old naive WT mice and embedded in Matrigel in 48-well plates together with cells, Abs, or supernatants to determine their angiogenic potential. Sprouting was observed after 72 h under a light microscope, as described (36).

Cytokine measurements

IL-1β levels were measured using a specific ELISA kit (BD Biosciences). To measure concentrations of VEGF, we used the DuoSet specific for mouse VEGF (R&D Systems).

Real-time PCR

Total mRNA was extracted using an RNA extraction kit (RNeasy; Qiagen, Valencia, CA). One microgram total RNA was annealed with random hexamers and reverse-transcribed using MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). Subsequent real-time PCR was performed with an ABI Prism 7500 sequence detection system (Applied Biosystems). The following primers were used: Actg, forward 5′-TGGATCTCTGTGAGCACCAC-3′ and reverse 5′-AGGCAACTAACAACCGATGG-3′; IL-1β, forward 5′-TTGGGAAACACAGGTCAGAA-3′ and reverse 5′-CCCTTGTGCTAGGATGGACT-3′; VEGF, forward 5′-ACTGGACCCTGGCTTTACTG-3′ and reverse 5′-AGCTTCGCTGGTAGACATCC-3′; angiopoietin 1, forward 5′-CAAATGCGCTCTCATGCTAA-3′ and reverse 5′-ATCATGGTGGTGGAACGTAAG-3′; basic fibroblast growth factor (bFGF), forward 5′-CCCGACGGCCGCGTGGAT-3′ and reverse 5′-ACTTAGAAGCCAGCAGCCG-3′; platelet-derived growth factor (PDGF), forward 5′-CGAAAGAAGCCCATCTTCAA-3′ and reverse 5′-CCTTGTCATGGGTGTGCTTA-3′; placental growth factor (PlGF), forward 5′-CTGCTGGGAACAACTCAA-3′ and reverse 5′-AGGACACAGGACGGACTG-3′; MMP9, forward 5′-ACTATGGAAACTCAAATGGTGC-3′ and reverse 5′-GAGCCACGACCATACAGATACT-3′; stromal cell–derived factor-1α, forward 5′-TGCCAGAGCCAACGTC-3′ and reverse 5′-CAGCCGGGCTACAATC-3′; CCL3, forward 5′-TTGAAACCAGCAGCCTTTG-3′ and reverse 5′-CAGGCATTCAGTTCCAGGTC-3′; CCL2, forward 5′-CCTGCTGCTACTCATTCACC-3′ and reverse 5′-CACAGACCTCTCTCTTGAGCTTG-3′; and Bv8, forward 5′-TGTGACAAGGACTCCCAATG-3′ and reverse 5′-AGACATGGGCAAGTGTGATG-3′.

Specificity of each primer was verified by the National Center for Biotechnology Information Blast module, and the primers were synthesized by IDT (Beit-Shemesh, Israel). PCR results were analyzed with SDS 2.02 software (Applied Biosystems). The level of target gene expression was calculated following normalization of the actin gene level in each sample and presented as relative units.

Immunohistochemistry (paraffin sections)

Samples from Matrigel plugs were embedded in paraffin and stained with H&E or subjected to immunohistochemistry, according to previously described protocols (36). Rabbit polyclonal anti–von Willebrand factor (vWF) (DakoCytomation, Glostrup, Denmark), rat anti-CD11b (Serotec, Oxford, U.K.), goat anti–IL-1β (R&D Systems), rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), and monoclonal mouse anti-human Smooth Muscle Actin (DakoCytomation) Abs were used for staining. The Vectastain Elite ABC Peroxidase kit (Vector Laboratories, Burlingame, CA) or the universal anti-mouse/rabbit IG ImmPRESS Reagent Kit (MP-7500; Vector Laboratories) was used for secondary Ab detection. Visualization was performed using AEC as a substrate (Zymed Laboratories, San Francisco, CA).

Immunohistochemistry (frozen sections)

Part of the Matrigel plugs were frozen at −80°C in frozen tissue matrix (Tissue-Tek OCT, Torrance, CA) for immunofluorescence staining. The following Abs were used: goat anti–IL-1β, goat anti-VEGF, rat anti-VEGFR1, and goat anti-VEGFR2 (all purchased from R&D Systems), rat anti-CD11b (eBioscience, San Diego, CA), and rabbit anti–hypoxia inducible factor-1α (HIF-1α; Bethyl Laboratories, Montgomery, TX). Sec-

ondary Abs conjugated with Cy-2 or Cy-3 (Jackson ImmunoResearch Laboratories, West Grove, PA) were used. Nuclei were counterstained with DAPI. Sections were examined under a Zeiss Laser Scanning Confocal Microscope (Carl Zeiss).

Microvessel density

The microvessel density (MVD) was calculated by counting blood vessels in areas of high vascularity, as described (24).

Flow cytometry

Single-cell suspensions obtained from Matrigel plugs were analyzed using flow cytometry (FACSCanto II; BD Biosciences). Datasets were analyzed using FlowJo software (Tree Star). mAbs conjugated to FITC, PE, PE-Cy7, PE-Cy5.5, allophycocyanin, allophycocyanin-Cy7, or Pacific Blue specific for the following Ags were used: anti-CD11b, anti-Gr1, anti-F4/80, anti-CD80, anti–I-A^b, anti-CD31, anti-VEGFR2 (eBioscience), and anti-VEGFR1 (R&D Systems). Dead cells were excluded using the Live/Dead Fixable Aqua Dead Cell Stain kit (Invitrogen). Intracellular cytokine staining in permeabilized cells was performed using anti–IL-1β (eBioscience) and anti-VEGF Abs (R&D Systems) as described (35).

Angiogenesis induced by recombinant cytokines in Matrigel plugs

The Matrigel plug assay, with either rIL-1β (0.5 μg/plug) or recombinant VEGF (rVEGF; 0.5 μg/plug) (PeproTech, Rocky Hill, NJ), was performed as indicated above. Neutralization assays were performed with anti–IL-1β or anti-VEGF, as indicated above. Anti-VEGFRI (1 μg/plug) and anti-VEGFRII (1 μg/plug) (R&D Systems) were also used.

Statistical analyses of the results

Each experiment was performed three to five times. In Matrigel plug assays, each experimental group consisted of four to five mice. Significance of results was determined using the two-sided Student *t* test.

