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Innate IFNs and Plasmacytoid Dendritic Cells Constrain Th2 Cytokine Responses to Rhinovirus: A Regulatory Mechanism with Relevance to Asthma

Antonia L. Pritchard,* Melanie L. Carroll,* Julie G. Burel,* Olivia J. White,* Simon Phipps,† and John W. Upham*‡

Human rhinoviruses (RV) cause only minor illness in healthy individuals, but can have deleterious consequences in people with asthma. This study sought to examine normal homeostatic mechanisms regulating adaptive immunity to RV in healthy humans, focusing on effects of IFN-αβ and plasmacytoid dendritic cells (pDC) on Th2 immune responses. PBMC were isolated from 27 healthy individuals and cultured with RV16 for up to 5 d. In some experiments, IFN-αβ was neutralized using a decoy receptor that blocks IFN signaling, whereas specific dendritic cell subsets were depleted from cultures with immune-magnetic beads. RV16 induced robust expression of IFN-α, IFN-β, multiple IFN-stimulated genes, and T cell-polarizing factors within the first 24 h. At 5 d, the production of memory T cell-derived IFN-γ, IL-10, and IL-13, but not IL-17A, was significantly elevated. Neutralizing the effects of type-I IFN with the decoy receptor B18R led to a significant increase in IL-13 synthesis, but had no effect on IFN-γ synthesis. Depletion of pDC from RV-stimulated cultures markedly inhibited IFN-α secretion, and led to a significant increase in expression and production of the Th2 cytokines IL-5 (p = 0.02), IL-9 (p < 0.01), and IL-13 (p < 0.01), but had no effect on IFN-γ synthesis. Depletion of CD1c* dendritic cells did not alter cytokine synthesis. In healthy humans, pDC and the IFN-αβ they secrete selectively constrain Th2 cytokine synthesis following RV exposure in vitro. This important regulatory mechanism may be lost in asthma; deficient IFN-αβ synthesis and/or pDC dysfunction have the potential to contribute to asthma exacerbations during RV infections. The Journal of Immunology, 2012, 188: 000–000.

Respiratory viruses are associated with the majority of severe asthma exacerbations, with human rhinovirus (RV) infections being the most common viruses identified in children and adults (1–3). Asthmatics do not appear to have more frequent viral infections than healthy individuals, but instead suffer more persistent and severe lower respiratory tract symptoms (4).

It has long been a puzzle why a commonly innocuous mucosal virus such as RV should cause such adverse clinical effects in asthma, although an increasing body of evidence points to a dysregulated immune or inflammatory response to respiratory viruses (5, 6). Some reports indicate that airway epithelial cells from asthmatics have a reduced capacity for synthesis of innate IFN following RV exposure in vitro, including IFN-α, IFN-β, and IFN-λ, compared with normal airway epithelial cells from healthy people (7, 8), although these findings have been disputed by others (9, 10).

The dysregulated antiviral immune response in asthma has also been demonstrated in migratory populations of immune cells derived from the bone marrow. RV-stimulated alveolar macrophages from asthmatics produce less IFN-λ than alveolar macrophages from healthy individuals (7). PBMC from asthmatic children and adults secrete less IFN-α following in vitro exposure to viruses (11, 12), and this is associated with reduced function of TLR7, the receptor for viral ssRNA (13). Plasmacytoid dendritic cells (pDC) are a potent source of type-I IFN synthesis during virus infections (14), and numerical changes in circulating pDC have been linked both to asthma development in young children (15) and to established asthma in adults (16). The function of pDC may also be abnormal in asthma, with reports demonstrating that pDC from allergic asthmatics are less able to synthesize IFN-α in response to influenza A (17) or TLR9 activation (18) than pDC from healthy subjects.

In addition to these facets of innate immune function, adaptive immunity to RV also appears abnormal in asthma. Several groups have reported that RV-stimulated PBMC from asthmatics exhibit greater synthesis of IL-5, IL-13, and IL-10 and reduced synthesis of IFN-γ (19–22). Cytokine dysregulation in circulating cells has been shown to be proportional to asthma severity, to the degree of bronchial hyperresponsiveness, and to the extent of viral shedding following experimental human RV infection in vivo, providing strong circumstantial evidence that these alterations in immune function are clinically relevant (19, 21, 22).

