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Leptin Modulates the Survival of Autoreactive CD4+ T Cells through the Nutrient/Energy-Sensing Mammalian Target of Rapamycin Signaling Pathway

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Chronic inflammation can associate with autoreactive immune responses, including CD4+ T cell responses to self-Ags. In this paper, we show that the adipocyte-derived proinflammatory hormone leptin can affect the survival and proliferation of autoreactive CD4+ T cells in experimental autoimmune encephalomyelitis, an animal model of human multiple sclerosis. We found that myelin olygodendrocyte glycoprotein peptide 35–55 (MOG35–55)-specific CD4+ T cells from C57BL/6J wild-type mice could not transfer experimental autoimmune encephalomyelitis into leptin-deficient ob/ob mice. Such a finding was associated with a reduced proliferation of the transferred MOG35–55-reactive CD4+ T cells, which had a reduced degradation of the cyclin-dependent kinase inhibitor p27kip1 and ERK1/2 phosphorylation. The transferred cells displayed reduced Th1/Th17 responses and reduced delayed-type hypersensitivity. Moreover, MOG35–55-reactive CD4+ T cells in ob/ob mice underwent apoptosis that associated with a down-modulation of Bcl-2. Similar results were observed in transgenic AND-TCR- mice carrying a TCR specific for the pigeon cytochrome c 88–104 peptide. These molecular events reveal a reduced activity of the nutrient/energy-sensing AKT/mammalian target of rapamycin pathway, which can be restored in vivo by exogenous leptin replacement. These results may help to explain a link between chronic inflammation and autoimmune T cell reactivity. The Journal of Immunology, 2010, 185: 7474–7479.

Sever al studies have shown that calorie restriction (CR) can modulate metabolic and physiologic changes that can be beneficial in cancer, metabolic syndrome, inflammation, and autoimmunity (1–3). Leptin, an adipocyte-derived hormone of the long-chain helical cytokine family (4, 5), has been proposed to link nutritional status and certain immune responses (6, 7). Leptin concentration is proportional to fat mass and is positively associated with overweight, obesity, and increased susceptibility to chronic inflammation and autoimmunity (6, 7). At the immune level, leptin promotes Th1 proinflammatory responses in autoimmunity (8, 9), although it can also induce the expression of IL-1 receptor antagonist in human monocytes (10) or IL-4 (11) and IL-10 (12) and suppress IFN-γ (12) or IL-6 production caused by endotoxin (13).

Leptin-deficient (ob/ob) mice and leptin receptor-deficient mice (db/db) mice display numerous immune abnormalities (14, 15), including CD4+ T cell lymphopenia, increased numbers of natural regulatory T cells, and a resistance to autoimmune disorders including experimental autoimmune encephalomyelitis (EAE), experimental colitis, Ag-induced arthritis, and type I diabetes (16). The cytokines IL-6, IL-7, IL-15, and IL-21 have all been implicated in the homeostatic control of lymphocyte numbers (17–19), and homeostatic proliferation of regulatory T cells has been observed during T cell lymphopenia. An uncontrolled expansion of autoreactive T cells could favor autoimmunity (20–24), but in ob/ob mice, CD4+ lymphopenia does not associate with homeostatic proliferation of T cells and autoimmunity (14–16).

In this paper, we show that leptin can affect the survival and proliferation of autoreactive T cells by modulating the activity of the survival protein Bcl-2, Th1/Th17 cytokines, and the nutrient/energy-sensing AKT-mammalian target of rapamycin (mTOR) pathway. Findings that link body fat mass and autoactivity also suggest the possibility to regard obesity as a disease with autoimmune components (25–29).