Results

Myeloid cell–derived IL-1β is essential for tumor angiogenesis

To evaluate the role of IL-1β in early events of the tumor-induced angiogenic response, we used the Matrigel plug assay that was routinely terminated on day 9. The gross morphology of a Matri-

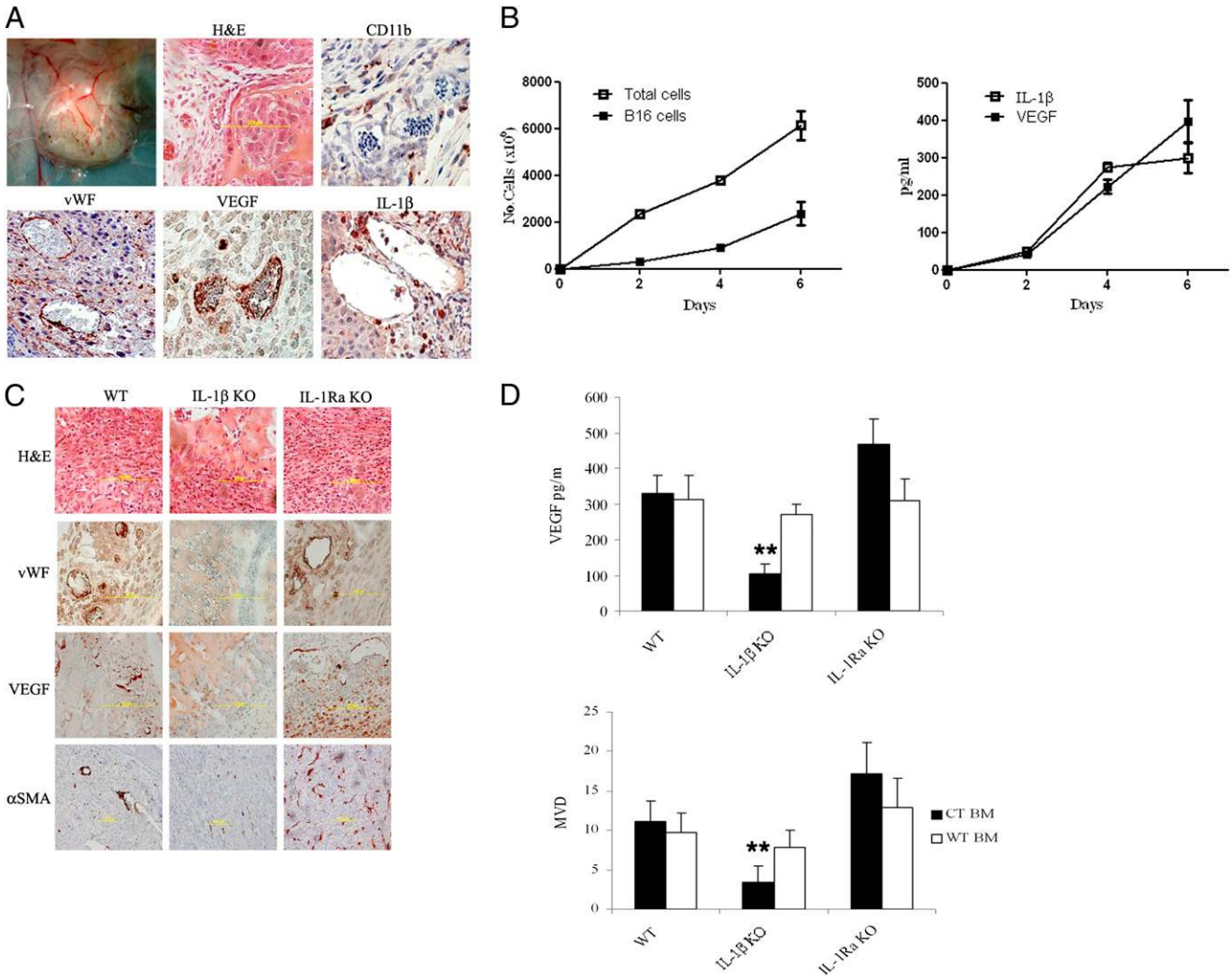

FIGURE 1. Microenvironment-derived IL-1β is involved in tumor angiogenesis. (**A**) Gross morphology and histological sections from day 9 Matrigel plugs stained with H&E (original magnification ×200), anti-CD11b, anti-vWF, anti-VEGF, or anti–IL-1β (original magnification ×400). Representative pictures from one out of six independent experiments. (**B**) Kinetics of tumor and inflammatory cell accumulation in Matrigel plugs. Total cell counts versus $CD45^{neg}/PE^{pos}$–B16-mCherry cells (*left panel*); kinetics of IL-1β and VEGF secretion (*right panel*). The data represent mean ± SEM from three independent experiments. (**C**) Microenvironment IL-1β dictates tumor cell–mediated angiogenic responses. Paraffin sections of Matrigel plugs containing B16 cells were obtained on day 9 from WT and IL-1β KO mice and stained with H&E (original magnification ×200), anti-vWF, anti-VEGF, or anti–α-smooth muscle Ab (αSMA) (original magnification ×400). Representative pictures from one out of five independent experiments. (**D**) BM-derived cells producing IL-1β are essential for the angiogenic response. VEGF levels (*top panel*) and MVD counts (*bottom panel*) in day 9 Matrigel plug extracts from WT and IL-1β KO mice lethally irradiated and rescued with self-BM (control [CT] BM) or BM cells from WT mice. The data represent mean ± SEM from five independent experiments. The *p* values relate to statistical significance compared with the relevant values in WT mice. $^{**}p < 0.005$ ($n = 4$–5 mice/group).

gel plug containing B16 melanoma cells is presented in Fig. 1A. In H&E and immunohistochemical stainings, the close proximity of myeloid cells (CD11b-positive) to blood vessels (vWF-positive) and tumor deposits is shown. It was found that VEGF is mostly expressed in cells lining blood vessels, whereas IL-1β is expressed in mononuclear cells near blood vessels (Fig. 1A). The number of B16 and infiltrating cells increased with time in Matrigel plugs (Fig. 1B, *left panel*) concomitantly with increasing levels of VEGF and IL-1β (Fig. 1B, *right panel*).

The necessity of IL-1 for the tumor-induced angiogenic response was further demonstrated using IL-1β and IL-1Ra KO mice. In B16-containing Matrigel plugs obtained from IL-1β KO mice, a minimal angiogenic response was evidenced by low numbers of blood vessels (vWF), decreased numbers of VEGF-producing cells, and pericyte-coated mature blood vessels that were α-smooth muscle actin–positive (Fig. 1C). In contrast, in IL-1Ra KO mice, which display unattenuated IL-1 activity, angiogenesis was more pronounced than in WT mice. Reconstitution of irradiated IL-1β KO mice with BM cells from WT mice resulted in a significant restoration of the angiogenic response in Matrigel plugs (Fig. 1D). BM transplantation of lethally irradiated WT mice and IL-1Ra KO mice with BM cells from WT mice was used as a control. These results clearly show that an IL-1β–induced inflammatory response, mediated by BM-derived myeloid cells, is essential for inducing the angiogenic switch in B16-containing Matrigel plugs.

Next, we characterized the composition of the cellular infiltrate in B16 melanoma cell–containing Matrigel plugs. All infiltrating cells were initially gated as PE-negative to exclude tumor cells from the analyses. As shown in Fig. 2A, *left panel*, most ECs displayed a mature phenotype ($CD11b^{neg}/CD117^{neg}/VEGFR2^{+}/CD31^{+}$), and their number increased with time. Immature ECs ($CD117^{+}/VEGFR2^{+}/CD31^{neg}$) infiltrated the plugs only in low numbers. Two dominant populations of myeloid cells infiltrated the Matrigel within hours, iMCs ($Gr1^{hi}/CD11b^{hi}/VEGFR1^{+}$/MHC class II [MHC II]^{neg}) and, to a lesser extent, mature macrophages ($CD11b^{+}/Gr1^{lo}/F4/80^{+}$/MHC $II^{+}/CD80^{+}$). We prefer to use the term iMCs, rather than MDSCs, as in this study, we did not examine immunomodulating functions of myeloid cells. The number of iMCs increased with time. An elevation in the number of macrophages was observed only at early time intervals (until day 6), after which their numbers decreased (Fig. 2A, *right panel*).

