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Tumor-Derived IL-35 Promotes Tumor Growth by Enhancing Myeloid Cell Accumulation and Angiogenesis

Zhihui Wang,*1 Jin-Qing Liu,*1 Zhenzhen Liu,* Rulong Shen,* Guoqiang Zhang,‡ Jianping Xu,§ Sujit Basu,* Youmei Feng,‡ and Xue-Feng Bai*1,‡

IL-35 is a member of the IL-12 family of cytokines that is comprised of an IL-12 p35 subunit and an IL-12 p40-related protein subunit, EBV-induced gene 3 (EBI3). IL-35 functions through IL-35R and has a potent immune-suppressive activity. Although IL-35 was demonstrated to be produced by regulatory T cells, gene-expression analysis revealed that it is likely to have a wider distribution, including expression in cancer cells. In this study, we demonstrated that IL-35 is produced in human cancer tissues, such as large B cell lymphoma, nasopharyngeal carcinoma, and melanoma. To determine the roles of tumor-derived IL-35 in tumorigenesis and tumor immunity, we generated IL-35–producing plasmacytoma J558 and B16 melanoma cells and observed that the expression of IL-35 in cancer cells does not affect their growth and survival in vitro, but it stimulates tumorigenesis in both immune-competent and Rag1/2-deficient mice. Tumor-derived IL-35 increases CD11b+Gr1+ myeloid cell accumulation in the tumor microenvironment and, therefore, promotes tumor angiogenesis. In immune-competent mice, spontaneous CTL responses to tumors are diminished. IL-35 does not directly inhibit tumor Ag–specific CD8+ T cell activation, differentiation, and effector functions. However, IL-35–treated cancer cells had increased expression of gp130 and reduced sensitivity to CTL destruction. Thus, our study indicates novel functions for IL-35 in promoting tumor growth via the enhancement of myeloid cell accumulation, tumor angiogenesis, and suppression of tumor immunity. The Journal of Immunology, 2013, 190: 000–000.

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IL-35 IN TUMOR GROWTH AND IMMUNITY

Materials and Methods

**Mice**

BALB/c, C57BL/6, and Rag1−/−C57BL/6 mice were purchased from The Jackson Laboratory. Rag2−/−BALB/c mice were purchased from Taconic Farms (Germantown, NY). Transgenic mice expressing a TCR specific for the tumor Ag P1A (P1CTL), whose TCR recognizes the H-2Ld−/P1A35-43 complex, were described (18). All animal experiments were performed after approval by The Ohio State University Institutional Animal Care and Use Committee.

**Cancer cell lines and tumor establishment in mice**

Mouse plasmacytoma J558 cells (H-2Ld+) were described (19). Mouse plasmacytoma J559 cells or B16F10 melanoma cells were cotransfected with an expression vector pORF9-mH-2L3-53Elasti (InvivoGen) and a selection vector (pCDNA3-neo) or the control expression vector pORF9 (InvivoGen) and pcDNA3-neo. Thereafter, stable cell lines resistant to G418 were generated. J558-IL-35, J558-Ctrl and B16-IL-35, B16-Ctrl cells were main-

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associated with EBV infection. In addition, we also examined IL-35 expression in non-EBV–related cancer tissues, such as melanoma. As shown in Fig. 1B, some large B cell lymphoma cells showed positive staining of IL-35. In other cancer types, IL-35+ staining was detected mainly in stromal cells rather than cancer cells (Fig. 1D, 1F, 1H). In addition, IL-35+ cells exhibited various shapes (Fig. 1D, 1F, 1H). Thus, IL-35 can be readily detected in various human cancer tissues and is likely of multiple cellular sources.