Materials and Methods

Mice

Eight- to 10-wk-old female C57BL/6J (B6), wild-type (WT), and C57BL/6J-ob/ob-leptin–deficient mice were purchased from Charles River Laboratories (Calco, Italy) and from Harlan (Correzzana, Italy). The B10.Cg.Tg (TcraAND)53Hed/J (AND-TCR transgenic [Tg]) pigeon cytochrome c (PCC3Na,10a)–specific transgenic mice were purchased from The Jackson Laboratory (Bar Harbor, ME). B6-WT and ob/ob mice were age-matched for individual experiments and were group-housed in the same cages according to different experimental condition, with a 12-h light-dark cycle. All experiments were performed under approved protocol in accordance with animal use guidelines of the Istituto Superiore di Sanità (Rome, Italy).
CD4+ T lymphocytes. Treated, and T cells to transfer EAE, DTH, and Th1/Th17 immunity in independent experiments with similar results, showing means data refer to 24-h DTH footpad swelling. Data are representative of two independent experiments showing means of similar results (6 means three independent experiments with similar results (n = 6–11/group) of ob/ob-leptin–deficient obese mice and one group (n = 6–10/group) of B6-WT age- and sex-matched control mice. For adaptively induced disease, mice were treated with r-leptin (0.5 μg/g initial body weight twice daily in 200 μl volume i.p.) starting 3 d before the transfer of MOG35–55–specific CD4+ T cells and continuing over a period of 30 d. Of the groups of leptin-deficient mice, one was injected with 200 μl PBS twice daily (at 10:00 AM and 6:00 PM); the second group was injected with murine r-leptin (0.5 μg/g initial body weight twice daily in 200 μl volume i.p.). For the group of B6-WT mice, PBS was injected twice daily according to the same schedule. All mice were weighed, and food intake was recorded daily. Rapamycin was dissolved in DMSO and then brought to volume with PBS. For in vivo treatment with rapamycin (Sigma-Aldrich, St. Louis, MO), B6-WT mice adaptively transferred with CFSE+ MOG35–55-specific CD4+ T cells were quantified at six time points after adoptive transfer and 1 d later. Individual mice were monitored daily for clinical signs of disease for up to 30 d after adoptive transfer, as described previously (30).

Treatment with leptin and rapamycin

Mouse r-leptin was purchased from R&D Systems (Oxon, U.K.); purity was >97%. The endotoxin level was <0.1 ng/μl, as determined by the Limulus amebocyte lysate method. Mice comprised two groups (n = 6–11/group) of ob/ob-leptin–deficient obese mice and one group (n = 6–10/group) of B6-WT age- and sex-matched control mice. For adaptively induced disease, mice were treated with r-leptin (0.5 μg/g initial body weight twice daily in 200 μl volume i.p.) starting 3 d before the transfer of MOG35–55–specific CD4+ T cells and continuing over a period of 30 d. Of the groups of leptin-deficient mice, one was injected with 200 μl PBS twice daily (at 10:00 AM and 6:00 PM); the second group was injected with murine r-leptin (0.5 μg/g initial body weight twice daily in 200 μl volume i.p.). For the group of B6-WT mice, PBS was injected twice daily according to the same schedule. All mice were weighed, and food intake was recorded daily. Rapamycin was dissolved in DMSO and then brought to volume with PBS. For in vivo treatment with rapamycin (Sigma-Aldrich, St. Louis, MO), B6-WT mice adaptively transferred with CFSE+ MOG35–55–specific CD4+ T cells were injected once (day 0) i.p. with 100 μg rapamycin/mouse (final volume, 200 μl). The control group received the same volume of vehicle once. Leptin-treated mice received one single dose of 100 μg r-leptin/mouse once. Mice were sacrificed 7 d posttreatment, and cells were analyzed by flow cytometry.

Delayed-type hypersensitivity

Delayed-type hypersensitivity (DTH) responses to MOG35–55 peptide by adaptively transferred MOG35–55–specific CD4+ T cells were quantitated using a time-dependent footpad swelling assay (32). Footpad thickness was measured at 7 d after transfer and at 12, 24, 48, and 72 h after challenge or at 1, 7, or 14 d by a “blinded” investigator, as described previously (30).

Proliferation assays

Spleen cells were obtained from mice at different time points after adoptive transfer and used for proliferation assays, as described previously (30). For experiments with AND-TCR-Tg mice, DCEK transfectants (murine fibroblasts cells transfected with the E8 mouse MHC class II molecule) (31) were used as APCs to activate in vitro CD4+ AND-TCR-Tg mice T cells. T cells were incubated and harvested as described previously (30).

Cytokine measurement

Leptin, IL-7, IL-15, and IL-21 were measured using ELISA kits from R&D Systems (Minneapolis, MN), BioScientific (Austin, TX), and BioLegend (San Diego, CA), respectively. Measurements were performed according to

Peptides

MOG35–55 peptide (MEVGWYRSPFSRVHLYRNGK) was used for the induction of EAE (30), and PCC88–104 peptide (KAERADLIALKQ-ATAK) was used to stimulate AND-TCR-Tg cells (31).

