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Seasonal FluMist Vaccination Induces Cross-Reactive T Cell Immunity against H1N1 (2009) Influenza and Secondary Bacterial Infections

Keer Sun,* Jianqiang Ye,†‡ Daniel R. Perez,†‡ and Dennis W. Metzger*

T cell epitopes have been found to be shared by circulating, seasonal influenza virus strains and the novel pandemic H1N1 influenza infection, but the ability of these common epitopes to provide cross-protection is unknown. We have now directly tested this by examining the ability of live seasonal influenza vaccine (FluMist) to mediate protection against swine-origin H1N1 influenza virus infection. Naïve mice demonstrated considerable susceptibility to H1N1 Cal/04/09 infection, whereas FluMist-vaccinated mice had markedly decreased morbidity and mortality. In vivo depletion of CD4+ or CD8+ immune cells after vaccination indicated that protective immunity was primarily dependent upon FluMist-induced CD4+ cells but not CD8+ T cells. Passive protection studies revealed little role for serum or mucosal Abs in cross-protection. Although H1N1 influenza infection of naïve mice induced intensive phagocyte recruitment, pulmonary innate defense against secondary pneumococcal infection was severely suppressed. This increased susceptibility to bacterial infection was correlated with augmented IFN-γ production produced during the recovery stage of H1N1 influenza infection, which was completely suppressed in mice previously immunized with FluMist. Furthermore, susceptibility to secondary bacterial infection was decreased in the absence of type II, but not type I, IFN signaling. Thus, seasonal FluMist treatment not only promoted resistance to pandemic H1N1 influenza infection but also restored innate immunity against complicating secondary bacterial infections.

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of fatal antibiotic-resistant bacterial infections (20). Furthermore, the efficiency of H1N1-specific vaccination in protecting the human population from secondary bacterial infection has yet to be established. To provide insight into effective prevention and treatment strategies, we have established mouse models to examine the ability of human S-OIV to enhance susceptibility to secondary bacterial infections and have determined the efficacy of seasonal live FluMist vaccine in protection against viral and secondary bacterial infections caused by S-OIV.

Materials and Methods

Murine model of viral and bacterial infection

Specific pathogen-free, 6- to 8-wk-old C57BL/6 and C3H/HeNcCr wild-type (WT) mice were purchased from Charles River Laboratories (Wilmington, MA). C57BL/6 IFN-γ- and IFN-αR−/− mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred at Albany Medical College following institutional animal care and use committee guidelines. BALB/c IFN-αR−/− mice were provided by Daniel Portnoy (University of California, Berkeley, CA) and bred at Albany Medical College.

Viral challenge was performed with H1N1 Cal/04/09 administered i.n. to anesthetized mice in 50 μl sterile PBS. Titers of virus were determined by plaque assays on Madin-Darby canine kidney cell monolayers. Bacterial co-infection was performed approximately 1 wk later, at the time when the mice began to recover from viral infection, as evidenced by weight regain. To induce bacterial pneumonia, anesthetized mice were inoculated i.n. with 50 μl containing Staphylococcus pneumoniae strain D39 or Staphylococcus aureus ATCC strain BAA-1692.

Mouse-adapted influenza virus

DBA mice were infected i.n. with 5.4 × 10^5 50% tissue culture infective dose of the H1N1 Cal/04/09 virus. Lung homogenates from the dead mice were used to infect BALB/c mice at an inoculum of 1.16 × 10^5 50% tissue culture infective dose. Lung homogenates from the dead BALB/c mice were then used to prepare a virus stock in Madin-Darby canine kidney culture infective dose. Lung homogenates from the dead BALB/c mice were used to infect BALB/c mice at an inoculum of 1.16 × 10^5 50% tissue culture infective dose. Lung homogenates from the dead BALB/c mice were then used to prepare a virus stock in Madin-Darby canine kidney cells.

Vaccination

Anesthetized mice were inoculated i.n. with 20 μl FluMist vaccine (2008–2009 formulation; MedImmune, Gaithersburg, MD) 4–5 wk before H1N1 influenza infection. BAL fluids were determined by ELISA at various days after H1N1 CA04 infection (Fig. 1).

