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J Immunol 2005; 175:2788-2792; doi: 10.4049/jimmunol.175.5.2788
http://www.jimmunol.org/content/175/5/2788

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Cutting Edge: Proangiogenic Properties of Alternatively Activated Dendritic Cells

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Angiogenesis plays an important role in tissue remodeling and repair during the late phase of inflammation. In the present study, we show that human dendritic cells (DC) that matured in the presence of anti-inflammatory molecules such as calcitriol, PGE2, or IL-10 (alternatively activated DC) selectively secrete the potent angiogenic cytokine vascular endothelial growth factor (VEGF) isoforms VEGF165 and VEGF121. No VEGF production was observed in immature or classically activated DC. Also, the capacity to produce VEGF was restricted to the myeloid DC subset. When implanted in the chick embryo chorioallantoic membrane, alternatively activated DC elicited a marked angiogenic response, which is inhibited by neutralizing anti-VEGF Abs and by the VEGFR-2 inhibitor SU5416. Therefore, alternatively activated DC may contribute to the resolution of the inflammatory reaction by promoting VEGF-induced angiogenesis. The Journal of Immunology, 2005, 175: 2788–2792.

D endritic cells (DC) are professional APC that play a crucial role in the onset and regulation of acquired immunity. The number of tissue DC rapidly increases during inflammation, and DC participate to the regulation of the inflammatory reaction through the release of cytokines and chemokines (1).

The balance between pro- and anti-inflammatory signals present in the inflammatory microenvironment determines the phenotype and the behavior of the immune cells at the site of inflammation (2). APC such as macrophages activated by IFN-γ and proinflammatory signals (e.g., LPS and TNF-α) are characterized by proinflammatory and cytotoxic functions. Conversely, exposure of macrophages to anti-inflammatory molecules, such as IL-10, IL-13, IL-4, calcitriol, and glucocorticoids, induces an alternative program of activation characterized by a peculiar membrane phenotype and function (2).

Alternatively activated macrophages are devoid of proinflammatory and cytotoxic functions; instead, they express high levels of MHC class II molecules and are potent endocytic cells. Alternatively activated macrophages play a relevant role during the resolution phase of inflammation by producing cytokines (e.g., IL-10, TGF-β, and IL-1 receptor antagonist) and scavenging cellular debris. DC may also be alternatively activated and induced to express a different profile of cytokines and functions. For instance, alternatively activated DC (AA-DC) were shown to produce CCL18 and to posses tolerogenic activity (3).

The resolution phase of inflammation is characterized by enhanced angiogenesis. Newly formed blood vessels provide nutrients to growing tissues and allow traffic of immune cells. This process is sustained by the early production of angiogenic factors, including basic fibroblast growth factor (FGF) (FGF-2), TNF-α, some "ELR" chemokines, such as CXCL8, followed by the release of vascular endothelial growth factor (VEGF), whose production peaks several days after injury (4, 5).

In this study, we investigated the angiogenic properties of DC in vitro and in vivo. The results show that alternatively activated myeloid DC, but not classically activated DC, release relevant levels of biologically active VEGF and posses proangiogenic activity in vivo.

Materials and Methods
DC preparation and culture

Highly enriched blood monocytes were obtained from buffy coats (through the courtesy of the Centro Trasfusionale) by Ficol and Percoll (Amersham Biosciences) gradients. DC were generated in vitro as described previously (6). DC maturation (106 DC/ml) was induced by incubation with 100 ng/ml LPS (Escherichia coli 055:B5; Sigma-Aldrich), 1/5000 dilution of Staphylococcus aureus Cowan 1 (SAC) (Calbiochem), 20 ng/ml TNF-α (BASF/Knoll), or CD40L-transfected J558 cells (1:4 ratio) for 24 h. Where indicated, DC were treated with 50 ng/ml human IL-10 (Schering-Plough), 50 ng/ml TGF-β1 (PeproTech), 10−6 M PGE2, or 10−6 M (unless otherwise specified) calcitriol (25-dihydroxyvitamin D3) (Sigma-Aldrich). Peripheral blood DC were obtained from PBMC by magnetic sorting with blood DC Ag 1 and blood DC Ag 4 kits (Miltenyi Biotec) (6). Cytokines were detected using specific Duo-Set kits (R&D Systems). DC conditioned medium (106 cells/ml) were concentrated on

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Received for publication May 17, 2005. Accepted for publication June 29, 2005.

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1 This work was supported by the Associazione Italiana per la Ricerca sul Cancro, the Ministero dell’Istruzione Università e Ricerca, Coln and Firb, Association for International Cancer Research Grant 04-223, the Fondazione Berlucchi, Istituto Superiore di Sanità (Programma Oncotecnologico), and Centro d’Eccellenza per l’Innovazione Diagnostica e Terapeutica (IDET).

