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Cytotoxic Dendritic Cells Generated from Cancer Patients

Daniela Lakomy,*† Nona Janikashvili,*† Jennifer Fraszczak,* Malika Trad,* Sylvain Audia,*† Maxime Samson,* Marion Ciudad,* Julien Vinit,* Catherine Vergely,* Denis Caillot,‡ Pascal Foucher,§ Laurent Lagrost,* Salem Chouaib,¶ Emmanuel Katsanis,||###,** Nicolas Larmonier,||†††‡‡‡ and Bernard Bonnotte*†,‡

Abbreviations used in this article: CMH, 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine; DC, dendritic cell; ESR, electron spin resonance; FasL, Fas ligand; FeTPPS, 5,10,15,20-tetrakis(4-sulfonatophenyl)porphyrinato iron (III) chloride; hKDC, human killer dendritic cell; KDC, killer dendritic cell; poly(L:C), polyinosinic-polycytidylic acid; poly(I:C), polyinosinic-polycytidylic acid.

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The possibility of improving the efficacy of DC-based cancer vaccines by triggering their direct tumor killing activity has not been evaluated. Several subsets of killer DCs (KDCs) have been described in mice (8–14), rats (15–22), and humans (23–49). However, few data concerning the killing ability of human DCs generated from cancer patients are available (26, 44, 49). In most human studies, KDCs were obtained by in vitro differentiation of monocytes from healthy donors in the presence of GM-CSF and IL-4 (23, 24, 27, 31, 32, 45, 46) followed by exposure to diverse inflammatory signals such as IFN-β (35), IFN-γ (27), LPS (27, 33, 46), imiquimod, polyinosinic-polycytidylic acid [poly(L:C)] (48), OK432 (penicillin-inactivated Streptococcus pyogenes) (30), or CD40L (33). The modes of induction (LPS, CpG, IFNs, and CD40L) and the effector mechanisms (Fas ligand [FasL], TNF family members) underlying the killing activity of these cells remain a subject of controversy (26, 29, 34, 35, 38, 44, 47, 48). Additionally, the ability of these KDCs to capture dead tumor cells and to induce specific T cell activation has not been fully elucidated. In terms of clinical applications, triggering of the cytotoxic activity of autologous DCs from cancer patients may further enhance their therapeutic potential by fostering the release of tumor Ags immediately available for capture and presentation to specific T cells. Clear evidence has been provided that cancers can inhibit the anti-tumor immune response by numerous mechanisms, including the induction of tolerogenic DCs. However, no information regarding the possible negative regulation of KDC cytotoxic activity by cancer cells is currently available. To address this question, in this study we investigated the possibility of generating human DCs endowed with tumor-killing activity from cancer patients.

Our results indicate that following activation with LPS, human monocyte-derived DCs generated from advanced-stage cancer patients are strongly cytotoxic against tumor cells. These human KDCs (hKDCs) were capable of triggering necrotic death of a wide variety of tumor cell lines through direct cell contact. The killing mechanism involves peroxynitrite release. Interestingly, immature but mature DCs exhibit cytotoxic activity against cancer cells.
Importantly, after killing tumor cells, these cytotoxic DCs were able to activate tumor Ag-specific T lymphocytes.

Materials and Methods

Cells

The following human cancer cell lines were purchased from the American Type Culture Collection (Manassas, VA): the cervix carcinoma cell line HeLa, the breast adenocarcinoma cell line MCF7, and the human colorectal cancer cell lines HT29, SW480, and HCT116. The T1 melanoma cell line and the LT12 CTL clone specific for the Melan-A26,1-26,1 peptide were provided by Dr. S. Chouaib (INSERM Unité 753, Villejuif, France). The human embryonic cells and the A7R5 rat vascular smooth muscle cells were provided by Dr. L. Lagrot (INSERM Unité 866, Dijon, France). HT29-expressing GFP was obtained by retroviral transfection (9). The percentage of GFP-expressing cells was measured by flow cytometry.

Patients

Ten patients with different confirmed cancers were enrolled in the clinical study before treatment after giving their written informed consent. Lymphoma (n = 2), pulmonary (n = 4), or colon cancer (n = 4) patients at stage IV incurable cancer were enrolled. None of the patients had received chemotherapy or any other immunosuppressive treatment during the previous 3 mo.