Results

Human swine-origin H1N1 influenza infection in C57BL/6 mice

To establish the kinetics of S-OIV infection in mice, we monitored body weight and pulmonary viral growth after intranasal inoculation of C57BL/6 mice with a sublethal dose (3 × 10^3 PFU) of H1N1 CA04 influenza virus obtained from a human isolate (21). Continuous weight loss was observed within the first week of H1N1 infection, together with significant increases in viral lung titers until day 7 postinfection, at which point the virus was cleared with no detectable virus remaining by day 11 (Fig. 1A). Viral clearance was accompanied by full recovery of body weight. This course of human H1N1 CA04 infection is very similar to infection of mice with other influenza A virus strains, such as the mouse-adapted influenza A/PR/8/34 (PR8) virus (22). Also similar was the expression of H1N1 CA04-induced IFN-γ in BAL fluids, which reached maximum expression on day 7. However, no appreciable levels of TNF-α or IL-1β were detected after viral infection (Fig. 1B–D).

FluMist induces protection against novel pandemic H1N1 infection

FluMist vaccine was initially designed for protection against seasonal influenza infection. To determine whether this vaccine could also mediate protection against S-OIV infection, mice were challenged with H1N1 CA04 approximately 5 wk after FluMist vaccination. It was found that vaccination enhanced both H1N1 infection. Each point represents the mean ± SD. Student t test (to compare two samples) or ANOVA (to compare multiple samples) was used for statistical analysis. Survival analyses were performed using the Kaplan–Meier log-rank test. A p value <0.05 was considered to be significant. Statistical procedures were performed using GraphPad InStat 3 software.

FIGURE 1. Kinetics of H1N1 CA04 infection in C57BL/6 mice. A, C57BL/6 mice were infected with 3 × 10^3 PFU H1N1 CA04 influenza virus. Total weight and viral PFU in the lungs were measured at the indicated time points. B–D, Levels of IFN-γ, TNF-α, and IL-1β in BAL fluids were determined by ELISA at various days after H1N1 CA04 infection. Each point represents the mean ± SD of five mice.
intranasal infection with 10^3 PFU H1N1 influenza virus that had been mixed before infection with FluMist. Ab levels in BAL fluids and sera (Fig. 2C) consisted of nearly equal proportions of CD4+ and CD8+ T cells (Fig. 2D). Transfer experiments were conducted using both serum and BAL fluid at the time of infection likewise did not increase pulmonary viral clearance nor prevent weight loss after infection with 10^3 PFU H1N1 influenza virus that had been mixed before infection with 10% serum from naive (NMS) or FluMist-treated (IMS) mice.

No significant induction of TNF-α or IL-1β was found after H1N1 CA04 infection regardless of vaccination status (Fig. 3B). Of interest, production of IFN-γ was increased after influenza infection of naive mice, but this was not observed in vaccinated mice. Significant, albeit limited, production of IFN-γ could be detected in mice depleted of CD4+ cells after FluMist vaccination (Fig. 3B). In these mice, because immune cell analysis was performed 3 wk after the final dose of depleting Ab, there was reappearance of T cells in the respiratory tract at levels similar to those seen in depleted, unvaccinated mice but less than the levels seen in nondepleted, vaccinated mice (Fig. 3A). Specifically, after H1N1 infection, similar levels of CD4 T cells were found in the respiratory tracts of FluMist-treated mice that were depleted of memory CD4 cells compared with those of unvaccinated, naive mice. This suggests that the naive CD4 T cell population had been replenished in the mice by the time of challenge. Nevertheless, these CD4 T cells failed to improve viral clearance, even in the presence of FluMist-induced memory CD8 T cells and non-neutralizing Abs. The CD8 T cell population was not fully replenished after depletion of memory CD8 T cells, but this did not compromise the effectiveness of viral clearance observed in these animals (Fig. 3A). Although all groups of FluMist-vaccinated mice maintained their body weight after viral challenge (Fig. 3C), viral burden was significantly greater in FluMist-treated mice depleted of memory CD4+ cells compared with that in control rat IgG-treated mice or mice depleted of memory CD8+ cells (Fig. 3D). Taken together, these results indicate that FluMist-
induced CD4+ T cells are the crucial effectors in mediating efficient viral clearance after H1N1 CA04 infection, whereas CD8+ cells appeared to be dispensable. This finding indicates that seasonal FluMist vaccination generates considerable levels of memory CD4 T cells that are cross-reactive with S-OIV.