To assess the cell source of IL-1 and VEGF at the tumor site, sections from day 9 Matrigel plugs were stained for both cytokines, along with VEGFR1, a functional marker for protumorigenic and proangiogenic myeloid cells, or VEGFR2, which is mainly expressed by ECs. IL-1β was predominantly coexpressed with

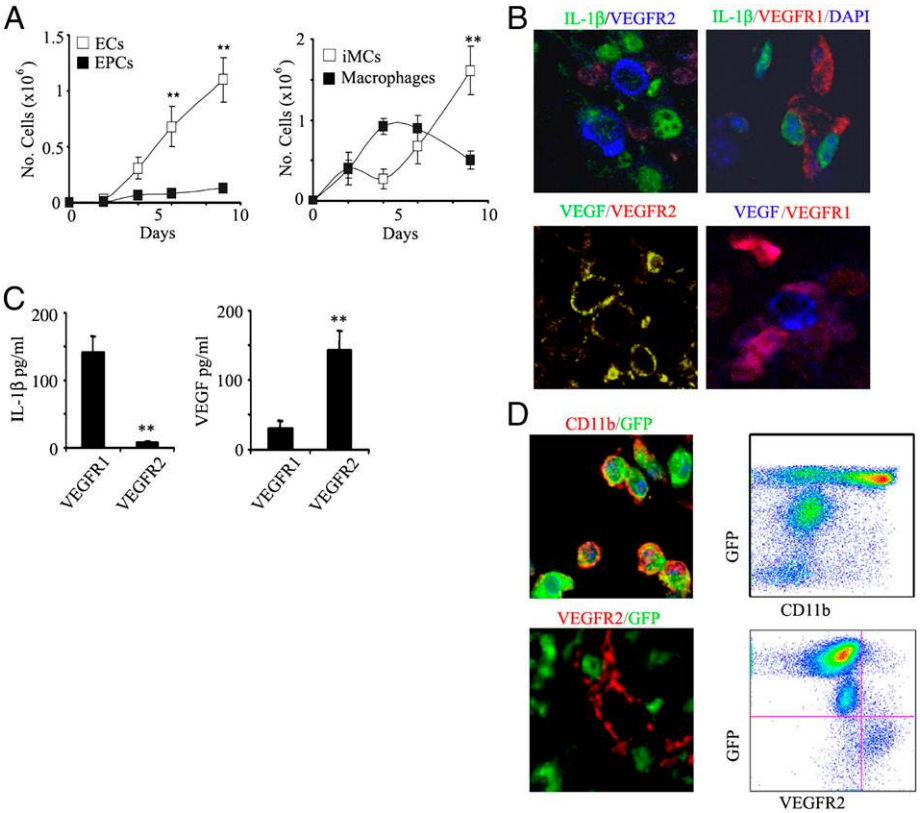

FIGURE 2. IL-1β and VEGF expression by different cells at early stages of the angiogenic response in Matrigel plugs. (**A**) Kinetics of myeloid cells, $Gr1^{lo}/CD11b^{+}/F4/80^{+}$/MHC II^{+} (mature macrophages) and $Gr1^{hi}/CD11b^{+}/VEGFR1^{+}$ (iMCs) (*right panel*), and ECs, $CD117^{+}/VEGFR2^{+}/CD31^{neg}$ (EPCs) and $CD11b^{neg}/CD117^{neg}/VEGFR2^{+}/CD31^{+}$ (mature ECs) (*left panel*), in B16 cell-containing Matrigel plugs. Data represent mean ± SEM from three independent experiments (n = 4–7 mice/group in each experiment). (**B**) Expression of IL-1β and VEGF in Matrigel plugs. Frozen sections of day 9 Matrigel plugs were stained with anti–IL-1β (green), anti-$VEGFR2^{+}$ (blue), and anti-$VEGFR1^{+}$ (red) (*top panel*) or with anti-VEGF (green) and anti-$VEGFR2^{+}$ (red) or anti-VEGF (blue) and anti-$VEGFR1^{+}$ (red) (*bottom panel*) (original magnification ×600). Representative pictures from one out of three independent experiments. (**C**) Levels of IL-1β and VEGF in 24-h supernatants of $VEGFR1^{+}$ and $VEGFR2^{+}$-enriched cells recovered with magnetic beads from day 9 Matrigel plugs. Data represent mean ± SEM from four independent experiments (n = 5–8 samples/group in each experiment). The p values relate to statistical significance comparing secretion patterns of $VEGFR1^{+}$- versus $VEGFR2^{+}$-enriched cells. (**D**) *Top panel*, Frozen sections (*left panel*) and FACS analysis (*right panel*) of Matrigel plugs containing B16 cells obtained from GFP-tagged BM chimeric mice stained for CD11b (red). *Bottom panel*, Frozen sections (*left panel*) and FACS analysis (*right panel*) of Matrigel plugs obtained from GFP-tagged BM chimeric mice stained for VEGFR2 (red) (original magnification ×60). Photomicrographs and FACS analyses are from one representative experiment out of three performed. $^{**}p < 0.005$.

VEGFR1-positive cells (Fig. 2B, *top panel*), whereas VEGF colocalized with VEGFR2-stained ECs (Fig. 2B, *bottom panel*). VEGFR2$^+$ or VEGFR1$^+$ cells were isolated from day 9 Matrigel plugs using magnetic beads. All VEGFR2$^+$ cells were also CD31$^+$, whereas 85% of VEGFR1$^+$ cells were CD11b$^+$. To determine if these cells secrete IL-1β and VEGF, isolated cells were cultured for 24 h, and supernatants were examined for cytokine production. IL-1β was detected only in supernatants from VEGFR1$^+$-enriched myeloid cells, whereas VEGF was mainly secreted by VEGFR2$^+$-enriched cells (Fig. 2C).

Finally, we demonstrated the origin of myeloid cells and ECs infiltrating B16-containing Matrigel plugs using GFP-tagged BM chimeric mice. Most myeloid cells (CD11b$^+$/VEGFR1$^+$cells) were colocalized with GFP (Fig. 2D, *top panel*), whereas VEGFR2$^+$ ECs were mainly GFP negative (Fig. 2D, *bottom panel*). This indicates that myeloid cells of BM origin and tissue-resident ECs participate in the early angiogenic response in Matrigel plugs containing B16 melanoma cells.

VEGFR2$^+$ endothelial cells and VEGFR1$^+$ myeloid cells perform different functions in the early angiogenic response of tumors. The expression patterns of other proinflammatory and angiogenic factors in enriched VEGFR1$^+$ myeloid and VEGFR2$^+$ ECs recovered from Matrigel plugs were further assessed. Expression levels of the major angiogenic factors, such as VEGF, bFGF, PlGF, and PDGF, and also of stromal cell–derived factor-1α, were 2–4-fold higher in VEGFR2$^+$ cells, compared with VEGFR1$^+$ cells (Fig. 3A, *top panel*), whereas proinflammatory mediators, such as IL-1β, Bv8, angiopoietin 1, MMP-9, and CCL2 were dominantly expressed in VEGFR1$^+$-enriched myeloid cells (Fig. 3A, *bottom panel*). Thus, myeloid cells and ECs complement each other's function in the early angiogenic response by expressing the essential proinflammatory and proangiogenic factors, respectively. To elucidate the distinct role of myeloid and ECs in the tumor-mediated early angiogenic response, we examined their direct effects on EC function, using the aortic ring-sprouting assay, which is inflammation independent. Coculture of aortic rings from control mice with VEGFR2$^+$-enriched cells induced marked EC sprouting, whereas only marginal sprouting was observed following coincubation of aortic rings with VEGFR1$^+$-enriched cells (Fig. 3B). These findings are consistent with the patterns of gene expression in myeloid cells and ECs.

