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The Role of Macrophage-Derived IL-1 in Induction and Maintenance of Angiogenesis

Yaron Carmi,* Elena Voronov,²* Shahar Dotan,* Nitzah Lahat,† Michal A. Rahat,† Mina Fogel,† Monika Huszar,‡ Malka R. White,* Charles A. Dinarello,§ and Ron N. Apte3*

Inflammation and angiogenesis are pivotal processes in the progression of many diseases, including malignancies. A hypoxic microenvironment often results in a milieu of proinflammatory and proangiogenic cytokines produced by infiltrating cells. We assessed the role of macrophage-derived hypoxia-associated cytokines in promoting inflammation and angiogenesis. Supernatants of macrophages, stimulated under hypoxia with or without an inflammatory stimulus (LPS), promoted angiogenesis when incorporated into Matrigel plugs. However, neutralization of IL-1 in the supernatants, particularly IL-1β, completely abrogated cell infiltration and angiogenesis in Matrigel plugs and reduced vascular endothelial growth factor (VEGF) levels by 85%. Similarly, supernatants from macrophages of IL-1β knockout mice did not induce inflammatory or angiogenic responses. The importance of IL-1 signaling in the host was demonstrated by the dramatic reduction of inflammatory and angiogenic responses in Matrigel plugs that contained macrophage supernatants from control mice which had been implanted in IL-1 receptor type I knockout mice. Myeloid cells infiltrating into Matrigel plugs were of bone marrow origin and represented the major source of IL-1 and other cytokines/chemokines in the plugs. Cells of endothelial lineage were the main source of VEGF and were recruited mainly from neighboring tissues, rather than from the bone marrow. Using the aortic ring sprouting assay, it was shown that in this experimental system, IL-1 does not directly activate endothelial cell migration, proliferation and organization into blood vessel-like structures, but rather activates infiltrating cells to produce endothelial cell activating factors, such as VEGF. Thus, targeting IL-1β has the potential to inhibit angiogenesis in pathological situations and may be of considerable clinical value. The Journal of Immunology, 2009, 183: 4705–4714.

The microenvironment of injured tissues includes low levels of oxygen as well as reductive metabolites and products of necrotic cells (1, 2). Macrophages play a pivotal role in restoring tissue homeostasis, due to their plasticity, as well as their remarkable ability to survive and function under harsh conditions (3–14). Monocytes are rapidly recruited into damaged tissue (i.e., ischemia, chronic inflammation, wounds, bacterial infection, and solid tumors), where they differentiate, redefining their transcriptome to express genes involved in adaptation to anaerobic glycolysis and tissue repair (5, 7, 15, 16). Angiogenesis, the growth of new capillaries from pre-existing blood vessels, is an integral process in tissue repair (17–19). Vasculogenesis, the recruitment and in situ differentiation of endothelial precursor cells (EPC) and myeloid cells from the bone marrow (BM), assists in this process (20, 21).

IL-1 is a macrophage-derived major proinflammatory “alarm” cytokine, which acts mainly through the induction of a network of cytokines, chemokines and small molecule mediators. IL-1 also induces the expression of adhesion molecules and integrins on leukocytes, endothelial and other cells (22–25), promoting cell infiltration, inflammation and tissue repair at damaged sites. Of the 11 members of the IL-1 family of ligands, IL-1β and IL-1α are the two major agonistic molecules; whereas the IL-1 receptor antagonist (IL-1Ra) is a physiological inhibitor of IL-1. In comparison to IL-1β, IL-1α is not secreted, remains mainly cell-associated and therefore induces less inflammation and angiogenesis. In experimental tumor models, we have previously reported that IL-1β is the primary mediator of tumor angiogenesis, invasiveness and metastasis (24–30). Specifically, we have shown that blockade of the IL-1 receptor type I or neutralization of IL-1β of host origin reduces tumor angiogenesis and invasiveness (31, 32). Nakao et al. (33) demonstrated that ectopic expression of IL-1β in the cornea or in malignant cells induces IL-1β-mediated angiogenesis, which is dependent on infiltration of COX-2-positive macrophages that activate the angiogenic process in a complex manner. In another study, nylon discs impregnated with vascular endothelial growth factor (VEGF) or basic

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4 Abbreviations used in this paper: EPC, endothelial precursor cell; BM, bone marrow; EC, endothelial cell; HIF-1α, hypoxia-inducible factor; IL-1ra, IL-1 receptor antagonist; KO, knockout; MPC, myeloid precursor cell; MVD, microvessel density; PEC, peritoneal exudates cell; VEGF, vascular endothelial growth factor; wV, von willebrand factor; WT, wild type; CT, control.
fibroblast factor were implanted in the rat cornea; an aggressive angiogenic response followed. Systemic treatment with IL-1Ra, but not with soluble TNFR, prevented the formation of new blood vessels (34). Thus, the mechanism by which IL-1 initiates angiogenesis is of considerable biological significance.

In the present study, we have assessed the role of hypoxia in the presence of an inflammatory signal (LPS) on the inflammatory and angiogenic effects of products secreted from primary macrophages. By using Matrigel plugs, containing macrophage supernatants, implanted in mice, we have characterized inflammatory and angiogenic responses. We demonstrate that the source of the direct angiogenic stimulus comes from cells of endothelial lineage, attracted to the Matrigel. Myeloid cells, attracted from the BM by macrophage-derived IL-1, mainly IL-1β, locally produce additional IL-1, which stimulates cells of endothelial lineage to secrete VEGF. Thus, the data establish IL-1β as the primary macrophage-derived product that initiates angiogenesis by recruitment of cells of myeloid and endothelial lineages. The findings also point to the potential of IL-1β neutralization as a general inhibitor of angiogenesis, not exclusively related to cancer.

Materials and Methods

Mice

IL-1α, IL-1β, and IL-1αβ knockout (KO) mice were generated by Y. Iwakura as described previously (35). GFP transgenic mice [C57BL/6-Tg (UBC-GFP) 30Sha/J] and IL-1R KO mice were purchased from The Jackson Laboratory. Wild-type (WT) C57BL/6 mice were obtained from Harlan. Animal studies were approved by the Animal Care Committee of Ben-Gurion University. Male, 6- to 8-wk-old mice were used in all experiments.

