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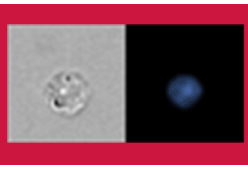
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Cutting Edge

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Cutting Edge: FcγRIII (CD16) and FcγRI (CD64) Are Responsible for Anti-Glycoprotein 75 Monoclonal Antibody TA99 Therapy for Experimental Metastatic B16 Melanoma

Marcello Albanesi,[*,†] David A. Mancardi,[*,†] Lynn E. Macdonald,[‡] Bruno Iannascoli,[*,†] Laurence Zitvogel,[§,¶] Andrew J. Murphy,[‡] Jeanette H. Leusen,[‖] and Pierre Bruhns[*,†]

mAb therapy for experimental metastatic melanoma relies on activating receptors for the Fc portion of IgG (FcγR). Opposing results on the respective contribution of mouse FcγRI, FcγRIII, and FcγRIV have been reported using the gp75-expressing B16 melanoma and the protective anti-gp75 mAb TA99. We analyzed the contribution of FcγRs to this therapy model using bioluminescent measurement of lung metastases loads, novel mouse strains, and anti-FcγR blocking mAbs. We found that the TA99 mAb-mediated effects in a combination therapy using cyclophosphamide relied on activating FcγRs. The combination therapy, however, was not more efficient than mAb therapy alone. We demonstrate that FcγRI and, unexpectedly, FcγRIII contributed to TA99 mAb therapeutic effects, whereas FcγRIV did not. Therefore, FcγRIII and FcγRI are, together, responsible for anti-gp75 mAb therapy of B16 lung metastases. Our finding that mouse FcγRIII contributes to Ab-induced tumor reduction correlates with clinical data on its human functional equivalent human FcγRIIIA (CD16A). *The Journal of Immunology*, **2012, 189: 5513–5517.**

Tumor cells can be specifically targeted using mAbs that bind to specific or overexpressed Ags on the tumor cell surface. Some of these mAbs can directly affect tumor growth or survival when their Fab portions are bound to their target, for example, trastuzumab targeting HER2/Neu. Some mAbs, however, target molecules that are not involved in tumor growth or survival. In most situations, however, mAbs bound to tumor cells enable the recruitment of phagocytic and cytotoxic immune cells bearing receptors for the Fc portion of IgG (FcγR). Activating FcγRs are indeed necessary for the protective effect of the mouse anti-gp75 mAb TA99 on the development of lung metastases of gp75-expressing B16F10 mouse melanoma cells (1). The contribution of FcγRs in the context of a therapy using a combination of chemotherapy and anti-gp75 mAb treatment has, however, not been investigated.

Activating FcγRs in humans comprise FcγRI (CD64), FcγRIIA (CD32A), FcγRIIC (CD32C), and FcγRIIIA (CD16A), whereas in mice, FcγRI, FcγRIII, and FcγRIV exist. All mouse activating FcγRs require the association of the FcRγ subunit to be expressed and functional at the cell surface. The generation of FcRγ$^{-/-}$ mice allowed for the discovery that activating FcγRs contribute primarily to the protective effect of mAb TA99 on B16 lung metastases (1). Since then, FcγRI$^{-/-}$ mice (2, 3), FcγRIII$^{-/-}$ mice (4), and FcγRIV$^{-/-}$ mice (5) have been reported; however, studies that have used these mice to identify the activating FcγR(s) responsible for the protective effect of mAb TA99 on B16 lung metastases report contradictory results. Whereas Nimmerjahn et al. (6) described a contribution of FcγRIV, but neither FcγRI nor FcγRIII, Bevaart et al. (7) described a mandatory role for FcγRI, but no detectable contribution of FcγRIII or, by deduction, of FcγRIV. These opposing results may be attributable to differences in techniques used to measure tumor load: Nimmerjahn et al. (6) counted the total number of lung surface metastases, whereas Bevaart et al. (7) scored lung surface metastases based on their number and size.

Using a luciferase-expressing variant of B16F10, allowing bioluminescent quantification of the metastases load, combined with single or multiple FcγR-deficient mice and FcγR-blocking mAbs, we reassessed the contribution of FcγRs to a therapy model of B16 lung metastases. Activating IgG receptors were necessary for the protective effect of mAb TA99 not

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Abbreviations used in this article: CTX, cyclophosphamide; wt, wild-type.