**H1N1 infection results in enhanced susceptibility to secondary bacterial infection**

To establish directly whether S-OIV infection predisposes individuals to lethal secondary bacterial infection, we followed an experimental protocol similar to our previous PR8 studies (22). C57BL/6 mice were infected i.n. with a sublethal dose of H1N1 CA04 and then were challenged i.n. 8 d later (i.e., during the beginning of the viral recovery phase) with *S. pneumoniae* strain D39 (24). We previously reported that this time point after PR8 influenza virus infection leads to significantly increased susceptibility to secondary pneumococcal infection (22). It was found that normal (non-influenza-infected) mice cleared nearly 99% of bacteria from the lungs within 4 h after pneumococcal challenge, whereas influenza-infected mice were defective in clearance, and this defect was particularly evident 24 h after bacterial infection (Fig. 4A). Furthermore, lethal synergy was observed after H1N1 CA04 and *S. pneumoniae* co-infection (Fig. 4B).

To determine whether IFN-γ signaling during H1N1 CA04 infection suppresses innate defenses against secondary bacterial infection, analogous to results observed in our previous studies with mouse-adapted PR8 influenza virus (22), C57BL/6 WT and IFN-γR−/− mice were co-infected with H1N1 CA04 and pneumococci. Similar viral titers were found in WT and IFN-γR−/− mice on day 8 after viral infection (Fig. 4C). However, significantly decreased bacterial burden was found in IFN-γR−/− mice 4 h after pneumococcal co-infection (Fig. 4D). In addition, survival studies showed that H1N1 CA04-infected IFN-γR−/− mice demonstrated no change in susceptibility to lethal viral infection alone compared with WT mice (Fig. 5A) but did show increased survival after viral and bacterial co-infection (Fig. 5B). Type I IFN signaling was recently reported to play a role in polymicrobial synergy, as mice deficient in expression of IFN-α/βR showed decreased susceptibility to secondary pneumococcal infection after PR8-mediated influenza (25). However, we found that IFN-α/βR−/− mice were not more resistant to secondary infection but were in fact significantly more susceptible to both H1N1 CA04 influenza infection (Fig. 5C) and bacterial co-infection (Fig. 5D). Taken together, these results indicate that lung IFN-γ signaling during H1N1 CA04 infection impairs innate defenses against secondary bacterial infection.

**FluMist induces protection against secondary bacterial infection**

The above results established that FluMist vaccination can mediate protection against H1N1 CA04 infection. To determine whether FluMist could also alleviate susceptibility to secondary bacterial infections, FluMist-vaccinated mice were infected with H1N1 CA04 and then challenged i.n. approximately 1 wk later with pneumococci. It was found that vaccination enhanced viral clearance from the respiratory tract (Fig. 6A). In addition, clearance of pneumococci was significantly increased in virus-infected mice that were previously treated with FluMist (Fig. 6B). Protection from morbidity and mortality in vaccinated mice was also established by monitoring weight loss (Fig. 6C) and survival after co-infection (Fig. 6D). Notably, vaccinated WT and nonvaccinated IFN-γR−/− mice showed comparable survival after co-infection (Fig. 6D). Thus, whereas FluMist mediated protection against both influenza virus (Fig. 6C) and bacterial co-infection, IFN-γ only played a role during secondary pneumococcal infection and was detrimental, not protective. Of note, although the attenuated live virus in FluMist induced significant T cell recruitment into the respiratory tract, this cold-adapted virus was unable to replicate in the lungs, induced little IFN-γ expression, and had no effect on bacterial clearance in non-influenza-infected mice (data not shown).