Reagents

LPS, flagellin, poly(I:C), gardiquimod, and crystal violet were purchased from Sigma-Aldrich (St. Louis, MO). 5,10,15,20-Tetrakis(4-sulfonato-phenyl)propiophenino iron (III) chloride (FeTPPS) was obtained from Calbiochem (San Diego, CA). IFN-γ and TNF-α were purchased from PeproTech (Rocky Hill, NJ). CpG was from InvivoGen (Carlsbad, CA), and PamCys-SK4 was from EMC (Tubingen, Germany). IL-6 and IL-1β were purchased from R&D Systems (Minneapolis, MN) and PGE2 was from Sigma-Aldrich.

Abs and flow cytometry analysis

The following human Abs were purchased from BD Biosciences (San Jose, CA): alpha4/CD1a, FITC-CD11c, FITC-CD40, FITC-CD80, FITC-CD83, FITC-CD86, FITC-HLA-DR, PE-CD14, PE-CD163, FITC-CD3, and FITC-CD56. For flow cytometry analysis, cells (5 × 10⁶) were washed in PBS with 0.5% BSA and 0.1% sodium azide, incubated with the appropriate Ab or isotype controls for 1 h, and then washed and analyzed by flow cytometry using an LSRII (BD Biosciences).

Generation of DCs

Human PBMCs were isolated from the blood of cancer patients or from buffy coats of healthy donors (EFS, Besançon, France) by Ficoll-Percoll density gradient centrifugation. Monocytes were purified from human PBMCs using CD14 microbeads (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer’s recommendations. CD14 purity was determined using flow cytometry analysis after staining with a PE-conjugated anti-CD14 Ab (BD Biosciences) and was routinely found to be >95%. Immature DCs were obtained by incubating monocytes (5 × 10⁵ cells/ml) in RPMI 1640 (BioWhittaker, Basel, Switzerland) supplemented with 10% FBS and recombinant human GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) (both from PromoCell, Heidelberg, Germany) according to the manufacturer’s recommendations (BD Biosciences). CD14 purity was determined using flow cytometry analysis after staining with a PE-conjugated anti-CD14 Ab (BD Biosciences) and was routinely found to be >95%. Immature DCs were obtained by incubating monocytes (5 × 10⁵ cells/ml) in RPMI 1640 (BioWhittaker, Basel, Switzerland) supplemented with 10% FBS and recombinant human GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) (both from PromoCell, Heidelberg, Germany) according to the manufacturer’s recommendations (BD Biosciences). CD14 purity was determined using flow cytometry analysis after staining with a PE-conjugated anti-CD14 Ab (BD Biosciences) and was routinely found to be >95%. Immature DCs were obtained by incubating monocytes (5 × 10⁵ cells/ml) in RPMI 1640 (BioWhittaker, Basel, Switzerland) supplemented with 10% FBS and recombinant human GM-CSF (100 ng/ml) and IL-4 (20 ng/ml) (both from PromoCell, Heidelberg, Germany) according to the manufacturer’s recommendations (BD Biosciences).

Cytokine assays

The concentration of IL-12 in cell culture supernatants was determined using ELISA kits according to the manufacturer’s procedures (Diacalone, Besançon, France, and Gen-Probe, San Diego, CA).

Transwell assays

The tumor cells were plated (10⁶ cells/well) in 24-well plates. Untreated or LPS-activated DCs (5 × 10⁶) were deposited in the inner chamber of a Transwell membrane (0.45 μm pore size) (Dutscher, Brumath, France). Cytotoxic assays were performed after 48 h.

Cytotoxic assays

DCs and target tumor cells were cocultured for 48 h. The number of residual adherent cells was then evaluated by crystal violet staining as previously described (50). Data are presented as the percentage of relative absorbance calculated from the formula Atest/Acontrol, where Atest is the absorbance of tumor cells cultured with DCs in the different conditions, and Acontrol is the absorbance of tumor cells cultured alone.