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only in single mAb therapy, but also in combination therapy with cyclophosphamide (CTX). Moreover, we identified a significant and unexpected contribution of FcγRIII, confirmed the contribution of FcγRI, and excluded any contribution of FcγRIV to TA99 mAb therapy.

Materials and Methods

Reagents and flow cytometry

B16F10 cells and hybridoma TA99 (anti-gp75) were from American Type Culture Collection, B16F10-Luc2$^+$ from Caliper-Life Sciences, hybridoma 9E9 (anti-FcγRIV) from J.V. Ravetch, anti-FcγRIII mAb 275003 (8) from R&D Systems, and CTX (Endoxan) from Baxter SAS. Staining by mAb TA99 (30 μg/ml) for 30 min at 0°C was revealed using FITC-labeled anti-mouse IgG.

Mice

C57BL/6J FcγRI$^{-/-}$ mice (3), FcγRIII$^{-/-}$ mice, and FcRγ$^{-/-}$ mice (Jackson Laboratories), FcγRIV$^{-/-}$ mice (5), and FcγRI/IIB/III FcεRI/II (5KO, also known as "FcγRIV-only") mice (9) have been reported previously. Mice deficient in FcγRIIB, FcγRIII, and FcγRIV ("FcγRI-only") were generated at Regeneron Pharmaceuticals by deletion of the entire mouse low-affinity FcγR locus (L. Macdonald, C. Gurer, K. Hosiawa, N. Tu, M. Zhong, D. Grote, F. Harris, V. Voronina, D. Chalothorn, L. Morton, D. Valenzuela, W. Poueymirou, W. Auerbach, A. Murphy, and G. Yancopoulos, manuscript in preparation) using Velocigene technology (10). All mice were used at 6–10 wk of age. A total of 1×10^6 B16 cells were injected i.v. on day 0. Where indicated, mice were injected with mAb TA99 (200 μg) or isotype control i.p. on days 0, 1, 2, 4, 7, and 9; CTX (100 mg/kg) i.p. on day 0; anti-FcγRIV (mAb 9E9, hamster IgG, 200 μg), anti-FcγRIII (mAb 275003, rat IgG2a, 100 μg), or respective isotype controls i.v. on days 0, 2, 4, and 6. Mice were shaved, anesthetized, and injected i.p. with 3 mg luciferin to acquire bioluminescence on the whole mouse. Alternatively, explanted lungs were exposed to luciferin (50 μl at 15 mg/ml) 2 min before bioluminescence acquisition (IVIS 100; Caliper Life Sciences), with settings of 2 min exposure time and medium binning. Total photon flux (photons/seconds) was calculated using Living Image software. Mouse protocols were approved by the Animal Care and Use Committees of Paris, France.

In vitro assays

For bioluminescence detection, B16-Luc2$^+$ cells (5000/well) were added to a black 96-well plate, incubated with D-luciferin (150 μg/ml), and bioluminescence acquired.

Statistical analyses

Data were analyzed using Student *t* test (NS: $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$). Error bars correspond to SEM. In vivo data were analyzed using two-way ANOVA with Bonferroni test.

Results and Discussion

An i.v. injection of B16-F10 cells leads to lung metastases of very different sizes (Fig. 1A, *left panel*). Notably, metastases developed also inside the lung parenchyma (Fig. 1A, *right panel*), a parameter that was not considered in previous studies (6, 7). To analyze the contribution of FcγR to the B16-TA99