Culture of peritoneal macrophages

Peritoneal exudate cells (PEC) were obtained according to routine procedures. Briefly, mice were injected i.p. with 3 ml of thioglycollate (Hy-Labs). After 4 days, PEC were collected by peritoneal lavage with PBS (Life Technologies). Cells were then washed with fresh PBS, resuspended in DMEM (Life Technologies) containing 10% FBS (Biological Industries), plated at 0.5 × 10⁶ cells/ml in 12-well plates (Greiner) and incubated overnight to enable adherence. The cultures were then washed to remove nonadherent cells, and adherent cells were incubated with or without 1 μg/ml LPS (Escherichia coli 055:B5; Sigma-Aldrich) in 1 ml of serum-free DMEM, under hypoxic or normoxic conditions. Cell viability was determined after 24 h using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilid kit (Biological Industries).

Normoxic and hypoxic conditions

Cells were incubated in a standard incubator (21% O₂, 5% CO₂, and 75% N₂) for 24 h. Cells were then washed with fresh PBS, resuspended in DMEM (Life Technologies) containing 10% FBS (Biological Industries), plated at 0.5 × 10⁶ cells/ml in 12-well plates (Greiner) and incubated overnight to enable adherence. The cultures were then washed to remove nonadherent cells, and adherent cells were incubated with or without 1 μg/ml LPS (Escherichia coli 055:B5; Sigma-Aldrich) in 1 ml of serum-free DMEM, under hypoxic or normoxic conditions. Cell viability was determined after 24 h using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilid kit (Biological Industries).

Chimeric mice model

BM cells were obtained from GFP transgenic mice or WT C57BL/6 mice. WT C57BL/6 mice were lethally irradiated (1300 rad γ-Cobalt per mouse) and after 24 h, mice were injected i.v. with GFP-tagged BM-derived cells (2.5 × 10⁶/mouse in 0.2 ml PBS). After 2 mo, these mice were injected with Matrigel and angiogenic responses in the plugs were assessed. Similarly, irradiation chimeras consisting of IL-1αβ KO or WT irradiated mice, which were reconstituted with BM cells from either of these strains, were prepared.

Cytokine measurements

Levels of VEGF, CCL5, CCL3, CCL12; IL-1α and CXCL10 were measured in 24-h supernatants using specific ELISA kits (Duolset; R&D Systems). TNF-α, IL-10, IL-12, IL-6, IL-1β, and TGF-β were measured in 24-h supernatants by specific ELISA kits (BD Biosciences). CCL2, CXCL8 and IFN-γ were measured by using the Qplex Mouse Cytokine array (Quansys Biosciences) according to the manufacturer’s instructions. The images were captured by Q-View Imager (Quansys Biosciences) using a cooled charge-coupled device camera (MicroMAX-1300B; Roper Scientific).

Angiogenesis in Matrigel plugs

Irradiated Matrigel (2.1 ml, growth factor reduced; R&D Systems) was mixed with 400 μl of macrophage supernatants at 4°C and then 0.5 ml of the 2.5-ml mixture was injected s.c. into the interscapular region of mice (31). Therefore, each Matrigel injection contained 80 μl of the original supernatant. As a control, Matrigel was mixed with serum-free DMEM and injected as above. In some experiments, anti-mouse IL-1αβ or/and anti-mouse IL-1α Abs (R&D Systems) were added to the mixture of Matrigel and incubated at 4°C for 2 h before injection into mice. Matrigel plugs were surgically removed on day 2 or 8 and their gross morphology examined. Some of the plugs were solubilized in 3 ml of HBSS (Life Technologies) containing 5 mg/ml collagenase type IV (Roche) for 1 h at 37°C to measure cytokine levels and assess the composition of infiltrating cells. The remainder of the plugs were fixed, embedded in paraffin, and stained with H&E or subjected to immunohistochemistry with specific Abs. Unless otherwise stated, we assessed angiogenesis and cytokine levels on day 8.

Microvessel density (MVD)

Microvessels in Matrigel plugs were visualized by using rat Abs to human von Willebrand factor (vWF) (DakoCytomation), which cross-react with murine vWF (31). The MVD was calculated by counting blood vessels in areas of high vascularity at a magnification of ×200. From each experiment, the average number of blood vessels from six fields was used as a single value and the data shown is the mean MVD of four separate experiments.

Immunohistochemistry

Samples from Matrigel plugs were fixed in 4% paraformaldehyde, dehydrated in alcohol, cleared in xylene and embedded in paraffin. Four-micrometer sections of the plugs were stained with H&E or were subjected to immunohistochemistry, according to previously described protocols (26). Rabbit polyclonal anti-vWF (Dako 1/200) Abs were used for staining endothelial cells. The Vectastain Elite ABC Peroxidase kit (Vector Laboratories) was used for secondary Ab application and detection. Visualization was performed using 3-amin-9-ethylcarbazole as a substrate (Zymed Laboratories). The examination of the slides was performed by a pathologist in a blind manner.

Flow cytometry

Single-cell suspensions obtained from Matrigel plugs were analyzed using flow cytometry (FACSCanto; BD Biosciences). Data sets were analyzed using FlowJo software (Tree Star). Mice were injected i.v. with FITC, PE, PE-Cy7, PE-Cy5.5, alkaliphycocyanin, PE-Cy7 or pacific blue specific for the following Abs: CD11b (M1/70), CD31 (390), CD34 (RAM34), CD80 (16-10A1), VEGFR2 (Avas121a), Gr-1 (RB6-8C5), CD45 (30-F11), F4/80 (BM8), and IL-1α (ALF-161) (eBioscience). Polyclonal IL-1β Abs were also purchased from eBioscience. Anti-CCR4 (FAB70), anti-VEGFR1 (FAB4711A), and polyclonal anti-mouse VEGF Abs were purchased from R&D Systems. Samples were suspended in FACS buffer, consisting of PBS with 3% FBS and 0.01% NaN₃. Dead cells were excluded by use of the Live/Dead Fixable Aqua Dead Cell Stain kit (Invitrogen).

Aortic ring assay

Aortas were removed from 6-wk-old C57BL/6 mice and immediately placed in ice cold PBS. Fatty tissues were removed by gentle scraping under a dissecting microscope and aortas were cut into thin rings, as previously reported (37). Forty-eight-well plates were coated with 100 μl of Matrigel; after gelation at 37°C, the rings were placed in the wells and sealed in place with an overlay of 100 μl of Matrigel. Conditioned media for testing were added to the wells of embedded aorta. Recombinant VEGF was added as a positive control.