Measurement of reactive oxygen and nitrogen species production by human DCs

The production of reactive oxygen and nitrogen species by cultivated human DCs was evaluated with electron spin resonance (ESR) spectroscopy using 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine (CMH; Noxygen, Elzach, Germany) as the spin probe. CMH is a cell membrane-permeable hydroxylamine that can be oxidized by superoxide anions, peroxynitrates, and transition metals into a paramagnetic nitroxide (CP), which forms a characteristic triplet electron paramagnetic resonance signal with a half-life of several hours (51-53). Briefly, activated and nonactivated human DCs (1 × 10⁶) were incubated for 30 min at 37 °C in a 5% CO₂ atmosphere with 10⁻⁴ mol/l CMH in 200 μl (pH 7.4) ESR Krebs-HEPES buffer containing 5 × 10⁻⁴ mol/l diethyldithiocarbamate and 2.5 × 10⁻⁵ mol/l desferoxamine as transition metal chelators, as described elsewhere (53). After incubation, the cells and medium were immediately frozen in liquid nitrogen in polyethylene tubes and kept in liquid nitrogen until measurement. ESR spectra were recorded at 100 K using a Bruker EMX-100 spectrometer (Bruker, Wissembourg, France). ESR spectra were obtained using the following instrument settings: center field, 3330.2 g-factor; sweep width, 100 g-factor; microwave frequency, 9.378 GHz; microwave power, 20 mW; modulation amplitude, 5 g-factor; conversion time, 40.96 ms; time constant, 327.68 ms; receiver gain, 1 × 10³. The amplitude of center field anisotropic signal was measured (arbitrary units) to determine CP nitroxide formation in activated or nonactivated human DCs as well as in buffer alone.

Annexin V propidium iodide

The percentage of apoptotic cells and necrotic cells was determined by using an FITC-conjugated Annexin V Apoptosis Detection Kit I according to the manufacturer’s recommendations (BD Biosciences).

T lymphocyte proliferation

T lymphocytes were obtained from buffy coats by using a Pan T Cell Isolation Kit (Miltenyi Biotec). T cells were then stained using a CellTrace Violet cell proliferation kit according to the manufacturer’s procedure (Innovernig, Carlsbad, CA). Labeled cells (1 × 10⁶ cells/well) were cocultured with DCs (1 × 10⁵) and cell division was detected by flow cytometry and analyzed by FlowJo software after 5 d, as indicated by the manufacturer (Verity Software House, Topsham, ME).

Statistical analyses

Unless specified, all experiments were reproduced at least three times and performed on samples from at least three different patients or healthy donors. Paired t tests were used to compare the different groups. When more than two groups were compared at the same time, we used repeated measures ANOVA tests. Results were considered statistically significant for p < 0.05. Analysis was performed with GraphPad Prism.

Results

LPS induces human monocyte-derived DC cytotoxic activity

We have previously reported that in rats or mice, DC killing activity can be triggered by the TLR4 ligand LPS (9, 19). To evaluate whether similar activation conditions may induce the cytotoxic potential of ex vivo-generated human monocyte-derived DCs, cells were exposed on day 5 to the indicated TLR ligands. Our results indicate that LPS was the only TLR agonist capable of triggering DC killing activity (Fig. 1A). LPS-activated day 5 DCs were able to kill several human tumor cell lines (HT29, MCF7, HeLa, HCT116, SW480) and some nonmalignant cells (Fig. 1B). Importantly, the viability of total T lymphocytes was minimally affected after culture with LPS-activated day 5 DCs (Sup-
Cells obtained after 3 d culture (early in the differentiation process toward DCs) did not exhibit significant tumoricidal activity after LPS activation (Supplemental Fig. 1C). Interestingly, even low concentrations of LPS were sufficient to trigger the cytotoxic function of DCs with a minimum optimal concentration between 0.01 and 0.1 μg/ml (Fig. 1C). A direct cytotoxic effect of LPS on tumor cells was excluded, as the survival of tumor cells was not affected by exposure to the TLR4 agonist for extensive periods of time (Supplemental Fig. 2A). Moreover, the pretreatment of tumor cells with LPS did not sensitize them to DC-mediated cytotoxicity (Supplemental Fig. 2B).