The normal homeostatic mechanisms regulating adaptive immune responses to RV in humans are not well understood. Type-I IFNs (IFN-αβ) play a well-established role in early innate immune defense against a wide range of viral pathogens (23). Experimental evidence also suggests that IFN-αβ can modulate adaptive immunity to many viruses, supporting the polarization and effector function of Th1 cells (24) and Th17 cells (25), while inhibiting Th2 commitment and secretion of potentially malad-
apart Th2 cytokines (26, 27). The extent to which these observations hold true in relation to RV has not been studied, but may be highly relevant to the pathophysiology of asthma where IFN-αβ synthesis appears to be deficient, and where a Th2 or Th17 response to the virus might worsen eosinophilic or neutrophilic airway inflammation, respectively.

In the current study, we characterized the innate immune response to RV in detail, in particular the type-I IFNs and associated IFN-stimulated genes, and the adaptive immune responses to RV, focusing on the secretion of Th1, Th2, Th17 cytokines, and the regulatory cytokine IL-10. Our experiments then focused on the extent to which type-I IFNs regulated Th2 adaptive immune responses to RV using rIFN-β and the type-I IFN receptor antagonist B18R. As pDC are a highly potent source of IFN-αβ, we also examined whether pDC modulate the adaptive immune responses to RV. Our results indicate that, in healthy individuals, IFN-αβ and pDC play critical roles in selectively constraining maladaptive Th2 cytokine responses to RV, but have little, if any, effects on synthesis of IFN-γ or IL-10 protein production or IL-17a gene expression.

**Materials and Methods**

**Patients**

Twenty-seven healthy adult volunteers were recruited for this study (49% female, average age 34.79 ± 14.2); given our previous findings that antiviral adaptive immunity varies with sex (28), it was important to have similar numbers of women and men in the study. Five of the participants were atopic, as determined by skin prick testing to a panel of common inhaled allergens (mean wheal diameter ≥4 mm), but none had clinical features of asthma or other respiratory diseases. No correlations were found between atopy and cytokine responses to RV, but patients with atopy had consistently lower adaptive IFN-γ responses to RV using rIFN-γ and IL-13 ELISAs were performed using commercially available paired Abs and recombinant cytokines (BD Biosciences, Franklin Lakes, NJ). The limit of detection was 15.6 pg/ml for IP-10 and IFN-γ, and 7.8 pg/ml for IL-13, IL-5, and IL-10. Both IFN-α (PBL IFN Source, Piscataway, NJ) and IFN-λ (eBioscience) were assayed via commercial ELISA kit, according to the manufacturer’s instructions; IFN-α multi-subtype kit detects all isoforms except IFNα-13 and IFNα-21. The limit of detection was 9.7 pg/ml for IFN-α and 15.6 pg/ml for IFN-λ.

**Quantitative real-time PCR**

RNA was reverse transcribed using Transcripter first-strand cDNA synthesis kit (Roche Applied Science), according to manufacturer’s instructions. Gene expression was investigated by quantitative real-time PCR (qPCR). Data generated were assessed using the methodology described by Pflaff (32), unless the cDNA was not detectable in the unstimulated cultures, in which case it was analyzed using the standard curve method. UBE2D2 was initially identified as a stable reference gene in CD4+ cells (33) and subsequently assessed in house to be stably expressed in total PBMC in the absence and presence of RV16, using the method described by Silve et al. (34). qPCR was performed using the LightCycler 480 (Roche Applied Science) with GoTaq SYBR Green (Promega). Supplemental Table Ia shows the primers used to amplify IFN-β, IFN-inducible MxA (also known as Mx1), 2′,5′-oligoadenylate synthetase (OAS1), IFN-stimulated gene 15 (ISG15), IL-17a, IL-9, IL-12p35, T-bet (also known as TBX21), GATA3, IFN regulatory factor 4 (IRF4), and class II MHC transactivator. Where data were analyzed using the Pfaff method, the results are expressed as a ratio of stimulated to control (unstimulated) samples, with a fold change of 1 representing unstimulated expression levels.