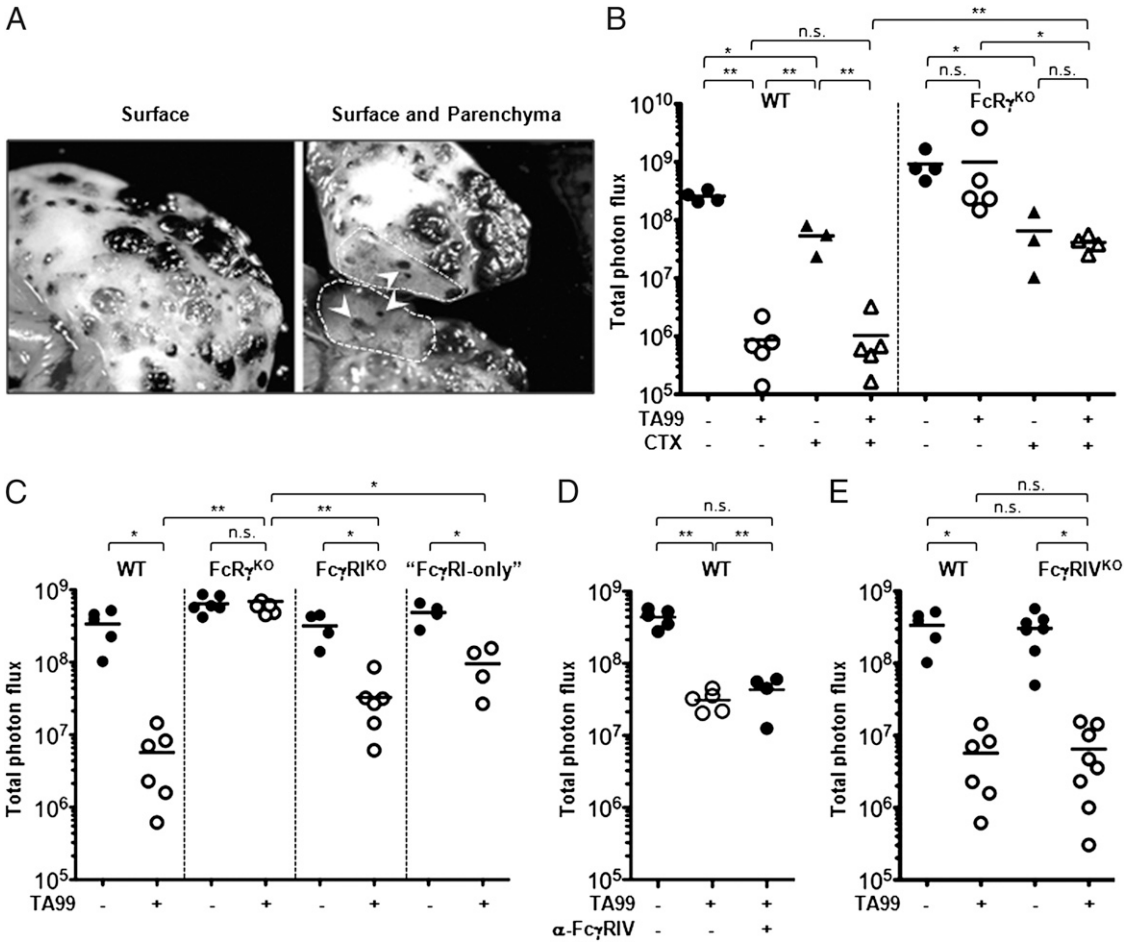

FIGURE 1. FcγRI, but not FcγRIV, contributes to mAb therapy of metastatic melanoma. (**A**) Representative picture of metastases on the lung surface (*left panel*) or on the surface and in the lung parenchyma (*right panel*) of wt mice at day 11 postinjection of B16F10 cells. Arrowheads indicate metastases in the parenchyma. (**B**–**E**) Mice were injected with B16-luc2$^+$ cells and with mAb TA99 when indicated. Quantification of tumor load on explanted lungs in (B) wt and FcRγ$^{-/-}$ mice that were additionally injected with CTX when indicated ($n \geq 3$); (C) wt, FcRγ$^{-/-}$, FcγRI$^{-/-}$, or "FcγRI-only" mice ($n \geq 4$); (D) wt mice injected with anti-FcγRIV mAb or not ($n \geq 4$); (E) wt and FcγRIV$^{-/-}$ mice ($n \geq 5$, compilation of two identical experiments). Data are representative of two to four independent experiments. Not significant (n.s.): $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$.