We next performed in vivo experiments to assess the effects of IL-1β neutralization on EC responses in Matrigel plugs. Anti–IL-1β Abs reduced the prevalence of ECs in Matrigel plugs by 5–8-fold, which is comparable with the effects induced by inhibition of VEGF (Fig. 4A). IL-1β was shown to affect the in vivo proliferation of ECs, as evidenced by BrdU incorporation into ECs in mice bearing Matrigel plugs with B16 melanoma cells alone or with anti–IL-1β. As seen in Fig. 4B, tumor growth in Matrigel plugs induced a potent proliferative response in ECs, which was significantly reduced upon IL-1β or VEGF neutralization. Control isotype matched Abs did not have any effects on these parameters.

To determine whether the inhibitory effect of anti–IL-1β on EC proliferation was direct or indirect, we preincubated extracts of day 9, B16-containing Matrigel plugs from WT mice with anti–IL-1β Abs and as a positive control with anti-VEGF and then added them to aortic rings. Extracts from plugs containing only B16 melanoma cells induced potent aortic ring sprouting, which was abolished following VEGF neutralization, whereas inhibition of IL-1β only modestly affected sprouting (Fig. 4C). This indicates that in contrast to VEGF, IL-1β has no direct mitogenic effect on ECs, but probably plays an accessory role in the angiogenic response.

IL-1β levels at the tumor site regulate the influx and maturation of infiltrating myeloid cells

Next, we assessed the effects of IL-1β on the recruitment and activation of VEGFR1$^+$ iMCs, which represent the major myeloid cell population present in the microenvironment of Matrigel plugs. GR-1^{high}/CD11b^{high} iMCs (population A) express higher levels of VEGFR1 and IL-1R1 compared with GR-1^{low}/CD11b^{high} myeloid cells (population B) (Fig. 5A), suggesting the possible responsiveness of iMCs to IL-1β and VEGF in the tumor microenvironment. iMCs in the peripheral blood express only background levels of these receptors (results not shown).

We further characterized the effects of IL-1β neutralization on myeloid cell recruitment into Matrigel plugs. The levels of iMCs

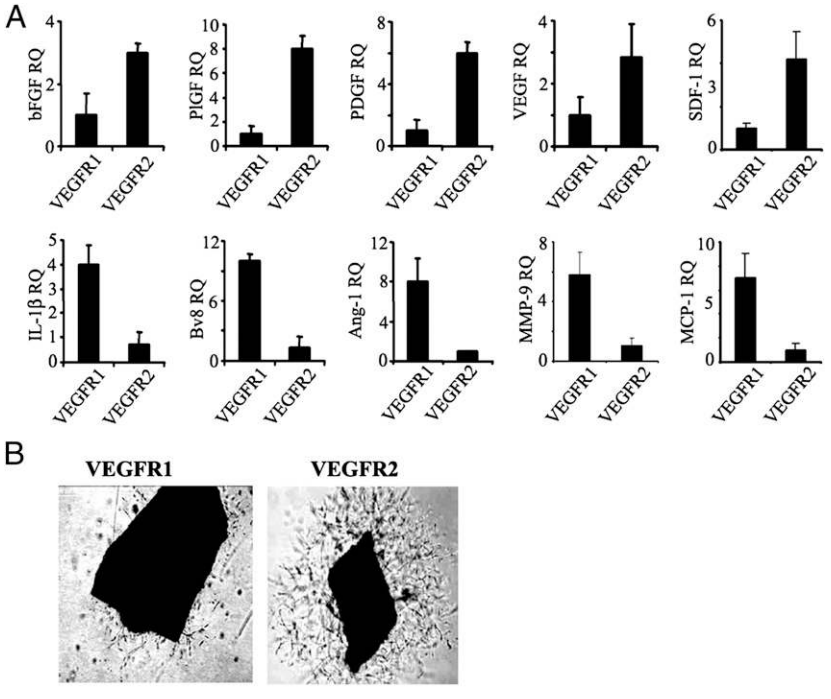

FIGURE 3. VEGFR2$^+$ and VEGFR1$^+$ cells express different patterns of angiogenic and inflammatory genes. (**A**) Relative quantification (RQ) of angiogenic and inflammatory transcripts in VEGFR1$^+$ and VEGFR2$^+$-enriched cells recovered from Matrigel plugs on day 9. The data represent mean values from five independent experiments. (**B**) Sprouting of aortic rings cocultured with VEGFR1$^+$ or with VEGFR2$^+$-enriched cells recovered from Matrigel plugs containing B16 cells (original magnification ×40). Photomicrographs are from one representative experiment out of five performed.