**IL-35 production in the tumor microenvironment stimulates tumor growth**

To determine the roles of IL-35 in the tumor microenvironment, we generated IL-35+ or IL-35− mouse plasmacytoma J558 cells and B16 melanoma cells by transfecting J558 and B16 melanoma cells with an IL-35 expression vector or a control expression vector. RT-PCR analysis revealed that J558–IL-35 and B16–IL-35 cells expressed both EBI3 and IL35A mRNA (Fig. 2A, 2E). Moreover, RT-PCR assay using EBI3 forward primer and P35 reverse primer detected the rRNA encoding both EBI3 and IL-35A subunits (Fig. 2A, 2E). Thus, the IL-35 molecule was stably expressed in J558– IL-35 and B16–IL-35 cells. Immunocytochemistry staining of cells and ELISA assay of supernatants from cell cultures revealed that IL-35 protein was produced by J558 (Fig. 2B) and B16 (Fig. 2F) cells. Expression of IL-35 in cancer cells did not alter the expression of MHC class I (Fig. 2C, 2G) and tumor Ag P1A (Fig. 2A) in J558 cells. MTT assay revealed that IL-35 expression did not affect J558 and B16 cell growth in vitro (Fig. 2D, 2H).

To determine whether the expression of IL-35 in tumor cells affects tumor growth in vivo, 5 × 10⁶ IL-35+ or IL-35− J558 cells were injected s.c. into each BALB/c mouse. As shown in Fig. 3A, IL-35− J558 cells induced tumors in normal BALB/c mice 10–14 d after tumor cell injection. J558–IL-35 tumors grew progressively in BALB/c mice; within 2 wk the tumors grew to a size that required euthanasia. Our results indicated that ex vivo J558–IL-35 tumors were significantly bigger than ex vivo J558-Ctrl

**FIGURE 1.** Expression of IL-35 in human cancer tissues. H&E staining and IHC were performed on paraffin-embedded serial tissue sections of human large B cell lymphoma (A, B), nasopharyngeal carcinoma (C, D), skin melanoma (E, F), and lymph node metastatic melanoma (G, H). The anti-human IL-35 mAb 15K8D10 was used to stain human cancer tissues. (A), (C), (E), and (G) show H&E staining; (B), (D), (F), and (H) show IL-35–specific staining. Scale bars, 100 μm.

**FIGURE 2.** Generation of IL-35–producing J558 and B16.F10 cells. Mouse plasmacytoma J558 cells or B16.F10 melanoma cells were cotransfected with an expression vector pORF9-IL-35 and a selection vector pCDNA-neo or the control expression vector pORF9 and pCDNA-neo. Stable cell lines that were resistant to G418 were generated. RT-PCR was used to detect the expression of transcripts for recombinant IL-35, IL-12A, EBI3, and tumor Ag P1A in J558 cells (A) and IL-35, IL-12A, and EBI3 transcripts in B16.F10 cells (E). Immunofluorescence staining and ELISA revealed that IL-35 protein was produced by the generated J558 (B) and B16.F10 (F) cells. Flow cytometry was used for the analysis of MHC class I expression on the generated J558 cells (C) and B16 cells (G). MTT proliferation assay was used to measure growth and proliferation of J558 cells (D) and B16 cells (H). Bars indicate SD of triplicates. Original magnifications ×400 for (B) and (F).
creased tumor angiogenesis was also found in B16–IL-35 tumors in comparison with J558-Ctrl tumors (Fig. 4A, 4B). Inhibition of VEGF, was significantly increased in J558–IL-35 tumors by day 15 after tumor cell injection, and a high concentration of IL-35 was detected in protein lysates of IL-35+ tumors (Fig. 3B).

To determine whether IL-35 enhances tumor growth via inhibition of the adaptive immune response, we injected J558–IL-35 or J558-Ctrl cells into Rag2-/-BALB/c mice that lack T and B lymphocytes. As shown in Fig. 3C, J558–IL-35 tumors grew much faster compared with J558-Ctrl cells in Rag2-/-BALB/c mice. To determine whether the tumor enhancement was IL-35 specific, an IL-35–neutralizing mAb or an isotype-matched control mAb was coinjected with J558–IL-35 cells into Rag2-/-BALB/c mice. We found that IL-35–neutralizing mAb abrogated tumor growth enhancement (Fig. 3D).

Similarly, we found that B16–IL-35 tumors grew much faster than B16-Ctrl tumors in both C57BL/6 mice (Fig. 3E) and Rag2-/-C57BL/6 mice (Fig. 3F). In addition, when injected i.v., B16–IL-35 cells established lung foci faster than did B16-Ctrl cells (Fig. 3G). Thus, IL-35 production in the tumor microenvironment stimulates tumor growth.