Induction of adoptive EAE and clinical assessment

The induction of EAE has been described previously (30). Ten female donor B6-WT mice (6–8 wk old) were primed s.c. with 300 μg MOG35–55 peptide in CFA distributed over four sites. Recipient syngeneic naive female ob/ob, PBS or recombinant leptin (r-leptin)–treated, and B6-WT control mice were injected i.v. with 5 × 106 CD4+ T cells in a final volume of 500 μl PBS. Mice received 200 ng pertussis toxin immediately after cell transfer and 1 d later. Individual mice were monitored daily for clinical signs of disease for up to 30 d after adoptive transfer, as described previously (30).

FIGURE 1. Impaired capacity of autoreactive CD4+ MOG35–55–reactive T cells to transfer EAE, DTH, and Th1/Th17 immunity in ob/ob mice. A. Mean clinical score of adaptively induced EAE in B6-WT, ob/ob-PBS–treated, and ob/ob-leptin–treated-treated mice. Only B6-WT and ob/ob-leptin–treated mice groups develop clinical signs of disease, with a similar disease score. Ob/ob mice treated with PBS are resistant to EAE induction when adaptively transferred with 5 × 106 encephalitogenic MOG35–55–specific CD4+ T lymphocytes. Ob/ob-leptin–treated group were injected with r-leptin starting 3 d before transfer until day 25 (1 μg/g body weight). Data are representative of three independent experiments with similar results (n = 5 mice/group). B. Kinetics of DTH responses at day 7 posttransfer in B6-WT, ob/ob-PBS–treated, and ob/ob mice treated with leptin. Seven days after adoptive transfer of MOG35–55–specific CD4+ T cells, mice were challenged with 50 μg MOG35–55 peptide into the footpad, and DTH response was measured in time kinetics (0–72 h); data are representative of three independent experiments with similar results (n = 5 mice/group; means ± SD of footpad-swelling responses); **p < 0.05 of ob/ob PBS versus B6-WT and ob/ob leptin treated. C. DTH response over time at 1, 7, and 14 d after adoptive transfer of MOG35–55–specific CD4+ T cells. Plotted data refer to 24-h DTH footpad swelling. Data are representative of two independent experiments with similar results, showing means ± SD of footpad-swelling responses; *p < 0.01 and **p < 0.05 of ob/ob PBS versus B6-WT and ob/ob leptin treated. D. Dose-dependent MOG35–55-specific in vitro proliferation of splenocytes pulsed with MOG35–55 from the three groups of mice previously transferred with B6-WT MOG35–55–specific CD4+ T cells. Data are representative of two independent experiments showing means ± SD of [3H]thymidine incorporation; *p < 0.01 of ob/ob PBS versus B6-WT and ob/ob leptin treated. E. In vitro cytokine release in supernatants from in vitro proliferation of splenocytes to MOG35–55 from the three groups of mice previously transferred with B6-WT MOG35–55–specific CD4+ T cells; *p < 0.01 and **p < 0.05 of ob/ob PBS versus B6-WT and ob/ob leptin treated.
the manufacturer’s instructions. Soluble IL-1α, IL-2, IL-4, IL-5, IL-10, IL-17A, IFN-γ GM-CSF, and TNF-α mouse cytokines were measured using beads-based Analyte Detection Assay (Th1/Th2 FlowCytomix Kit; Bender MedSystems, Vienna, Austria), according to the manufacturer’s instructions.