**FIGURE 4.** H1N1 CA04 influenza infection decreases resistance of mice to subsequent bacterial co-infection. C57BL/6 mice were infected with 3 × 10^5 PFU H1N1 CA04 influenza virus. A, Numbers of bacteria in the lungs at 4 and 24 h after challenge of naive (−H1N1) or day 8 postinfluenza-infected (+H1N1) mice (5 mice/group) with 10^6 CFU *S. pneumoniae*. The data represent means ± SD. **p < 0.01; ***p < 0.001 (compared with mice not infected with influenza virus). B, Survival of naïve mice after infection with 10^6 CFU *S. pneumoniae* alone (D39) or 8 d after H1N1 CA04 infection (H1N1 plus D39) (5–11 mice/group). Survival of mice infected with H1N1 CA04 and challenged with PBS alone is also shown (H1N1 plus PBS). C and D, Numbers of viral PFU (C) and bacterial CFU (D) in lungs 4 h after infecting day 8 postinfluenza C57BL/6 WT and IFN-γR−/− mice with 10^6 CFU *S. pneumoniae* D39. *p < 0.05 (compared with influenza-infected WT mice).

**FIGURE 5.** Role of type II, but not type I, IFN in enhanced susceptibility to secondary pneumococcal infection. Survival of C57BL/6 WT, IFN-γ−/−, and IFN-γR−/− mice after infection with (A) 2000 PFU mouse-adapted H1N1 CA04 influenza virus only or (B) 100 PFU mouse-adapted H1N1 CA04 influenza virus followed by 10^5 CFU *S. pneumoniae* D39 8 d later. Survival of BALB/c WT and IFN-α/βR−/− mice with infection with (C) 2000 PFU mouse-adapted H1N1 CA04 influenza virus only or (D) 100 PFU mouse-adapted H1N1 CA04 influenza virus followed by 10^5 CFU *S. pneumoniae* D39 8 d later. Of note, there were differences in the susceptibility of C57BL/6 WT mice used in A and B compared with BALB/c WT mice in C and D.
lungs 24 h after infection of naive (mice with 10^7 CFU of influenza virus 5 wk later. Infection with 10^5 CFU represents the mean body weight of eight mice. Superinfection with pneumococci was performed on day 9 when the infected mice challenged with MRSA.

Because staphylococcal infection is likewise significantly complicated by preceding H1N1 influenza infection (16, 17), we determined whether FluMist could also prevent secondary staphylococcal infection. Viral and bacterial burdens in lungs were determined whether FluMist could also prevent secondary staphylococcal infection. Viral and bacterial burdens in lungs were examined 24 h after staphylococcal infection. The ability of FluMist vaccination to protect against H1N1 CA04 infection was not affected by methicillin-resistant *Staphylococcus aureus* (MRSA) co-infection (Fig. 7A). In addition, FluMist vaccination restored innate immune protection against MRSA close to the levels seen in mice not previously infected with virus (Fig. 7B). Thus, comparable protection by FluMist was observed in H1N1 CA04-infected mice challenged with MRSA.

**FIGURE 6.** Seasonal FluMist vaccination protects against H1N1 CA04 influenza and secondary pneumococcal infection. C57BL/6 mice were inoculated i.n. with 20 μl FluMist and 4 wk later were infected with 3 × 10^5 PFU H1N1 CA04 influenza virus. A and B, Viral (A) and bacterial (B) burdens in BAL fluid 4 h after infection of naive (−FluMist) and FluMist-treated (+FluMist) mice with 10^5 CFU *S. pneumoniae* 8 d after influenza infection. C and D, C57BL/6 WT and INF-γR−/− mice, either naive or FluMist-treated, were infected i.n. with 3 × 10^5 PFU H1N1 CA04 influenza virus. Superinfection with pneumococci was performed on day 9 when the mice began to regain weight after primary viral infection (dashed vertical line). C, Animal weights after H1N1 influenza infection; each point represents the mean body weight of eight mice. D, Survival of mice after infection with 10^5 CFU *S. pneumoniae* D39 9 d after influenza infection. *p < 0.05; ***p < 0.001 [compared with WT mice in the absence of FluMist treatment (A, B)].

**FIGURE 7.** Seasonal FluMist vaccination protects against H1N1 CA04 influenza and secondary MRSA infections. C3H/HeNcr mice were inoculated i.n. with 20 μl FluMist and infected with 3 × 10^5 PFU H1N1 CA04 influenza virus 5 wk later. A and B, Viral PFU (A) and bacterial CFU (B) in lungs 24 h after infection of naive (−FluMist) or FluMist-treated (+FluMist) mice with 10^5 CFU *S. aureus* BAA-1692 (MRSA) on day 7 after influenza infection. Also shown in A is viral PFU in influenza-infected mice challenged with PBS instead of *S. aureus* as a control for the effect of bacterial infection on viral clearance, and also shown in B is bacterial CFU in the absence of preceding influenza infection. Data represent means ± SD. ***p < 0.001 (compared with WT mice in the absence of FluMist treatment).

