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Cutting Edge

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Cutting Edge: The RIG-I Ligand 3pRNA Potently Improves CTL Cross-Priming and Facilitates Antiviral Vaccination

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Protective immunity against intracellular pathogens involves the induction of robust CTL responses. Vaccination with protein Ags establishes such responses only when combined with immune-stimulatory adjuvants. In this study, we compared different adjuvants and identified triphosphate RNA (3pRNA) as especially effective at inducing CTL responses. 3pRNA sensing required IPS-1/MAVS signaling and induced type I IFN in plasmacytoid dendritic cells and macrophages, with the latter being more important for the adjuvant effect. Type I IFN acted on CD11c$^+$ cells, especially on CD8α$^+$ Batf3-dependent dendritic cells. Vaccination with OVA in combination with 3pRNA protected mice from a subsequent OVA-encoding adenovirus infection in a CD8$^+$ cell–dependent manner and more efficiently than other adjuvants. In summary, 3pRNA is a superior adjuvant for CTL activation and might be useful to facilitate antiviral immunization strategies. ***The Journal of Immunology*****, 2016, 196: 2439–2443.**

The induction of protective CTL responses by vaccination requires adjuvants that activate dendritic cells (DCs) to express costimulatory molecules. Effective adjuvants mimic the presence of pathogens by triggering immune sensing receptors. TLR ligands like CpG, polyinosinic-polycytidylic acid (polyI:C), or LPS are particularly well studied immune-sensing receptor ligands (1). Another signal indicating the presence of intracellular microbes is 5′ triphosphate RNA (3pRNA), which is produced by eukaryotic cells, but normally does not reach the cytosol due to nuclear posttranscriptional processing. During viral infection and replication, however, 3pRNA is present in the cytosol, where it binds to the cytosolic RNA helicase retinoic acid–inducible gene I (RIG-I) (2). RIG-I is expressed by many cell types and especially in immune cells (3). Upon ligand binding, RIG-I signals via the adaptor mitochondrial antiviral-signaling protein (MAVS, also known as IPS-1, Cardiff, or VISA) (4–6), which stimulates production of type I IFN (IFN I), a mediator critical for CTL responses (7).

CTL responses are induced by CD8α$^+$ DCs, which can cross-present exogenous Ag in the context of MHC class I molecules (8). Development of these DCs under homeostatic conditions requires the transcription factor Batf3, and Batf3-deficient mice have been used to unravel the role of CD8α$^+$ DCs in immune responses (9). In prolonged inflammation, the transcription factors Batf2 and Batf can compensate for Batf3 and induce CD8α$^+$ DC differentiation (10).

In this study, we tested different adjuvants and identified 3pRNA as especially competent in inducing CTL responses, with implications for antiviral immunization strategies.

Materials and Methods

Reagents and mice

Reagents were from Sigma-Aldrich unless specified otherwise. Mice were bred under specific pathogen-free conditions at the animal facility of Bonn University Clinic and used at 8–12 wk of age. Mixed bone marrow chimeras were generated as described (11). OVA (200 μg) was combined with 20 μg of CpG ODN1668 (MolBiol), 20 μg of polyI:C (Invitrogen), 5 μg of 3pRNA (TranscriptAid T7 High Yield Transcription Kit; Fermentas), or 5 μg of pCA (Biomers) complexed to 0.8 μl in vivo JetPEI (Polyplus Transfection). CD8$^+$ cells were depleted by injecting 300 μg of YTS 169.4 Ab (BioXCell), which caused a reduction of ∼90% of CTL.

Miscellaneous measurements

Flow cytometry was performed as described (11, 12). To measure in vivo cytotoxicity, splenocytes were either pulsed with 2 μg/ml SIINFEKL and labeled with 1 μmol of CFSE (CFSE^{high} cells), or unpulsed splenocytes were labeled with 0.1 μmol of CFSE (CFSE^{low} cells). A total of 1×10^7 of both target cell types was coinjected i.v., and after 4 h, their survival was analyzed by flow cytometry using the formula: percent specific

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Abbreviations used in this article: DC, dendritic cell; IFN I, type I IFN; MAVS, mitochondrial antiviral-signaling protein; pDC, plasmacytoid dendritic cell; polyI:C, polyinosinic-polycytidylic acid; 3pRNA, triphosphate RNA; RIG-I, retinoic acid–inducible gene I; wt, wild-type.