Flow cytometry, cell sorting, and biochemical analyses
Untouched CD4+ T cells (isolated by negative selection using the Miltenyi Biotec T Cell Isolation kit and an AutoMACS cell separator) from donor mice were stained with the fluorescent dye CFSE from Molecular Probes (Eugene, OR) used at 1 μg/ml. A total of 5 × 10^6 CD4+CFSE+ labeled T cells were injected into the tail vein of B6-WT, ob/ob, and leptin-treated ob/ob mice. For flow cytometric analyses of CFSE+CD4+ T cells 7 and 14 d after transfer, spleens of three mice groups were harvested, and 1 × 10^6 cells were analyzed using a FACS-Calibur (BD Biosciences, San Jose, CA) and CellQuest software (BD Biosciences). For biochemical analyses, 0.5–1 × 10^6 CD4+CFSE+ cells were obtained from the spleen of each group of WT, ob/ob, and ob/ob-leptin–replaced mice using High-Speed Cells Sorting (MoFlo; DakoCytomation, Glostrup, Denmark); cells were >99% pure. For Western blotting, sorted cells were lysed as described previously (33). The Abs used were the following: anti-p27Kip-1, anti-phospho (P)-AKT, anti-AKT and anti–Bcl-2, and anti-S6 ribosomal protein (S6) and anti–P-S6 (all from Cell Signaling Technology, Beverly, MA). All filters were quantified by densitometric analysis of the bands using the program ScionImage 1.63 for Macintosh (Scion, Frederick, MD). FACS analyses for intracellular signaling on ex vivo CFSE+ cells fixed and permeabilized were performed by intracellular staining of P-S6 using PE-conjugated P-S6 Ab (Cell Signaling Technology).

Statistical analyses
Analyses were performed using Mann-Whitney U test (for unpaired two group analyses) and Kruskal-Wallis ANOVA test (for three or more group analyses). Results are expressed as mean ± SD; p < 0.05 was considered statistically significant.

Results
Autoreactive CD4+ T cells do not transfer EAE in ob/ob mice
We adaptively transferred 5 × 10^6 encephalitogenic MOG35–55–specific CD4+ T lymphocytes into leptin-deficient ob/ob mice treated or not with mouse r-leptin. As shown in Fig. 1A, MOG35–55–specific CD4+ T cells did not transfer EAE when injected into ob/ob recipients. In contrast, T cells did induce EAE when transferred into B6-WT and ob/ob mice treated with r-leptin with a similar frequency of disease and clinical score in the groups.

Transferred MOG35–55–specific CD4+ T cells were studied in vivo and in vitro. Seven days after transfer of MOG35–55–reactive CD4+ T cells, mice were challenged with 50 μg MOG35–55 peptide in the footpad, and DTH response was measured. Ob/ob mice had delayed DTH (12–72 h) as compared with B6-WT and r-leptin–treated ob/ob mice (Fig. 1B). The monitoring of DTH after adoptive transfer (1, 7, and 14 d) indicated that the DTH was lost when MOG35–55–specific CD4+ T cells were transferred into ob/ob mice (Fig. 1C).

To also define whether leptin deficiency affected the proliferation of transferred MOG35–55–specific CD4+ T cells, we performed dose-dependent in vitro stimulation with MOG35–55 in autologous serum (to preserve leptin deficiency). Reduced cell proliferation to MOG35–55 peptide of B6-WT cells from ob/ob mice was restored by r-leptin treatment (Fig. 1D). Also, secretion of proinflammatory cytokines IL-1α, IL-2, IL-6, IL-17A, GM-CSF, IFN-γ, and TNF-α from B6-WT MOG35–55–specific CD4+ T cells from ob/ob mice was reduced and restored by r-leptin administration (Fig. 1E). Leptin deficiency did not alter the production of IL-4 and IL-10 from B6-WT T cells in response to MOG35–55, and IL-5 was reduced similarly to Th1 cytokines. Finally, in adaptively transferred CD4+ B6-WT T cells stimulated in vitro with MOG35–55, the levels of IL-15 and IL-21 were significantly reduced (Fig. 1E), and IL-7 was undetectable (data not shown). To exclude that the observed

![Figure 2.](http://www.jimmunol.org/Downloadedfrom)
differences were due to quantitative effects of cells recovery, we normalized the in vitro experiments for the same number of CFSE+ CD4+ T cells in each well and confirmed that autoreactive CD4+ T cells exposed to a leptin-free microenvironment secrete less Th1/Th17 inflammatory and prosurvival cytokines.

