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A New Look at Immune Privilege of the Eye: Dual Role for the Vision-Related Molecule Retinoic Acid

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The eye is an immunologically privileged and profoundly immunosuppressive environment. Early studies reported inhibition of T cell proliferation, IFN-γ production, and generation of regulatory T cells (Tregs) by aqueous humor (AH) and identified TGF-β as a critical factor. However, T cell subsets including Foxp3+ Treg and Th17 were unknown at that time, as was the role of retinoic acid (RA) in Treg induction. Consequently, the effect of the ocular microenvironment on T cell lineage commitment and function, and the role of RA in this process, had not been explored. We now use gene-manipulated mice and highly purified T cell populations to demonstrate that AH suppresses lineage commitment and acquisition of Th1 and Th17 effector function of naive T cells, manifested as reduction of lineage-specific transcription factors and cytokines. Instead, AH promoted its massive conversion to Foxp3+ Tregs that expressed CD25, GITR, CTLA-4, and CD103 and were functionally suppressive. TGF-β and RA were both needed and synergized for Treg conversion by AH, with TGF-β–enhancing T cell expression of RA receptor α. Newly converted Foxp3+ Tregs were unstable, but were stabilized upon continued exposure to AH or by the DNA demethylating agent 5-aza-2’-deoxycytidine. In contrast, T cells already committed to effector function were resistant to the suppressive and Treg-inducing effects of AH. We conclude that RA in the eye plays a dual role: in vision and in immune privilege. Nevertheless, primed effector T cells are relatively insensitive to AH, helping to explain their ability to induce uveitis despite an inhibitory ocular microenvironment. The Journal of Immunology, 2011, 187: 000–000.

Vision is without a doubt the single most important sense that we possess and the one most affecting survival ability. The process of inflammation, although important to eradicate infectious agents, can cause significant collateral damage to the tissue. Because even small perturbation of the integrity of the light-sensing structures can have very deleterious consequences to vision, the eye resists inflammatory processes, a phenomenon known as immune privilege of the eye. These include sequestration of retinal Ags behind an efficient blood–retina barrier (BRB), absence of lymphatic drainage of the interior of the intact globe (although once the BRB is breached, the eyes are drained by submandibular lymph nodes), a paucity of resident class II+ APC in the healthy retina, and an immunosuppressive ocular microenvironment composed of soluble and cell-bound inhibitory factors. This includes 500–2250 pg/ml TGF-β (mainly as TGF-B2) and immunoinhibitory neuropeptides in ocular fluids, as well as constitutive expression of Fas ligand, PD-L1, galectins, CTLA-2α, and so on, on ocular cells (1–3). Finally, under some circumstances, the eye is able to influence immunity at the systemic level, through anterior chamber-associated immune deviation, and postrecovery tolerance (2, 4). These evolutionary adaptations limit induction and expression of immunity in the eye in the event of influx into the eye of immune-competent cells from the circulation as a result of damage to retinal vasculature as a result of an abnormality or trauma (2). Nevertheless, despite immune privilege, the eye is subject to autoimmune inflammation triggered by retina-specific T cells activated in the periphery by innate or cross-reactive antigenic stimuli (5).

Early studies reported that aqueous humor (AH), through the activity of TGF-β, could inhibit IFN-γ production in culture by T cells obtained from CFA-primed mice and converted them to TGF-β–producing regulatory T cells (Tregs) (6). Additional factors identified in the AH that contribute to its immunosuppressive properties are the neuropeptides α-melanocyte–stimulating hormone, vasoactive intestinal peptide, calcitonin gene-related peptide, and somatostatin (7–10). These early experiments provided important evidence that the ocular fluids could promote local Treg generation, but tools were simply not available at that time to dissect the phenomenon at a mechanistic level made possible by today’s state of knowledge. The studies predated the discovery of Foxp3 as a marker for Tregs. The experiments were done with mixed lymph node cell populations containing newly primed and naive T cells as well as natural Tregs (nTregs) and induced Tregs. Thus, they could not distinguish whether Tregs arose from primed or from naive precursors and could not distinguish proliferation of preexisting Tregs from de novo induction. Retinoic acid (RA) as a Treg inducer had not yet been recognized. RA is highly abundant in the eye because of its role in the visual cycle (25 pmol/ml in human AH) (11), but its role, if any, in immune privilege is unknown. Finally, Th17 cells as pathogenic effectors with a central role in ocular pathology had not yet been discovered, so effects on Th17 induction could not be studied.