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metastasis immunotherapy model with an accurate measure of tumor load, we took advantage of a B16-F10 variant expressing the enhanced firefly luciferase (luc2) that allows assessment of tumor growth by bioluminescence. The expression of luc2 in B16-F10 cells led to photon release in the presence of luciferin but did not alter the expression of gp75 (Supplemental Fig. 1A). In vitro B16-luc2$^+$ cell growth was not affected by anti-gp75 mAb TA99 whether bound or aggregated at the cell membrane (Supplemental Fig. 1B), as expected (11). When injected i.v., B16-luc2$^+$ cells formed metastases specifically in the lung that could be noninvasively monitored in vivo and quantified over time by bioluminescence. Performing the same analysis on explanted lungs from the same mice, however, increased the sensitivity 20-fold (Supplemental Fig. 1C). Subsequent analyses were therefore performed on explanted lungs at day 11 postinjection of B16-luc2$^+$ cells.

B16-luc2$^+$ metastases formed to a greater extent in FcR$\gamma^{-/-}$ mice than in wild-type (wt) mice (Fig. 1B), as previously reported for wt B16F10 metastases (12). This difference was not due to different homing of tumor cells to the lungs after B16-luc2$^+$ cell injection (Supplemental Fig. 1D, insert). mAb TA99, but not an isotype control mAb (Supplemental Fig. 1E), reduced the tumor load in wt mice, but not in FcR$\gamma^{-/-}$ mice (Fig. 1B), an effect reliant on the FcRγ ITAMs, as described previously (13). Notably, the quantification of B16-luc2$^+$ metastases using bioluminescence therefore reproduces data from previous studies using metastasis counts by eye (1, 6, 7), and may thus be applied to analyze the contribution of specific FcγRs to the mAb therapy of metastatic melanoma. CTX administration reduced the tumor load to a similar extent in both wt and FcR$\gamma^{-/-}$ mice (Fig. 1B). Importantly, the combination treatment (CTX+mAb TA99) was more efficient than the CTX treatment alone in wt mice, but not in FcR$\gamma^{-/-}$ mice. The combination treatment, however, was not more efficient in wt mice than the administration of TA99 mAb alone. These results indicate that FcγRs contribute to both single mAb therapy and CTX+mAb combination therapy in this model.

First, we examined the contribution of FcγRI to mAb TA99 therapy of B16-luc2$^+$ metastases. Injection of B16-luc2$^+$ cells led to similar tumor loads in wt and FcγRI$^{-/-}$ mice that were significantly reduced after mAb TA99 injections in both mouse strains (Fig. 1C). The TA99-mediated reduction in tumor load was, however, less profound in FcγRI$^{-/-}$ mice compared with wt mice. This result indicates that FcγRI contributes to TA99-mediated reduction of B16 lung metastases, in accordance with the report by Bevaart et al. (7). Although no contribution of FcγRI was identified by Nimmerjahn et al. (6), our method of measuring tumor load may show in this study subtle differences that could not be revealed previously. To confirm our finding, we also investigated the contribution of FcγRI to this mAb therapy model in novel mice deficient for all IgG receptors except FcγRI (FcγRIIB/III/IV deficient). These "FcγRI-only" mice had partial reduction of tumor load after mAb TA99 injections (Fig. 1C). Whereas the kinetics of tumor growth was similar in both types of mice, TA99-mediated reduction in tumor load tended to be less efficient in "FcγRI-only" mice than in wt mice (Supplemental Fig. 2A). FcγRI therefore partially mediates the protective effect of mAb TA99.

We then reassessed the contribution of FcγRIV to this model. The injection of anti-FcγRIV mAbs had no significant effect on TA99-mediated tumor reduction in wt mice (Fig. 1D), similar to its isotype control (Supplemental Fig. 2B). Various FcγRIV-dependent disease models were abolished using the same dose of the same batch of anti-FcγRIV mAbs (data not shown and Refs. 8, 14), indicating that FcγRIV was efficiently and systemically blocked in vivo. Notably, this mAb does not, by itself, induce FcγRIV-mediated cell activation in vitro (9). Our in vivo result is unexpected because injections of the same dose of this blocking mAb were reported to partially impair the protective effect of TA99 (6). To ascertain the lack of contribution of FcγRIV to this Ab

