The Journal of Immunology

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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

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

THE JOURNAL OF IMMUNOLOGY

CUTTING EDGE

Cutting Edge: Proangiogenic Properties of Alternatively Activated Dendritic Cells[1]

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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, PGE_2, or IL-10 (alternatively activated DC) selectively secrete the potent angiogenic cytokine vascular endothelial growth factor (VEGF) isoforms $VEGF_{165}$ and $VEGF_{121}$. 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 elicit 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.**

Dendritic cells (DC)[3] 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 Ficoll and Percoll (Amersham Biosciences) gradients. DC were generated in vitro as described previously (6). DC maturation (10^6 DC/ml) was induced by incubation with 100 ng/ml LPS (*Escherichia coli* 055:B5; Sigma-Aldrich), 1/5000 dilution of *Staphylococcus aureus* Cowan I (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^{-5} M PGE_2, or 10^{-6} M (unless otherwise specified) calcitriol (1,25-dihydroxyvitamin D_3) (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 (10^6 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, Cofin and Firb, Association for International Cancer Research Grant 04-223, the Fondazione Berlucchi, Istituto Superiore di Sanitá (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 I; PAE-KDR, KDR-transfected porcine aortic endothelial; CAM, chorioallantoic membrane; CA-DC, classically activated DC.

0022-1767/05/$02.00

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′-GTCTC CTACCAGACCAAGGTCAA-3′, and hTNF-α reverse, 5′-CAAAGTA GACCTGCCCAGACTCG-3′; hCXCL8 forward, 5′-CGATGTCAGTG CATAAAGACA-3′, and hCXCL8 reverse, 5′-TGAATTCTCAGCCCT CTTCAAAAA-3′; hIL-12p40 forward, 5′-CCACATTCCTACTTCTC-3′, and hIL-12p40 reverse, 5′-GTCTATTCCGTTGTGTC-3′; hFGF-2 forward, 5′-AAGCGGCTGTACTGCAAAAACG-3′, and hFGF-2 reverse, 5′-AACT GGTGTATTTCCTTGACCGGTA-3′; hVEGF forward, 5′-TGGATGTC TATCAGCGCAGCT-3′, and hVEGF reverse, 5′-TGTTTTTGCAGGAA CATTTACACG-3′; and hβ-actin forward, 5′-GAAGAGCTACGAGCTG CCTGA-3′, and hβ-actin reverse 5′-TGATCTTCATTCTGCTGGGTG-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 pellet within the focal plane of the CAM were photographed at ×20 magnification and counted (8). In some experiments, 200 ng/egg 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, PGE_2, 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. 1*A* 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, PGE_2, 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. 1*A*). 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, PGE_2, and IL-10, with IL-10 being the weakest agonist. Conversely, FGF-2 protein

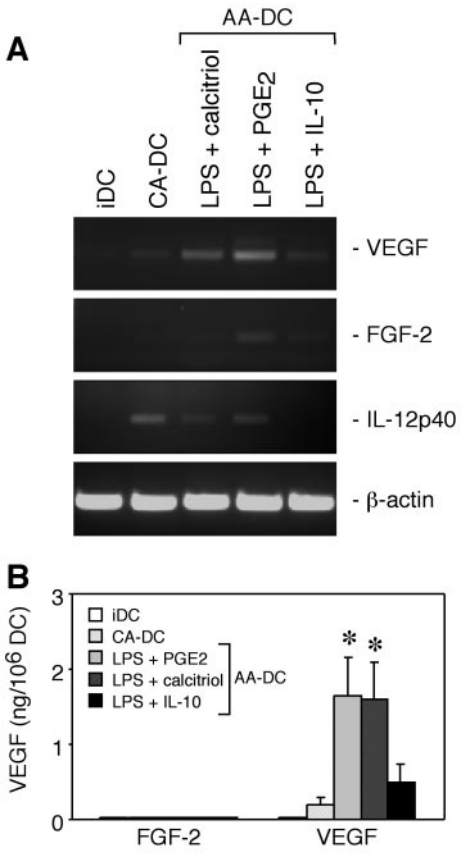

FIGURE 1. VEGF production by alternatively activated DC. *A*, DC were incubated in the absence (iDC) or in the presence of LPS (CA-DC) for 6 h. AA-DC were incubated with LPS in the presence of calcitriol, PGE_2, or IL-10 for 6 h. mRNA expression was analyzed by RT-PCR. *B*, Cytokines were tested by ELISA in supernatants of DC incubated for 24 h. Data are expressed as mean ± SEM (n = 3–6); *, $p < 0.05$ by Student's *t* test.

levels were undetectable in all the experimental conditions used (Fig. 1*B*). 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 PGE_2, 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 10^6/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