In the current study, we fill these critical gaps in knowledge through the use of gene-manipulated mice and highly purified T cell populations. To our knowledge, we demonstrate for the first time that not only Th1 but also Th17 lineage commitment is suppressed by AH, and instead, these cells are shunted to the
Foxp3+ Treg pathway. RA has a critical role in this process, as does TGF-β, which upregulates RA receptor (RAR)α in the differentiating T cells and synergizes with RA. However, T cells that had initially been primed in the absence of AH are relatively resistant to these effects, and if anything, their effector function appears to even be stabilized by exposure to AH. This may explain why uveitogenic T cells, after being activated outside the eye and acquiring the ability to cross the BRB, are able to induce uveitis in the face of an inhibitory ocular microenvironment.

Materials and Methods

Mice

C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Foxp3-GFP reporter mice on C57BL/6 background were a gift from Drs. V.K. Kuchroo and M. Oukka (Brigham and Women’s Hospital, Cambridge, MA) (12). All mice were housed under specific pathogen-free conditions, fed standard laboratory chow ad libitum, and used at 6–10 wk of age. Treatment of animals was in compliance with Institutional Guidelines, and all animal study protocols were approved by the National Eye Institute Animal Care and Use Committee.

Cytokines and Abs

Recombinant mouse IL-6 and human TGF-β1 were from R&D Systems (Minneapolis, MN); recombinant human IL-2 and mouse IL-12 were from PeproTech (Rocky Hill, NJ); anti–IFN-γ (clone R4-6A2) was from Bio-XCell (West Lebanon, NH); and anti–IL-4 (11B11) was from National Cancer Institute-Frederick Biological Resources Branch Preclinical Repository (Frederick, MD). Abs used for flow cytometry analysis were all from eBioscience (San Diego, CA), labeled with fluorochromes as specified. All primary Abs for Western blotting were from Cell Signaling Technology (Danvers, MA).

Aqueous humor preparation

Fresh bovine eyes were purchased from J.W. Treuth and Sons (Baltimore, MD). AH was aspirated by syringe using 25-gauge needle. After being sterilized by filtration, AH was stored at −20°C. For thymidine uptake, CD4+ T cells were stimulated with anti-CD3/CD28 in medium containing 10 ng/ml recombinant human IL-2 in presence or absence of AH for 72 h. The incorporated radioactivity was measured by a MicroBeta TriLux scintillation counter (PerkinElmer Life Sciences, Waltham, MA). Repeated cultures were performed as described previously (13). After 4–5 h, cells were fixed with 4% paraformaldehyde, permeabilized with PBS containing 0.1% BSA and 0.05% Triton X-100, and stained with PE-conjugated anti-CD4, anti-CD8, anti–Foxp3, or anti–IFN-γ, and allophycocyanin-conjugated anti–IL-17. Up to 100,000 events were acquired using BD CellQuest software and analyzed using FlowJo software (Tree Star, Ashland, OR).

RNA isolation and real-time PCR

Total RNA from cells or tissues was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA), and cDNA was synthesized using SuperScript III First Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed with a TaqMan 7500 sequence detection system (Applied Biosystems, Foster City, CA) using endogenous control for 18S rRNA or GAPDH and primer/probe sets from Applied Biosystems. Data were normalized to 18S rRNA or GAPDH and expressed relative to Th0 or spleen control (set as 1).

Western blotting

After cell polarization for 12 or 48 h, whole-cell lysates were prepared in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100, supplemented with a protease inhibitor mix). Lysates were centrifuged at 14,000 rpm for 5 min at 4°C, supernatants were collected, and protein concentration was measured. A total of ∼5 μg protein was resolved on a 10% SDS-PAGE gel (Invitrogen Life Technologies) and transferred to a nitrocellulose membrane (Whatman, Sanford, ME) using a semidry transfer cell apparatus (Bio-Rad, Hercules, CA). The membranes were treated with 5% nonfat milk for 2 h to block nonspecific binding, rinsed, and incubated with anti–phospho-STAT3 (Tyr705, p-STAT3), anti-STAT3, anti–phospho-STAT1 (Tyr701, p-STAT1), or anti-STAT1. Signals were detected with HRP-conjugated anti-rabbit IgG using the ECL system (Amersham Biosciences, Piscataway, NJ).