**Discussion**

Our results directly show that vaccination with seasonal FluMist vaccine can significantly enhance T cell protective immunity against novel H1N1 CA04 influenza virus despite the distinct antigenic properties of the pandemic virus compared with those of seasonal H1N1 virus. The protection was dependent upon the presence of FluMist-induced CD4+ cells but not CD8+ cells or Abs. Moreover, FluMist protected mice against secondary pneumococcal and MRSA infections, which can be exacerbated in humans by previous H1N1 influenza infection. We have demonstrated previously that T cell-derived IFN-γ expressed during adaptive immune responses to PR8 influenza virus infection modifies alveolar macrophage scavenger receptor expression and temporarily inhibits innate pulmonary defenses against pneumococcal infection (22). Results from the current study suggest that similar mechanisms are used by the pandemic H1N1 CA04 influenza virus. Importantly, as a result of controlling virus replication and inhibiting local IFN-γ production, seasonal FluMist vaccine was found to provide efficient immune protection against co-infection, which occurred through preservation of lung innate immune defense mechanisms.

Mucosal immunization, especially respiratory tract exposure to cold-adapted live virus, has proved to be an effective route for induction of adaptive immunity against influenza virus. FluMist vaccine was initially designed for protection against seasonal influenza infection (3, 4). The results from the current study indicate that this seasonal vaccine can likewise mediate efficient protection against 2009 H1N1 influenza virus infection. Notably, we found that FluMist-primed CD4+ T cells, but not CD8+ cells or Abs, were required for optimum protection. Our findings are consistent with recent suggestions that infection with seasonal influenza strains can confer immunity against S-OIV (9). However, it has been shown that vaccination with recent seasonal influenza vaccines only induces low levels of cross-reactive Ab responses to S-OIV (1). Nevertheless, Steel and colleagues (9) showed that previous exposure to a seasonal influenza A virus regardless of subtype conferred some protection against H1N1 CA04, suggesting that the basis for protection was not neutralizing Ab against viral surface proteins. Ge et al. (6) reported that seasonal influenza-specific memory CD4 T cells in humans may cross-react with S-OIV. There are several possible explanations for the ability of memory CD4 T cells to mediate protection. First, CD4 effector cells have been demonstrated to possess Ab-independent, perforin-mediated cytolytic activity (26). Second, FluMist-primed CD4+ T cells might facilitate stimulation of naive B cells for generation of new neutralizing Abs against S-OIV (27), even though FluMist-specific Ab failed to confer direct protection. Third, FluMist-primed CD4+ T cells may augment cell-mediated responses through enhanced stimulation of naive CD8 precursors (28).

Typically, the very young and the elderly are most susceptible to influenza infection. Curiously, during the 2009 H1N1 pandemic, older children and young adults demonstrated noticeable susceptibility, a pattern similar to what was observed during the 1918 influenza pandemic. It was recently reported that cross-reactive Abs induced against the 1918 viral strain were likely responsible for protection of the elderly against S-OIV (1). Our results suggest an additional protective mechanism that would be operative in the very young (i.e., seasonal FluMist-induced T cell cross-reactivity). This mechanism would apply only to those eligible to receive this vaccine (i.e., individuals aged 2–49 y of age). It is known that T cell memory in the lung is relatively short-lived compared with humoral immunity. In addition, preexisting neutralizing Ab in the respiratory tract may remove cold-adapted live
virus before induction of effective T cell responses, and therefore, it is likely that only those recently immunized with seasonal FluMist, primarily the very young, contained a sufficient level of pulmonary T cell-mediated protection. Conversely, we propose that such protection would have waned in the older pediatric and young adult population and failed to adequately protect them against the novel H1N1 influenza virus strain.

Despite viral replication in the respiratory tract, isolates of H1N1 CA04 caused only mild and self-limited illness in our mouse model, similar to that seen in most human cases. There was limited weight loss and scarce induction of proinflammatory cytokines such as TNF-α, IL-1β, and IL-17 after challenge with >1000 PFU virus. However, our results clearly demonstrate that H1N1 CA04 infection significantly enhances susceptibility to secondary bacterial infections.