The maturation state of cytotoxic DCs remains a subject of controversy. To address this issue, we investigated the cytotoxic potential of day 5 DCs and day 7 DCs that had been matured from day 5 to day 7 with different reagents such as LPS, IFN-γ, TNF-α plus poly(I:C), or IL-1β plus IL-6 plus TNF-α plus PGE2 (Supplemental Fig. 3A). These day 7 DCs were activated with LPS and cultured for 48 h with tumor cells, and then survival of the tumor cells was assessed. The data presented in Fig. 1E indicate that LPS failed to trigger the killing activity of day 7 mature DCs regardless of the maturation mixture used to induce DC maturation (Fig. 1E). Only immature day 5 DCs were capable of triggering tumor cell death after activation with LPS (Fig. 1E). We therefore focused our investigation on the killing properties of LPS-activated day 5 monocyte-derived DCs (hereafter referred to as hKDCs) from healthy donors and from cancer patients.

hKDCs generated from cancer patient blood monocytes exhibit cytotoxic activity against tumor cells

The inhibition of DCs by tumors and their maintenance at a non-functional stage are two of the numerous mechanisms used by cancer cells to escape the antitumor response. These “tolerogenic” DCs, typically characterized by an immature or semi-mature phenotype, are capable of anergizing effector T lymphocytes and/or driving the differentiation of immunosuppressive Foxp3+ regulatory T cells (54, 55). It was therefore fundamental and clinically relevant to determine whether the tumoricidal activity of
DCs generated from cancer patients was impaired. Monocyte-derived DCs generated from 10 patients with stage IV cancers (lung and colon cancers or lymphomas) were characterized by the same immature phenotype as DCs from healthy volunteers (Supplemental Fig. 3Aa, 3Ab). Upon LPS stimulation, DCs generated from cancer patients or healthy donors exhibited a similar mature phenotype and functional properties (Supplemental Fig. 3Ac–f, Supplemental Fig. 3B) and significant cytotoxic activity against tumor cells (40–80%) when activated with LPS (Fig. 2A). These results therefore indicate that the tumor killing ability of DCs generated from individuals with stage IV cancer is similar to that in DCs from healthy volunteers (Fig. 2B).

**hKDC cytotoxic activity depends on peroxynitrite**

We next investigated the mechanism underlying hKDC cytotoxicity. We first determined that the tumoricidal activity of hKDCs was dependent on direct cell–cell contact, as separation from their targets by a microporous membrane prevented killing of cancer cells (Fig. 3A). Death receptor ligands such as TRAIL, or FasL have been reported to play a role in the cytotoxic activity of DCs (26, 32, 35, 36, 38, 43–46, 48, 49). We therefore evaluated whether the tumor killing activity of hKDCs was dependent on similar processes. Our results indicate that hKDCs were capable of eliminating tumor cell lines that are resistant to TNF, TRAIL, or FasL (data not shown). Consistent with these results, LPS-activated day 5 DCs did not secrete TRAIL or FasL (data not shown). In line with this result, annexin V and propidium iodide staining indicated that hKDCs induced the necrosis of tumor cells as evidenced by early plasma membrane disruption (Fig. 3B). Consistent with these results, z-VAD-fmk, a broad-spectrum caspase inhibitor, failed at inhibiting hKDC-induced tumor cell death, thus excluding a conventional apoptotic-related process (Fig. 3C).

We and others have previously reported that NO or peroxynitrates represent critical effector molecules required for the tumoricidal activity of both rat and mouse DCs (9, 12, 19, 56). To define whether these cytotoxic products may also play a role in tumor cell killing by hKDCs, we analyzed reactive oxygen species production by these killer cells using ESR spectroscopy. ESR spectroscopy is one of the few techniques that allows the direct measurement of free radical species. However, most biological reactive oxygen and nitrogen species of interest are too short-lived to be measured directly with ESR spectroscopy. Hydroxylamine spin probes such as CMH can be oxidized by superoxide anion or peroxynitrates, giving rise to a more stable long-lived ESR-detectable CP• nitroxide. This allows the detection of these unstable species in cell cultures or isolated organs (57, 58). Our results indicate that both immature (day 5) and mature (day 7) DCs oxidize CMH into CP• nitroxide. However, after 6 h stimulation with LPS, immature day 5 DCs produced a significantly increased reactive oxygen and nitrogen species quantity, a characteristic that was lost in mature day 7 DCs (Fig. 3D). Moreover, a peroxynitrite inhibitor, FeTPPS, significantly abrogated the cytotoxic activity of hKDCs against several human tumor cell lines (Fig. 3E). We also confirmed that the cytotoxic activity of monocyte-derived DCs generated from cancer patients was inhibited by FeTPPS (Fig. 3F). These results therefore strongly suggest that hKDCs generated either from healthy donors or from cancer patients kill tumor cells by a mechanism involving peroxynitrite production.

