Microbial Heat Shock Protein 65 Attenuates Airway Hyperresponsiveness and Inflammation by Modulating the Function of Dendritic Cells

Yoo Seob Shin, Katsuyuki Takeda, Yoshiki Shiraishi, Yi Yeong Jeong, Joanne Domenico, Yi Jia, Junyan Han, Ralf Spallek, Mahavir Singh, Joseph J. Lucas and Erwin W. Gelfand

J Immunol published online 29 August 2012
http://www.jimmunol.org/content/early/2012/08/29/jimmunol.1201138

Subscription Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Microbial Heat Shock Protein 65 Attenuates Airway Hyperresponsiveness and Inflammation by Modulating the Function of Dendritic Cells

Yoo Seob Shin,*,1 Katsuyuki Takeda,*,1 Yoshiki Shiraishi,*, Yi Yeong Jeong,*, Joanne Domenico,*, Yi Jia,*, Junyan Han,*, Ralf Spallek,† Mahavir Singh,† Joseph J. Lucas,* and Erwin W. Gelfand*

Heat shock proteins (HSPs), produced in response to stress, are suppressive in disease models. We previously showed that Mycobacterium leprae HSP65 prevented development of airway hyperresponsiveness and inflammation in mice. Our goal in this study was to define the mechanism responsible for the suppressive effects of HSP. In one in vivo approach, BALB/c mice were sensitized to OVA, followed by primary OVA challenges. Several weeks later, HSP65 was administered prior to a single, provocative secondary challenge. In a second in vivo approach, the secondary challenge was replaced by intratracheal instillation of allergen-pulsed bone marrow-derived dendritic cells (BMDCs). The in vitro effects of HSP65 on BMDCs were examined in coculture experiments with CD4+ T cells. In vivo, HSP65 prevented the development of airway hyperresponsiveness and inflammation. Additionally, Th1 cytokine levels in bronchoalveolar lavage fluid were increased. In vitro, HSP65 induced Notch receptor ligand Delta1 expression on BMDCs, and HSP65-treated BMDCs skewed CD4+ T cells to Th1 cytokine production. Thus, HSP65-induced effects on allergen-induced airway hyperresponsiveness and inflammation were associated with increased Delta1 expression on dendritic cells, modulation of dendritic cell function, and CD4+ Th1 cytokine production. The Journal of Immunology, 2012, 189: 000–000.

Asthma is the most common chronic airway disease in industrial countries; despite advances in disease management, the socioeconomic burden caused by disease exacerbations or steroid-refractory asthma remains high (1). The introduction of new therapeutic modalities in asthma has been limited by an absence of benefits in major proportions of asthmatics, highlighting the need for better targeting of relevant pathophysiologic pathways.

Asthma is a chronic airway disease characterized by persistent airway hyperresponsiveness (AHR) and airway inflammation as a result of cellular and molecular responses (2). Because Th2-type CD4+ T cells were shown to be a dominant cell type in the airways of asthmatics, allergenic asthma has been viewed as an imbalance between Th1 and Th2 cells (3), although this notion is not universally accepted (4). APCs, dendritic cells (DCs) in the lung, govern the direction of T lymphocyte differentiation and cytokine responses through Notch-signaling pathways (5, 6). In mammals, four Notch receptors (Notch1-4) have been found on T lymphocytes, and two ligand families, Delta-like family (Delta1, Delta3, and Delta4) and the Jagged family (Jagged1 and Jagged2) have been identified on the surface of APCs (5, 6). In experimental models of asthma, the ligation of Notch receptor on CD4+ T cells by Jagged1 expressed on DCs augmented IL-4 production and resulted in the development of AHR and airway inflammation. In contrast, Delta-like ligands have been associated with suppression of lung allergic responses (7–9).

Heat shock proteins (HSPs) are a highly conserved group of functional proteins produced by prokaryotic and eukaryotic cells in response to a variety of stressors, including inflammation. Their primary function is folding and unfolding of protein substrates (10–13). HSPs also recognize cellular abnormalities and deliver them to APCs to generate peptide-specific T lymphocyte responses by binding pathogen-associated molecular pattern receptors or by modulating pathogen-associated molecular pattern-induced stimulation (14). HSPs are classified on the basis of their monomeric molecular weights (15). HSP60 and HSP70 were shown to be involved in binding and presenting Ags to the immune system (16, 17). HSPs were shown to modulate the function of APCs, including DCs (18, 19).