Recovering cells from Matrigel plugs

Matrigel plugs were incubated in 4 ml of HBSS containing 5 mg/ml collagenase D (Roche) and stirred for 1 h at 37°C. Recovered cells were washed three times in PBS and transferred through 70-μm cell strainers. Recovered cells were then used for either FACS analysis or in some experiments for preparation of conditioned medium. Cytokines were assessed in Matrigel extracts by commercial ELISA kits. For the preparation of
Serum-free DMEM. After 24 h, supernatants were collected and assayed.

CCTATGGGTCTCAAACATGATCTGGG; IL-6, GAAGTAGGGAAGGC.

anti-mouse p65 subunit of NF-

quently, cells were incubated overnight at 4°C with mAbs of either rabbit

5% BSA (Sigma-Aldrich) in PBS for 2 h at room temperature. Subse-

hyde containing 0.1% Triton X-100 in PBS for 10 min and blocked with

room temperature. Cells were then permeabilized with 4% paraformalde-

and fixed in 4% freshly prepared paraformaldehyde in PBS for 20 min at

under hypoxic or normoxic conditions. Cells were washed twice with PBS

cultured PEC from WT mice were plated onto culture slides and allowed

Immunofluorescence staining for p65 and HIF-1α

Cultured PEC from WT mice were plated onto culture slides and allowed
to adhere overnight in DMEM supplemented with 10% FBS. Cells were
then washed with PBS and incubated for 6 h, with or without 1 μg/ml LPS,
under hypoxic or normoxic conditions. Cells were washed twice with PBS
and fixed in 4% freshly prepared paraformaldehyde in PBS for 20 min at
room temperature. Cells were then permeabilized with 4% paraformalde-
hyde containing 0.1% Triton X-100 in PBS for 10 min and blocked with
5% BSA (Sigma-Aldrich) in PBS for 2 h at room temperature. Subse-
cquently, cells were incubated overnight at 4°C with mAbs of either rabbit
anti-mouse p65 subunit of NF-κB (Santa Cruz Biotechnology) or rabbit
anti-mouse HIF-1α (Delta Bio labs). After washing, cells were incubated at
room temperature with donkey anti-rabbit Cy-3 secondary Ab (Jackson
Immunoresearch Laboratories), washed extensively with PBS and
mounted on slides with an anti-fade reagent. All Abs were diluted in a
blocking solution (5% BSA in PBS).

Imaging and calculation of fluorescent intensity

Imaging was performed with an Olympus Fluoview FV1000 confocal mi-
croscope (Olympus). Laser energy and parameters of intensity detection
were equivalent for all slides. The images shown represent typical cells
from at least three independent experiments. The relative intensity of nu-
clear fluorescence was calculated using Velocity software (Improvision)
with the following equation: \( F(n)/F(n+c) \), where \( F(n) \) is the intensity of fluorescence in the nucleus, \( F(n+c) \) is the
intensity of fluorescence in the cytoplasm and \( n \) is the number of cells
examined.

Real-time PCR

Total RNA was extracted from PEC using the RNeasy kit (Qiagen).
cDNA was prepared using the Reverse Transcription System kit (Pro-
mega). All real-time PCR reactions were performed in a 20-μl mixture,
containing 10 ng/ml cDNA, 1× Ready Mix SYBR Green Master Mix
(Applied Biosystems) and the relevant primers. Real-time quantifica-
tions were performed using the ABI 7500 System. The relative amount
of transcript was calculated by the CT method, using the ABI 7500
System Software (version 1.2) and was normalized to an endogenous
reference gene, as indicated in the manufacturer’s instructions (Applied
BioSystems). Real time quantitative PCR primers targeting murine cyto-
kines were designed using Primer Express software (Applied Bio-
systems), as follows: gene reverse forward, β-actin, GGGTGCAAGAGAAG
CTTATGGCTCACAACATGATCCGG; IL-6, GAAATGGGAAGGG
CGTGCGCTCAAGAGACTTCCATCCAGTT; and VEGF, AGCTTGC
TGTTAGACATCCACTGGACCTGGCCTTTACTG.

Statistical analyses

Each experiment was performed three to five times. In Matrigel plug as-
says, each experimental group consisted of three to five mice. Significance
of the results was determined using the two-sided Student’s \( t \) test.

Results

Supernatants of LPS-stimulated macrophages induce
inflammation and angiogenesis in Matrigel plugs

We studied the effects of hypoxia and inflammation on proangi-
ogenic properties of secreted macrophage products. Peritoneal
macrophages were cultured under normoxic (5–8% \( O_2 \)) or hypoxic
(0.3% \( O_2 \)) conditions, with or without LPS stimulation. Hypoxia or
hypoxia with LPS for 24 h, under the experimental conditions
described here, did not result in a significant death of macro-
phages, as assessed by 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-
2H-tetrazolium-5-carboxanilid and lactate dehydrogenase assays
(results not shown). The 24-h macrophage supernatants were
mixed with Matrigel and injected into mice. The inflammatory
and angiogenic responses in the plugs were routinely assessed after 8
days. As shown in Fig. 1A, Matrigel plugs containing supernatants
of normoxic macrophages (indicated as control, CT) were trans-
parent, those of hypoxic macrophages were reddish in color,
whereas plugs containing supernatants of LPS-stimulated hypoxic
or normoxic macrophages were turbid and red, suggesting a pro-
nounced inflammatory and angiogenic response. Angiogenesis
was further demonstrated by immunohistochemistry staining of
Matrigel sections with anti-vWF (Fig. 1B) and quantified by MVD
count (Fig. 1C). No blood vessels were observed in plugs contain-
ing supernatants of macrophages cultured under normoxic condi-
tions, yet some vessels were present in plugs containing superna-
tants of hypoxic macrophages (\( p \leq 0.05 \)). In contrast, there were
numerous vessels in plugs containing supernatants of LPS-stimu-
lated macrophages cultured under hypoxic and normoxic condi-
tions (\( p \leq 0.001 \)) (Fig. 1, A and C, left).