Statistics and experimental reproducibility

Values are presented as mean ± SD where indicated. Statistical differences were calculated with an unpaired Student t test, two-tailed (GraphPad Prism version 5.0b). Statistical significance was set at p ≤ 0.05. Experiments were repeated at least three, and usually more, times, with number of repetitions stated in the figure legends. Results were highly reproducible.

Results

Aqueous humor favors Foxp3+ Treg induction at the expense of Th17 and Th1 differentiation

Although lymphocytes are mostly excluded from the healthy eye, bleeding into the eye can occur as a result of a minor trauma or a vascular abnormality. T cells that find themselves in the eye would...
immediately be bathed in ocular fluids. The composition of ocular fluids is evolutionarily conserved among species (1, 14–16). Therefore, we used bovine AH, transiently acidified to activate TGF-β and highly purified CD4+Foxp3+ T cells stimulated with anti-CD3/CD28, to examine direct effects of ocular fluid on T cell responses. Rabbit and rat AH had similar activity (data not shown).

T cell proliferation and acquisition of effector function were markedly inhibited by AH. Comparison of proliferation dye eFluor 670 dilution with [³H]thymidine uptake (Fig. 1A, 1B) suggested that proliferation in presence of AH was induced, but ceased early, because despite the cells having undergone several divisions, there was little uptake of [³H]thymidine, which was added on the third day for the last 6 h of culture.

Acquisition of Th17 and Th1 effector function (measured as secretion of IL-17 and IFN-γ) was dramatically inhibited by AH (Fig. 2A, 2E, respectively). Examination of Th17 and Th1 lineage-specific gene expression revealed that the respective effector programs were largely shut down. Message levels for IL-17A, IL-21, IL-22, IL-23R, and the Th17-associated transcription factor retinoic acid receptor-related orphan receptor γt (RORγt) were all reduced (Fig. 2D). Interestingly, although IL-6–induced STAT3 plays a key role in Th17 development, IL-6Rx expression, phosphorylation of STAT3, and its total protein level were not affected (Fig. 2C), suggesting that the effect of AH was downstream of STAT3 induction. Under Th1 conditions IFN-γ, IL-2, and the Th1 transcription factor T-bet were all reduced, as was the phosphorylation of STAT1 (Fig. 2G, 2H). Instead, AH promoted massive Foxp3 expression, with 56% of the cells converting to Foxp3 positivity was even higher (∼90% in rabbit AH) (1). It was not well established that in vitro, TGF-β converts conventional T cells into Foxp3+ Tregs and that RA strongly enhances this process (17–23). To address the respective roles of RA and TGF-β in AH-induced Foxp3 expression, RAR antagonist LE540 and neutralizing Abs to TGF-β2 were added to CD4+ T cells undergoing TCR stimulation in the presence of AH. Induction of Foxp3 by AH was reduced 4-fold by LE540 (from 79 to 19.3%) despite presence of TGF-β (Fig. 4A). Similarly, there was a 6-fold reduction of Foxp3 expression in the presence of anti–TGF-β2–neutralizing Ab (from 79 to 12.5%), even though RA was present. A combination of LE540 with anti–TGF-β2 had a synergistic inhibitory effect.

Neutralization of all TGF-β isoforms completely prevented induction of Foxp3 (data not shown).

Because the complex effects of RA on T cells are at least in part mediated by the nuclear RARs (24, 25), we examined the effect of AH on the expression of this receptor. There was a marked increase of RARs mRNA expression in cells activated in the presence of AH, compared with control. Notably, this induction was blocked by neutralization of TGF-β (Fig. 4B), suggesting that TGF-β contributes to the effects of AH at least in part by up-regulating RARα.