In a mouse model of experimental asthma, we previously showed that HSP65 derived from Mycobacterium leprae prevented the development of allergic inflammation and AHR (20). Thus, in the context of asthma, HSPs may modulate DC function and skew the lymphocyte response from a Th2 type to one in which Th1 cytokines predominate.

Based on these descriptions, we hypothesized that HSP65 may prevent allergen-induced airway inflammation and AHR, even in established disease, by modulating DC regulation of Th differ-

*Division of Cell Biology, Department of Pediatrics, National Jewish Health, Denver, CO 80260; and 1LIONEX Diagnostics and Therapeutics, GmbH, Braunschweig, Germany 38126

1Y.S.S. and K.T. contributed equally to this work.

Received for publication April 20, 2012. Accepted for publication July 25, 2012.

This work was supported by the State of Colorado Bioscience Discovery Evaluation Grant Program and National Institutes of Health Grants HL-36577 and AI-77609.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Heart, Lung, and Blood Institute or the National Institutes of Health.

Address correspondence and reprint requests to Dr. Erwin W. Gelfand, Division of Cell Biology, National Jewish Health, 1400 Jackson Street, K801, Denver, CO 80206.

E-mail address: gelfande@njhealth.org

Abbreviations used in this article: AHR, airway hyperresponsiveness; BAL, bronchoalveolar lavage; BMDC, bone marrow-derived dendritic cell; DC, dendritic cell; HSP, heat shock protein; Mch, methacholine; PAS, periodic acid-Schiff; Rl, lung resistance.

Copyright © 2012 by The American Association of Immunologists, Inc. 0022-1767/12/$16.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1201138
entiation through Notch ligand expression. In the current study, we defined the efficacy of HSP65 derived from M. leprae in a secondary allergen challenge model, linking attenuation of lung allergic responses to alterations in Notch ligand expression on DCs.

Materials and Methods

Animals

Female BALB/c mice, 6–8 wk of age and free of pathogens, were purchased from Harlan Laboratory (Indianapolis, IN). All mice were housed under specific pathogen- and OVA-free conditions and maintained on a 12 h light–dark cycle with food and water ad libitum. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of National Jewish Health.

Sensitization and challenge to allergen

The experimental protocol for sensitization and challenge to allergen was modified from previously described procedures (21). Briefly, BALB/c mice were sensitized i.p. with 10 μg OVA (BP2535-5; Fisher Scientific, Pittsburgh, PA) emulsified in 1 mg alun (Injlect Alum; Pierce, Rockford, IL) or sham sensitized with PBS on days 0 and 14. Mice received airway primary allergen challenges by exposure to OVA aerosols (0.2% in saline) for 20 min on days 28, 29, and 30 with an ultrasonic nebulizer (NE-U07; OMRON, Kyoto, Japan). This was followed by a single, secondary, or provocative allergen challenge (1% OVA in saline for 20 min) 2 wk after the last primary allergen challenge. Forty-eight hours after the last allergen challenge, airway responsiveness was measured, followed by collection of samples.

Preparation and treatment with HSP65

The recombinant M. leprae HSP65 (LIONEX Diagnostics and Therapeutics) was purified from Escherichia coli to >98% purity, as confirmed by SDS electrophoresis, immunoblotting, and N-terminal sequencing and was without flagellin contamination. Endotoxin was removed by endotoxin-removing gel (Thermo Scientific, Rockford, IL); after removal, the endotoxin levels were <0.2 EU/mg HSP protein as detected using the ToxinSensor Chromogenic LAL Endotoxin Assay Kit (GenScript, Piscataway, NJ).

In in vivo experiments, purified HSP65 (100 μg) was administered by i.p. injection 2 h prior to secondary OVA challenge. For in vitro experiments, 1 or 10 μg/ml HSP65 was added to the cultures of bone marrow-derived DCs (BMDCs) for 24 h.