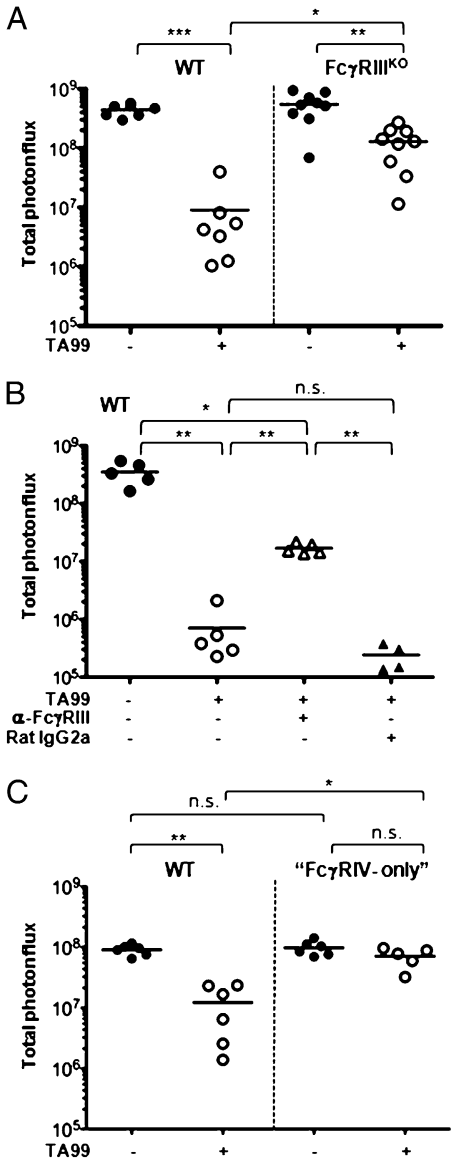

FIGURE 2. FcγRIII and FcγRI are responsible for mAb therapy to metastatic melanoma. Indicated mice were injected with B16-luc2$^+$ cells on day 0 and with mAb TA99 when indicated. Quantification of tumor load on explanted lungs at day 11 in (**A**) wt ($n \geq 7$) and FcγRIII$^{-/-}$ mice ($n = 9$), two identical experiments were compiled; (**B**) wt mice injected or not with anti-FcγRIII mAb ($n = 5$) or isotype control ($n = 4$); (**C**) wt ($n = 6$) and "FcγRIV-only" mice ($n \geq 5$). Data are representative of two (A, C) or four (B) independent experiments. Not significant (n.s.): $p > 0.05$, $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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therapy, we compared wt and FcγRIV$^{-/-}$ mice. No difference in tumor rejection efficacy between wt and FcγRIV$^{-/-}$ mice could be detected after mAb TA99 injections (Fig. 1E). These results demonstrate that FcγRIV does not contribute to this model of Ab therapy, in agreement with results reported by Bevaart et al. (7), but in contradiction to Nimmerjahn et al. (6).

Considering that FcγRI contributes only partially to the reduction of tumor load (Fig. 1C) and because FcγRIV does not contribute (Fig. 1D, 1E), we investigated whether the last mouse activating IgG receptor FcγRIII may contribute to TA99-mediated reduction of B16 lung metastases. mAb TA99 injections reduced tumor loads in both wt and FcγRIII$^{-/-}$ mice, but this effect was significantly impaired in FcγRIII$^{-/-}$ mice (Fig. 2A). This result is unexpected because it has been reported that wt and FcγRIII$^{-/-}$ mice display similar numbers/scores of B16 metastases after mAb TA99 injections (6, 7). To validate our finding, we took advantage of anti-FcγRIII mAbs that we have described previously, which specifically block FcγRIII in vivo (8), and do not induce FcγRIII-mediated cell activation in vitro (Supplemental Fig. 2C, 2D). Treatment with anti-FcγRIII mAb, but not isotype control, significantly impaired, but did not abolish, TA99-mediated reduction of tumor load in wt mice (Fig. 2B). These results indicate that FcγRIII contributes to TA99-mediated therapy of B16 lung metastases.