**IL-35 production in the tumor microenvironment increases myeloid cell accumulation and tumor angiogenesis**

To determine the mechanisms by which IL-35 expression promote tumor growth, we first examined tumor angiogenesis in established J558–IL-35 and J558-Ctrl tumors collected from Rag2-/-BALB/c mice. Tumor angiogenesis, as determined by CD31 expression and expression of VEGF, was significantly increased in J558–IL-35 tumors in comparison with J558-Ctrl tumors (Fig. 4A, 4B). Increased tumor angiogenesis was also found in B16–IL-35 tumors (Fig. 4C, 4D).

IL-35+ tumors also contained increased numbers of myeloid cells in comparison with J558-Ctrl tumors. Fluorescence staining of sections from J558–IL-35 tumors grown in Rag2-/- mice revealed accumulation of more myeloid-derived suppressor cells (MDSCs; CD11b+Gr1+) (Fig. 5A). In contrast, the numbers of CD11b single-positive cells were low in J558–IL-35 tumors compared with control tumors (Fig. 5A). However, the numbers of Gr1 single-positive cells were low and did not differ between the two types of tumors (Fig. 5A). Flow cytometry analysis of dissociated tumor cells verified the fluorescence staining results obtained from tumor sections (Fig. 5B). Furthermore, increased numbers of CD11b+Gr1+ cells were also found in J558–IL-35 tumors grown in BALB/c mice (Fig. 5C) and B16–IL-35 tumors from C57BL/6 mice (Fig. 5D) in comparison with their controls. To determine whether increased MDSC accumulation was responsible for enhanced tumor growth, we injected three doses of anti-Gr1 mAb (250 µg/dose) into J558–IL-35 or J558-Ctrl cell–inoculated Rag2-/-BALB/c mice. We previously showed that this regimen could completely deplete Gr1+ cells (22). We found that anti-Gr1 mAb treatment largely eliminated the tumor growth difference between the two groups of mice (Fig. 5E). Thus, MDSC accumulation is largely responsible for the enhanced growth of J558–IL-35 tumors in Rag2-/-BALB/c mice.

**IL-35 does not enhance migration of myeloid cells in vitro**

The increased accumulation of myeloid cells in IL-35+ tumors suggests that IL-35 may play a chemotaxis effect on myeloid cells. To test this possibility, we performed a transmigration assay using a Transwell system in the presence or absence of IL-35.
different lineages of myeloid cells, including macrophage cell lines Raw264.7 (H-2^b) and P388D1 (H-2^d), MDSCs purified from the spleen of J558-tumor bearing mice, and bone marrow–derived CD11b^Gr1^ cells, were used in the transmigration assay. As shown in Fig. 6, similar numbers of Raw264.7 cells (Fig. 6A), P388D1 cells (Fig. 6B), spleen MDSCs (Fig. 6C), and bone marrow

FIGURE 4. IL-35 production in the tumor microenvironment enhances angiogenesis. J558 tumors (A, B) from Rag2^−/−^ mice and B16 tumors from C57BL6 mice (C, D) were analyzed for the expression of CD31 and VEGF by immunofluorescent staining and microscopy. Scale bars, 200 μm. Mean vessel wall area and numbers of VEGF^+^ cells for each tumor were analyzed and quantified using ImageJ software. Three random fields from each slide/tumor were analyzed, and each symbol represents data from one microscope field. **p = 0.001, ***p < 0.0001, Student t test.

FIGURE 5. Increased numbers of CD11b^Gr1^ myeloid cells in the tumor microenvironment of IL-35^+^ tumors. (A) J558–IL-35 and J558–Ctrl tumors from Rag2^−/−^ mice were analyzed for the infiltration of myeloid cells by immunofluorescence staining and microscopy. Scale bars, 200 μm. Frozen tissue sections were colabeled for CD11b (Alexa Fluor 488) and Gr1 (Texas Red), and images were analyzed and quantified using ImageJ software. Three random fields from each slide/tumor were analyzed, and each symbol represents data from one microscope field. Arrows indicate CD11b and Gr1 double positive MDSCs. **p < 0.001, Student t test. (B–D) Flow cytometry was also used for the analysis of myeloid cells in IL-35^+^ and IL-35^−^ tumors. Single-cell suspensions were prepared from tumors grown in Rag2^−/−^ (B), BALB/c (C), and C57BL6 (D) mice and stained for CD11b and Gr-1, followed by flow cytometry analysis. Each symbol represents data from a single tumor. *p < 0.05, **p < 0.01, ***p < 0.001, Student t test. (E) A total of 5 × 10^6^ J558–IL-35 or J558–Ctrl cells was injected s.c. into each BALB/c mouse, followed by treatment with 250 μg/mouse of anti-Gr1 mAb (RB6-8C5; Bio X Cell) i.p. on days 0, 5, and 10. Mice were observed for tumor growth over time. Five mice/group were used for this experiment, and the data shown represent two experiments with similar results.
CD11b+Gr1+ cells (Fig. 6D) migrated to chambers containing IL-35 or the control medium. Thus, IL-35 does not directly increase migration of myeloid cells.