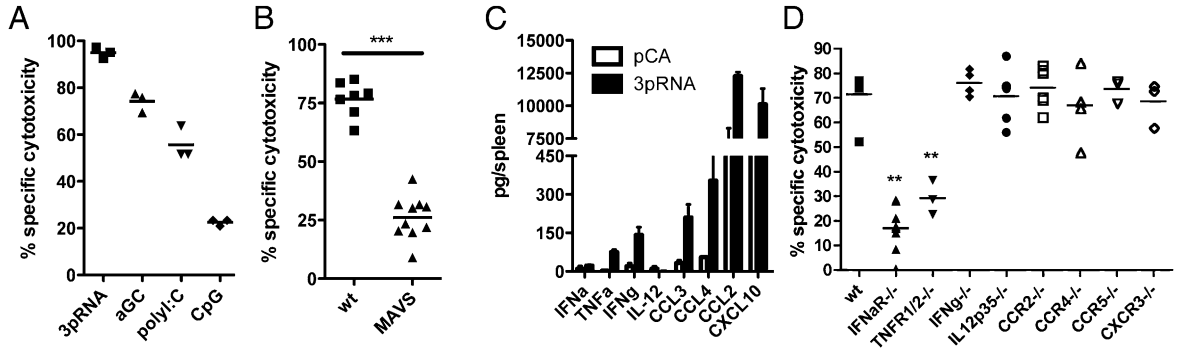

FIGURE 1. Immunization with 3pRNA induces a strong CTL response that depends on MAVS and IFN I. (**A**) wt mice were immunized with OVA and different adjuvants (5 μg 3pRNA, 0.2 μg anti-GC, 50 μg polyI:C, or 20 μg CpG), and the CTL response was assessed on day 5. (**B**) wt and MAVS$^{-/-}$ mice were immunized with OVA and 5 μg 3pRNA, and the CTL response was determined on day 5. (**C**) Mice were immunized with 5 μg pCA or 3pRNA after 24 h, and cytokine concentrations were determined in spleen homogenates. (**D**) wt and mice lacking cytokines or chemokine receptors were analyzed as in (B). Data were combined from or represent at least two individual experiments with three to five mice per group. Statistical significance was tested with the unpaired *t* test (B) or one-way ANOVA with Dunnett posttest (D). $^{**}p < 0.01$, $^{***}p < 0.001$.

cytotoxicity = 100 − (100 × [CFSE^{high}/CFSE^{low}]$_{primed}$/[CFSE^{high}/CFSE^{low}]$_{control}$). Cytokines were measured by Flow Cytomix Kits (Bender MedSystems) or ELISA (PBL Assay Science). OVA-specific serum IgG titers were analyzed by a home-made ELISA 20 d after immunization (13). For RT-PCR, RNA from FACS-sorted splenocyte subsets was extracted and reverse transcribed into cDNA using High Capacity RT kit (Applied Biosystems). Quantitative PCR was performed for 40 cycles using SYBR Green in triplicates and normalized to GAPDH.

Recombinant adenovirus infection and analysis

E1- and E3-deleted adenoviral vectors expressing fusion proteins of enhanced GFP, OVA, and click-beetle luciferase (AdLGO) were generated by Cre-lox recombination as described (14). AdLGO was propagated on HEK 293 cells and purified by cesium chloride density-gradient centrifugation. Mice were i.v. infected with 5×10^6 PFU. Infection was monitored by i.p. injection of Luciferin (50 mmol) and measuring luminescence in anesthetized mice with an IVIS 200 as described (15). Data were analyzed with Living Image 2.50.1 software (Caliper LifeSciences).