Preparation of BMDCs and protocol for transfer of OVA-pulsed BMDCs

BMDCs were generated from bone marrow of naive BALB/c mice, as described previously (22). Briefly, bone marrow cells were obtained from femurs and iliac bones of mice and placed in DC culture medium (RPMI 1640 containing 10% heat-inactivated FCS, 50 μM 2-ME, 2 mM l-glutamine, 100 μM penicillin, 100 μg/ml streptomycin [Life Technologies, Carlsbad, CA], 10 ng/ml recombinant mouse GM-CSF, and 10 ng/ml recombinant mouse IL-4 [R&D Systems, Minneapolis, MN]). On day 8, nonadherent cells were recovered. These cells were >95% CD11c+.

BMDCs were pulsed with OVA (200 μg/ml) in the presence or absence of HSP65 for 24 h and washed three times with PBS. As controls, OVA-pulsed BMDCs were cultured with PBS instead of HSP65 or were not pulsed with OVA. BMDCs (2 × 105 cells) were administered intratracheally into sensitized and primary allergen-challenged mice in lieu of secondary allergen challenge. To determine the direct effects of HSP65 on BMDC function, the cells were cultured with HSP65 (0.1, or 10 μg/ml), or LPS (11 μg/ml) as a positive control, for 24 h, followed by assays of cells or culture supernates.

CD4+ T cell preparation and coculture with BMDCs and HSP65

CD4+ T cells were isolated, as previously described (23). Spleen cells from sensitized and secondary allergen-challenged mice were harvested by mincing the tissues and passing them through a stainless steel sieve. After washing with PBS, mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO). Purification of CD4+ T cells was conducted by negative selection using a mouse CD4+ T cell recovery column kit (Cedarlane Laboratories, Burlington, NC), in accordance with the manufacturer’s instructions. Purity of CD4+ T cell populations after purification exceeded 95%, as assessed by flow cytometry.

BMDCs were treated with HSP65 (1 or 10 μg/ml) or vehicle alone for 24 h and then cocultured with isolated CD4+ cells in the presence of OVA (200 μg/ml) at a ratio of 1:10 for 24 h. Supernates were collected and evaluated by ELISA.

Assessment of airway responsiveness, bronchoalveolar lavage fluid, and lung histology

Airway responsiveness was assessed as previously described by measuring changes in airway resistance in response to increasing doses of inhaled methacholine (MCh; Sigma-Aldrich) in anesthetized and ventilated mice (21). Data are expressed as the percent change from baseline lung resistance (Rl) values obtained after inhalation of saline. Immediately after measurement of AHR, lungs were lavaged via the tracheal tube, as described (21). Numbers of total leukocytes in bronchoalveolar lavage (BAL) fluid were determined, and cell differentiation was performed on cytospin slides prepared with Wright-Giemsa stain. After BAL fluid was obtained, the lungs were fixed in 10% formalin, embedded in paraffin, and cut into 5-μm sections. The number of inflammatory and mucus-containing cells was quantitated, as previously described, with some modification (24). Tissue sections were evaluated using NIH ImageJ (Version 1.45; available at http://rsbweb.nih.gov/ij/download.html). For detection of inflammatory cells, sections were stained with H&E, and the number of inflammatory cells/μm2 of perivascular and peribronchial areas was determined. In addition, mucus-containing cells stained with periodic acid-Schiff (PAS) were quantitated and expressed as PAS-positive areas/μm of basement membrane.

Preparation of RNA and real-time PCR

Total RNA was extracted from HSP65 or LPS-treated BMDCs using a total RNA isolation kit (Macherey-Nagel, Bethlehem, PA). One microgram of total RNA was used in each reaction primed with oligonucleotide deoxythymidine to obtain cDNA. Then, 3 μl cDNA was used as the template for real-time PCR (9). Real-time cDNA primers for Deltal and Jagged1 were obtained from the TaqMan gene expression assay (Applied Biosystems, Carlsbad, CA). The real-time PCRs were performed on an ABI 7700 sequence detection system (Applied Biosystems) with cycling parameters of 50°C for 2 min, 95°C for 10 min, and 40 repeats at 95°C for 15 s and 60°C for 1 min. The cycle threshold method was performed for relative quantification of mRNA expression (7).

