Cutting Edge

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Cutting Edge: NADPH Oxidase Modulates MHC Class II Antigen Presentation by B Cells

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Phagocyte NADPH oxidase plays a key role in pathogen clearance via reactive oxygen species (ROS) production. Defects in oxidase function result in chronic granulomatous disease with hallmark recurrent microbial infections and inflammation. The oxidase's role in the adaptive immune response is not well understood. Class II presentation of cytoplasmic and exogenous Ag to $CD4^+$ T cells was impaired in human B cells with reduced oxidase $p40^{phox}$ subunit expression. Naturally arising mutations, which compromise $p40^{phox}$ function in a chronic granulomatous disease patient, also perturbed class II Ag presentation and intracellular ROS production. Reconstitution of patient B cells with a wild-type, but not a mutant, $p40^{phox}$ allele restored exogenous Ag presentation and intracellular ROS generation. Remarkably, class II presentation of epitopes from membrane Ag was robust in $p40^{phox}$-deficient B cells. These studies reveal a role for NADPH oxidase and $p40^{phox}$ in skewing epitope selection and T cell recognition of self Ag. ***The Journal of Immunology*****, 2012, 189: 3800–3804.**

The phagocyte NADPH oxidase plays a critical role in microbial killing by catalyzing electron transfer from NADPH to molecular oxygen, giving rise to superoxide and other forms of reactive oxygen species (ROS) (1). This oxidase contains several *phox* subunits, including $gp91^{phox}$ and $p22^{phox}$, which comprise flavocytochrome b_{558} as well as cytoplasmic $p40^{phox}$, $p47^{phox}$, and $p67^{phox}$. The binding of ligands to phagocyte receptors stimulates cytoplasmic subunit translocation to membrane-bound flavocytochrome b_{558}, facilitating superoxide production (1). *Phox* subunit mutations are associated with chronic granulomatous disease (CGD), characterized by the absence of or marked reduction in ROS production, recurrent pathogenic infections, and granulomatous inflammation (2, 3).

Whereas the microbicidal role of phagocyte NADPH oxidase during the innate immune response to pathogenic infections is established (2), its function in APC/T cell interactions is less well understood. In $gp91^{phox}$-deficient dendritic cells, but not macrophages, NADPH oxidase tempers phagosome acidification, preserving internalized Ags for efficient MHC class I-mediated cross-presentation to $CD8^+$ T cells (4, 5). In contrast, $CD4^+$ T cell activation was enhanced in response to murine macrophages with $p47^{phox}$ mutations (6). Thus, mutation of distinct oxidase subunits may differentially affect cellular immune responses.

Evidence is conflicting regarding a direct role for NADPH oxidase in regulating MHC class II (MHC II) Ag presentation. Presentation of exogenous OVA but not tetanus toxoid Ag was altered in APCs from CGD patients (7, 8). Neither study identified the defective oxidase subunits in the CGD patient-derived APCs. ROS produced by NADPH oxidase can regulate autophagy in phagocytes (9), and oxidase subunits are detected in endosomes and phagosomes (10). Presentation of exogenous Ag via MHC II requires Ag transit and proteolysis in endosomes and lysosomes to yield peptide ligands (11). Cytoplasmic or nuclear Ag can also access MHC II via several autophagy pathways (12). MHC II αβ complexes are directed via the invariant chain (Ii) to endosomes where proteases fragment Ii (13). HLA-DM, whose function is regulated by HLA-DO, facilitates the removal of these fragments and antigenic peptide capture by MHC II. The resulting peptide/MHC II complexes then traffic to the cell surface for immune surveillance by $CD4^+$ T cells.