Statistical analysis

Results are expressed as mean ± SEM and compared by unpaired Student *t* test, Mann–Whitney *U* test, or one-way ANOVAs (one-way ANOVA) with Dunnett posttest. The *p* values were expressed as: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

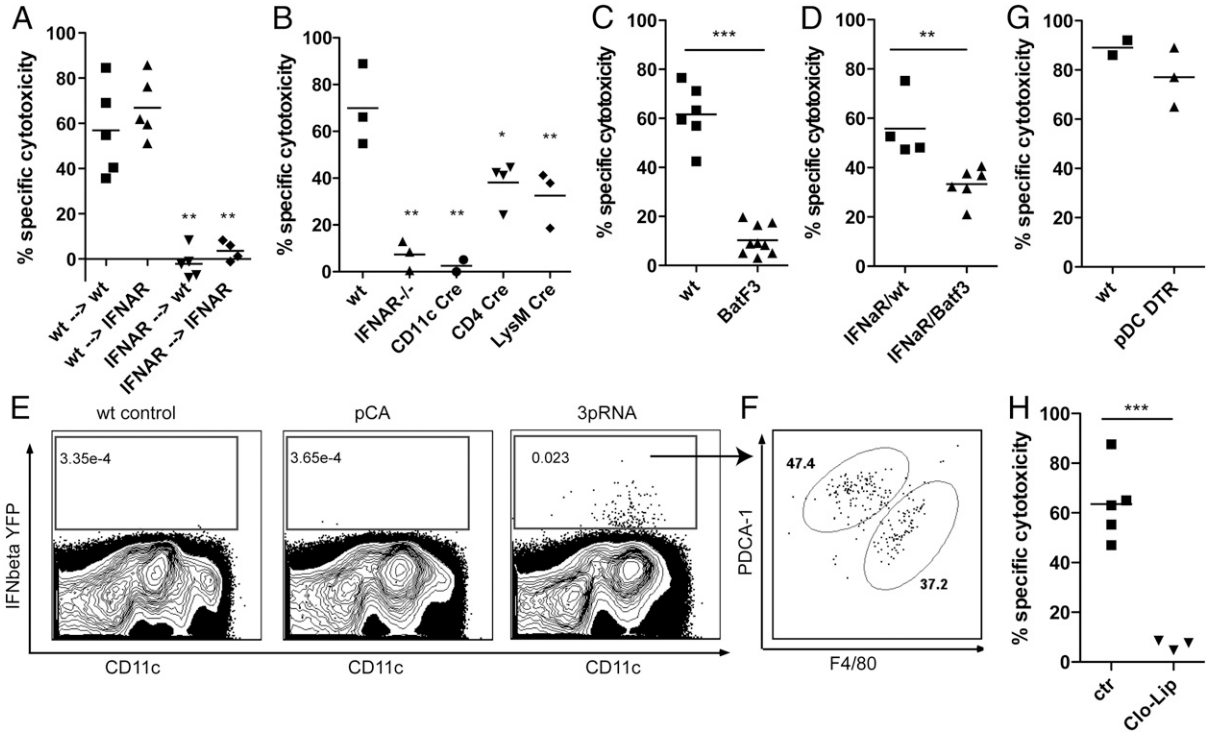

FIGURE 2. Adjuvanticity of 3pRNA requires IFN I by macrophages sensed by CD11c^{+} cells. Bone marrow chimeras (**A**), cell type–specific IFNaR$^{-/-}$ mice (**B**), or Batf3$^{-/-}$ mice (**C**) were vaccinated with OVA and 3pRNA, and CTL response was assessed on day 5. (**D**) Sublethally irradiated IFNaR$^{-/-}$ mice were reconstituted with a 1:1 mixture of IFNaR$^{-/-}$ and Batf3$^{-/-}$ bone marrow or of IFNaR$^{-/-}$ and wt bone marrow as control. Cytotoxicity after immunization with OVA and 3pRNA was assessed. (**E**) wt and IFN-β$^{mob/mob}$ mice were immunized with pCA or 3pRNA. After 24 h, YFP expression in spleens was analyzed by flow cytometry, excluding autofluorescent CD3^{+} and CD19^{+} cells. (**F**) YFP^{+} cells include pDC Ag-1 (PDCA-1)$^{+}$ and F4/80^{+} cells. (**G**) pDC-DTR mice and non-transgenic littermates were injected with 1 ng diphtheria toxin on days −2 and −1, immunized on day 0, and the CTL response was analyzed on day 5. (**H**) wt mice were injected with 200 μl clodronate liposomes (Clo-Lip) 7 d prior to immunization, and the CTL response was analyzed on day 5. Data combined from or represent at least two individual experiments with three to five mice per group. Statistical significance was tested with the unpaired *t* test (C, D, and H) or one-way ANOVA with Dunnett posttest (A and B). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. ctr, control.