Measurement of cytokines

Cytokine levels in the BAL fluid and cell culture supernatants were measured by ELISA, as previously described (24). IL-5, IL-10, IL-12, IL-13, and IFN-γ cytokine ELISAs were performed according to the manufacturers’ instructions (eBioscience, San Diego, CA). The lower limits of detection were 4 pg/ml for IL-5 and IL-13, 10 pg/ml for IL-10, IFN-γ, and TNF-α, and 15 pg/ml for IL-12.

Statistical analysis

Results are expressed as mean ± SEM. The t test was used to determine differences between two groups. The Tukey–Kramer test was used for comparisons between multiple groups. Nonparametric analysis using the Mann–Whitney U test or Kruskal–Wallis test was also used to confirm that the statistical differences remained significant, even if the underlying distribution was uncertain. The p values for significance were set to 0.05 for all tests.

Results

HSP65 treatment inhibits the development of AHR and airway inflammation following secondary allergen challenge

HSP65 or PBS were administered by i.p. injection just prior to secondary allergen challenge of sensitized and primary allergen-challenged mice. As shown in Fig. 1A and 1B, PBS-treated mice developed increases in Rl to inhaled MCh and eosinophil numbers in BAL fluid. Mice treated with HSP65 developed significantly lower airway responsiveness and BAL eosinophilia, accompanied by significantly increased levels of IL-10, IL-12, and IFN-γ and decreased levels of IL-4, IL-5, and IL-13 in BAL fluid, compared with vehicle-treated mice (Fig. 1C).

Histopathological analysis of lung tissue sections stained with H&E stain revealed that the number of inflammatory cells, including eosinophils in the peribronchial and perivascular areas, was increased in vehicle-treated mice after secondary allergen challenge
Compared with sham-sensitized and challenged mice (Fig. 1Da); these cell numbers were significantly decreased in HSP65-treated mice (Fig. 1Dc). Similar to the changes in inflammatory cell numbers, goblet cell metaplasia developed in vehicle-treated mice following secondary allergen challenge, and HSP65 treatment significantly reduced the number of goblet cells (Fig. 1E).

In view of the increases in levels of IL-10, IL-12, and IFN-γ in BAL fluid following HSP65 treatment, we examined the effect of HSP65 on BMDC function. BMDCs were cultured in the presence (or absence) of HSP65 (1 or 10 μg/ml) or LPS (1 μg/ml), a potent Th response inducer.
stimulus of DC cytokine production (25), as a positive control. Addition of 1 or 10 μg/ml HSP65 triggered significant increases in DC production of TNF-α, almost to the levels induced by LPS (Fig. 2A). Similarly, production of IL-12 and IL-10 was also increased following addition of HSP.

Because expression of the Notch ligands, such as Delta1 and Jagged1, on BMDCs has been associated with the differentiation fate of CD4+ Th cells (7–9), we analyzed their expression in BMDCs cultured with HSP65 by real-time PCR. Expression levels of Delta1 were significantly higher in BMDCs cultured with HSP65 compared with BMDCs cultured with vehicle, similar to the levels detected after culture with LPS (Fig. 2B). This contrasted with levels of expression of Jagged 1, where little induction was seen following culture with HSP65 or LPS.

**HSP65 treatment of BMDCs modifies cytokine production from CD4+ T cells**

Because CD4+ T cells are potent effector cells in the development of allergic inflammation, their function was examined after coculture with BMDCs in the presence of allergen and HSP65. CD4+ T cells isolated from secondary allergen-challenged mice were incubated with OVA and BMDCs pretreated with HSP65 (0, 1, or 10 μg/ml) for 24 h. Supernates were collected and evaluated by ELISA. The results are representative of three independent experiments and are expressed as means ± SEM. *p < 0.05 versus vehicle-treated BMDCs.

**HSP65 treatment of BMDCs results in significantly lower AHR and inflammation following transfer into previously sensitized and challenged recipients**

To determine the consequences of HSP65 treatment on BMDC function in an in vivo model, Ag-pulsed BMDCs were administered intratracheally to previously sensitized and challenged recipients as a substitute for secondary allergen challenge. As shown in Fig. 4, recipients of OVA-pulsed, vehicle-treated BMDCs developed increased airway resistance to MCh and greater eosinophil numbers in BAL fluid. In contrast, recipients of OVA-pulsed, HSP65-treated BMDCs failed to develop AHR and BAL eosinophilia. Cytokine levels in BAL fluid did not differ between groups (data not shown), similar to previously reported findings in this short-term DC-transfer model (26).