**hKDCs are capable of capturing tumor cell debris and of activating tumor-specific T cells**

Whether hKDCs that had killed cancer cells are also capable of presenting tumor Ags and activating specific lymphocytes has remained controversial. A prerequisite for tumor Ag presentation to T cells is their uptake and processing by DCs. Using GFP-transfected HT29 tumor cells, we demonstrated that hKDCs were able to engulf tumor fragments from the tumor cells they had killed (Fig. 4A). Additionally, after tumor cell killing in presence of LPS, hKDCs (generated from healthy donors or cancer patients) exhibited a phenotype consistent with that of mature DCs (Fig. 4B). Following killing and the uptake of tumor cell fragments, these hKDCs retained their functional properties, as they were capable of inducing the proliferation of autologous T lymphocytes (Fig. 4C). Moreover, using the human melanoma cell line T1, we demonstrated that after killing cancer cells, KDCs were capable of inducing the activation of the tumor Ag (Melan- A25–36)-specific human CD8+ T cell clone (Fig. 4D). These results thus indicate that hKDCs generated ex vivo from healthy volunteers or cancer patients that had killed tumor cells are efficient at presenting tumor Ag from the cancer cells they have killed.

**Discussion**

We have previously reported that KDCs can be generated from rat (19) and mouse bone marrow (9). In the present study we provide evidence that human peripheral blood monocytes from both healthy individuals and, more clinically relevant, from patients with advanced cancer can also be driven to differentiate into potent killer DCs. The killing mechanism mediated by these hKDCs involves the production of peroxynitrates. After killing cancer cells, hKDCs are capable of capturing necrotic tumor cells and of differentiating into activated and fully functional APCs that produce IL-12, express high levels of HLA-DR and costimulatory molecules, and induce T cell proliferation. Moreover, these ex
vivo-generated hKDCs are capable of activating tumor Ag-specific CTLs, which strongly advocates for their therapeutic use in cancer patients. To facilitate the clinical application of hKDCs, we also demonstrated that KDCs could be generated in serum-free medium (Supplemental Fig. 4).

The cytotoxic activity of human immature peripheral blood monocyte-derived DCs has already been reported (27, 35, 37, 45, 46), but no or only a low killing activity at an E:T ratio of 5:1 was observed. Additionally, in these studies, LPS was used at high concentrations, ranging from 5 (27, 43) to 10 μg/ml (37). In turn, the use of LPS at high concentrations may not be suitable for clinical applications due to its potential side effects. To overcome this challenge, we explored the use of alternative stimuli for DC generation and activation.