Proliferation accompanied acquisition of Foxp3 expression but was not a prerequisite for conversion and Foxp3 expression was also induced on nondivided cells (Fig. 4C). Compared with control cells stimulated in the absence of AH, the cells stimulated in the presence of AH appeared to undergo fewer proliferations cycles, in agreement with data shown in Fig. 1. Induction of activation markers shared by effector and by Tregs, including CD25, GITR (Fig. 5), as well as of CD44 and CTLA-4 (data not shown), was

Induction of Foxp3 and Treg-specific markers by AH is dependent on RA and requires TGF-β to upregulate RARα

In addition to TGF-β, ocular fluids contain high levels of RA, which functions in the chemistry of the visual cycle (25 pg/ml in human AH) (11). Most of the TGF-β in ocular fluids is in the form of TGF-β2 (∼90% in rabbit AH) (1). It is now well established that in vitro, TGF-β converts conventional T cells into Foxp3+ Tregs and that RA strongly enhances this process (17–23). To address the respective roles of RA and TGF-β in AH-induced Foxp3 expression, RAR antagonist LE540 and neutralizing Abs to TGF-β2 were added to CD4+ T cells undergoing TCR stimulation in the presence of AH. Induction of Foxp3 by AH was reduced 4-fold by LE540 (from 79 to 19.3%) despite presence of TGF-β (Fig. 4A). Similarly, there was a 6-fold reduction of Foxp3 expression in the presence of anti–TGF-β2–neutralizing Ab (from 79 to 12.5%), even though RA was present. A combination of LE540 with anti–TGF-β2 had a synergistic inhibitory effect.

Neutralization of all TGF-β isoforms completely prevented induction of Foxp3 (data not shown).
unaffected by presence of AH. Expression shifted from Foxp3\(^{-}\) to Foxp3\(^{+}\) population and back to the Foxp3\(^{+}\) population upon blockade of RA and/or TGF-\(\beta\) signaling, but levels of expression remained largely unaltered. AH promoted the induction of CD103 on Foxp3\(^{+}\) cells. Presence of LE540 or anti–TGF-\(\beta\) Ab significantly reduced or completely abrogated CD103 induction, respectively (Fig. 5).

AH-induced Foxp3 expression is initially unstable but is stabilized by DNA demethylation or by continued exposure to AH

In contrast to natural Tregs, induced Tregs were reported to be unstable and to lose Foxp3 expression (26). Therefore, we examined the stability of the AH-induced Tregs. Toward that end, CD4\(^{+}\) T cells were converted to Foxp3\(^{+}\) Tregs by AH (Fig. 6A, left panel). Foxp3\(^{+}\) T cells were subsequently isolated, and upon restimulation in medium alone, only 34% of the cells retained Foxp3 expression. However, Foxp3 expression continued to be stable (99.1%) in the presence of AH. Foxp3 expression could also be stabilized in the presence of the DNA demethylating agent Aza, which interferes with DNA methylation by inhibiting the function of DNA methyltransferase-1 (27, 28). The presence of Aza largely prevented the loss of Foxp3 expression (Fig. 6A), supporting the notion that the instability of Foxp3 expression was due to incomplete demethylation of the Foxp3 locus after a single exposure to AH.

To examine how long Tregs would need to remain in the presence of AH for Foxp3 expression to fully stabilize, Foxp3\(^{+}\) T cells were isolated after one and two rounds of culture with AH, and each time Foxp3\(^{+}\) T cells were tested for stability by restimulation without AH. Foxp3 expression became progressively more stable, and after three rounds of stimulation with AH, Foxp3 expression was retained during the fourth round of stimulation in absence of AH (Fig. 6B).

Unlike naive cells, effector and memory T cells are relatively resistant to conversion by AH

The data described above are compatible with the interpretation that a T cell that enters the eye and sees its cognate Ag there will be induced to adopt a Treg rather than a T effector fate. If so, the question immediately arises, how can uveitis develop? Because uveitogenic T cells are primed in the periphery before they encounter the ocular microenvironment, we decided to examine the effect of ocular fluid on already activated T cells.