The $p40^{phox}$ subunit has been associated with Crohn's disease and rheumatoid arthritis in genome-wide association

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Abbreviations used in this article: B-LCL, B lymphoblastoid cell line; CGD, chronic granulomatous disease; GAD, glutamate decarboxylase; $h\beta_2m$, human β_2-microglobulin; HSA, human serum albumin; Ii, invariant chain; MHC II, MHC class II; PI3P, phosphatidylinositol 3-phosphate; ROS, reactive oxygen species; shRNA, short hairpin RNA; WT, wild-type.

studies (14–16), but its role in Ag presentation has not been investigated. In this study, we examine how loss of $p40^{phox}$ in human B cells affects MHC II Ag presentation and oxidase function. In the absence of functional $p40^{phox}$, human B cells displayed a reduced capacity for cytoplasmic Ag presentation. Perturbations in $p40^{phox}$ also disrupted MHC II exogenous Ag presentation, yet presentation of membrane autoantigens was efficient. Disruption in $p40^{phox}$ compromised intracellular but not extracellular ROS production by B cells. These results suggest roles for NADPH oxidase and its regulatory subunit $p40^{phox}$ in skewing epitope selection by MHC II.

Materials and Methods

Cell lines

Human B lymphoblastoid cell lines (B-LCLs) and T cells have been described (17, 18). Lentiviral short hairpin RNA (shRNA) targeting human $p40^{phox}$ (19) or β_2-microglobulin ($h\beta_2m$) transcripts (Sigma-Aldrich) were used to transduce B-LCLs to generate $p40^{phox}$- or β_2m-deficient cells. Institutional approval was obtained for human blood collection. A B-LCL, AR40 from a $p40^{phox}$-deficient patient (3) (DRβ1*0101, DRβ1*0701), was generated and transduced to express DRβ1*0401 (17). Retroviruses encoding $p40^{phox}$ wild-type (WT) or $p40^{phox}$ R105Q were used to transduce AR40.DR4 (3). Frev and AR40 B-LCLs synthesize Igλ-chains but not Igκ-chains.

Western blotting

Cell lysates were analyzed by immunoblotting (10, 17) using Abs for $p22^{phox}$ (10), $p40^{phox}$ (Upstate Biotechnology), $p47^{phox}$, $p67^{phox}$ (BD Biosciences, San Jose, CA), $gp91^{phox}$ (10), and glutamate decarboxylase (GAD; Sigma-Aldrich). The mAb DA6.147 detects HLA-DRα and HLA-DR$\alpha\beta$ dimers (20). The mAb PIN1.1 detects Ii (21). Membranes were stripped and reprobed for β-actin (Sigma-Aldrich) or GAPDH (Chemicon) as controls for sample loading. Quantity One one-dimensional analysis software (Bio-Rad) was used to quantify protein expression.

Ag presentation

APCs were incubated with or without 10–20 μM human serum albumin (HSA), human IgG Ag (Sigma-Aldrich), or peptides $HSA_{64–76}$, $\kappa I_{188–203}$, and $\kappa II_{145–159}$ (Quality Controlled Biochemicals) for 6 h at 37°C. APCs were washed and incubated with epitope-specific T cells for 24 h at 37°C prior to analysis for T cell activation (17). Data shown are the averages of triplicate samples for each assay, and the error bars indicate the mean T cell activation ($\pm$SD). Statistical comparisons between two groups were performed using an unpaired *t* test, whereas comparison among three groups was performed using a one-way ANOVA. In each case, $p \leq 0.01$ was considered to be significant. Adjustment for multiple comparisons was made using the Bonferroni correction.

Flow cytometry and ROS production

Cells were fixed, permeabilized, and incubated with the mAb MaP.DM1 or HLA-DO (BD Biosciences) (17). To detect intracellular ROS, viable cells were incubated with 5 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (Invitrogen) and stimulated with or without 10 μg/ml PMA (Sigma-Aldrich) for 30 min at 37°C (22). Cellular ROS production was sensitive to the oxidase inhibitor diphenyleneiodonium.