IL-35 production in the tumor microenvironment suppresses CTL responses

Because the IL-35+ tumors contained higher numbers of myeloid suppressor cells, which are known to suppress CTL responses in tumors (23, 24), we hypothesized that IL-35 production in the tumor microenvironment inhibits CTL responses. To test this hypothesis, we compared T cell responses in the J558–IL-35 and J558-Ctrl tumors grown in normal BALB/c mice. As shown in Fig. 7A, we found that total leukocyte numbers (CD45+) were increased in J558–IL-35 tumors. However, among the CD45+ leukocyte population, the numbers of CD8+ T cells were greatly reduced in J558–IL-35 tumors (Fig. 7A, 7B). CD8+ T cells from J558–IL-35 tumors also were less capable of producing IFN-γ in comparison with CD8+ T cells collected from J558-Ctrl tumors (Fig. 7C). However, the percentages of CD4+ T cells (Fig. 7D) and CD4+Foxp3+ Tregs (Fig. 7E) were not affected significantly. Similar to the J558 tumors, we also observed that B16–IL-35 tumors grown in C57BL6 mice contained significantly reduced numbers of CD8+ T cells (Fig. 7F, 7G). In contrast, the numbers of CD4+ T cells were not significantly affected (Fig. 7F, 7H).

IL-35 does not directly inhibit tumor Ag-specific CTL proliferation and effector functions

To determine whether IL-35 production directly inhibits tumor Ag-specific CD8+ T cell proliferation and effector function, we cultured P1CTL transgenic T cells that recognize tumor Ag P1A in the presence of IL-35 produced by J558 cells. There were no differences in the proliferation and growth of P1CTL cells either in the presence or absence of IL-35, as determined by [3H]thymidine-incorporation assay (Fig. 8A) and MTT assay (Fig. 8B). IL-35–stimulated P1CTL cells also expressed similar levels of IFN-γ and granzyme B (Fig. 8C) and exhibited similar cytotoxicity to P1A+ P815 target cells (Fig. 8D).

IL-35 induces resistance of cancer cells to destruction by tumor Ag-specific CTL

Despite expressing similar levels of P1A Ag and MHC class I (Fig. 2), J558–IL-35 cells were more resistant to P1CTL destruction.

**FIGURE 6.** IL-35 does not increase migratory activity of myeloid cells. Migration assay using a Transwell system, with or without IL-35, was performed to test the migration capacity of Raw264.7 cells (A), P338D1 cells (B), spleen MDSCs (C), and bone marrow Gr1+ cells (D). Cells that migrated to the bottom side of the Transwell membrane were stained with DAPI, and random fields from each well were photographed under a fluorescence microscope. The numbers of cells in each field were quantified using ImageJ software. Each symbol represents data from one microscopic field, and data shown represent three experiments with similar results. Original magnification for (A): ×200.