Results and Discussion

3pRNA induces particularly strong CTL responses that depend on MAVS-1 and IFN I signaling

We compared the adjuvanticity of 3pRNA to different immune-sensing receptor ligands in a vaccination model based on the model Ag OVA. We first determined by titration a dose of 5 μg 3pRNA as the lowest dose achieving the maximal CTL response (Supplemental Fig. 1A). This response was greater that the maximal response achieved with optimal doses of other adjuvants (16, 17) (Fig. 1A). 3pRNA had to be applied through an in vivo transfection reagent (Supplemental Fig. 1B), because it is recognized by the cytosolic helicase RIG-I. The transfection reagent itself did not show adjuvant activity (Supplemental Fig. 1C). CTL responses were strongly diminished in MAVS$^{-/-}$ mice (Fig. 1B), consistent with RIG-I signaling through this adaptor.

Many proinflammatory cytokines and chemokines were increased in spleen homogenates from 3pRNA-injected mice (Fig. 1C). However, CTL responses were unimpaired in mice deficient for most of these mediators, notably IFN-γ or ligands of the chemokine receptors CCR2, CCR4, CCR5, or CXCR3 that are induced by 3pRNA (Fig. 1D), probably because of redundancy. This is in contrast to other adjuvants such as CpG or anti-GC that depend on CCR5 and CCR4, respectively (16, 18), and hinted at an especially broad adjuvanticity of 3pRNA. IL-12 was not induced by 3pRNA, and accordingly, CTL responses were not reduced in IL-12–deficient mice (Fig. 1C, 1D). These responses were reduced partially in TNFR1/2-deficient mice and completely depended on IFN I signaling (Fig. 1D). CTL responses were unchanged in TLR7$^{-/-}$, Asc$^{-/-}$, and Nlrp3$^{-/-}$ mice (Supplemental Fig. 1D). Of note, IFN I levels in serum and spleen were significantly higher in mice immunized with OVA and 3pRNA compared with mice immunized with OVA and polyI:C or CpG (Supplemental Fig. 1E, 1F), which might explain its superior adjuvant activity, given that IFN I stimulates CTL responses (7).

IFN I signaling in CD11c hematopoietic cells is important

We next identified the IFN I target cell(s). We first generated bone marrow chimeras in which the receptor was either lacking on radio-resistant stromal cells or radio-sensitive cells of the hematopoietic compartment. CTL cytotoxicity was strongly reduced in mice lacking IFNaR on hematopoietic cells, whereas IFNaR unresponsiveness of stroma cells did not influence it (Fig. 2A). To further characterize the responsive hematopoietic cells, we used cell type–specific IFNaR$^{-/-}$ mice, lacking IFNaR on T cells (CD4-Cre), macrophages and granulocytes (LysM-Cre), or DCs (CD11c-Cre). Cytotoxicity after priming with 3pRNA was moderately diminished in IFNaR$^{flox/flox}$ × CD4-Cre and IFNaR$^{flox/flox}$ × LysM-Cre mice, consistent with previously shown direct effects of IFN I on T cells and macrophages (19, 20). Notably, cytotoxicity was completely abrogated in IFNaR$^{flox/flox}$ × CD11c-Cre mice (Fig. 2B), suggesting that DC were the main IFN I targets. This is in line with a study showing that IFNaR expression by CD11c^{+}, but not on LysM^{+}, cells was necessary for CTL-mediated tumor rejection (21). However, CD11c is expressed also on some macrophages and on some activated CTL, suggesting that effects on these cells might also contribute in our model, consistent with previous findings (7, 22). DC maturation after 3pRNA vaccination depended on IFN I signaling and mainly affected cross-presenting CD8α^{+} DCs (Supplemental Fig. 2A, 2B). In line with this, CD8α^{+} DCs were essential in our setting, as Batf3$^{-/-}$ mice failed to mount a specific CTL response in response to 3pRNA vaccination (Fig. 2C).