Results and Discussion

Diminished cytoplasmic Ag presentation in B-LCLs with reduced $p40^{phox}$ expression

Autophagy promotes cytoplasmic Ag presentation by MHC II (12, 23). To test whether $p40^{phox}$ plays a role in cytoplasmic GAD Ag presentation, $p40^{phox}$ expression was disrupted in the B cell line PriessGAD using shRNA. Expression of $p40^{phox}$ was reduced ~80% in these cells compared with parental PriessGAD, whereas GAD Ag expression was unperturbed (Supplemental Fig. 1A, 1B). The ability of $p40^{phox}$-deficient B-LCLs to present GAD epitopes was substantially reduced (Fig. 1A). PriessGAD cells transduced with control $h\beta_2m$ shRNA (Supplemental Fig. 1C) stimulated GAD-specific

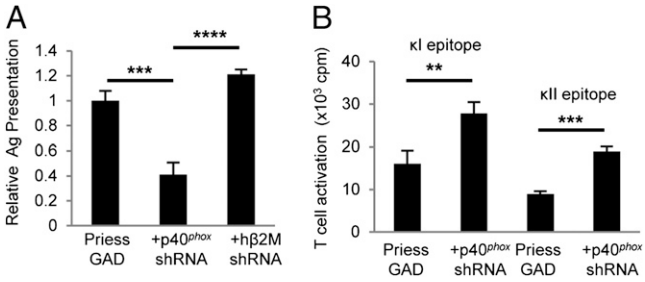

FIGURE 1. Altered Ag presentation in B-LCLs with reduced $p40^{phox}$ expression. (**A**) PriessGAD, PriessGAD plus $p40^{phox}$ shRNA, or PriessGAD plus $h\beta_2m$ shRNA cells were cultured with GAD-specific T cells to measure cytoplasmic GAD presentation. (**B**) PriessGAD and PriessGAD plus $p40^{phox}$ shRNA cells were cultured with either κI- or κII-specific T cells to measure endogenous Igκ presentation. In data not shown, APCs were titrated from 1000 to 125 cells/well to determine the effect of reduced $p40^{phox}$ expression on endogenous Ig κ presentation. Data in (A) and (B) are representative of three independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

T cells comparably with parental PriessGAD (Fig. 1A). Levels of LC3-II, a marker of autophagosome formation (24), were similar in each B cell line tested, suggesting little change in autophagy with disruption of $p40^{phox}$ (Supplemental Fig. 1D). Surface expression of HLA-DR4 was equivalent for PriessGAD and shRNA-treated cells (Supplemental Fig. 1E, 1F). Notably, PriessGAD cells with reduced $p40^{phox}$ levels stimulated T cells specific for endogenous Ag Igκ more efficiently than did the parental PriessGAD cells (Fig. 1B). These results suggest that reduced $p40^{phox}$ expression in B-LCLs may compromise cytoplasmic Ag presentation while favoring epitope presentation from endogenous Igκ.

Reducing B-LCL $p40^{phox}$ expression disrupted MHC II exogenous Ag presentation

To evaluate the role of $p40^{phox}$ in exogenous Ag presentation, $p40^{phox}$ expression was diminished ~80% by treating Frev B-LCL with a $p40^{phox}$-specific shRNA (Supplemental Fig. 1G). T cell responses to the exogenous Ag HSA and Frev cells with diminished $p40^{phox}$ levels were significantly reduced compared with Frev cells transduced with $h\beta_2m$ shRNA (Fig. 2A, Sup-