**FIGURE 7.** Expression of IL-35 contributes to an immune-suppressive microenvironment. J558 cells or B16 cells, with or without IL-35 expression, were injected s.c. into each BALB/c or C57BL6 mouse. When tumors were fully established (~1 cm in length), mice were sacrificed, and T cell responses in tumors were evaluated by flow cytometry. (A–E) T cell responses in IL-35+ and IL-35− J558 tumors from BALB/c mice. (F–H) T cell responses in IL-35+ and IL-35− B16 tumors from C57BL6 mice. Each symbol represents data from a single mouse/tumor. *p < 0.05, **p < 0.01, Student t test.
compared with J558-Ctrl cells (Fig. 9A). Similarly, IL-35–stimulated P815 cells were also found to be more resistant to P1CTL lysis (Fig. 9B) compared with control P815 cells. Consistent with this observation, established J558–IL-35 tumors in BALB/c mice contained less apoptotic tumor cells compared with J558-Ctrl tumors (Fig. 9C). Thus, IL-35 renders tumor target cells more resistant to CTL destruction. To determine whether cancer cell resistance to CTL destruction was due to IL-35R signaling, we determined the expression of IL-35R subunits (IL-12Rβ2 and gp130) in IL-35+ and IL-35− tumor cells by RT-PCR. As shown in Fig. 9D, although expression of IL-12Rβ2 and gp130 was barely detectable in B16 cells, increased gp130 expression was detected in J558–IL-35 cells compared with J558-Ctrl cells. To determine whether induction of gp130 was IL-35 specific, J558 and P815 cells were cultured or not with IL-35 for 24 h, and we determined gp130 expression by RT-PCR. As shown in

**FIGURE 8.** IL-35 does not directly affect differentiation of tumor Ag–specific CTL. Splenocytes from P1CTL-transgenic mice were activated with P1A peptide (0.2 μg/ml) in the presence or absence of IL-35. [3H]tritium-incorporation assay (A) and MTT assay (B) were used to determine cell proliferation and survival. (C) Intracellular staining and flow cytometry were used to determine IFN-γ and granzyme B expression in activated P1CTL cells. (D) [51Cr] release assay was used to determine cytotoxicity of activated P1CTL cells to P815 target cells. Data shown represent at least three experiments with similar results.

**FIGURE 9.** IL-35 induces tumor cell resistance to CTL destruction. P1CTL cells were activated with P1A peptide (0.2 μg/ml) for 5 d. [51Cr] release assay was used to determine cytotoxicity of activated P1CTL cells to J558–IL-35/J558-Ctrl cells (A) and P815 cells treated or not with IL-35 (B). Data shown represent three experiments with similar results. (C) Frozen tissue sections from J558–IL-35 or J558-Ctrl tumors grown in BALB/c mice were labeled for TUNEL; images were photographed under a fluorescent microscope and quantified using ImageJ software (left panels). Scale bars, 200 μm. Three random fields from each slide/tumor were analyzed, and each symbol represents data from one microscope field (right panel). RT-PCR was used to detect IL-35R subunits in B16–IL-35/B16-Ctrl and J558–IL-35/J558-Ctrl cells (D) or in J558 and P815 cells treated or not with IL-35 (E). Data shown in (D) and (E) represent three experiments with similar results. *p < 0.05, Student t test.
Fig. 9E, IL-35 increased gp130 expression in both J558 and P815 cells.

Discussion

Our study validates the hypothesis that IL-35 is produced in human cancer tissues, and tumor-derived IL-35 plays important roles in tumor progression and tumor immune surveillance.

First, we demonstrated that IL-35 is produced in human cancer tissues. Among the three types of human cancer tissues examined, IL-35 is mainly found in tumor stromal cells. Thus, it will be interesting to determine the types of cells in cancer stromas that produce IL-35. Previous studies revealed that TIDCs (12, 15) express EBI3; therefore, TIDCs could be a source of IL-35. Additionally, tumor-infiltrating FOXP3+ Treg cells could be another source of IL-35. EBI3 is known to be expressed in EBV-associated Hodgkin lymphoma, diffuse large B lymphoma, nasopharyngeal carcinoma (12, 25), and EBV+ malignant tumors (14). In this study, we observed that some diffuse large B lymphoma cells are also positive for IL-35. Therefore, IL-35 is abundantly produced in many human cancer tissues and multiple cell types, including some cancer cells.