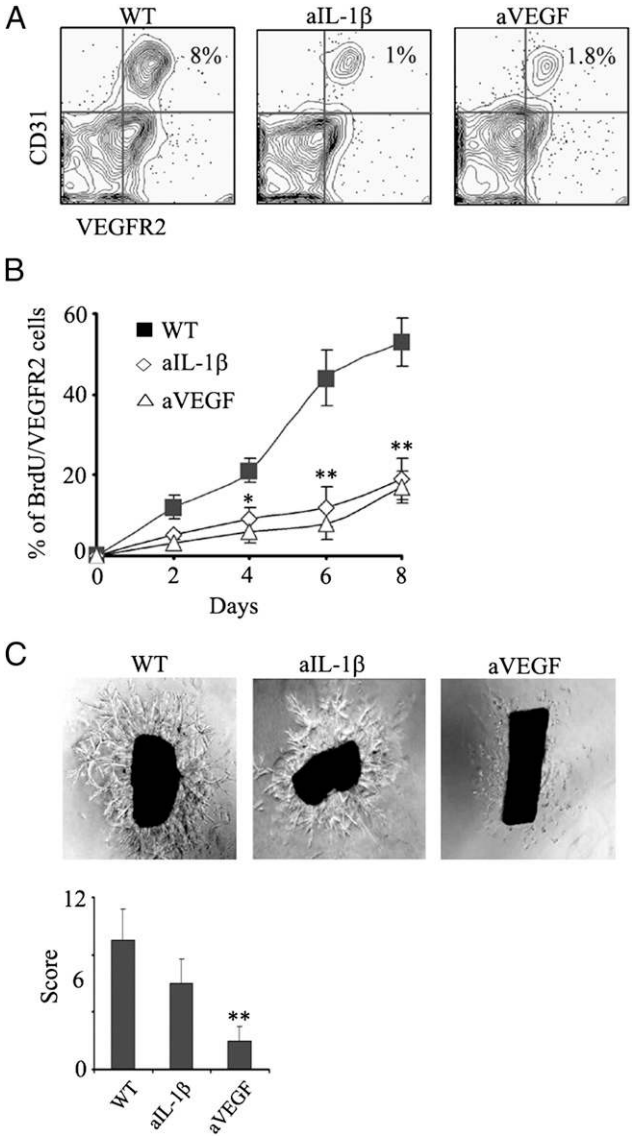

FIGURE 4. Neutralization of IL-1β reduces the recruitment and proliferation rate of ECs in vivo. (**A**) FACS analyses of cells obtained from day 9 Matrigel plugs containing B16 melanoma cells. Shown is a representative picture of the percentage of $CD31^+/VEGFR2^+$ ECs from the total number of cells. The data are from one of four independent experiments. (**B**) Levels of proliferating ECs in Matrigel plugs containing B16 cells mixed with control (WT) or neutralizing Abs for IL-1β or VEGF. Shown is the percentage of BrdU incorporated into $VEGFR2^+/CD31^+$ cells. The results shown in this figure represent mean values from three independent experiments (± SEM; n = 5–6 samples/group in each experiment). The p values relate to statistical significance compared with the relevant values in mice treated with control isotypic Abs (WT). (**C**) Photographs of aortic rings incubated with extracts of day 9 Matrigel plugs as such or after depletion of IL-1β or VEGF from the extracts by preincubation with the relevant Abs (original magnification ×40). The graph (*bottom panel*) indicates the score of aortic ring sprouting in arbitrary units. The results shown in this figure represent mean values from five independent experiments (± SEM, n = 5–6 samples/group per each experiment). Photomicrographs are from one representative experiment out of five performed. $*p < 0.01$, $**p < 0.005$.

and mature macrophages were evaluated at different time intervals. IL-1β neutralization leads to fewer iMCs (Fig. 5B, *top panel*) and concomitantly to a significant elevation in the number of mature macrophages (Fig. 5B, *bottom panel*). These results suggest the possibility that local IL-1β may affect not only the recruitment of iMCs, but also their differentiation state at the tumor site. To confirm this observation, we isolated enriched GFP-tagged $VEGFR1^+$cells from GFP-transgenic mice bearing Matrigel plugs containing B16 melanoma cells. These cells were subsequently injected into secondary mice together with Matrigel mixed with B16 cells, with or without anti–IL-1β Abs. Two days later, plugs were removed, and the maturation state of GFP-tagged $VEGFR1^+$ cells was assessed. Upon IL-1β neutralization, the composition of GFP-tagged cells in the Matrigel was shifted toward mature macrophages in comparison with plugs without IL-1β neutralization, where iMCs dominated (Fig. 5C).

The effect of IL-1 signaling on the composition of myeloid cells was also investigated using mice deficient in IL-1R1. Cells obtained from Matrigel were gated as $PE^-CD11b^+VEGFR1^+$ and then assessed for myeloid maturation markers, such as F4/80, MHC II, CD80, and CD86. In WT mice, 20% of myeloid cells were $Gr1^+CD11b^+F4/80^{low}$MHC II^-CD80^- cells, compared with only 8% in IL-1R1 KO mice (Fig. 5D, *top panel*). In contrast, at least three times more mature macrophages were found in IL-1R1 KO mice than in WT mice.

As another approach to demonstrate the effects of IL-1β on maturation of myeloid cells in Matrigel plugs containing tumor cells, we used isolated BM-derived $CD11b^+/VEGFR1^+$ myeloid cells from WT or IL-1R1 KO mice. BM-derived cells were labeled in vitro with CFSE and subsequently injected i.v. into WT mice bearing B16 cell–containing Matrigel plugs that already displayed tumor-mediated inflammation (day 7). After 2 additional days, the maturation of CFSE-tagged cells was assessed in excised plugs. When $CD11b^+/VEGFR1^+$ myeloid cells from WT mice were injected into WT mice, 32% of cells displayed an immature phenotype and 40% a phenotype of mature macrophages. However, injection of $CD11b^+/VEGFR1^+$ myeloid cells from IL-1R1 KO mice into WT mice leads to a decrease in iMCs (7%) and an increase in mature macrophages (63%) (Fig. 5D, *bottom panel*). These results support the hypothesis that IL-1β serves as a negative regulator of iMC differentiation at tumor sites.

Neutralization of IL-1β inhibits tumor progression more effectively than VEGF neutralization

Based on our finding that IL-1β is a key molecule in the tumor-induced angiogenic response, we next compared the antitumor effects of IL-1β neutralization to that of VEGF neutralization that is already in use to treat cancer patients. Thus, WT mice were injected intrafoot pad with B16 melanoma cells and treated weekly with neutralizing Abs. Neutralization of either of these molecules initially inhibited the rate of growth of B16 tumors. Nevertheless, a late burst in tumor growth was observed after VEGF neutralization, whereas treatment with anti–IL-1β Abs induced a more stable growth inhibition during the experiment (Fig. 6A, *left panel*). Similar results were observed in Matrigel plugs containing B16 cells injected into WT mice with neutralizing Abs (Fig. 6A, *right panel*).

Neutralization of VEGF shifts the phenotype of $VEGFR1^+$ myeloid cells into proangiogenic cells

Next, we assessed the effects of neutralization of VEGF or IL-1β in Matrigel plugs on the expression of proinflammatory and angiogenic factors in $VEGFR1^+$ iMCs, which are important myeloid accessory cells for the angiogenic response. Neutralization of VEGF in Matrigel plugs resulted in a significant and consistent elevated expression of direct angiogenic factors, such as VEGF, bFGF, and PDGF in $VEGFR1^+$ myeloid cells (Fig. 6B). In contrast, IL-1β neutralization inhibited proinflammatory genes, such as Bv8, CCL2, and CCL3 in myeloid cells and did not significantly change the expression of angiogenic factors.