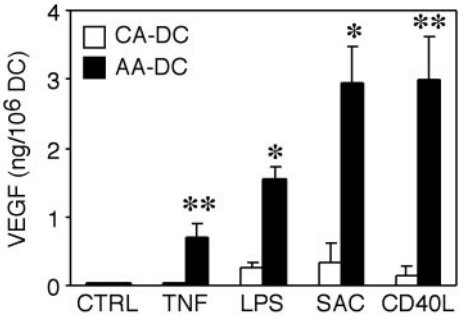

FIGURE 2. VEGF production by DC alternatively activated by different maturative stimuli. DC were incubate with different maturative stimuli in the absence (CA-DC) or in the presence of 10^{-6} M calcitriol (AA-DC) for 24 h. VEGF production was evaluated by ELISA. Data are expressed as mean ± SEM ($n = 3$–5); *, $p < 0.05$; **, $p < 0.01$ by Student's t test.

presence of calcitriol or PGE_2 (data not shown). Thus, the production of VEGF is apparently restricted to the myeloid DC subset.

Angiogenic properties of alternatively activated DC

The regulation of VEGF production by AA-DC was further characterized. The effect of calcitriol was concentration dependent (Fig. 3*A*) and time dependent (Fig. 3*B*). VEGF is produced in multiple isoforms by alternative splicing of a single gene, with $VEGF_{121}$ and $VEGF_{165}$ being the two most common variants (12). Western blot analysis was performed to identify the nature of the splicing isoforms produced by AA-DC. Fig. 3*C* shows that two major bands, corresponding to $VEGF_{165}$ and $VEGF_{121}$, were detectable in the supernatants from AA-DC but not LPS-activated DC. VEGF exerts its biologically activity on endothelial cells through the interaction with tyrosine kinase receptors, namely VEGFR-1 (also known as Flt-1) and VEGFR-2 (also known as KDR or Flk-1) (12). An endothelial cell line transfected with VEGFR-2 (PAE-KDR) was used to evaluate the biological activity of VEGF-containing AA-DC supernatants. The activation of the MAPK ERK1/2 was used as readout for VEGFR-2 activation. Fig. 3*D* shows that the conditioned medium of DC matured with LPS in the presence of calcitriol-induced ERK1/2 phosphorylation in PAE-KDR cells, whereas no activation was observed using conditioned medium from immature or LPS-activated DC. ERK1/2 activation in PAE-KDR cells was specifically mediated by VEGFR-2 signaling because AA-DC conditioned medium was inactive in mock-transfected PAE cells (data not shown).

On the basis of the in vitro results, proangiogenic activity of AA-DC was investigated in vivo using the chicken embryo CAM assay. As shown in Fig. 4 calcitriol-AA-DC exert a potent angiogenic response when delivered on the top of the CAM via an alginate-pellet implant. Conversely, immature or LPS-activated DC (Fig. 4*B*) do not exert any proangiogenic effect in this assay. To assess the contribution of VEGF to the proangiogenic activity of calcitriol-AA-DC, CAM assay was performed in the presence of a VEGF-blocking Ab or SU5416, a VEGFR-2 inhibitor. As reported in Fig. 4*B*, AA-DC-induced neo-angiogenesis was reduced by 53% in the presence of a blocking anti-VEGF mAb and by 74% in the presence of SU5416. These results indicate that AA-DC induce angiogenesis in vivo mainly through the production of VEGF.