To test whether IFNaR on CD8α^{+} DCs was important, we generated mixed bone marrow chimeras in which all cell types except Batf3-dependent CD8α^{+} DCs expressed IFNaR. To this end, we reconstituted irradiated IFNaR$^{-/-}$ mice with a 1:1 mixture of IFNaR$^{-/-}$ and Batf3$^{-/-}$ bone marrow. CTL activation was indeed diminished in IFNaR/Batf3 compared with IFNaR/wild-type (wt) chimeras (Fig. 2D). However, the CTL response was not as markedly reduced as in IFNaR$^{flox/flox}$ × CD11c Cre mice (Fig. 2B), suggesting that IFN I acts partially on Batf3-independent CD11c^{+} cells. The transcription factors Batf and Batf2 can compensate for the lack of Batf3 in prolonged inflammation (10), and irradiation causes cell death and thereby inflammation (23), suggesting that such compensation might occur upon bone marrow chimera generation. To investigate this idea, we generated congenic mixed bone marrow chimeras (CD45.1/CD45.2.Batf3$^{-/-}$ → CD45.1). After 8 wk, we detected CD45.1^{+}CD8α^{+} DCs,

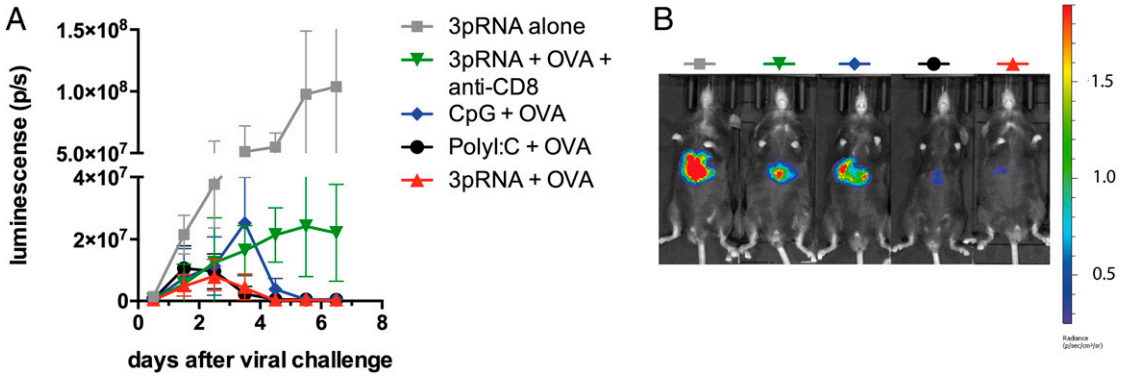

FIGURE 3. Vaccination with OVA and 3pRNA protects against adenoviral hepatitis. (**A**) Mice were vaccinated with 3pRNA alone (gray), CpG+OVA (blue), polyI:C+OVA (black), 3pRNA+OVA (red), or 3pRNA+OVA plus CD8^{+} cell depletion 3 d before infection (green) and challenged with adenovirus expressing OVA and Luciferase (5×10^{6} PFU) on day 40. Viral clearance was monitored daily by injecting luciferin and measuring luminescence by IVIS. (**B**) Representative IVIS images of mice from (A) on day 3.5 after viral challenge. Luminescence scale is 10^{6} photons/s/cm^{2}/sr. Data represent two individual experiments with four mice per group. p/s, photons per second.

which can be of host or wt donor origin, but also some CD45.1$^-$CD8α^+ DCs (Supplemental Fig. 2C), which must be derived from Batf3$^{-/-}$ donors. This indicated that the procedure of bone marrow chimera generation induced differentiation of CD8α^+ DCs also from Batf3$^{-/-}$ cells. If this had occurred also in our IFNaR$^{-/-}$Batf3$^{-/-}$ mixed bone marrow chimeras, some of their CD8α^+ DCs would still be able to sense IFN I, and these cells might have caused the small residual CTL response we detected (Fig. 2D). In this case, our results would underestimate the importance of IFN I signaling in CD8α^+ DCs. This issue may be generally relevant in irradiation chimeras using Batf3-deficient bone marrow. In conclusion, IFN I signaling in CD8α^+ DCs was particularly important for the adjuvanticity of 3pRNA.