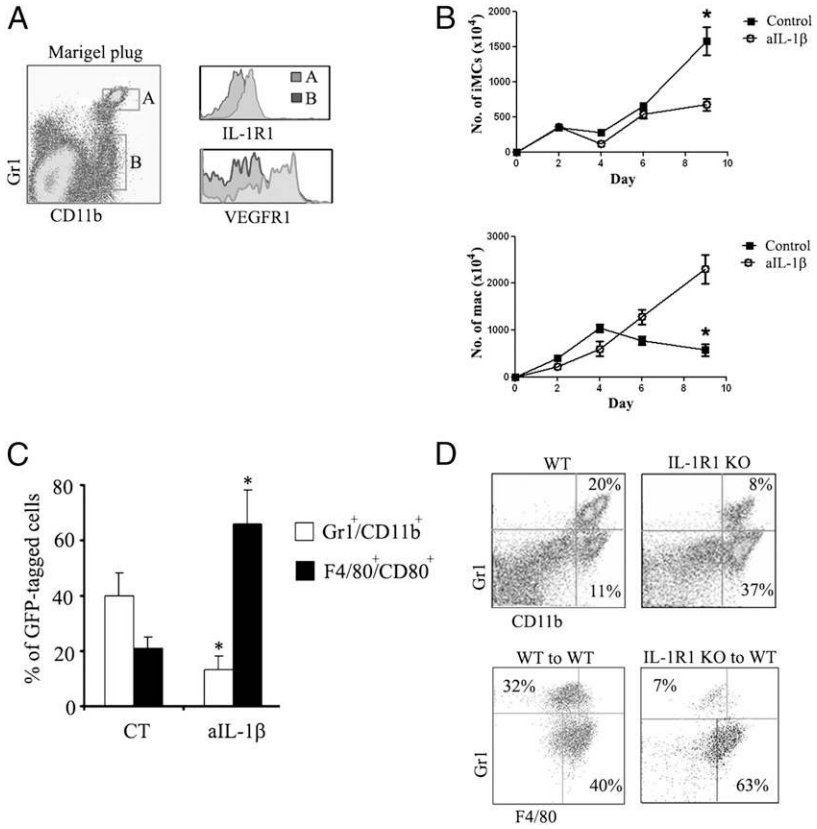

FIGURE 5. Deficiency of IL-1β or IL-1R signaling leads to modulation of myeloid cell subpopulations during the early angiogenic response in B16 cell–containing Matrigel plugs. (**A**) FACS analyses of myeloid cells obtained from day 9 Matrigel plugs containing B16 melanoma cells (*left panel*). Expression levels of IL-1R1 (*right top panel*) and VEGFR1 (*right bottom panel*) on $Gr1^{hi}/CD11b^{hi}$ cells (population "A") and $Gr1^{lo/neg}/CD11b^{+}$ cells (population "B") in Matrigel plugs containing B16 cells. Data are from one of three representative experiments. (**B**) Time-course kinetics of $Gr^{hi}/CD11b^{hi}/VEGFR1^{+}$ (iMCs) (*top panel*) and $Gr1^{lo}/CD11b^{+}/F4/80^{+}/MHC\ II^{hi}/CD80^{+}$ (mature macrophages [mac]) (*bottom panel*) in Matrigel plugs containing B16 melanoma cells, with and without IL-1β neutralization. The data represent mean ± SEM from three independent experiments. (**C**) GFP-tagged/$VEGFR1^{+}$ cells obtained from day 9 Matrigel plugs containing B16 cells were reinjected into WT mice, together with new Matrigel-containing B16 cells, with or without Abs to IL-1β. The graph indicates the percentage of GFP-tagged cells from Matrigel plugs obtained on day 2, characterizing iMCs ($Gr1^{hi}/CD1b^{hi}/VEGFR1^{+}$) and mature macrophages ($Gr1^{lo}/CD11b^{+}/F4/80^{+}/MHC\ II^{hi}/CD80^{+}$). The data represent mean ± SEM from two independent experiments. (**D**) *Top panel*, Levels of $Gr^{hi}/CD11b^{hi}$ iMCs and $Gr^{lo/neg}/CD11b^{+}$ myeloid cells in Matrigel plugs from WT and IL-1R1 KO mice. *Bottom panel*, BM-derived $CD11b^{+}/VEGFR1^{+}$ myeloid cells were isolated from naive WT or IL-1R1 KO mice and labeled in vitro with CFSE. CFSE-labeled cells were injected i.v. into WT mice bearing B16-containing Matrigel plugs (day 7). After 2 additional days, the maturity of CFSE-tagged cells removed from the Matrigel was assessed. FACS analyses are from a representative experiment out of three performed. $*p < 0.01$; p values relate to the statistical significance between cells obtained from control and IL-1β–depleted Matrigel plugs.

Next, we assessed whether $VEGFR1^{+}$ cells from tumor cell–containing Matrigel plugs after IL-1β or VEGF inhibition could stimulate EC sprouting. As already shown (Fig. 3), $VEGFR1^{+}$ cells recovered from plugs containing only B16 cells failed to induce potent sprouting. However, $VEGFR1^{+}$ cells recovered from plugs containing B16 cells in the presence of anti-VEGF Abs potently stimulated aortic ring sprouting (Fig. 6C). $VEGFR1^{+}$ cells from Matrigel plugs in which IL-1β was inhibited induced only a weak sprouting response. Thus, VEGF neutralization induces reprogramming of $VEGFR1^{+}$ myeloid cells into active proangiogenic cells, which directly stimulate ECs. This reprogramming was also accompanied by increased expression of HIF-1α in $VEGFR1^{+}$ iMCs (Fig. 6D). Thus, in Matrigel plugs containing B16 melanoma cells obtained from WT mice, only relatively low numbers of $VEGFR1^{+}$ cells expressed HIF-1α. Neutralization of IL-1β in such plugs reduced the number of $VEGFR1^{+}$ cells, but did not significantly alter expression of HIF-1α, as observed by immunofluorescence. Upon VEGF neutralization, HIF-1α is activated in $VEGFR1^{+}$ myeloid cells, which results in the capacity to directly stimulate EC proliferation through expression of direct angiogenic factors.

Cross talk between IL-1β and VEGF also occurs in the early angiogenic response in Matrigel plugs supplemented with recombinant cytokines

In this study, we illustrated the mutual interactions between IL-1β and VEGF in early tumor-mediated angiogenesis. To confirm that this interaction is also important in nontumor inflammation-dependent angiogenic responses, we injected mice with Matrigel containing either rIL-1β or rVEGF. We found that both rIL-1β and rVEGF induce a potent angiogenic response in Matrigel plugs (Fig. 7A, 7B). Both molecules induce each other's production (Fig. 7C). Furthermore, neutralization of VEGF abrogates angiogenesis in Matrigel plugs injected with rIL-1β. Moreover, IL-1β–induced angiogenesis was VEGFR1 dependent, whereas inhibition of VEGFR2 signaling in Matrigel caused a burst in the angiogenic response (Fig. 7A, 7B). Neutralization of IL-1β also abrogates angiogenesis induced by rVEGF in Matrigel plugs (Fig. 7A, 7B). The importance of the interactions between IL-1β and VEGF was also demonstrated by the weak angiogenic response, as well as by decreased amount of infiltrating cells into Matrigel plugs in mice lacking IL-1β, which were injected with Matrigel containing rVEGF (Fig. 7D–F).