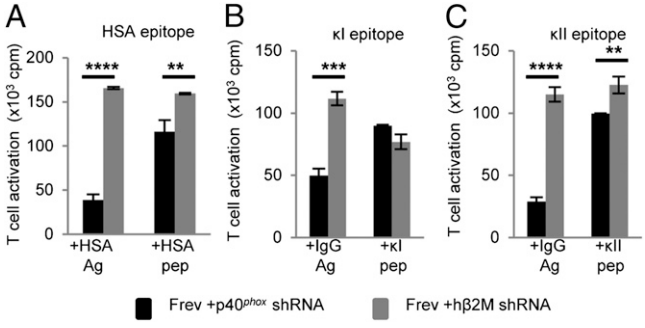

FIGURE 2. Reduced exogenous Ag presentation in B-LCLs with diminished $p40^{phox}$ expression. (**A**) Frev plus $p40^{phox}$ shRNA or Frev plus $h\beta_2m$ shRNA cells were incubated with HSA Ag or $HSA_{64–76}$ peptide and cultured with HSA-specific T cells to measure MHC II presentation. Data are representative of two independent experiments. (**B** and **C**) Frev plus $p40^{phox}$ shRNA or Frev plus $h\beta_2m$ shRNA cells were incubated with human IgG Ag, $\kappa I_{188–203}$ peptide, or $\kappa II_{145–159}$ peptide and cultured with either κI-specific T cells (B) or κII-specific T cells (C) to measure MHC II presentation. Minor differences in peptide presentation were observed using B-LCLs treated with $p40^{phox}$ shRNA based on statistical analyses. Data in (B) and (C) are representative of three independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

plemental Fig. 1H) or the parental line Frev (data not shown). The ability of Frev cells with reduced p40^{phox} expression to present exogenous IgG Ag was also evaluated. Frev cells with reduced p40^{phox} levels were less effective in presenting exogenous IgG to T cells compared with Frev transduced with control hβ_2m shRNA (Fig. 2B, 2C) or the parental line Frev (data not shown). Surface expression of HLA-DR4 was equivalent in each cell line (Supplemental Fig. 1I, 1J). Only minor differences were observed in exogenous peptide presentation by Frev cells with diminished p40^{phox} expression compared with cells treated with hβ_2m shRNA (Fig. 2A–C). These results suggest that a reduction in p40^{phox} expression in B-LCLs may perturb multiple routes for Ag presentation by MHC II.

Analysis of the MHC II pathway in B-LCLs with mutations in p40^{phox}

A new genetic subgroup of CGD with mutations in the gene *NCF4* encoding p40^{phox} was described in a patient and linked to functional defects in the neutrophil NADPH oxidase (3). In this patient, one *NCF4* allele harbors a frame-shift mutation with a premature stop codon, whereas the other allele encodes a point mutation (R105Q) resulting in a nonfunctional form of p40^{phox}. A B-LCL from this patient (AR40) expressing HLA-DR4 was further transduced with either WT p40^{phox} (AR40.DR4.p40^{phox} WT) or the R105Q mutant allele (AR40.DR4.p40^{phox} R105Q) to evaluate the effects of p40^{phox} mutation and reconstitution. Immunoblots demonstrated reduced p40^{phox} expression in AR40.DR4 cells, consistent with the frame-shifted *NCF4* allele as seen in patient neutrophils (3), and higher levels of WT or mutant p40^{phox} in the reconstituted cells (Fig. 3A). Levels of gp91^{phox} and p67^{phox} were comparably reduced in the reconstituted cells relative to the patient line, likely due to clonal variation. Extracellular ROS production upon PMA stimulation was similar in the patient and WT p40^{phox}-reconstituted B cells (Supplemental Fig. 2A) as observed in p40^{phox}-deficient neutrophils (3). However, basal and PMA-inducible intracellular ROS production were reduced in the AR40.DR4 cells compared with cells expressing WT p40^{phox} (Fig. 3B), thus suggesting a defect in the ability of the oxidase in the patient B cells to produce intracellular ROS similar to p40^{phox}-deficient neutrophils (3).

Whether the absence of functional p40^{phox} in AR40.DR4 B-LCLs influenced the expression of molecules in the MHC II Ag presentation pathway was tested. A slight reduction in the levels of total HLA-DRα, HLA-DR$\alpha\beta$, and Ii were observed in AR40.DR4.p40^{phox} WT and AR40.DR4.p40^{phox} R105Q compared with AR40.DR4 (Fig. 3C). Maturation of Ii was not impaired in AR40.DR4 as detected by the presence of mature glycosylated forms of Ii (Fig. 3C, asterisk) in the p40^{phox}-deficient and reconstituted B-LCLs. Changes in HLA-DM and HLA-DO can alter Ag presentation without perturbing T cell responses to synthetic peptides (25–27). AR40.DR4, AR40.DR4.p40^{phox} WT, and AR40.DR4.p40^{phox} R105Q expressed equivalent levels of HLA-DM and HLA-DO (Fig. 3D). Taken together, these results suggest that the absence of functional p40^{phox} in AR40.DR4 did not substantially alter the levels of HLA-DR, HLA-DM, HLA-DO, and Ii.