Leptin controls the survival of autoreactive CD4+ T cells

To define whether leptin deficiency influences in vivo survival and proliferation of autoreactive MOG35–55-reactive T cells, CD4+ T cells purified from MOG35–55-immunized B6-WT mice 7 d after immunization were activated in vitro for 3 d with MOG35–55 peptide, CFSE labeled, and adoptively transferred into B6-WT, ob/ob PBS, and ob/ob-leptin–treated mice 7 d postadoptive transfer. The graphs show quantity of each specific protein. Data are shown as average value ± SD (n = 3; *p < 0.05 and **p < 0.01). Immunoblot for P-ERK1/2 (D) and for P-S6 (E) on FACS-sorted B6-WT CFSE+ MOG35–55-specific CD4+ T cells isolated from B6-WT, ob/ob PBS, and ob/ob-leptin–treated mice 7 d postadoptive transfer. Graphs show quantity of each protein. Data are shown as average value ± SD (n = 3; *p < 0.05). F, Flow cytometric analysis of S6 phosphorylation on CFSE+ CD4+ T cells in splenocytes from B6-WT transferred into B6-WT (left panel), ob/ob PBS (middle panel), and ob/ob-leptin–treated (right panel) mice. Representative of three independent experiments; *p < 0.05.

Leptin deficiency associates with a decrease of Bcl-2 expression, P-ERK1/2, and reduced degradation of the cell-cycle inhibitor p27kip1 in CD4+ T cells

The pathways for impaired survival of MOG35–55-specific CD4+ T cells in leptin-deficient ob/ob mice were studied. Western blot analysis for Bcl-2 (A), P-ERK1/2 (B), and p27kip1 (C) in FACS-sorted B6-WT CFSE+ MOG35–55-reactive CD4+ T cells isolated from B6-WT, ob/ob PBS, and ob/ob-leptin–treated mice 7 d postadoptive transfer. The graphs show quantity of each specific protein. Data are shown as averaged value ± SD (n = 3; *p < 0.05 and **p < 0.01). Immunoblot for P-AKT (D) and for P-S6 (E) on FACS-sorted B6-WT CFSE+ MOG35–55-specific CD4+ T cells isolated from B6-WT, ob/ob PBS, and ob/ob-leptin–treated mice 7 d postadoptive transfer. Graphs show quantity of each protein. Data are shown as averaged value ± SD (n = 3; *p < 0.05).

FIGURE 4. Inhibition of the leptin/mTOR signaling with rapamycin decreases autoreactive MOG35–55-specific CD4+ T cells and leptin secretion. A total of 5 × 10^6 CFSE+ MOG35–55-specific CD4+ T cells were FACS sorted from spleen suspensions of MOG35–55-immunized B6-WT mice and adoptively transferred into naive B6-WT mice treated with one dose at day 0 of PBS, rapamycin, r-leptin, or rapamycin plus r-leptin (see Materials and Methods and diagram for details). After 7 d, enumeration of adoptively transferred CFSE+ CD4+ MOG35–55-specific CD4+ T cells was performed (●) in association with quantification of circulating serum leptin (○). Data show averaged values ± SD; *p < 0.05.
 blot analysis showed that FACS-sorted B6-WT MOG35-55-specific CD4+ T cells, isolated from ob/ob mice, had lower levels of Bcl-2 and P-ERK1/2; these data were associated with a reduced degradation of the cell cycle inhibitor p27kip1 as compared with B6-WT CD4+ T cells from B6-WT and leptin-treated ob/ob mice (Fig. 3A–C). These findings suggest an increased apoptosis, reduced proliferation, and cell cycle arrest in the absence of leptin.

Leptin modulates the survival of autoreactive CD4+ T cells through the nutrient/energy-sensing AKT-mTOR pathway

We tested whether leptin affects the expression of the protein kinase B (AKT) and its downstream energy-sensing mTOR pathway (34–36). We found that FACS-sorted MOG35-55-specific CD4+ T cells from ob/ob mice had lower levels of phosphorylation of both AKT and S6, which is downstream of the mTOR pathway (Fig. 3D, 3E). These results were confirmed by FACS for P-S6 in B6-WT CFSE+ MOG35-55-specific CD4+ T cells (Fig. 3F).