Cellular infiltration into Matrigel plugs was observed in H&E-
stained histological sections (Fig. 1D). There was no infiltrate in
plugs containing supernatants of normoxic macrophages, except

FIGURE 1. LPS-stimulated macrophage supernatants induce inflammation and angiogenesis in Matrigel plugs. Supernatants from normoxic or hypoxic macrophages, as such or stimulated with LPS, were mixed with Matrigel and injected s.c. into WT mice. Mice were sacrificed on day 8, and Matrigel plugs embedded in para-
affin were stained with H&E or anti-vWF (×200). A, Gross morphology of Matrigel plugs. B, vWF staining of paraaffin sections. C, Mean MVD and total cell count (in thousands) (±SEM, \( n = 4 \)) in Matrigel plugs. D, H&E staining of paraaffin sections.
for low numbers of cells in the peripheral areas of the plugs. Similarly, supernatants of macrophages cultured only under hypoxic conditions induced moderate invasion of cells into the periphery of the plugs. In contrast, plugs containing supernatants from LPS-stimulated macrophages in normoxic or hypoxic conditions showed a dense infiltrate, which invaded deeply into the Matrigel and partially digested the plugs creating large areas of vacuolation. Quantification of the number of cells infiltrating into the plugs is shown in Fig. 1C, right.

Infiltration of cells into Matrigel plugs was progressive with time. For example, on day 2, the number of infiltrating cells in Matrigel plugs was approximately one third of that observed on day 8 and consisted primarily of myeloid lineage cells (results not shown). Infiltration of inflammatory cells into the Matrigel preceded angiogenesis, as on day 2 no blood vessels were observed.

Cytokines/proinflammatory mediators and transcription factors in hypoxia and LPS-stimulated macrophages

We next assessed cytokines and proinflammatory molecules in macrophage supernatants (Table I). Macrophages cultivated under hypoxia or normoxia, without LPS (CT), produced low or undetectable levels of most proinflammatory cytokines, chemokines and PGE$_2$. Moderate levels of IL-1α were present in supernatants of hypoxic, but not normoxic, macrophages. As anticipated, high levels of cytokines/chemokines were secreted by macrophages cultured with LPS, but there were no differences in the levels of TNF-α, IL-6, IL-10, IL-12, CCL5, CXCL10, or CCL3 between LPS-stimulated normoxic and hypoxic macrophages. CCL2 secretion was markedly decreased in LPS-stimulated hypoxic macrophages vs normoxic macrophages (Table I). Unexpectedly, there was ~50% less IL-1β in LPS-stimulated macrophages under hypoxic conditions compared with normoxia ($p < 0.005$), concomitantly to a similar increase in IL-1α secretion ($p < 0.001$).

A dramatic increase in PGE$_2$ secretion and a decrease in NO secretion were observed in supernatants of LPS stimulated hypoxic vs normoxic macrophages (Table I). It is notable that hypoxia itself induced secretion of significant levels of NO that did not increase upon addition of LPS, whereas under normoxia, NO secretion was strictly LPS dependent.

In contrast to the secretion of pro-inflammatory cytokines, VEGF was induced primarily by hypoxia with a nonsignificant further increase with LPS stimulation. Similar observations were found for mRNA expression of other direct angiogenic factors, such as endothelial cell growth factor and platelet-derived growth factor (results not shown). Levels of basic fibroblast factor in macrophage supernatants were undetectable.

Hypoxia-inducible factor 1α (HIF-1α) and NF-κB are characteristic transcription factors of hypoxic and inflammatory microenvironments, where they affect expression of pro-inflammatory and angiogenic molecules (reviewed in Refs. 1 and 38). It was thus of considerable interest to assess nuclear translocation of HIF-1α and the p65 subunit of NF-κB complexes in macrophages stimulated under hypoxic conditions with or without LPS stimulation. Marked nuclear localization of HIF-1α was induced by hypoxia and increased further by addition of LPS ($p < 0.005$), whereas the effects of LPS stimulation under normoxia were less dramatic (supplemental Fig. 1A).5 In contrast, p65 nuclear translocation was mainly affected by LPS rather than by hypoxia (supplemental Fig. 1B).5 mRNA levels of VEGF (right) and the quantification of fluorescence intensity per pixel of HIF-1α in the nucleus (left) are presented in supplemental Fig. 1C.5 mRNA levels of IL-6 (right) and the quantification of fluorescence intensity per pixel of p65 in the nucleus (left) are shown in supplemental Fig. 1D.5 The levels of VEGF and IL-6 mRNA correlated with nuclear localization of HIF-1α and p65, respectively, indicating their transcriptional activity.

Angiogenesis induced by macrophages is IL-1 dependent

We next asked whether IL-1 of macrophage origin is essential for hypoxia-associated angiogenesis. For this purpose, either IL-1β or IL-1α was first neutralized in the macrophage supernatants before their incorporation into Matrigel plugs. As shown in Fig. 2A, neutralization of IL-1β completely prevented angiogenesis induced by supernatants of LPS-stimulated hypoxic and normoxic macrophages ($p < 0.001$). Neutralization of IL-1α mainly inhibited the angiogenic capacity of supernatants from LPS-stimulated hypoxic macrophages ($p < 0.001$). A less significant reduction was observed upon neutralization of IL-1α in supernatants of LPS-stimulated normoxic macrophages. There was a complete absence of angiogenesis when both anti-IL-1α and anti-IL-1β Abs were used, demonstrating that IL-1β is the cytokine primarily responsible for angiogenesis in LPS-activated hypoxic macrophage supernatants. In contrast, neutralization of TNF-α did not affect angiogenesis, as manifested by MVD counts in Matrigel plugs (Fig. 2B). Thus, these data demonstrate that of the many cytokines and mediators present in the different macrophage supernatants, each of which might have effects on angiogenesis, only neutralization of IL-1β completely abrogated the angiogenic response.