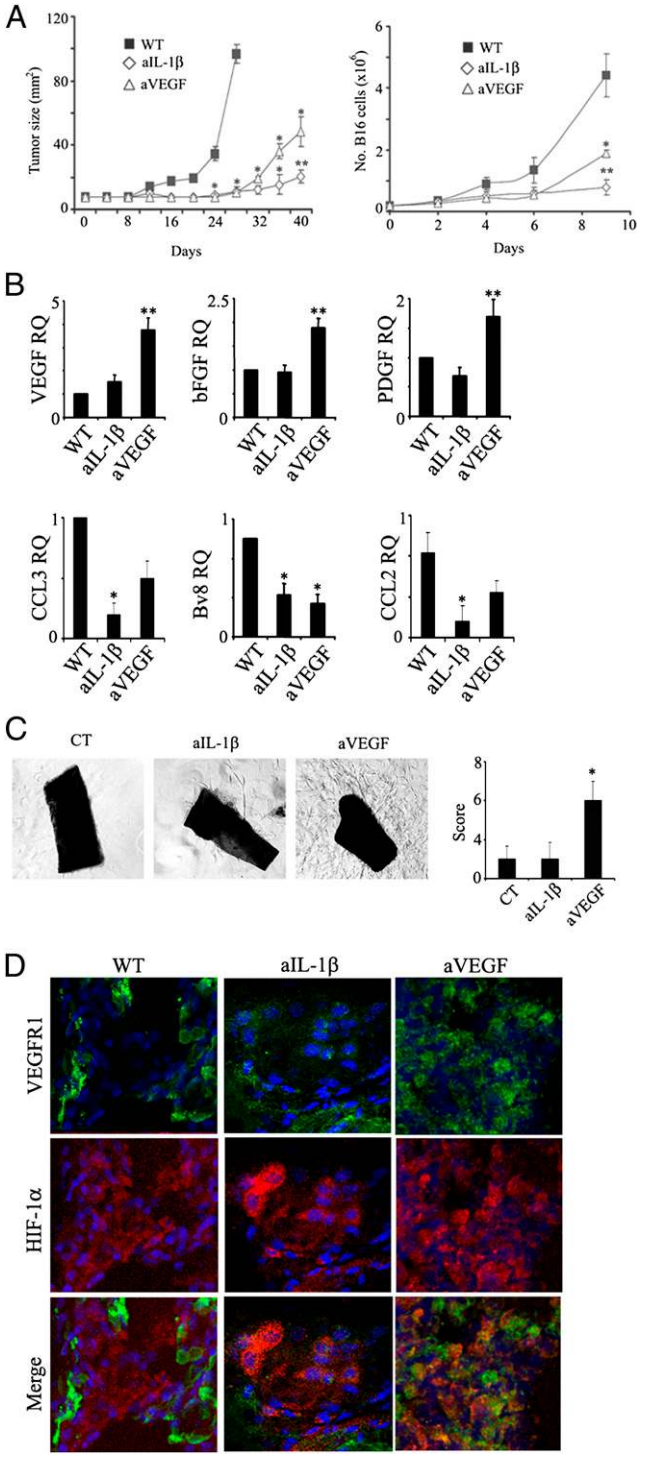

FIGURE 6. Differential effects of anti–IL-1β and anti-VEGF treatment on tumor invasiveness and reprogramming iMCs. (**A**) Mice were injected intrafoot pad with B16 cells together with control Abs (WT), anti–IL-1β, or anti-VEGF Abs. The diameter of tumors is shown (*left panel*). Matrigel plugs containing B16-mCherry cells mixed with CT, anti–IL-1β, or anti-VEGF Abs were injected into WT mice. The number of B16-mCherry cells, calculated as the percentage of the $CD45^{neg}/PE^{pos}$ fraction out of the total number of cells in the plugs is shown (*right panel*). The results shown in this figure represent mean values from five independent experiments (± SEM; n = 7–10 mice/group in each experiment). (**B**) Relative quantification of transcripts of angiogenic factors and proinflammatory molecules in $VEGFR1^+$-enriched cells recovered from Matrigel plugs containing B16 cells are shown. (**C**) Aorta rings incubated for 4 d with $VEGFR1^+$-enriched cells recovered from day 9 Matrigel plugs containing B16 melanoma cells together with control anti-mouse IgG (CT), anti–IL-1β, or anti-VEGF Abs. Data represent mean ± SEM. The *right panel* indicates the score of aorta ring sprouting in arbitrary units. Photomicrographs are from one representative experiment out of three performed. (**D**) Immunohistochemistry staining for VEGFR1 and HIF-1α (red) in day 9 Matrigel plugs containing B16 cells in the presence of anti-VEGF or anti–IL-1β Abs (original magnification ×60). Photomicrographs are from one representative experiment out of three performed. $^*p < 0.01$, $^{**}p < 0.005$.

Discussion

In this study, we have demonstrated, for the first time to our knowledge, cross talk between VEGF and IL-1β in the early angiogenic response during tumor development. We also found that IL-1β plays an essential role in the recruitment and maturation of myeloid cells. In this study, we show that VEGF and IL-1β have unique, indispensable, yet complementary, functions in the early angiogenic response. VEGF and IL-1β are both present in Matrigel plugs containing tumor cells (Fig. 1) and induce each other, even though VEGF is mainly produced by ECs, whereas IL-1β is produced by myeloid infiltrating cells (Fig. 2). Furthermore, in IL-1β–deficient mice or upon IL-1β neutralization in WT mice, the angiogenic potential of VEGF is completely abrogated in Matrigel plugs containing B16 melanoma cells (Fig. 1) and also in a nontumor model of angiogenesis induced by rVEGF (Fig. 7). IL-1β was shown to be essential for in vivo responses of ECs, such as their recruitment and proliferation. IL-1β acts directly or through induction of other proinflammatory molecules in the tumor microenvironment (Fig. 4). Previously, in a transgenic model of Myc-dependent carcinogenesis, IL-1β has been characterized as the principal effector molecule in the onset of angiogenesis via MMP-mediated sequestration of extracellular matrix–associated VEGF followed by its ligation to VEGFR2 on ECs (37). In this study, we have shown that active IL-1β secretion by myeloid cells activates the initial angiogenic response of tumors by inducing a cascade of proinflammatory mediators.

We found that in tumor cell–supplemented Matrigel plugs, the myeloid cell infiltrate was primarily of BM origin and consisted mainly of $VEGFR1^+$ iMCs and, to a lesser extent, mature macrophages (Fig. 2). iMCs were shown to be the dominant cells that secrete IL-1β (Fig. 2) and other proinflammatory molecules, such as Bv8, CCL2, and CCL3 (Fig. 3), but not direct angiogenic factors. In contrast, the primary source of VEGF were tissue-resident $VEGFR2^+$ ECs (Fig. 2), which also express other proangiogenic factors, such as PlGF, bFGF, and PDGF (Fig. 3). Traditionally, ECs were thought to be activated by VEGF in the angiogenic response, but produce only low levels of VEGF (17). In this study, we show that in the early angiogenic response, ECs secrete VEGF, which probably synergizes with proangiogenic factors of tumor cell origin. In diverse experimental tumor systems, infiltrating myeloid cells, such as tumor-associated macrophages or MDSCs, were shown to be a major source of VEGF and other proangiogenic mitogens (reviewed in Refs. 1, 7–14, 38). In these studies, large, established tumors were analyzed, whereas in our experiments, we assessed the early angiogenic response induced by dispersed malignant cells, which concomitantly recruit their microenvironment in the Matrigel plugs.

iMCs at tumor sites express both IL-1R1 and VEGFR1 at higher levels than in the peripheral blood, suggesting that their signaling might be essential for the protumorigenic function of iMCs. Furthermore, iMCs were the dominant cell type in the myeloid cell infiltrate in B16-supplemented Matrigel plugs in WT mice, whereas in IL-1R1 KO mice or following IL-1β neutralization, their number was reduced in parallel to an increase in the number of mature M1 macrophages. In BM transplantation experiments,