To examine the effect on activated effector T cells, polyclonal CD4\(^{+}\) T cells were polarized under neutral (Th0), Th17, or Th1...
conditions in the absence of AH. The cells were rested and were exposed to AH during the second stimulation (Fig. 7A). Irrespective of the polarization conditions in primary culture, production of the respective effector cytokine was reduced under nonpolarizing conditions in secondary control cultures. Strikingly, addition of AH to the secondary culture appeared to stabilize effector cytokine production, despite inducing measurable Foxp3 expression in a part of the population, in sharp contrast to the profoundly inhibitory effect of AH on effector cytokine production in naive T cells (compare with Fig. 2B, 2F).

Stabilization of effector function was not observed in control cultures with a mix of TGF-β plus RA in place of AH (data not shown), underscoring the much more complex effects of ocular fluids.

To examine the effect of AH on memory T cells, we used the criterion of high (memory) versus low (naive) CD44 expression to isolate polyclonal memory and naive CD4 T cells from conventional Foxp3-GFP reporter mice. Both T cell populations were exposed to stimulation with CD3/CD28 in presence or absence of AH. Whereas the naive population efficiently acquired Foxp3 expression, only one-third of the memory population was converted (Fig. 7A). Notably, however, memory cells were less resistant to conversion than the more recently activated effector T cells (compare with Fig. 7A).

That these pathways may be relevant to ocular immune privilege and to uveitis in vivo is suggested by preliminary data that retina-specific T cells injected into the healthy eye behave similarly to T cells stimulated in vitro in presence of AH (R. Zhou, R. Horai, P.B. Silver, M.J. Mattapallil, C.R. Zarate-Blades, W.P. Chong, J. Chen, R. Villasmil, and R.R. Caspi, submitted for publication).

Taken together, these data suggest that effector T cells, and to a somewhat lesser extent memory T cells, are relatively resistant to the immunosuppressive and Treg-inducing effects of the ocular fluids.
microenvironment. This can help to explain the ability of autoaggressive T cells that had been primed outside the eye to induce uveitis, as well as the chronicity of uveitis fueled by autoreactive memory T cells, despite ocular immune privilege.

Discussion

The present study uses highly purified T cells, Foxp3-GFP reporter mice, and analyses at the cellular and molecular level to reexamine the paradigm of local immune privilege in the eye. Our current data considerably extend what has been known about the suppressive ability of ocular fluids and provide new information on the likely fate of a T cell that enters the eye and undergoes TCR ligation in the ocular environment. The entire differentiation program for Th1 as well as for Th17 was shut down and diverted toward de novo Foxp3+ Treg induction. Interestingly, although phosphorylation of STAT1 and its target, the Th1 lineage-specific transcription factor T-bet, were both inhibited, phosphorylation of STAT3, which is triggered by IL-6R ligation and induces the Th17 lineage-specific transcription factor RORγt (29), was not affected, and neither was expression of IL-6Rα. This suggests that inhibition of RORγt by AH was not through the IL-6–induced STAT3 pathway. Because Foxp3 was shown to bind to RORγt (30), high expression of Foxp3 induced by AH could have directly repressed RORγt activity, leading to the inhibition of Th17 responses. Our data also uncover a previously unappreciated role for RA, which functions in the visual cycle, in Treg induction by the eye. Although the effects of RA produced by dendritic cells (DCs) on Treg induction has been well described in organs such as the gut (18–23), the case of the eye is a bit different, because RA is free in ocular fluids. In the current study, we are reporting direct effects of RA on T cells, in the absence of DCs and any modifying effects of RA on DCs. The RAR pathway may be important in EAU, as systemic treatment of mice with RA ameliorated induction of disease and its associated immunological responses (Supplemental Fig. 2), and similar data were published by others (31, 32). We show that the massive switch in the AH-treated T cells from Th1 or Th17 programs to Foxp3+ Tregs required RA and was dependent on TGF-β–driven upregulation RARα. Proliferation accompanied acquisition of Foxp3 expression but was not necessary for conversion and was curtailed after a few proliferation cycles. Expression of Foxp3 was initially unstable (apparently because of incomplete DNA methylation, as it could be maintained by 5-Aza) and

FIGURE 5. Expression of selected cell surface markers in AH-induced Tregs. CD4+ T cells from Foxp3-GFP reporter mice were activated with anti-CD3/CD28 in the presence of IL-2, and either LE540 and/or anti–TGF-β2. After 2 h, AH was added where specified and the culture was continued for 72 h. The expression of Foxp3, CD25, GITR, and CD103 in CD4+ T cells was detected by flow cytometry. All assays were done under serum-free conditions to avoid possible contribution of RA and TGF-β from serum. Data are representative of four repeat experiments.