**T cell proliferation assay using CFSE**

T cell proliferation was assessed using flow cytometry by labeling cells with the vital dye CFSE. Briefly, 10 × 10⁶ cells and 10% FCS (6/24 h, innate immune studies) or 5% autologous plasma (5 d, adaptive immune studies). Extensive quantitative real-time PCR was acquired for the assessment of basophil activation. Data were then analyzed with FlowJo software version 7.6.

**Statistics**

Statistical analysis was performed using SPSS 18 (IBM SPSS, Chicago, IL) and GraphPad Prism 5 for Windows (GraphPad Software, San Diego, CA).
The data were not normally distributed, so results are presented as medians and interquartile ranges and analyzed using the nonparametric Wilcoxon paired signed rank test.

Results
Characterization of the early immune responses to RV
We first undertook a detailed characterization of the early immune response to RV16 using PBMC from 27 healthy individuals. RV led to a 100-fold induction of IFN-β expression at 6 h together with several IFN-stimulated genes known to have antiviral properties, including MxA, OAS1, and ISG15 (Fig. 1A). By 24 h, high concentrations of IFN-α and the chemokine IP-10 (CXCL10) were present in supernatant from RV-stimulated cells. RV also induced the expression of additional immune response genes within the first 24 h, including the IFN pathway regulator IRF4, the Th1-polarizing cytokine IL-12p35, and the Th1-associated transcription factor T-bet, as detailed in Table I. To our surprise, expression of CITA, the master regulator of MHC-II Ag presentation, and the Th17-polarizing cytokine IL-23p19 was not induced at either 6 or 24 h post-RV stimulation relative to unstimulated cultures (Table I). Moreover, type-III IFN (IFN-λ1, and IFN-λ2/3) responses to RV16 were not detectable by qPCR (Table I) or by ELISA at multiple time points up to 5 d; in contrast, PBMC stimulated with the TLR3 ligand poly(I:C) secreted IFN-λ1 (Supplemental Table Ia).

Characterization of the later, adaptive immune responses to RV
The experiments next examined the adaptive immune response to RV at day 5. At this later time point, the cytokine response was dominated by high concentrations of the Th1 cytokine IFN-γ, and only modest concentrations of IL-13 and IL-10 (Fig. 1B). Although IL-17a mRNA was induced at 5 d post-RV stimulation (Fig. 1B), IL-17a protein was not detectable in supernatant at 5 d (data not shown). These changes in cytokine production were associated with RV-induced proliferation in both the CD4 and CD8 T cell compartments at 5 d stimulation, as shown in Supplemental Fig. 1a.

The cellular source of the cytokine synthesis at day 5 was investigated by depleting CD45RO-positive Ag-experienced T cells from PBMC prior to addition of RV. This produced a marked reduction in IFN-γ and IL-13 synthesis relative to sham-depleted cells (98.5% decrease and 92.77% decrease, respectively; n = 5, data not shown).

Basophils, when activated, are capable of producing IL-13 (35), and it was therefore important to assess the role played by these cells. We found that the Lymphoprep gradient decreased the amount of basophils in PBMC compared with whole blood (0.115% total cells in whole blood versus 0.07% in freshly extracted PBMC; n = 4; Supplemental Fig. 2a). It was also found that over 5 d of culture, the number of basophils within the culture decreases further to 0.04% of total cells. Interestingly, the number of basophils in culture at 5 d appeared to decrease further in the presence of RV16, to 0.008% (Supplemental Fig. 2a). We found very little evidence of basophil activation in any of our culture conditions; in the presence of RV16 at day 5, some CD69+ cells were observed, but these were very low in number (Supplemental Fig. 2b).

Modifying effects of type-1 IFN
To determine the extent to which IFN-αβ modulates the immune response to RV, PBMC were cultured with RV16 in the presence or absence of B18R. This purified protein originally isolated from vaccinia virus acts as a decoy receptor, inhibiting the binding of all subtypes of the type-I IFN family to their common receptor (type-I IFN receptor). As expected, addition of B18R resulted in a significant decrease in synthesis of the type-I IFN-sensitive chemokine IP-10 at 24 h (Fig. 2A). Whereas B18R had no significant effect on IFN-γ at day 5, B18R induced a significant increase in IL-13 synthesis, compared with PBMC cultured with RV16 alone (Fig. 2A).