**Discussion**

In recent decades there has been an increase in the prevalence of allergic diseases in developed countries (27, 28). One factor proposed to explain this increase in allergic diseases is the decline

![FIGURE 3. Cytokine profile following culture of CD4+ T cells with HSP65-treated BMDCs. Isolated CD4+ T cells from secondary OVA-challenged mice were incubated with OVA and BMDCs pretreated with HSP65 (0, 1, or 10 μg/ml) for 24 h. Supernates were collected and evaluated by ELISA. The results are representative of three independent experiments and are expressed as means ± SEM. *p < 0.05 versus vehicle-treated BMDCs.](http://www.jimmunol.org/)

![FIGURE 4. Effect of HSP65 on OVA-pulsed BMDC function following transfer into previously sensitized and challenged mice. The in vivo effects of HSP65 on OVA-pulsed BMDCs were determined. All groups of mice were sensitized to OVA, followed by primary OVA challenge. Two weeks after primary OVA challenge, mice received (intratracheal instillation) BMDCs pretreated with HSP65 and pulsed with OVA (OVA/HSP65) or PBS as control (PBS/vehicle) or BMDCs pretreated with vehicle and pulsed with OVA (OVA/vehicle) or pulsed with PBS (PBS/vehicle). (A) Changes in airway resistance (R_{L}). (B) Cell composition in BAL fluid. (n = 8). *p < 0.05 versus OVA/vehicle.](http://www.jimmunol.org/)
in the incidence of many infectious diseases in developed countries as the result of improved living standards and vaccination (29). Although it is possible that increased microbial exposure could lead to a decline in allergic diseases, such as asthma (30), the mechanisms are not defined. To address this issue, we hypothesized that treatment with Mycobacterium-derived HSP may inhibit the development of allergen-induced airway inflammation and AHR in a secondary allergen challenge model through modulation of DC function. Administration of HSP65 inhibited the development of AHR and inflammation when administered prior to secondary challenge of previously sensitized and challenged mice. In addition, transfer of HSP65-treated BMDCs into previously sensitized and challenged mice as a substitute for secondary allergen challenge failed to trigger airway inflammation and AHR. Upregulation of the Notch ligand Delta1 was seen following HSP65 incubation with BMDCs, and in coculture experiments with CD4⁺ T cells, levels of IFN-γ were increased, whereas levels of IL-5 and IL-13 were decreased relative to cultures of CD4⁺ T cells with untreated BMDCs. Together, these findings demonstrated that DCs are an important target of the HSP65 suppressive effects that are mediated, at least in part, through increased Delta1 expression on DCs and Th1 skewing of CD4⁺ T cell responses.

HSPs are molecular chaperones whose predominant function is the folding and unfolding of protein substrates in response to stressors, such as in an infectious disease (12, 14). Elevated HSP levels are associated with many infectious diseases (31–33). Recently, upregulation of HSP was demonstrated in patients with bronchial asthma (34, 35). In an earlier study designed to link asthma suppression and HSPs (20), we used HSP65 derived from Mycobacterium leprae to define a mechanism for the observed reductions in allergen-induced AHR and airway inflammation. In the current study, we used a secondary allergen challenge model to demonstrate the efficacy of HSP65 on a background of pre-existing disease. As demonstrated in this study, HSP65 administration prior to a single provocative (secondary) allergen challenge to previously sensitized and challenged mice significantly reduced AHR and airway inflammation. These outcomes of HSP65 treatment were associated with significant increases in BAL cytokine levels of IL-10, IL-12, and IFN-γ, as well as decreased levels of IL-4, IL-5, and IL-13. It was reported that Mycobacterium tuberculosis chaperones stimulated IL-10 and IL-12 production (36), HSP70L1 induced the production of IL-12p70 (37), and mycobacterial HSP70 treatment increased IL-10 levels in an arthritits model (38). Although these particular HSPs have differences in structure and cellular localization compared with HSP65, they share the common feature of stimulating APCs. As examples, HSP60 strongly stimulated DC maturation whereas HSP70L1 activated DCs (25, 37). The results in each case were increases in production of IL-10 and IL-12. IL-12 is thought to be a major APC-derived factor promoting Th1 differentiation (39) whereas IL-10 activity, although complex, was associated with anti-inflammatory activities (40).