We next observed that tumor-derived IL-35 considerably increased tumorigenesis in both immune-competent and immune-deficient mice. The protumor effect is very rapid, which necessitates sacrifice of mice injected with IL-35+ cancer cells within 2–3 wk after tumor cell injection. This protumor effect of IL-35 was confirmed in two tumor models: plasmacytoma J558 and B16.F10 melanoma. Previous studies reported that IL-35–producing Tril5 cells can inhibit tumor growth via suppression of antitumor immune responses (7). Because the protumor effect of IL-35 was also observed in Rag1/2-deficient mice in this study, the rapid tumor growth of IL-35–producing tumors is not solely due to suppression of adaptive immunity. The IL-35 effect in tumors also differs from another EBI3-containing cytokine, IL-27, which has potent tumor-inhibiting effects (26–31). Expression of EBI3 in human lung cancer cells was reported to promote lung cancer cell growth in vitro (14). However, in this study we showed that IL-35 expression did not affect tumor growth and proliferation in vitro. It is not known whether the discrepancy of the results is due to a lack of IL-35 heterodimer formation in lung cancer cells. To our knowledge, this is the first study to show that tumor-derived IL-35 has a protumor effect in vivo.

We demonstrated that IL-35 production in the tumor microenvironment increased CD11b+Gr1+ myeloid cell accumulation and tumor angiogenesis (reflected by increased density of CD31+ blood vessels and VEGF production in the tumor bed). Because IL-35 production by cancer cells does not affect their growth and proliferation in vitro, it is likely that IL-35–producing cancer cells are capable of inducing host cells to promote tumor growth. In this regard, increased numbers of CD11b+Gr1+ MDSCs were present in IL-35+ tumors. MDSCs are a known cell type that can promote tumor angiogenesis via production of VEGF and other proangiogenesis factors (32–34). Thus, it is likely that increased angiogenesis and tumor growth in IL-35+ tumors are due to increased accumulation of CD11b+Gr1+ myeloid cells. Indeed, depletion of Gr1+ myeloid cells abrogated tumor growth enhancement by IL-35. Our in vitro migration assay revealed that tumor-produced IL-35 does not have a direct role in MDSC chemotaxis. Quantitative RT-PCR analysis also revealed that VEGF expression was not altered in J558–IL-35 and B16–IL-35 cells in comparison with their relative control cells (data not shown). Thus, it remains to be determined what signal pathways are activated in tumor cells by IL-35, which are responsible for initially accumulating CD11b+Gr1+ myeloid cells into tumors and, thereby, inducing tumor angiogenesis.

Finally, in this study we demonstrated that tumor-derived IL-35 induces a suppressive tumor microenvironment, which, in turn, inhibits tumor immunity. MDSCs induce immune suppression and inhibit CTL responses (23, 24). Consistent with this observation, we found that spontaneous CTL responses in IL-35+ tumors were inhibited in immune-competent mice. However, IL-35 does not directly inhibit CTL proliferation, differentiation, and effector functions in vitro, and this is in contrast to other IL-12 family cytokines, such as IL-12 and IL-27, which can significantly affect CTL differentiation (35–37). Thus, it is likely that the inhibition of CTL responses in IL-35+ tumors is due to the accumulation of MDSCs therein, which subsequently inhibits CTL responses.

Although IL-35 does not have direct effects on CTL activation and effector functions, we showed that IL-35–treated target cells were less susceptible to CTL-mediated destruction. Consistent with this effect, we found that less apoptotic tumor cells were observed in IL-35+ tumors than in IL-35− tumors grown in immune-competent mice. This effect is unlikely mediated by downregulation of MHC class I or Ag expression, because IL-35–expressing tumor cells have similar levels of MHC class I and Ag. Our RT-PCR results suggest that IL-35 upregulates gp130 expression in tumor cells. Interestingly, gp130 signaling was shown to mediate cancer cell resistance to chemotherapy (38). Because IL-35 is known to signal through the gp130 dimer (4), it is highly suggestive that IL-35 signaling via gp130 induces cancer cell resistance to CTL destruction. Thus, IL-35–induced cancer cell resistance to CTL destruction could be one mechanism by which cancer cells escape CTL destruction in tumors.

Taken together, our investigation determining the role of tumor-derived IL-35 on tumor growth and immunity revealed novel functions for IL-35 in promoting tumor growth and inhibition of antitumor CTL responses. Because IL-35 can be produced by both cancer cells and tumor-infiltrating stromal cells, further investigations elucidating the cross-talk among cancer cells, tumor-infiltrating stromal cells, and MDSCs via IL-35 may help us to better understand tumor progression and immune evasion. Accordingly, targeting IL-35–mediated cross-talk may be a novel immunotherapeutic approach for the treatment of cancer patients.

Disclosures

The authors have no financial conflicts of interest.

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