To further examine the modifying effects of type-I IFN on adaptive immune responses to RV, cultures were supplemented with rIFN-β. In this instance, exogenous IFN-β inhibited both IFN-γ and IL-13 synthesis by RV-stimulated cultures at day 5 (Fig. 2B).

Differential influence of DC subsets on Th1 and Th2 responses to RV
Given the ability of type-I IFNs to regulate Th2 responses in RV-stimulated cells, the next experiments concentrated on pDC. This DC subset is thought to be a major source of type-I IFN synthesis in many virus infections, although little information is currently available on the role of pDC in relation to RV. PBMC from 16 healthy donors were depleted of pDC using anti-BDCA-4–coated magnetic beads prior to addition of RV16; sham-depleted cells served as controls. The removal of pDC led to a >98% reduction in IFN-α secretion, and a lesser, but still significant reduction in IP-10 secretion at 24 h (Fig. 3A). Depletion of pDC did not change the Th1 response at 5 d with similar concentrations of IFN-γ observed in pDC-depleted and sham-depleted cultures (Fig. 3B); nor did it alter IL-10 production or expression of IL-17a mRNA (Fig. 3B). Significantly, pDC depletion was associated with significant increases in Th2 responses to RV at day 5 with enhanced secretion of IL-13 and IL-5 proteins and greater IL-9 mRNA expression (Fig. 3C).

In contrast, when CD1c+ DC were depleted from PBMC using anti-CD1c–coated magnetic beads, this did not alter the innate (IP-10) response to RV at 24 h nor the adaptive (IFN-γ and IL-13) response to RV at 5 d (Supplemental Fig. 1b).

Discussion
This study sought to provide detailed information on the immune response to RV and how it is regulated in healthy people, with the specific objective of identifying those aspects of the early innate response that are critical for shaping the adaptive immune response. Predictably, RV induced a strong early immune response dominated by IFN-α, IFN-β, and several molecules involved in the type-I IFN pathway (MxA, OAS1, ISG15, and IRF4). Viruses generally induce an adaptive immune response dominated by Th1 cytokines, and the current study indicated that this was the case in relation to RV with an immune response dominated by Th1 cytokines, and the current study indicated that this was the case in relation to RV with an immune response dominated by Th1 cytokines, and the current study indicated that this was the case in relation to RV with an immune response dominated by Th1 cytokines, and the current study indicated that this was the case in relation to RV with an immune response dominated by Th1 cytokines, and the current study indicated that this was the case in relation to RV with an immune response dominated by T-bet. In contrast, RV induced IL-13, IL-17, and IL-10 to a far lesser extent.

The significant and unexpected finding to emerge from this study was the importance of type-I IFNs and pDC in constraining Th2 cytokine production by RV-stimulated PBMC. When the activity of type-I IFNs was blocked, the cytokine response pattern changed, with significantly greater IL-13 secretion (Fig. 2A), whereas parallel experiments showed that supplementation of cultures with rIFN-β inhibited IL-13 secretion (Fig. 2B). There is evidence that IFN-α and IFN-β can inhibit Th2 immune responses, including eosinophilia (36) and cytokine secretion by committed Th2 cells (26, 27). To the best of our knowledge, the current investigation is the first report examining the effects of IFN-α and IFN-β on RV-specific immune responses in humans. Our findings indicate that inhibition of Th2 responses is an important regulatory property of type-I IFNs in healthy individuals. Blocking the activity of endogenous type-I IFNs had no effect on IFN-γ secretion (Fig. 2A),
suggesting that type-I IFNs have little, if any, role in supporting Th1 secretion in response to RV. Although this is an area of some controversy, most authors hold the view that type-I IFNs are much less important than IL-12 in supporting Th1 cytokine secretion [as reviewed by Huber and Farrar (37)]; in fact, we found that supplementing RV-stimulated cultures with IFN-β inhibited IFN-γ secretion.