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3 Abbreviations used in this paper: DC, dendritic cell; AA-DC, alternatively activated DC; FGF, fibroblast growth factor; VEGF, vascular endothelial growth factor; SAC, Staphylococcus aureus Cowan 1; PAE-KDR, KDR-transfected porcine aortic endothelial; CAM, chorioallantoic membrane; CA-DC, classically activated DC.
Centricon YM-10 filters (Millipore), and 500 μg of proteins were subjected to 15% SDS-PAGE, followed by Western blotting using an anti-VEGF Ab (Santa Cruz Biotechnology). Immunoreactivity was detected using the SuperSignal West Pico Chemiluminescent Substrate (Pierce).

**RT-PCR**

Total RNA was purified with TRIzol (Invitrogen Life Technologies). RNA samples (1 μg) were reverse transcripted using the SuperScript II RNase H− Reverse Transcriptase (Invitrogen Life Technologies). PCR were performed on cDNA samples using the following primers: hTNF-α forward, 5′-GTCTCC GTACCAGACCAAGGTCA-3′, and hTNF-α reverse, 5′-CAAGTA GACGTCCGACAATCGG-3′; hCXCL8 forward, 5′-CGATGTCAGTG CATAAAGCA-3′, and hCXCL8 reverse, 5′-TGAATTTCTGACGCCCT CTCTAAA-3′; hIL-12p40 forward, 5′-CCACATTCTCCTACTGTC-3′, and hIL-12p40 reverse, 5′-GTCTATCGTGGTTGTC-3′; hEGF-2 forward, 5′-AAGCTTCTTGACTGCAAAACG-3′, and hFGF-2 reverse, 5′-AACT GGTGTATTTCCTTGACCGGTA-3′; hVEGF forward, 5′-TGGATAGTC TATACGGCAGCT-3′, and hVEGF reverse, 5′-TGTGTATTTCGAGGA CATTTCAGCAG-3′; and hβ-actin forward, 5′-GAAGACCCAGAAGCTG CCTGAA-3′, and hβ-actin reverse 5′-TGAATTTCTTCTTACTGCGTG TTG-3′. Amplified products were subjected to electrophoresis on agarose gels and stained with ethidium bromide.

**VEGFR-2 transfectants**

VEGFR-2/KDR-transfected porcine aortic endothelial (PAE/KDR) cells (7) were kindly provided by Dr. S. Mitola (University of Brescia, Brescia, Italy). Serum-starved cells were incubated at 37°C for 15 min with DC conditioned medium. Western blots were performed using anti-phospho-ERK1/2 Ab (Santa Cruz Biotechnology).

**Chicken embryo chorioallantoic membrane (CAM) assay**

Untreated DC, DC treated with 100 ng/ml LPS, or DC treated with 100 ng/ml LPS in the presence of 10−6 M calcitriol for 6 h were washed, entrapped in a 3% alginate pellet (3-μl suspension, 40,000 cells/pellet), and placed on top of the CAM of fertilized White Leghorn chicken eggs on day 11 of incubation. After 72 h, blood vessels entering the fetal plane of the CAM were photographed at ×20 magnification and counted (8). In some experiments, 200 ng of a blocking anti-VEGF Ab (R&D Systems) or 300 ng/egg of SU5416 [3-[(2,4-dimethylpyrrol-5-yl)methyllidenyl]-indolin-2-one] (Calbiochem-Merk) were added to DC-loaded pellets. Pellets containing vehicle alone were used as negative control.

**Statistical analysis**

Statistical significance between the experimental groups was determined using unpaired Student’s t test or one-way ANOVA with Dunnet’s post hoc test where appropriate.