It is possible that Abs added to the Matrigel, to neutralize IL-1 in the supernatants, also neutralize IL-1 produced by infiltrating host cells. To rule out this possibility, LPS-stimulated macrophage supernatants from IL-1α- or IL-1β-deficient mice were assessed for their angiogenic capacity. As shown in Fig. 2B, supernatants from IL-1β-deficient mice did not induce angiogenesis in Matrigel plugs. There was only a minor reduction in MVD induced by supernatants of normoxic macrophages from IL-1α-deficient mice, whereas a significant decrease in MVD was found in Matrigel plugs containing supernatants of hypoxic IL-1α-deficient macrophages. Thus, in LPS-stimulated macrophage supernatants, IL-1β

<table>
<thead>
<tr>
<th>Cytokine/chemokine levels in macrophages supernatants*</th>
<th>Hypoxia</th>
<th>Normoxia</th>
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<tbody>
<tr>
<td></td>
<td>CT</td>
<td>LPS</td>
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<tr>
<td>IL-1α</td>
<td>91 ± 31</td>
<td>310 ± 54</td>
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<tr>
<td>IL-1β</td>
<td>254 ± 79</td>
<td>510 ± 91</td>
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<tr>
<td>IL-6</td>
<td>26,450 ± 1,780</td>
<td>23,360 ± 2,230</td>
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<td>TNF-α</td>
<td>8,977 ± 1,049</td>
<td>8,870 ± 849</td>
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<td>IFN-γ</td>
<td>3,700 ± 604</td>
<td>3,950 ± 440</td>
</tr>
<tr>
<td>IL-12</td>
<td>3,700 ± 604</td>
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<tr>
<td>IL-10</td>
<td>208 ± 62</td>
<td>174 ± 80</td>
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<tr>
<td>PGE$_2$</td>
<td>732 ± 57</td>
<td>181 ± 94</td>
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<tr>
<td>NO</td>
<td>5,620 ± 870</td>
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<td>CCL2</td>
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<td>CCL3</td>
<td>764 ± 143</td>
<td>812 ± 105</td>
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<td>CCL5</td>
<td>3,760 ± 654</td>
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<td>CXCL8</td>
<td>55 ± 21</td>
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<td>CXCL10</td>
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<tr>
<td>CXCL12</td>
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</tr>
<tr>
<td>FGF2</td>
<td>420 ± 62</td>
<td>512 ± 77</td>
</tr>
</tbody>
</table>

* A total of 0.5 × 10$^6$ thioglycollate-induced macrophages were seeded onto 12-well culture dishes with or without 1 μg/ml LPS in 1 ml of serum-free DMEM, under hypoxic or normoxic conditions. After 24 h, supernatants were collected and assessed for cytokine and chemokine levels by commercial ELISA kits or Quansys custom cytokine and chemokine array. CT-plugs supplemented with supernatants of either hypoxic or normoxic macrophages. LPS-plugs supplemented with supernatants of LPS-stimulated hypoxic or normoxic macrophages. It is notable that 80 μl of each supernatant was incorporated in each Matrigel plug.

References

5 The online version of this article contains supplemental material.
is a necessary component for angiogenesis, whereas IL-1α contributes to the angiogenic potential of macrophages under hypoxia. Indeed, as shown in Table I, secretion of IL-1α increased in hypoxic macrophages stimulated by LPS. Supernatants of LPS-activated hypoxic macrophages from IL-1β KO mice had little angiogenic activity, despite the presence of an intact IL-1α gene. However, IL-1β is a strong inducer of IL-1α and thus levels of IL-1α in macrophages from IL-1β-deficient mice are much lower than those of macrophage supernatants from WT mice and are possibly below the threshold needed for stimulation of angiogenesis (Ref. 23 and our unpublished results).

Because of the central role of VEGF in angiogenesis (17, 19, 20, 39, 40), we measured levels of VEGF in Matrigel plugs. As shown in Fig. 2C, extracts of Matrigel plugs contained elevated levels of VEGF, 5–10 fold higher than VEGF levels measured in the original supernatants that were mixed with Matrigel before injection into mice (see Table I; ~50 pg/plug). Thus, host-derived infiltrating cells produce VEGF in response to the macrophage supernatants. The induction of VEGF in Matrigel plugs appears to be IL-1 dependent, as anti-IL-1 Abs (anti-IL-1α and anti-IL-1β) reduced the quantity of VEGF to background levels (p ≤ 0.001). Similarly, IL-1-dependent IL-1 induction by host cells was also observed in Matrigel plugs containing macrophage supernatants (p ≤ 0.001). As shown in Fig. 2D, elevated levels of IL-1β (right) and IL-1α (left), much higher (x5–10) then the initial amounts in macrophage supernatants, were observed in Matrigel plugs. However, ~60–75% of the total IL-1 detected in Matrigel plugs was IL-1β, further attesting to its dominant role in inflammation-mediated angiogenesis. Further analysis of angiogenic and inflammatory mediators in day 8 extracts of Matrigel plugs showed an abundance of cytokines/chemokines (see supplemental Table I). Interestingly, increased levels of cytokines/chemokines (IL-6, IL-12, CCL2, CCL5, CXCL8, and VEGF) were observed in plugs containing supernatants of only hypoxic macrophages. In contrast, low or undetectable levels of these cytokines/chemokines were detected in plugs containing supernatants of normoxic macrophages alone. Extracts of Matrigel plugs supplemented with supernatants of LPS-activated hypoxic and normoxic macrophages contained high levels of most cytokines/chemokines tested. Neutralization of IL-1 (IL-1α and IL-1β) in LPS-activated macrophage supernatants abrogated or reduced cytokine/chemokine levels. These results further attest to the important role of IL-1 in inducing and activating inflammatory responses which potentate angiogenesis.

To further demonstrate the importance of IL-1 of the host’s microenvironment in promoting angiogenesis, macrophage supernatants derived from WT mice were mixed with Matrigel following injection into IL-1R deficient mice. In each case, there was a dramatic reduction in blood vessel count (Fig. 2A), levels of VEGF (Fig. 2C) and IL-1 (Fig. 2D) (p ≤ 0.001). Thus, host-derived IL-1 in the microenvironment is required for a potent angiogenic response.

We next examined the direct angiogenic effect of supernatants from normoxic and hypoxic LPS-stimulated macrophages using the aortic ring-sprouting assay. This in vitro assay is inflammation independent and mainly assesses direct effects on EC migration and proliferation. As shown in Fig. 2E, there was no significant sprouting of EC from aortic rings stimulated by supernatants from LPS-stimulated hypoxic or normoxic macrophages. In contrast, recombinant VEGF (25 ng/ml) induced marked sprouting. Thus, IL-1-dependent angiogenesis in Matrigel plugs is mainly due to its capacity to induce cell infiltration and activate cytokine production by inflammatory cells, rather than the direct effect of VEGF present in the macrophage supernatants.