Depletion of pDC from cultures produced a marked reduction in IFN-α secretion at 24 h (Fig. 3A), showing that pDC provide an important and immediate source of IFN-α secretion in response to

FIGURE 1. Characterization of the early (innate) and late (adaptive) immune responses in PBMC exposed to RV16 in vitro. qPCR data are shown relative to unstimulated cultures (indicated by broken red line). (A) RV16 induces early expression of type I IFNs and IFN-stimulated genes. IFN-β, MxA (Mx1), OAS1, and ISG15 were measured by qPCR at 6 h, and IFN-α and IP-10 supernatant proteins were measured by ELISA at 24 h. RV16 induces marked upregulation of gene expression and protein synthesis relative to unstimulated cultures. ***p < 0.0001. (B) RV16 induces synthesis of Th1, Th2, Th17, and the regulatory cytokine IL-10. IFN-γ, IL-13, and IL-10 supernatant proteins were measured by ELISA at 5 d post-RV16 stimulation. IL-17a was measured by qPCR 5 d post-RV stimulation; levels were undetectable in unstimulated cultures, so qPCR was analyzed using the standard curve method. IL-17 supernatant protein was not detectable by ELISA at 5 d post-RV16 stimulation. Statistically significant increases in expression were observed. *p < 0.05, **p < 0.01, ***p < 0.0001.
Although other circulating cell types may produce IFN-α at later times, our findings emphasize the importance of pDC in initiating the type-I IFN response. Secretion of the chemokine IP-10 was also partly dependent on pDC (Fig. 3A), although clearly other PBMC populations are also sources of IP-10 production; the decreased type-I IFN production upon pDC depletion was most likely the cause of the decreased IP-10 (38). A key observation in this study was that depletion of pDC from PBMC cultures had no effect on IFN-γ or IL-10 protein production or IL-17α mRNA synthesis, but augmented the RV-stimulated Th2 immune response at day 5, with increased IL-13 and IL-5 protein synthesis and higher mRNA expression of IL-9 (a Th2 cytokine that acts on mast cells; see Fig. 3C). Our findings therefore highlight the regulatory properties of pDC, especially their ability to inhibit Th2

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Influence of type-I IFN on RV16-stimulated PBMC. (A) Effects of blocking type-I IFN signaling. IP-10 supernatant protein was measured by ELISA at 24 h post-RV16 stimulation. IFN-γ and IL-13 supernatant proteins were measured by ELISA at 5 d post-RV16 stimulation. *p < 0.05, **p < 0.01. (B) Addition of IFN-β inhibits IFN-γ and IL-13 synthesis. IFN-γ and IL-13 supernatant proteins were measured by ELISA at 5 d post-RV16 stimulation. **p < 0.01.

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Gene expression was determined by qPCR using cDNA from 6 h (IL-12p35 and IL-23p19), 24 h (CIITA, IRF4, and T-bet), and 5 d (IRF4, T-bet, and GATA3) post-RV16–stimulated cultures. Results are shown as fold change (median ± interquartile range) relative to unstimulated cultures and are from at least 25 healthy donors. ND, Not detected.
pDC selectively regulate cytokine synthesis by RV16-stimulated PBMC. Graphs show the innate response to RV16-stimulated sham-depleted and pDC-depleted cultures. Sham-depleted PBMC go through the identical protocol as the pDC-depleted culture, without the depletion beads. Unstimulated cultures were measured for both sham-depleted and pDC-depleted cultures, showing minimal induction of IFN-α or IP-10 at 24 h or of IFN-γ, IL-13, IL-5, or IL-9 at 5 d (data not shown). (A) pDC depletion reduces IFN-α and IP-10 synthesis. IFN-α and IP-10 supernatant proteins were measured by ELISA at 24 h post-RV16 stimulation. ***p ≤ 0.0005. (B) pDC depletion has no effect on IFN-γ or IL-10 production, or IL-17a mRNA expression. IFN-γ and IL-10 supernatant proteins were measured by ELISA at 5 d post-RV16 stimulation. IL-17a was measured by qPCR 5 d post-RV stimulation; levels were undetectable in unstimulated cultures, so qPCR was analyzed using the standard curve method. (C) pDC depletion augments Th2 cytokine synthesis. IL-5 and IL-13 supernatant proteins were measured by ELISA at 5 d post-RV16 stimulation. IL-9 was measured by qPCR; levels were undetectable in unstimulated cultures, so qPCR was analyzed using the standard curve method. *p < 0.05.
cytokine production by RV-stimulated PBMC. This appears to be mediated in part via the secretion of type-I IFNs by RV-stimulated pDC, although this does not preclude the additional involvement of other regulatory molecules expressed on the surface of pDC. In contrast, CD1c+ DC appear to have little, if any, role in regulating innate and adaptive (recall) responses to RV, providing evidence that the adaptive cytokine responses were most likely due to memory T cells, not activation of naive T cells by Ag presentation of RV-specific Ags.