The mechanism(s) responsible for enhanced susceptibility to secondary bacterial infections after viral infection is an area of active investigation because of the significant implications for world health. Most mechanistic studies of influenza and bacterial synergy have been performed using the mouse-adapted PR8 virus and S. pneumoniae co-infection. Several possible mechanisms have been proposed, including interactions between viral, bacterial, and host factors (29–32). It has been found that sustained desensitization to bacterial TLR ligands after resolution of respiratory influenza infection can result in reduced TNF-α production by alveolar macrophages, which in turn can contribute to secondary bacterial infection due to decreased neutrophil recruitment. This is a long-term effect that lasts for several months after resolution of influenza (33). However, in the current H1N1 virus and pneumococcal co-infection model, increased neutrophil recruitment was still observed around 1 wk after influenza infection despite low TNF-α production. Expression of the 1918 influenza A virus PB1-F2 protein has been shown to enhance the pathogenesis of viral and secondary bacterial pneumonia, which may explain the high incidence of fatal pneumonia that occurred during the 1918 influenza pandemic (32). However, PB1-F2 is absent in the current S-OIV, due to two stop codons present in the genome (34). Type I IFN signaling was recently reported to be responsible for influenza-pneumococcus synergy (25). In this latter study, IFN-α/βR−/− mice did not show any change in susceptibility to PR8 viral infection but did demonstrate >40% increased resistance to secondary pneumococcal infection, which correlated with increased neutrophil recruitment. Whereas we have similarly observed no influence of IFN-α/β on PR8 viral infections (data not shown), we did observe significantly more susceptibility to virulent H1N1 CA04 influenza infection in IFN-α/βR−/− mice, consistent with the findings of Steel et al. (9), who reported that type I IFN treatment improved protection against S-OIV infection. Because almost all IFN-α/βR−/− mice succumbed to secondary pneumococcal infection after a sublethal dose of H1N1 infection, we could not distinguish the protective role of type I IFN during primary influenza infection from a potential influence during secondary bacterial infection in the coinfection model. Although the requirement for type I IFN for immune defense against influenza infection is still not completely established, it is clear that IFN-α/β plays a protective rather than detrimental role during H1N1 CA04 viral–bacterial co-infections.

Our results further showed that H1N1 CA04 influenza virus can increase local IFN-γ expression and leukocyte recruitment into alveolar spaces. The timing of susceptibility to bacterial infection is closely correlated with production of this T cell-derived IFN-γ, which occurs at the recovery phase of influenza infection, as demonstrated in our previous study (22). Generation of IFN-γ can change the properties of resident alveolar macrophages that are required for initial clearance of non-opsinized pneumococci (22, 35). We have now shown that deficient IFN-γ signaling results in increased bacterial clearance and survival of H1N1 CA04/bacteria co-infected mice. These results indicate that pulmonary expression of IFN-γ during the response of the host to S-OIV viral infection mediates increased susceptibility to bacterial infection.

Notably, FluMist vaccination was found to suppress H1N1 CA04-induced local IFN-γ expression. Thus, we speculated that vaccination might provide protection against secondary pneumococcal infection in addition to enhancing viral clearance. Indeed, FluMist-treated mice had decreased viral and bacterial burdens, minimal weight loss, and no mortality after H1N1 CA04 and pneumococcal co-infection compared with those in unvaccinated mice. This FluMist-enhanced protection was also observed after H1N1 CA04 and MRSA co-infection, although further studies are necessary to establish fully the mechanism of susceptibility to secondary staphylococcal infection.

In summary, to our knowledge, our findings are the first to provide direct evidence that live seasonal vaccine can stimulate efficient CD4 T responses for protection against a pandemic influenza strain even in absence of effective CD8 T cell or neutralizing Ab responses. More importantly, presence of these memory T cells suppresses local IFN-γ production associated with primary influenza infection, which otherwise could impair innate antibacterial defense. Thus, we conclude that seasonal FluMist vaccination can be an important preventive strategy to induce T cell cross-protection against newly emergent influenza viruses and associated bacterial infections during future influenza pandemics.

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Disclosures

The authors have no financial conflicts of interest.

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