**IL-1-induced infiltration of myeloid and endothelial lineage cells into Matrigel plugs**

We next assessed the nature of infiltrating cells in Matrigel plugs containing supernatants of LPS-stimulated hypoxic or normoxic
Macrophages. As shown in Fig. 3A, neutralization of IL-1 activity markedly reduced the total number of infiltrating cells into the Matrigel.

A further characterization of the infiltrating cells was made by FACS analysis. Cells recovered from Matrigel plugs were analyzed for lineage surface markers. As shown in Fig. 3B, there was an accumulation of mature macrophages (F480$^{high}$/CD11b$^{+}$/CD115$^{+/−}$/GR-1$^{−}$), myeloid precursor cells (MPC) (CD45$^{+}$/CD11b$^{hig}/GR-1^{high}/CXCR4^{+}$), neutrophils (Ly-6C$^{−}/Gr-1^{high}/CD115^{−}$), and cells of endothelial cell (EC) lineage (CD11blow/neg/CD34dull/VEGFR-2$^{−}$). Macrophages accounted for 25% of the total cell infiltrate, MPC 20%, neutrophils 25%, and 10% were cells of endothelial lineage. The recruitment of these cell populations by macrophage supernatants was dependent on the presence of IL-1, as well as on IL-1 signaling in the host’s microenvironment, as evidenced in Matrigel plugs in IL-1R KO mice (p ≤ 0.001) (Fig. 3B).

The origin of cells infiltrating into the Matrigel was assessed in chimeric mice, consisting of lethally irradiated WT mice that were hematopoietically reconstituted with BM cells from GFP-transgenic mice. FACS analyses indicated that 50% of all infiltrating cells into the Matrigel were of BM origin. As shown in Fig. 3C, the majority of myeloid cells were of BM origin, whereas only 10% of cells of endothelial lineage were of BM origin (Fig. 3C, right). Most cells of myeloid and endothelial lineages in the blood were GFP tagged (Fig. 3C, left). These findings indicate that cells of endothelial lineage, including EPC, are indeed recruited from the BM; however, their presence in the Matrigel comprises only a minor fraction of the total endothelial lineage cell population. In contrast, most myeloid cells in the plugs are of BM origin.

**Cells of endothelial lineage are the primary source of VEGF in Matrigel plugs whereas myeloid cells produce IL-1**

As VEGF and IL-1 are important cytokines in the induction of inflammation-mediated angiogenesis in Matrigel plugs containing macrophage supernatants, it was of interest to know which of the infiltrating cells secrete these cytokines. Using anti-VEGFR-2- or anti-CD11b-coated beads, we depleted the infiltrating cells recovered from Matrigel plugs of endothelial or myeloid lineage cells, respectively. The recovered cells were further cultured in serum-free medium for 24 h, and the supernatants were assessed by ELISA for VEGF and IL-1 content. In addition, the 24-h supernatants were tested for their ability to induce sprouting of aortic rings, as a functional assay for direct activation of endothelial cells. As shown in Fig. 4A, depletion of VEGFR-2-positive cells (cells of the endothelial lineage), almost completely abolished VEGF secretion (Fig. 4A, right panel), whereas depletion of CD11b-positive myeloid cells, impaired secretion of IL-1 (Fig. 4A, left and middle panels). Aortic ring sprouting was promoted only by supernatants of nonseparated total infiltrating cells or supernatants from enriched cells of endothelial, but not myeloid, lineage (Fig. 4B).
EPC were reported to express the CD11b integrin, which is lost upon maturation; however, its expression is dull and 10- to 100-fold lower than expression patterns on myeloid cells. It is notable that the CD11b-conjugated magnetic beads, which were used to separate cells in our experiments, do not absorb CD11bdull cells. Intracellular staining of VEGF was consistent with these observations; 90% of VEGF-producing cells were CD34 dull and VEGFR-2-positive (Fig. 4C, right panel), but negative for CD11b. It was shown by intracellular staining that 90% of IL-1α, IL-1β, and VEGF (±SEM, n = 3) secreted by cells recovered from Matrigel plugs and further incubated for 24 h in culture. Aorta rings from WT mice were incubated with supernatants of cells recovered from Matrigel plugs (see A), and sprouting of EC from the aorta rings was tested on day 4 (n = 3) (×40). C, FACS analysis of VEGF and IL-1-producing cells recovered from Matrigel plugs on day 8. D, Mean VEGF levels (±SEM, n = 3) in day 8 Matrigel plugs extracts obtained from BM chimeric mice.

EPC were reported to express the CD11b integrin, which is lost upon maturation; however, its expression is dull and 10- to 100-fold lower than expression patterns on myeloid cells. It is notable that the CD11b-conjugated magnetic beads, which were used to separate cells in our experiments, do not absorb CD11bdull cells. Intracellular staining of VEGF was consistent with these observations; 90% of VEGF-producing cells were CD34 dull and VEGFR-2-positive (Fig. 4C, right panel), but negative for CD11b. It was shown by intracellular staining that 90% of IL-1β and 70% of IL-1α expressing cells are of myeloid lineage. Most IL-1β (55%) was found to be expressed in CD11b and F4/80-positive cells, yet other cells of myeloid lineage also expressed IL-1β (Fig. 4C, middle panel). IL-1α was expressed in a variety of cells, including macrophages (30%) and MPC (35%), but also in CD11b-negative cells (Fig. 4C, left panel).

Since VEGF production in Matrigel plugs is IL-1 dependent, we examined whether IL-1 expressing cells of BM origin affect VEGF secretion. For this purpose, we used chimeras consisting of lethally irradiated WT or IL-1-deficient mice, which were hematopoietically reconstituted with BM cells from either mouse strain. Thereafter, Matrigel with supernatants of either LPS-stimulated hypoxic or normoxic macrophages was injected into mice and the VEGF content was assessed in solubilized Matrigel plugs excised on day 8. As shown in Fig. 4D, VEGF levels were significantly reduced in Matrigel plugs from IL-1-deficient mice (rescued with BM from IL-1-deficient mice) compared with WT mice (rescued with BM from WT mice) (p ≤ 0.005). In contrast, upon rescue of IL-1 deficient mice with BM cells from WT mice, production of VEGF was restored (p ≤ 0.005). Consistent with these observations, in irradiated WT mice injected with BM cells from IL-1-deficient mice, reduced VEGF production was observed. Similar findings were observed for IL-1 production in Matrigel plugs (results not shown), further suggesting that IL-1 signaling is needed for IL-1 production. Thus, VEGF production by cells of endothelial lineage is dependent on IL-1 production by BM-derived cells.