Reconstitution of p40^{phox}*-deficient B-LCLs partially restored MHC II exogenous Ag presentation*

Naturally occurring mutations in p40^{phox} impacted the ability of B-LCLs to efficiently present Ag to MHC II-restricted T cells (Fig. 4). Reconstitution of the patient-derived AR40.

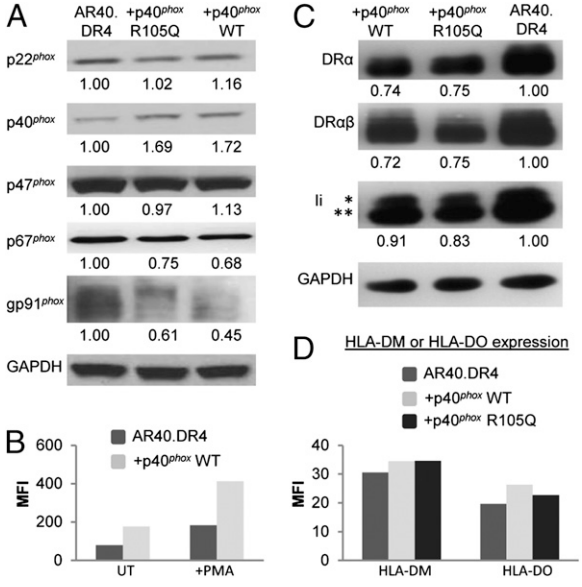

FIGURE 3. Expression of oxidase subunits and MHC II in p40^{phox}-deficient and reconstituted B-LCLs. (**A**) Cell lysates were immunoblotted to detect oxidase subunits and GAPDH. Values indicate the ratio of oxidase subunit levels to a loading control. (**B**) B cells were incubated with 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester to detect intracellular ROS production and left untreated (UT) or PMA stimulated. (**C**) Cell lysates were immunoblotted for HLA-DRα, HLA-DR$\alpha\beta$, Ii, and GAPDH. Values indicate the ratio of MHC II or Ii to GAPDH. (**D**) B cells were stained to detect HLA-DM or HLA-DO. Data in panels (A)–(D) are representative of at least three independent experiments.

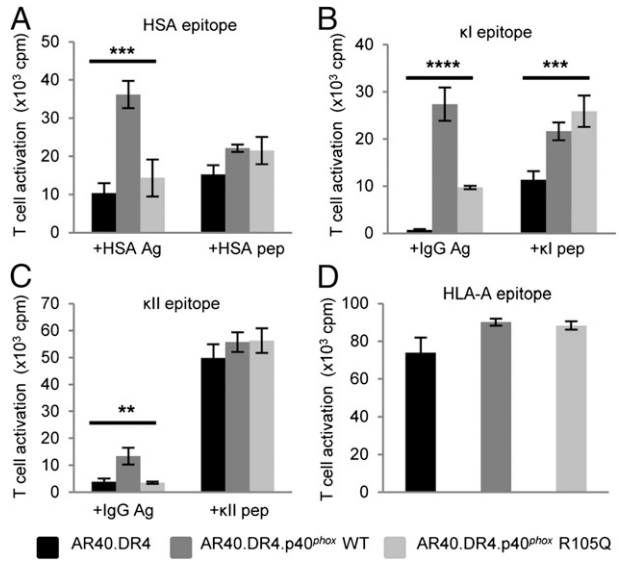

FIGURE 4. Exogenous but not endogenous Ag presentation was defective in p40^{phox}-deficient B-LCLs. (**A**–**C**) B cells were incubated with HSA Ag or $HSA_{64–76}$ peptide (A), human IgG Ag (B, C), $\kappa I_{188–203}$ peptide (B), or $\kappa II_{145–159}$ peptide (C) as in Fig. 2 to measure MHC II presentation. In data not shown, cells without Ag or peptide failed to activate epitope-specific T cells. (**D**) B cells were cultured with T cells to measure endogenous HLA-A epitope presentation. Data in (A)–(D) are representative of three independent experiments. $^{**}p \leq 0.01$, $^{***}p \leq 0.001$, $^{****}p \leq 0.0001$.