**Results**

**Induction of VEGF in alternatively activated DC**

Human monocyte-derived DC were matured in vitro in the presence of LPS (classically activated DC (CA-DC)) or a combination of LPS and calcitriol, PGE2, or IL-10 (AA-DC). Mature DC were then evaluated for their proangiogenic potential by measuring the mRNA steady-state levels of VEGF and FGF-2, two potent angiogenic cytokines. Fig. 1A shows that by RT-PCR, CA-DC did not express FGF-2 and expressed barely detectable levels VEGF transcripts. Conversely, CA-DC showed increased mRNA levels, compared with immature DC, for TNF-α and CXCL8, two cytokines that are known to be up-regulated during DC maturation (data not shown) (6). Of interest, VEGF mRNA levels were strongly induced in AA-DC generated in the presence of calcitriol, PGE2, or IL-10, whereas FGF-2 transcript was induced only weakly in the same experimental conditions. As expected, based on previous studies (9), IL-12p40 was inhibited in AA-DC (Fig. 1A). The results obtained at the mRNA level were confirmed by ELISA. Induction of VEGF protein production was easily detectable in AA-DC generated in the presence of calcitriol, PGE2, and IL-10, with IL-10 being the weakest agonist. Conversely, FGF-2 protein levels were undetectable in all the experimental conditions used (Fig. 1B). Neither VEGF nor FGF-2 was released by immature DC. TGF-β, a cytokine known for its ability to promote angiogenesis and tissue repair (10), was inactive in inducing VEGF production by DC (data not shown). Collectively, these results show that AA-DC are characterized by the production of high levels of VEGF, a potent proangiogenic cytokine.

The effect of calcitriol on VEGF production was investigated in the presence of agonists, such as TNF-α, SAC, and CD40L, which induce DC maturation by pathways that are different from that used by LPS. Fig. 2 shows that calcitriol was active in inducing VEGF in all the experimental conditions tested. Parallel experiments performed with PGE2, rather than calcitriol, provided similar results (data not shown).

Circulating DC comprise two main DC populations: myeloid and plasmacytoid (11). Consistent with the results reported above, blood myeloid DC secreted VEGF in a dose-dependent manner when stimulated with LPS in the presence of calcitriol, although at levels that were lower than those observed with monocyte-derived DC (0.34 ± 0.2 vs 1.64 ± 0.53 ng/ml 106/DC at 1 μM calcitriol, respectively). No VEGF production was observed with immature DC of classically activated myeloid blood DC (data not shown). Conversely, plasmacytoid DC did not produce VEGF in any of the experimental conditions tested, including activation with influenza virus in the
The production of VEGF was selectively observed in AA-DC obtained by in vitro DC maturation in the presence of calcitriol, PGE₂, or IL-10. Conversely, VEGF was not produced by immature DC nor by DC activated in the presence of the proinflammatory or immune signals LPS, TNF-α, SAC, or CD40L (i.e., CA-DC). It is interesting to note that AA-DC, at variance with CA-DC, are inhibited in their ability to produce IL-12, a cytokine with potent antiangiogenic activity (9). Therefore, it appears that in AA-DC the balance between pro- and antiangiogenic proteins favors angiogenesis.

Two subsets of human circulating blood DC were defined based on the expression of CD11c, namely CD11c⁺ myeloid DC and CD11c⁻ plasmacytoid DC (11). Myeloid DC express...
VEGF is known to inhibit the ability of hemopoietic progenitor cells to differentiate into functional DC (17). VEGF was also reported to inhibit IL-12 production and Th1 differentiation by LPS-activated DC (18). Therefore, VEGF production by DC might be seen as a potential autocrine negative loop of DC functions. This possibility was ruled out in this study by the use of VEGF blocking Abs during the in vitro maturation of AA-DC; in these experimental conditions, the inhibition of VEGF in the supernatants did not change the generation of mature DC (data not shown).

It was shown recently that in DC, IL-10, and PGE$_2$ up-regulate the production of thrombospordin 1, an inhibitor of angiogenesis (19). Therefore, AA-DC apparently produce both pro- and antiangiogenic cytokines. However, the in vivo proangiogenic activity of AA-DC reported in our study suggests that, at least in our experimental conditions, the balance of these two activities favors angiogenesis.

Signals associated with a type 2-polarized immune response (IL-4, IL-10, IL-13, calcitriol, and PGE$_2$) are known to be induced during the resolution phase of inflammation and to be responsible for the inhibition of inflammatory cytokines, the promotion of tissue remodeling and repair, the scavenging of cellular debris, and the inhibition of Th1 responses (2). Type 2-polarizing signals are also produced in other pathological conditions, including cancer (20). Solid tumors are infiltrated by DC that usually lack the phenotype of CA-DC (2, 3). Deregulated VEGF expression has been implicated in the development of solid tumors by supporting tumor angiogenesis (12). The data presented in this study suggest that within the tumor microenvironment AA-DC may represent a source of angiogenic factors contributing to tumor neo-vascularization and growth.

DC activated in the presence of anti-inflammatory agents as calcitriol or IL-10 are known to have tolerogenic properties and a reduced proinflammatory potential (3, 21). This study extends these observations providing evidence that AA-DC also possess a proangiogenic activity in vitro and in vivo through the production of VEGF.

**Acknowledgments**

We thank Felice De Ceglie for help in the preparation of digital art.

**Disclosures**

The authors have no financial conflict of interest.

**References**