Discussion

The design of this study was to mimic the microenvironment of most solid tumors, where insufficient oxygenation leads to tumor cell death and products of necrotic cells serve as an inflammatory trigger. Resident as well as infiltrating macrophages respond by producing cytokines and other mediators under the hypoxic conditions of tumor, which results in angiogenesis. Such a scenario is also typical of other physiological or pathological situations of ischemia characterized by tissue damage and inflammation (17–21). Thus, to understand the angiogenic response of the hypoxic
environment, we incorporated supernatants derived from hypoxic and normoxic macrophages without or after stimulation with an inflammatory signal (i.e., LPS) into Matrigel implanted into mice. This enabled us the characterization the supernatants properties and nature/origins of infiltrating cells, cytokines as well as angiogenic patterns in Matrigel plugs.

We observed that supernatants of hypoxic macrophages induced moderate inflammation and angiogenesis, whereas there was no reaction to supernatants of normoxic macrophages (Fig. 1). Hypoxia by itself modestly stimulated macrophage-induced inflammatory and angiogenic responses, but both were augmented by the inflammatory signal of LPS. Thus, the secretion of all pro-inflammatory cytokines by hypoxic and normoxic macrophages was induced by LPS. Nevertheless, VEGF, and most probably other proangiogenic factors, were induced by the hypoxic signal and did not further increase following LPS stimulation (Table I). Interestingly, the addition of LPS under hypoxic conditions resulted in a marked reduction in NO production and an increase in PGE2. This may indicate a shift in the macrophage phenotype toward alternative differentiation (M2 macrophages), which is involved in wound healing and tumor growth (3–13). Importantly, upon stimulation (hypoxia and LPS) macrophages secrete both pro- and anti-inflammatory/angiogenic mediators, VEGF, IL-1, IL-6, and chemokines like CCL3 and CCL5 are examples of proangiogenic mediators, whereas CXCL10, IL-12, and IL-10 are anti-inflammatory and angiostatic. However, the net balance between these multiple factors favors angiogenesis. These results were also confirmed when cytokine levels were assessed in day 8 Matrigel extracts, where pro- and antiangiogenic cytokines coexist.

Pronounced differences in the transcriptome and patterns of secretion of cytokines/mediators by hypoxic monocytes/macrophages were observed in different studies (5, 7, 15, 16). In this study, only selected cytokines were shown to change their pattern of expression/secretion. This disparity may stem from differences in the source of the macrophages, their initial activation state, the degree of hypoxia and its duration, or the presence of additional stimuli or culture conditions.

HIF-1α has been suggested as a transcription factor regulating macrophage differentiation, inflammation and cytokine secretion mainly in hypoxic microenvironments, but also under normoxia (4, 41). Indeed, the addition of LPS to hypoxic, and in a lesser extent to normoxic, macrophages potentiated HIF-1α nuclear translocation (supplemental Fig. 1). NF-κB activation, as evidenced by p65 nuclear translocation, was induced mainly by LPS, regardless of the environmental conditions. NF-κB and HIF-1α, when activated concomitantly in hypoxic inflamed areas, can act synergistically to amplify and sustain inflammatory and angiogenic responses (41, 42). Indeed, NF-κB was shown to be a critical transcriptional activator of HIF-1α in macrophages, as basal NF-κB activity is required for HIF-1α protein accumulation under hypoxia or after an encounter with a bacterial infection (42).

The overall production of IL-1 in LPS-stimulated hypoxic and normoxic macrophages was similar; however, differences in the pattern of expression/secretion of IL-1α vs IL-1β were observed. We noted a rapid decrease in IL-1β expression and secretion concomitantly to an increase in IL-1α (Table I). Changes in expression and secretion of IL-1 were rapid, peaking within 2–5 h with persistent high levels of transcripts/secreted IL-1 for 24–48 h, at which time the experiment was terminated (data not shown). A database search of the promoter region of IL-1α and IL-1β revealed a classical hypoxia response element in position −10 upstream from the initiation site of IL-1α, but there is no hypoxia response element in the IL-1β gene (data not shown). However, neutralization of IL-1 in macrophage supernatants has shown that IL-1β is more important for the induction of inflammation and angiogenesis in Matrigel plugs, compared with IL-1α that is mainly present in hypoxic macrophages (Fig. 2). This corresponds to previous studies using IL-1 KO mice, which demonstrated a stronger association of IL-1β in mediating inflammation compared with IL-1α, most probably due to better secretion of IL-1β, whereas IL-1α is mainly cell associated (24–30). Thus, IL-1β is the major IL-1 agonist molecule in the control of inflammation and angiogenesis in Matrigel plugs.

IL-1β of macrophage origin, which subsequently induces further production of IL-1 as well as other cytokines/chemokines by infiltrating cells, accounts for the initiation and perpetuation of inflammation and angiogenesis in Matrigel plugs. Most of the IL-1 (~70%), which was induced by host-derived myeloid cells and was detected in day 8 Matrigel plug extracts, was IL-1β, further attesting to its dominant role in inflammation-mediated angiogenesis (Fig. 2). The central role of IL-1β of macrophage origin was demonstrated by the dramatic reduction in cell recruitment and in angiogenesis following specific neutralization, as well as by the lack of angiogenic responses induced by macrophage supernatants derived from IL-1β-deficient mice. Remarkably, although some reports suggested a role for TNF-α in supporting angiogenesis (43), its neutralization in the supernatants did not alter the angiogenic process. Moreover, in contrast to IL-1β, TNF-α was not detected in extracts of Matrigel plugs supplemented with supernatants of either hypoxic or normoxic macrophages (supplemental Table I).