**FIGURE 3.** Human monocyte-derived DC killing activity depends on peroxynitrites. A, HT29 cells were cultured alone (Medium) or with monocyte-derived DCs from healthy donors in the presence (DC + LPS) or not of LPS (DC). The cells were cultured together (Control) or separated by a Transwell insert (Transwell). Tumor cell killing was determined after 48 h. B, HT29 tumor cells were cultured alone (Medium), with LPS, with day 5 monocyte-derived DCs from healthy donors with (DC + LPS), or without LPS (DC). After 48 h, the cells were stained with CD1a Ab and with annexin V-FITC/propidium iodide (PI). The percentage of PI−annexin V+ (apoptotic) or PI+annexin V+ (necrotic) tumor cells was determined after gating on CD1a-negative HT29 tumor cells. C, HT29 tumor cells were cultured alone (Medium), with LPS (100 ng/ml) (LPS), with day 5 monocyte-derived DCs (DC), with DCs in the presence of LPS (DC + LPS), or with DCs in the presence of LPS plus z-VAD-fmk (40 μM) (DC/tumor cell ratio of 5:1). Tumor cell survival was determined after 48 h. D, Production of reactive oxygen species by nonactivated or LPS-activated human monocyte-derived DCs was determined by ESR. CMH was used as the spin probe. Day 5 monocyte-derived DCs from healthy donors were cultured with (DC + LPS) or without LPS (DC) for 6 h. Activated and nonactivated human DCs (2 × 10⁶) were harvested and incubated with 1 mmol/l CMH. The amplitude of center field anisotropic signal was quantified in arbitrary units to determine CP• nitroxide formation in LPS-activated or nonactivated human DCs at days 5 and 7. This experiment was done three times with DCs from three different healthy donors and from three cancer patients. *p < 0.05, **p < 0.005. E, HT29 tumor cells were cultured alone (Control) or with day 5 monocyte-derived DCs from healthy donors (DC): without LPS (Medium), with LPS, or with LPS and FeTPPS (50 μM) (LPS + FeTPPS). Tumor cell viability was determined after 48 h. F, Different human tumor cell lines (HT29, HeLa, HCT116, SW480) were cocultured with LPS-activated human monocyte-derived DCs from healthy volunteers or from cancer patients in the presence (DC + LPS + FeTPPS) or absence (DC + LPS) of FeTPPS (50 μM) (DC/tumor cell ratio of 5:1). Tumor cell viability was determined after 48 h. Similar results were obtained with DCs generated from six healthy donors and four cancer patients. *p < 0.05 when compared with tumor cells cultured with activated DCs in the absence of FeTPPS.
contrast to these high concentrations, we now report that LPS induces hKDC killing activity even at doses as low as 10 ng/ml. The mechanisms underlying KDC-mediated cytotoxic function have been related to death receptors and their ligands (25–27, 29, 32–36, 42, 44, 46, 47). Our results indicate that hKDCs induce tumor cell necrosis by a process that is independent of the above-mentioned mechanisms of cytotoxicity. By using the ESR technique and specific inhibitors, we identified peroxynitrites as the primary molecule responsible for KDC tumoricidal function. The inhibition of KDC killing activity by the peroxynitrite catalyst FeTPPS (59–63) confirmed the primary role of these molecules.

Importantly, although the mechanism of cytotoxicity used by these hKDCs is not specific to tumor cells and may affect normal cell survival, we confirmed that no significant killing of T lymphocytes was detectable in the presence of hKDCs. This can be explained by the fact that T lymphocyte killing might be a minor phenomenon compared with the high proliferation rate of T cells induced by these DCs. Another explanation may stem from the fact that the high levels of proinflammatory cytokines produced by hKDCs can support T cell survival by activating anti-apoptotic mechanisms that render T lymphocytes resistant to DC-mediated cytotoxicity. In support of this hypothesis, recent studies have reported on cytokine driven upregulation of Bcl2 or related anti-apoptotic molecules in subsets of effector and memory T cells, allowing them to survive (64–66).

Whether KDCs can be generated from cancer patients has not been previously elucidated. This is a relevant question insofar as the tumor microenvironment is able to suppress many aspects of the anti-cancer immune response to escape from recognition by the immune system (67, 68). One of the mechanisms of tumor-induced immunosuppression consists in inhibiting the ability of DC precursors to differentiate into functional cells (5, 68, 69).
therefore reasoned that the tumor killing activity of DCs generated from cancer patient monocytes may be impaired. Our results indicate that KDCs generated from all the cancer patients evaluated in this study were able to kill tumor cells, indicating that this cytotoxic potential was not inhibited by the tumor environment. Moreover, whatever the type of cancer, hematological or solid tumors, hKDCs from cancer patients were as cytotoxic as those from healthy donors. This is a clinically important finding that opens new perspectives for the use of autologous hKDCs in cancer patients. The use of hKDCs could circumvent extensive ex vivo manipulations during DC vaccine protocols, such as tumor Ag preparation and loading, and DC activation. Autologous DCs from cancer patients may be differentiated into hKDCs and activated ex vivo and then used to kill autologous tumor cells and to acquire and process released tumor Ag in vitro before being reinjected into the patient. These novel findings may be relevant for the design of new improved DC-based vaccines.

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Disclosures
The authors have no financial conflicts of interest.

References