Notch ligand–Notch receptor interactions also play a major role in directing APC function and, in turn, the differentiation profile of Th cells. The Notch-signaling pathway is a highly conserved program for cell fate decisions, such as apoptosis, cell cycle arrest, and cellular polarization in all organisms (6, 41–43). Mammals express five genes that encode ligands for Notch receptors from two conserved families, Jagged (Jagged1 and Jagged2) and Delta-like (Delta1, Delta3, and Delta4) (6), and both types of ligands appear to transduce similar signaling pathways through Notch receptors (44). These ligands promote differentiation of naive CD4⁺ T cells into distinct effector cells; Delta promotes Th1 responses (45), and Jagged induces naive CD4⁺ T cells to differentiate into Th2 lineage (46). In experimental models of asthma, we showed that the Delta1-like ligand inhibited development of allergen-induced changes in lung function and inflammation, whereas the Jagged 1 ligand enhanced responsiveness (7, 8). In view of these findings and the associated cytokine changes, we examined Notch ligand expression on BMDCs and found that Delta1 expression on HSP65-treated BMDCs was significantly elevated, with little change in Jagged1 expression. These data supported a functional link among HSP65, Notch ligand expression, and production of the allergy-suppressive cytokines IL-10, IL-12, and IFN-γ, with some reduction in levels of the allergy-promoting cytokines IL-4, IL-5, and IL-13.

Although the target cell or cell surface receptor for HSP has not been completely elucidated, there is accumulating evidence for interactions with cells of the innate immune system (47, 48). The stimulatory activity of HSP60, HSP70, HSP90, and gp96 on the release of proinflammatory cytokines by cells of the innate immune system has been well described (25, 49–51). HSP-like protein 1 interacts with DCs, promoting DC maturation and polarizing responses toward a Th1 pattern (37). To pursue the interactions of HSP65 on DC function, we first examined their impact on DC–T cell interactions in vitro. CD4⁺ Th2 cells play a major effector role in the development of allergic inflammation through the release of cytokines, such as IL-4, IL-5, and IL-13 (52, 53). The transfer of Th2 cells, followed by airway allergen challenge in mice, was sufficient to induce airway eosinophilia and AHR (54, 55). When HSP65-treated, Ag-pulsed DCs were cocultured with CD4⁺ T cells, increases in Th1 cytokines were detected in culture supernates with lower levels of Th2 cytokines, establishing a basis for the in vivo findings in secondary challenged mice. These observations were extended to in vivo studies examining DC function in an adoptive transfer model. We previously showed that Ag-pulsed BMDCs could restore the development of AHR and eosinophilic inflammation following transfer into nonsensitized mice prior to allergen challenge (26). In this study, OVA-pulsed BMDCs treated with HSP65 were transferred into previously sensitized and challenged mice, as a means for exposing the mice to allergen in lieu of direct allergen challenge. Although recipients of untreated, OVA-pulsed BMDCs developed AHR and airway inflammation, recipients of HSP65-treated cells showed no such responses. One of the outcomes of this DC-transfer model was the ability to elicit AHR after allergen challenge in the apparent absence of an accompanying robust BAL eosinophilia or elevation of Th2 cytokine levels (26). This dissociation of AHR and airway eosinophilia/Th2 cytokines was previously reported under varying experimental conditions (56–59).

In summary, HSP65 reduced AHR and airway inflammation when administered prior to secondary allergen challenge of previously sensitized and challenged mice. The in vivo results, together with the in vitro findings, position DCs as a prominent target of the HSP65-suppressive effects that are mediated, at least in part, through increased Delta1 expression on DCs and Th1 skewing of CD4⁺ T cells. These results identify HSP65 as a potent modulator of allergen-induced lung allergic responses in established disease.

Acknowledgments
We thank Diana Nabighian for assistance with the preparation of this manuscript.

Disclosures
The authors have no financial conflicts of interest.