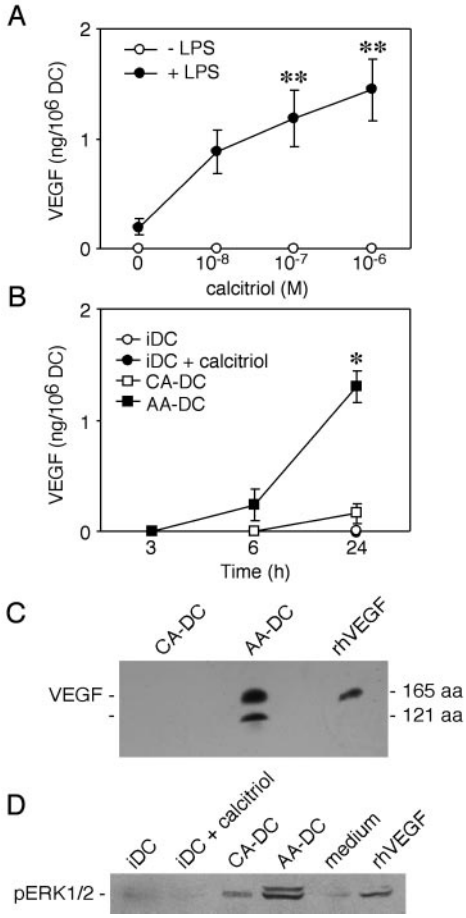

FIGURE 3. Characterization of VEGF production by AA-DC. *A*, DC were incubated in the presence of different concentrations of calcitriol for 24 h. *B*, DC were matured with LPS in the absence (CA-DC) or in the presence of 10^{-6} M calcitriol (AA-DC). *C*, VEGF isoforms present in the supernatants of 24-h treated DC were detected by Western blotting ($n = 3$). *D*, Western blot analysis ($n = 3$) of PAE-KDR cells stimulated with conditioned medium of DC. *, $p < 0.05$; **, $p < 0.01$ by one-way ANOVA with Dunnet's post hoc test.

Discussion

Angiogenesis is a process that characterizes tissue remodeling and repair during the late phase of inflammation (13). The resolution of the inflammatory process is characterized by the presence of anti-inflammatory signals, such as IL-10 and corticosteroids, that induce an "alternative/type 2" state of activation of infiltrating leukocytes (2, 3). This study reports that AA-DC release biologically active concentrations of VEGF, in vitro, and possess angiogenic activity in vivo.

The production of VEGF was selectively observed in AA-DC obtained by in vitro DC maturation in the presence of calcitriol, PGE_2, 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

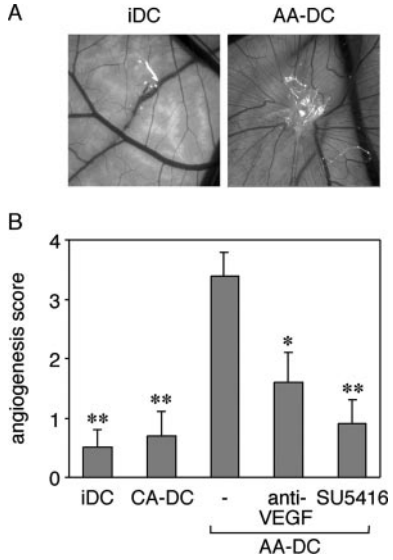

FIGURE 4. In vivo proangiogenic activity of alternatively activated DC. *A*, DC were entrapped in an alginate pellet and placed on top of the CAM of fertilized chicken eggs. After 72 h, CAM were photographed at ×20 magnification. *B*, Where indicated, the alginate pellet contained a monoclonal anti-VEGF Ab or the VEGFR-2 inhibitor SU5416. After 72 h, angiogenic response was graded on an arbitrary scale of 0–4+, with 0 representing no angiogenic response and 4+ representing the strongest activity. Data are expressed as mean ± SEM ($n = 5–8$); *, $p < 0.02$; **, $p < 0.01$ by Student's t test.

myeloid markers, such as CD13 and CD33, and produce high levels of IL-12 (11). Conversely, plasmacytoid DC have morphology resembling plasma cells, express high levels of CD4, CD62 ligand, and CD123 and produce high levels of IFN-α (11). These two DC subsets have distinct roles in the induction and regulation of immune response. The data reported in this study, showing that VEGF production is restricted to myeloid DC, further points to this dichotomy.

AA-DC release two of the many VEGF-spliced variants, namely $VEGF_{165}$ and $VEGF_{121}$, and, most importantly, AA-DC-derived VEGF is biologically active, as assessed by its ability to activate VEGFR-2. Accordingly, AA-DC, but not immature or CA-DC, have proangiogenic activity in vivo in the chick embryo CAM assay. Although the contribution of additional angiogenic factors cannot be formally excluded, experiments performed in the presence of a VEGF blocking mAb and SU5416, an inhibitor of VEGFR-2 activity, strongly point to VEGF as the major angiogenic factor produced by AA-DC in vivo.

The production of VEGF is known to be induced by hypoxia, by activated oncogenes, and by a variety of cytokines. In osteoblasts, synovial fibroblasts, monocytic THP-1 cells, and murine macrophages, VEGF production is mediated by increased levels of intracellular cAMP (14). It is interesting to note that both PGE_2 and calcitriol induce cAMP accumulation (15, 16). Consistently with these results, we have observed that 8-(4-chlorophenylthio)-adenosine 3′,5′-cAMP, a membrane permeable analog of cAMP, induces VEGF production in LPS-activated DC (E. Riboldi and S. Sozzani, unpublished observation). The effect of calcitriol in angiogenesis is currently unclear with both positive and negative effects reported (9). It is likely that these seemingly divergent results may be due to the use of different cell types and to differences in the experimental protocols used.

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 thrombospondin 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.

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