Results obtained using FcγRI$^{-/-}$ mice or "FcγRI-only" mice (Fig. 1C, Supplemental Fig. 2A), and FcγRIII$^{-/-}$ mice or anti-FcγRIII mAbs (Fig. 2A, 2B) suggest that FcγRI and FcγRIII may contribute nonredundantly to the protective effect of mAb TA99. The intermediate effect of mAb TA99 on the tumor load observed in FcγRI$^{-/-}$ or in FcγRIII$^{-/-}$ can be deduced to rely on the remaining activity of FcγRIII and FcγRI, respectively. Unfortunately, no mAb exists that blocks FcγRI to test this assumption in FcγRIII$^{-/-}$ mice. To investigate whether FcγRI and FcγRIII may be together responsible for TA99-mediated reduction in tumor load, we used mice expressing no activating IgG receptors except FcγRIV, "FcγRIV-only" mice (9). These mice were unable to mediate TA99-mediated reduction in tumor load (Fig. 2C). Furthermore, FcγRI$^{-/-}$ FcγRIIB$^{-/-}$ FcγRIII$^{-/-}$ triple-deficient mice were also unable to mediate the protective effect of mAb TA99 (data not shown).

Altogether, our results provide compelling evidence that FcγRI and FcγRIII are both responsible for the protective effect of anti-gp75 mAb on mouse B16 melanoma lung metastases. Using mice deficient for activating FcγRs, or blocking anti-FcγR mAbs, we identified a contribution by FcγRIII, reaffirmed the contribution of FcγRI (7), and by three different approaches excluded any contribution of FcγRIV to this immunotherapy model. Indeed, FcγRIII$^{-/-}$ mice and "FcγRIV-only" mice express higher levels of FcγRIV (5, 8, 15); however, these mice demonstrated reduced or undetectable TA99-mediated reduction in lung metastases load, also implying that FcγRIV does not contribute. Interestingly, it has been reported that the mAb TA99-mediated reduction of B16-F10 liver metastases relies on redundant functions of FcγRI and FcγRIV (16), whereas that of B16-F10 solid s.c. tumors relies only on FcγRIV (5). In particular, the mAb TA99-mediated effect was unaffected in both reports in mice deficient for both FcγRI$^{-/-}$ and FcγRIII$^{-/-}$. It remains to be determined why the s.c. solid tumor model relies on FcγRIV (5), the liver metastases model relies on either FcγRI or FcγRIV (16), and the lung metastases model relies on additive roles of FcγRI and FcγRIII (this report), but these discrepancies may be explained by different effector populations mediating tumor clearance in skin, liver, and lung, respectively, and therefore on the IgG receptors they express.

The contribution of FcγRI (CD64) to lung metastases clearance has been reported earlier (7) and is confirmed in this study using FcγRI$^{-/-}$ mice. Importantly, we demonstrate in this article for the first time, to our knowledge, that "FcγRI-only" mice retain the ability to reduce tumor load in this mAb therapy model. A role for FcγRIII (CD16) in contributing to metastases clearance was, however, unexpected based on previous reports (6, 7) but is supported by a report on the contribution of FcγRIII to the protective effect of anti-CD20 mAbs on the growth of CD20-expressing lymphoma (17). In concordance with these results obtained in the mouse, polymorphisms in the gene encoding human FcγRIIIA (CD16A), the human functional homolog of mouse FcγRIII, indeed correlate with the therapeutic efficacy of rituximab (a chimeric anti-CD20 mAb) on non-Hodgkin's lymphoma (18), and of trastuzumab (a humanized anti–HER-2 mAb) on HER-2^{+} metastatic breast cancer (19). Two of our findings have important repercussions for the validity of the mouse as an animal model to study Ab-mediated cancer immunotherapy: 1) that FcγRs contribute to both single mAb therapy and combination (CTX+mAb) therapy; and 2) that mouse FcγRIII, like human FcγRIIIA, contributes to tumor clearance. Furthermore, our result supports the current trend in therapeutic Ab engineering of aiming to improve specific binding to human FcγRIIIA (CD16A) to increase Ab antitumor efficacy.

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Disclosures

L.E.M. and A.J.M. are employees of Regeneron Pharmaceuticals, Inc. The other authors have no conflicts of interest.

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Corrections

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The seventh author was omitted from the article. An affiliation was also omitted from the article. The corrected author and affiliation lines are below.

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