FIGURE 6. Stability of AH-induced Foxp3 expression. A, Foxp3 expression is initially unstable but can be stabilized by DNA demethylation or continued presence of AH. CD4+ T cells from C57BL/6 Foxp3-GFP reporter mice were activated with anti-CD3/CD28 in the presence of AH for 72 h (first culture). GFP+ cells from first culture were restimulated for another 72 h in the absence or presence of AH or Aza (second culture). B, Foxp3 expression stabilizes after three cycles of stimulation in AH. CD4+ T cells were restimulated for another 72 h in the absence or presence of AH or Aza (second culture). 5-Aza, Foxp3 expression stabilized after three cycles of stimulation in AH. CD4+ T cells were restimulated for another 72 h in the absence or presence of AH or Aza (second culture). B, Foxp3 expression stabilized after three cycles of stimulation in AH. CD4+ T cells were restimulated for another 72 h in the absence or presence of AH or Aza (second culture).
required continued presence of AH through several cycles of TCR ligation to stabilize.

A question that our in vitro study is not able to address is the identity of cells that might present Ag within the eye to support this differentiation process. The healthy eye has few mature APC (33). However, in a trauma situation, local APC could become activated, and/or cells from the circulation with Ag-presenting capability could enter the eye. An analogous situation on a more local scale with subsequent amplification might occur after entry of activated uveitogenic T cells. In support of this, indirect evidence suggests that Ag recognition in the eye does occur in that situation and is necessary for autoimmune uveitis to develop (34, 35).

Importantly, our data demonstrate a very different susceptibility to the inhibitory and Treg-inducing effects of the ocular fluids of naive as compared with effector T cells, supporting the conclusion that T cells, which had been exposed to Ag shortly before entering the eye, resist inhibition by ocular fluids. These findings can help to clarify the apparent contradiction that although immune privilege is effective in protecting the eye from the consequences of minor insults and traumas and critically contributes to the success of corneal grafts that enjoy close to 90% success at the 1-y mark (2, 36), immune privilege is nevertheless unable to prevent an autoimmune attack on the eye. In trauma and first-time corneal transplantation, the T cells with which the eye must deal are largely naive and thus easily converted. In contrast, autoimmune uveitis is elicited by activated autoreactive T cells that had encountered Ag outside the eye and acquired the ability to penetrate the BRB (37), which are resistant to conversion. Rather, some aspects of ocular immune privilege may even predispose to uveitis, which then paradoxically becomes the “price of privilege”: first, because sequestration of retinal Ags behind the BRB impedes peripheral tolerance (38), and second, by “stabilizing” rather than inhibiting the effector function of recently activated effector T cells (present data; Fig. 7A). The relative resistance of in vivo-generated memory cells to conversion might help to explain the chronic-relapsing nature of human uveitis in the face of ocular immune privilege, which is not abolished by ocular inflammation (39). Still, memory cells clearly are less resistant to conversion than recently primed effector cells, whose transcriptome and metabolome are in a highly activated state. It is therefore conceivable that, both in uveitis and in high-risk corneal transplantation, where memory cells might represent at least part of the population recruited to the eye, the ability to convert a proportion of them to Tregs might mitigate an inflammation that would otherwise be even more severe.

Our data do not exclude direct suppressive effects of ocular resident cells, such as ocular pigment epithelia and retinal glial Müller cells, on T cells that might be undergoing activation in the eye in vivo. These resident cells are in fact reported to act on already activated T cells and their function may be even enhanced by the inflammatory process itself (40, 41). However, whether and how often infiltrating T cells might come in direct contact with ocular resident cells, and therefore the extent of their contribution, would be difficult to estimate.

In summary, it is becoming increasingly clear that tissues, among them the eye, are not merely the passive victims of inflammatory and autoimmune attack, but that they can actively regulate and control immune responses taking place in their territory. A thorough understanding of immune privilege mechanisms as well as their limitations might point the way to modulating these processes to help control disease and limit tissue damage.

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Disclosures

The authors have no financial conflicts of interest.