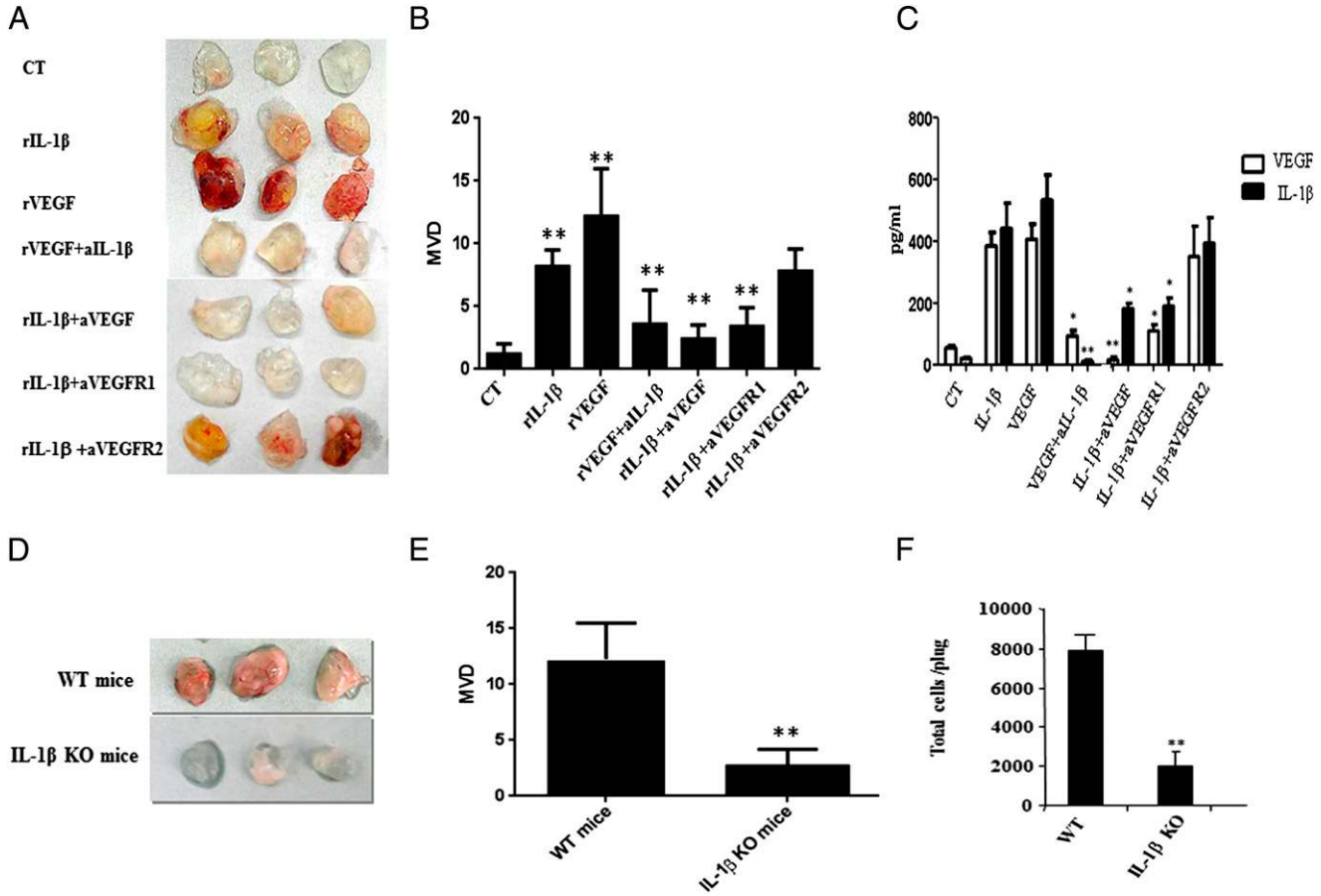

FIGURE 7. Patterns of angiogenesis in Matrigel plugs supplemented with rIL-1β or rVEGF. (**A**) Gross morphology of day 8 Matrigel plugs containing recombinant cytokines obtained from WT mice, with or without neutralization of cytokines. (**B**) MVD counts are also shown. (**C**) Mean levels of VEGF and IL-1β in Matrigel plug extracts. (**D**) Gross morphology of day 8 Matrigel plugs containing rVEGF obtained from WT and IL-1β KO mice. (**E**) MVD counts are also shown. (**F**) Total count of cells from Matrigel plugs containing rVEGF obtained from WT and IL-1β KO mice. The results shown in this figure represent mean values from five pooled experiments (± SEM; n = 6–9 mice/group). Photomicrographs are from a representative experiment out of five performed. In all cases, p values relate to statistical significance compared with the relevant values in WT mice. $*p < 0.01$, $**p < 0.005$. CT, Control.

we found that a deficiency of IL-1β in the microenvironment resulted in an increase in maturation of myeloid cells in the Matrigel model (Fig. 5). These results suggest that IL-1β may also affect the maturation state of iMCs, apart from its ability to affect recruitment of myeloid cells (22, 25, 39).

Similar interactions between iMCs and ECs, mediated by VEGF and IL-1β in the early angiogenic response of B16 cells, were observed in Matrigel plugs containing other types of tumor cells (results not shown) and in other systems of inflammation-dependent angiogenesis, such as in Matrigel plugs supplemented with either rIL-1β or rVEGF or in Matrigel plugs supplemented with supernatants of hypoxic macrophages (35). This may indicate that the described cross talk between VEGF and IL-1β is characteristic of early inflammation-dependent angiogenic responses.

Based on the interactions between IL-1β and VEGF in the angiogenic switch described in this study, and in light of the clinical use of anti-VEGF therapy in cancer patients, we compared the effects of neutralization of either IL-1β or VEGF on the growth of B16 melanoma cells. VEGF neutralization results in only an initial tumor inhibition followed by tumor recurrence, whereas blocking IL-1β decreased tumor growth for extended periods of time (Fig. 6A). Tumor relapse after anti-VEGF therapy has been described in multiple experimental tumors and in cancer patients treated with Avastin (9, 15). Some mechanisms of rebound angiogenesis after anti-VEGF therapy, mainly involving the generation of alternative angiogenic factors by malignant or microenvironment-derived cells, have been described. These include increased expression of redundant angiogenic factors, such as bFGF or PlGF by the tumor cells (40), induced secretion of Bv8 by MDSCs (41), and a subsequent VEGF-independent angiogenic response or expression of PDGF-C in cancer-associated fibroblasts (42). In this study, we describe a novel mechanism of rebound angiogenesis after VEGF inhibition (i.e., the rapid reprogramming of VEGFR1$^+$ iMCs for expression of direct angiogenic factors, such as VEGF, bFGF, and PlGF, thus acquiring the ability to directly stimulate ECs) (Fig. 6B, 6C). In contrast, in uninterrupted angiogenesis (without anti-VEGF treatment), ECs, rather than myeloid cells, are the major cells that produce VEGF and other direct angiogenic factors (Fig. 3). Initial characterization of reprogrammed VEGFR1$^+$ myeloid cells showed upregulation of HIF-1α, a key transcription factor regulating angiogenesis, especially in hypoxic microenvironments (Fig. 6D). In contrast, IL-1β inhibition during tumor cell development in Matrigel plugs led to downregulation of proinflammatory factor expression in myeloid cells and to some inhibition of production of angiogenic factors by ECs. The dramatic response of the tumor microenvironment to VEGF inhibition is possibly due to the key role VEGF plays in normal cell viability and tissue homeostasis (15, 18–20, 43). Furthermore, in Matrigel plugs supplemented with rVEGF and anti-VEGFR2 Abs, an enhanced angiogenic response was observed, possibly due to imposing a stressful situation on ECs. In contrast, anti-VEGR1 Abs abrogated angiogenesis (Fig. 7A, 7B).

IL-1β neutralization is more tolerated possibly because it is not a survival factor for cells. In addition, IL-1β neutralization creates the conditions for iMC maturation, thus reducing the invasiveness potential of tumors.

Finally, the data presented in this study provide new insight into the rationale for anticancer therapies based on IL-1β neutralization. We and others have demonstrated the value of IL-1Ra or anti–IL-1β Ab treatment in cancer therapy (24, 28, 44, 45). In the tumor microenvironment, IL-1β represents a major upstream cytokine, which controls the local proinflammatory cascade and thereby also affects the balance between protective immunity and destructive inflammation. IL-1 affects the activation of diverse cells in the tumor microenvironment and thus its neutralization should affect protumorigenic cells. Further characterization of the optimal conditions for IL-1 neutralization should lead to the application of anti–IL-1 approaches in cancer therapy.

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Disclosures

The authors have no financial conflicts of interest.

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