Depletion of CD45RO memory T cells resulted in a marked decrease in both Th1 and Th2 cytokine production, suggesting that little, if any, cytokine is produced by naive T cells or other cellular sources of Th1/Th2 cytokines following RV exposure. CD45RO cells could be a source of IL-3, which in turn could activate basophils; upon activation, these cells can be a source of IL-13. To investigate the activation of basophils, this was assessed in our culture conditions by flow cytometry. Given the extremely low number of cells found to become activated in the presence of RV16, it seems unlikely that these were the main source of IL-13 measured in the PBMC cultures and that the CD45RO memory T cells were the major cells producing IL-13.

Various authors report higher Th2 cytokine production by RV-stimulated cells from asthmatics, relative to healthy control subjects (20, 22, 39). The findings of the current study shed light on potential mechanisms by which this might occur. Evidence for deficient IFN-α/β secretion in asthma relative to healthy subjects has arisen from studies using different viruses and various circulating cell populations, including bronchial epithelial cells (8), PBMC (12, 19), and purified pDC (17). Asthma is also associated with numerical changes in circulating pDC, with lower numbers of blood pDC reported in infants and children (15, 40), although studies in adult asthma suggest that blood pDC numbers may be increased (16).

The main consequence of deficient IFN-α/β secretion in asthma has been thought to be impaired host defense and slow viral clearance. Given the findings reported in this work, we suggest that an additional and equally important consequence of deficient IFN-α/β secretion and pDC dysfunction is the expansion of Th2 responses to RV Ags, which might then intensify pre-existing Th2-mediated airway inflammation during infection. This is one of several mechanisms by which RV infection might precipitate acute asthma exacerbations. Animal models support this hypothesis, with multiple reports that pDC function to inhibit immunopathology associated with both allergic airway inflammation and viral lung infections (41–44). Further studies are clearly required using asthmatic and atopic cohorts; these investigations must, however, take into consideration the studies of Grayson et al. (45) and Subrata et al. (5), who describe type-I IFN induction of higher expression of the high-affinity IgE receptor (FceRI) on DC and monocytes. FceRI is not present on monocytes and myeloid DC from healthy, nonallergic individuals (46), so did not influence our findings described in this work.

Experimental evidence indicates that RV is not capable of replication within PBMC (47); therefore, the responses observed in this study were likely to have been initiated by viral detection and not dependent on viral replication. Using PBMC allowed us to develop a model in which an innate type-I IFN response was elicited, which was then available to interact with the adaptive responses. Whereas it should be acknowledged that the experiments outlined in this work were performed with a single strain of RV, the mechanisms by which all strains of RV activate pDC and induce type-I IFN synthesis are largely mediated by receptor recognition of conserved molecular patterns (e.g., viral ssRNA). We therefore propose that the capacity of type-I IFNs and pDC to inhibit Th2 cytokine production by T cells is likely to be shared across multiple RV strains and across many other respiratory viruses. Testing this hypothesis with a range of RV strains and other RNA viruses such as influenza and respiratory syncytial virus will thus be a high priority for the future. Although others have reported that RV can induce low-level expression of type-III IFNs in PBMC at both 8 and 24 h (48), we were only able to detect IFN-λ1 or IFN-λ2/3 in poly(I:C) (TLR3)-stimulated cultures and not in RV-stimulated cultures. Whereas the differences in findings may be explained by different methods of viral purification, we are conscious of the need to extend these studies to a wider range of viruses.

In conclusion, our dissection of the link between innate IFN responses and the Th1- and Th2-type adaptive cytokine responses to RV in healthy individuals has revealed a novel key role for pDC in controlling a maladaptive Th2 response. These findings might provide a link between two key observations in asthma, as follows: deficient pDC function (with subsequent decreased type-I IFN response) and an increased Th2 cytokine response. These findings could therefore potentially provide a vital clue as to the mechanism of RV-induced asthma exacerbation, as well as suggesting that type-I IFN is a vital therapeutic target to decrease maladaptive responses to this usually innocuous virus.

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Disclosures
The authors have no financial conflicts of interest.

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