The cytokines present in macrophage supernatants did not induce significant aortic ring sprouting, probably due to insufficient concentrations of direct angiogenic factors, such as VEGF (Fig. 2). The aortic ring-sprouting assay is inflammation independent and involves direct effects of angiogenic factors on EC, such as stimulation of migration, proliferation and organization into tube-like structures. Thus, the major function of IL-1β in supporting angiogenesis in Matrigel plugs is to induce migration of infiltrating cells into the plugs and activate them in situ to secrete cytokines. The role of IL-1 of the host’s microenvironment in perpetuating inflammatory and angiogenic responses was demonstrated by the absence of a response in Matrigel plugs containing macrophage supernatants which were implanted in IL-1R deficient mice (Fig. 2). On day 8, IL-1 and VEGF levels in Matrigel plugs were 5–10 times higher than the initial amounts incorporated into the plugs, indicating production of these cytokines by infiltrating cells. Expression of IL-1 and VEGF by host-derived cells in Matrigel plugs was dependent on IL-1 originating primarily from infiltrating cells of BM origin, as indicated by BM transplantation assays in chimeric mice (Fig. 4). In addition, day 8 Matrigel extracts contained high levels of cytokines/chemokines; the secretion of most of them is IL-1 dependent, because anti-IL-1 Abs present in Matrigel plugs dramatically reduced their levels (supplemental Table I).

The nature of the cells infiltrating into Matrigel plugs was characterized by FACS analyses, assessing macrophages, neutrophils, MPC and cells of endothelial lineage (Fig. 3). Similar infiltrating cell populations, differing in the ratio of the various cell types, were detected in Matrigel plugs supplemented with the different macrophage supernatants. Chimeric mice, consisting of lethally irradiated control mice, hemopoietically reconstituted with BM cells from GFP-transgenic mice, were used to determine whether cells infiltrating into Matrigel plugs were of BM origin. The vast majority of myeloid cells (macrophages, neutrophils and MPC) originate in the BM, whereas only ~10% of endothelial lineage cells are of BM origin. Thus, in our experimental system, the vast majority of endothelial lineage cells originate from tissue-resident...
blood vessel formation. Sufficient amounts of VEGF are produced and subsequently support the migratory response, or by IL-1-induced inflammation, during which sufficient cytokines secreted by them. Our data support these findings and demonstrate that myeloid cells could generate VEGF and that IL-1β-positive myeloid cells are the major source of IL-1, whereas VEGFR2-positive cells of endothelial lineage mainly secrete VEGF. After recovery from the plugs, infiltrating cells were activated and did not need any additional signals for the secretion of IL-1 and VEGF upon in vitro culture. These patterns of IL-1 and VEGF secretion were also corroborated by the aortic ring-sprouting assay. Sprouting was induced by supernatants of nonseparated infiltrating cells or cells enriched in VEGFR-2-positive cells but not by CD11b-positive cells. Although it was reported that myeloid cells could generate VEGF and that IL-1β is secreted by EC (44, 45), these cells did not represent the major source of these cytokines in Matrigel plugs. The BM origin of EPC, which following maturation incorporating into newly formed blood vessels, has been demonstrated in some experimental systems (20, 46). In contrast, other reports have shown that EPC of BM origin do not play a major role in tumor angiogenesis (47–49). The issue of differentiation of EPC and their incorporation into growing blood vessels was beyond the scope of this study.

Grunewald et al. (37), using an experimental system of organ culture, have demonstrated the necessity of inflammation for angiogenesis. Thus, VEGF activity must be complemented by inflammatory myeloid cells of BM origin, which possibly act, at least in part, through mediators secreted by them. Our data support these findings and demonstrate that inflammation-mediated angiogenesis can be initiated either by excess VEGF, which will summon an inflammatoryatory response, or by IL-1-induced inflammation, during which sufficient amounts of VEGF are produced and subsequently support blood vessel formation.

In conclusion, IL-1β of macrophage origin plays an essential role in the initiation and propagation of angiogenesis, mainly by recruitment of inflammatory cells into the affected site and their in situ activation for secretion of cytokines/chemokines. IL-1β and VEGF were shown to have a dominant role in inducing inflammation-mediated angiogenesis in Matrigel plugs. IL-1α contributes to angiogenesis in hypoxia, when IL-1β secretion is reduced to suboptimal levels. We have previously reported that tumor-mediated angiogenesis is inhibited in vivo by the IL-1ra, which also results in a reduction of tumor invasiveness (31, 32). The observations reported here are applicable also to non-tumor-related conditions, such as ischemia-reperfusion, chonic inflammation and wound repair. Additional experiments on the antiangiogenic potential of IL-1 neutralization are in progress.

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Disclosures
The authors have no financial conflict of interest.

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Supplemental Figure 1: Cytokines and transcription factors in hypoxia and LPS-stimulated macrophages Thioglycollate-induced macrophages (0.1x10^6) were seeded on slides for 6 h in serum free DMEM with or without LPS under hypoxic or normoxic conditions. A. Confocal microscopy of HIF-1α (red) and nuclear staining (blue). B. Confocal microscopy of p65 (red) and nuclear staining (blue). C. Right graph presents arbitrary values of fluorescence intensity per pixel of HIF-1α in the nucleus. Left graph presents the relative quantification (RQ) of VEGF mRNA expression after 6 h of incubation under the different experimental conditions (± SEM, n= 3). D. Right graph presents arbitrary values of fluorescence intensity per pixel of p65 in the nucleus. Left graph presents the relative quantification (RQ) of IL-6 mRNA under the different conditions compared to the normoxia control (CT) after 6 h (± SEM, n= 3).
Matrigel plugs, containing macrophage supernatants, were recovered on day 8 and were solubilized prior to cytokine/chemokine measurements. Shown are levels of cytokines/chemokines pg/ml/plug. In all experimental groups, plugs of similar size were observed (400 ±100 mg per plug). CT- plugs supplemented with supernatants of either hypoxic or normoxic macrophages; LPS- plugs supplemented with supernatants of LPS-stimulated hypoxic or normoxic macrophages; LPS+αIL-1- plugs supplemented with supernatants of LPS-stimulated hypoxic/normoxic macrophages, pretreated with anti-IL-1 antibodies (anti-IL-1α and anti-IL-1β). Cytokine/chemokine levels were assessed by ELISA or Quansys custom cytokine and chemokine array.