Because P-S6 appeared reduced by leptin deficiency in B6-WT autoreactive T cells, we performed in vivo experiments using rapamycin, an mTOR inhibitor. Interestingly, acute/short-term rapamycin treatment in vivo resembled the effects of leptin deficiency in terms of recovery of MOG35-55-specific CD4+ T in B6-WT mice. B6-WT CFSE+ MOG35-55 pathogenic CD4+ T cells were analyzed 7 d after adoptive transfer into B6-WT mice treated with a single dose of PBS, rapamycin, r-leptin, or r-leptin plus rapamycin (Fig. 4, ■) on day 0. Rapamycin treatment significantly reduced the number of B6-WT CFSE+ MOG35-55 T cells; these effects were prevented by coinjection of r-leptin with rapamycin (Fig. 4, □). In this last condition, r-leptin could partially sustain mTOR activation, as demonstrated by P-S6 levels in CD4+ T cells from rapamycin plus r-leptin–treated mice. These results also correlated with the capacity of rapamycin to reduce serum leptin levels in B6-WT mice (Fig. 4, △), and these effects were not secondary to weight loss (data not shown).

The serum leptin levels were unchanged in leptin-treated mice as compared with controls, likely because they were analyzed after 7 d of a single dose of r-leptin whose half-life is ∼180 min (37).

Discussion

Several reports have suggested that leptin can affect survival of immune cells in vitro (38, 39). To our knowledge, this study is the first to report that leptin has a key role in the survival and proliferation of autoreactive CD4+ T cells in vivo. Leptin-deficient ob/ob mice were protected from adoptively transferred EAE, and the protection was associated with a progressive decline in the survival of adoptively transferred autoreactive MOG35-55-specific CD4+ T cells and reduced secretion of Th1/Th17 proinflammatory cytokines. Also, CD4+ autoreactive T cells had a significant downregulation of the survival protein Bcl-2, reduction in P-ERK1/2, and cell cycle arrest associated with reduced degradation of the cell cycle inhibitor p27kip1. Importantly, a significant impairment occurred at the level of the nutrient/energy-sensing AKT-mTOR/S6 signaling pathway. These phenomena were all reversed in vivo by r-leptin administration, suggesting that leptin can activate the mTOR pathway in autoreactive CD4+ T cells (Supplemental Fig. 2).

Studies published by Piccio et al. (3) and by our group (40) have shown that nutritional deprivation or CR can reduce the magnitude and disease score of EAE. The so-called “frugal phenotype,” in which the survival of chronically food-restricted mice is higher than that of ad libitum-fed mice, fits well into our current findings of reduced T cell autoreactivity in the absence of leptin. Also, chronic rapamycin treatment is known to increase significantly retard survival (41). Although the precise mechanisms for the effects of rapamycin need to be elucidated, through mTOR inhibition, rapamycin can reduce the absorption of amino acids and glucose and dampen proinflammatory cytokines (including leptin) (42). This is interesting because rapamycin can improve disease course and progression in EAE and type 1 diabetes by increasing regulatory T cell responses and dampening Th1/Th17 responses (43–45).

Leptin represents a link with the environment by informing about available energy reserves as stored fat (5). Microenvironmental requirements for immune cells, including autoreactive CD4+ T cells, are not well known, although many cytokines, chemokines, and other mediators have been described as capable of significantly affecting survival and homeostasis of the T cells. In this paper, we describe a mechanism of leptin-based modulation of autoreactive CD4+ T cell survival through the nutrient energy-sensing mTOR pathway and the expression of Bcl-2. The reduced secretion of cytokines such as IL-6, IL-15, IL-21, and GM-CSF also appeared important. B6-WT myelin-specific CD4+ T cells in the leptin-free microenvironment showed a reduced secretion of IL-6, IL-15, IL-21, and GM-CSF when restimulated in vitro with MOG35-55. Other cytokines such as IL-1α, IL-2, IL-17A, IFN-γ, and TNF-α were also downmodulated in autoreactive MOG35-55-specific CD4+ B6-WT cells when transferred into leptin-deficient ob/ob mice. Incidentally, the fact that IL-1α and IL-17A were reduced by leptin deficiency is in line with a recent report of a key role for mTOR in the maintenance of Th17 cell proliferation and cytokine secretion by IL-1 in EAE (46).

In summary, this study shows a previously unappreciated role of leptin in the promotion of the survival of CD4+ autoreactive T cells. The implications of these findings in relation to nutritional deprivation or CR can suggest targeting of leptin-based pathways of intervention for the modulation of autoreactive T cell activity.

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Disclosures

The authors have no financial conflicts of interest.

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