Macrophage-derived IFN I is important

To identify the IFN I–producing cell type(s), we used IFN-$\beta^{mob/mob}$ mice, which report IFN-β–producing cells by YFP fluorescence (24). Twenty-four hours after 3pRNA injection, spleens of IFN-$\beta^{mob/mob}$ mice harbored some YFP$^+$ cells, as opposed to 3pRNA-treated wt and pCA (control RNA)-treated IFN-$\beta^{mob/mob}$ mice (Fig. 2E). YFP$^+$ cells showed intermediate CD11c expression and included plasmacytoid DC (pDC) Ag-1$^+$ and F4/80$^+$ cell populations (Fig. 2F), representing pDCs and macrophages, respectively. Quantification of IFN-β–encoding mRNA by RT-PCR in sorted splenocyte subsets confirmed IFN I expression by CD11c^{int}B220$^+$Ly6C$^+$ pDCs and F4/80$^+$ macrophages, but not by CD11c$^+$CD8α^+ DCs, CD11c$^+$CD8α^- DCs or NK1.1$^+$CD3$^-$ NK cells (Supplemental Fig. 2D). This is consistent with previously reported IFN I production in response to microbial stimuli by these cell types (25, 26). Thus, pDCs and macrophages responded to 3pRNA by IFN I production.

To clarify the importance of IFN I production by these cells, we first depleted pDCs using pDC-DTR mice and immunized them with 3pRNA and OVA. After 5 d, specific cytotoxicity was not significantly reduced compared with diphtheria toxin–injected controls (Fig. 2G). In contrast, depletion of macrophages by clodronate liposomes prior to immunization significantly reduced CTL cross-priming (Fig. 2H), indicating that IFN I from macrophages was more important than that from pDCs for the adjuvant effect by 3pRNA.

Vaccination with 3pRNA accelerates adenoviral infection clearance

Finally, we assessed the efficacy of 3pRNA immunization in an infection model. We used a replication-deficient adenovirus expressing luciferase and OVA in infected hepatocytes (15). We immunized wt mice with 3pRNA+OVA or 3pRNA alone. To compare protective immunity of 3pRNA to that of other adjuvants and assess dependence on CTL, we additionally immunized wt mice with polyI:C+OVA or CpG+OVA or depleted wt mice of CD8 T cells and infected them 40 d later. Mice immunized with 3pRNA alone still showed strong bioluminescence as a sign of ongoing infection on day 5.5 postinfection, whereas mice immunized with 3pRNA+OVA had lost bioluminescence on day 3.5 (Fig. 3A, 3B), indicating viral clearance. CpG was clearly inferior as adjuvant than 3pRNA (Fig. 3A, 3B). Poly I:C, which possesses very strong adjuvant activity in CTL priming (27), was able to clear the virus, but it was inferior to 3pRNA with respect to controlling peak bioluminescence (Fig. 3A, 3B, Supplemental Fig. 2E). Also, the specific CTL response at the time of analysis was stronger after 3pRNA+OVA than after PolyI:C+OVA (Fig. 3B), supporting the conclusion that this vaccination scheme induced antiviral protection most potently (Supplemental Fig. 2F).

3pRNA was ineffective when CD8$^+$ cells were depleted 3 d before viral infection (Fig. 3A, 3B, Supplemental Fig. 2E), indicating dependence on CTL. Even though OVA-specific Abs were elevated in mice immunized with 3pRNA and OVA (Supplemental Fig. 2G), a role of Abs is unlikely in our model, because OVA is not packaged into the virus during assembly (15), so that OVA-specific Abs cannot neutralize the adenovirus we used. Lysis of infected hepatocytes by OVA-specific Abs would require their binding to the hepatocyte surface, which is unlikely because OVA is a secreted protein. This does not exclude that 3pRNA can enhance Ab responses in other models.

In conclusion, we provided the first in vivo evidence, to our knowledge, that 3pRNA enhances CTL responses and demonstrated its potent adjuvant effect in antiviral vaccination. As 3pRNA emerged as superior to the other adjuvants tested in our vaccination model, our findings may have implications for clinical immunization strategies.

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Disclosures

G.H. is a founder of Rigontec. The other authors have no financial conflicts of interest.

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