DR4 cells with WT p40^{phox}, but not the mutant p40^{phox} R105Q allele, enhanced exogenous HSA Ag presentation (Fig. 4A). AR40.DR4 cells were unable to present either Ig κI or κII epitopes to T cells (Fig. 4B, 4C). Reconstitution of AR40.DR4 cells with p40^{phox} WT restored κI epitope presentation to a greater extent than with p40^{phox} R105Q expression (Fig. 4B). Only reconstitution of AR40.DR4 with p40^{phox} WT facilitated presentation of the κII epitope from exogenous human IgG (Fig. 4C). Although AR40.DR4 cells were able to present exogenously added synthetic peptides to T cells (Fig. 4), the level of κI peptide presentation was reduced compared with either AR40.DR4.p40^{phox} WT or AR40.DR4.p40^{phox} R105Q (Fig. 4B). Surface expression of HLA-DR4 was equivalent in each cell line (Supplemental Fig. 2B). Exogenous tetanus toxoid Ag presentation was reduced not only in AR40.DR4 but in B cells deficient in another oxidase subunit, gp91^{phox} (Supplemental Fig. 2C), consistent with perturbations in oxidase function modulating MHC II Ag presentation. Reconstitution of AR40.DR4 with WT p40^{phox} restored tetanus presentation (Supplemental Fig. 2C). Addition of an extracellular source of ROS failed to reconstitute tetanus presentation by B cells deficient in p40^{phox} or gp91^{phox} (Supplemental Fig. 2D). The ability of AR40.DR4 to endocytose a model exogenous Ag, FITC-albumin, was equivalent for p40^{phox}-deficient or reconstituted B-LCLs (Supplemental Fig. 2E). In data not shown, we observed a similar persistence of the FITC-albumin at longer time points (6–18 h) in each cell line. These results suggest that p40^{phox} deficiency does not substantially affect the internalization or initial degradation of a model exogenous protein.

The presence of assembled HLA-DRαβ dimers on the surface of AR40.DR4 suggested that these MHC II may acquire peptides from a source other than exogenous Ag. The ability of these cells to present antigenic peptides derived from an endogenous transmembrane protein was evaluated using an HLA-DR4–restricted T cell recognizing an epitope from MHC class I HLA-A. AR40.DR4 cells activated the HLA-A–specific T cells whereas reconstitution with either the p40^{phox} WT or R105Q mutant allele did not enhance this Ag presentation (Fig. 4D). Total MHC class I expression in each of the AR40.DR4-derived cells was equivalent (data not shown). These results suggest that although MHC II-restricted exogenous Ag presentation was impaired in the p40^{phox}-deficient cells, the presentation of an endogenous transmembrane protein in the context of MHC II could be readily detected.

In conclusion, the microbicidal role of phagocyte NADPH oxidase in neutrophils and macrophages during the innate immune response to pathogenic infections is well established, but the role of the oxidase in APCs during the adaptive immune response is less clear. In this study, functional p40^{phox} was shown to be required for the efficient presentation of cytoplasmic GAD and multiple exogenous Ags by MHC II in B cells. Additionally, studies suggest that functional gp91^{phox} is important for MHC II presentation of exogenous Ags. In phagocytes, cytoplasmic p40^{phox} binds to membrane phosphatidylinositol 3-phosphate (PI3P) and promotes assembly of the NADPH oxidase complex on phagosomes (19). Association of p40^{phox} and the oxidase with PI3P found in endosomal/lysosomal membranes (3) could influence MHC II Ag processing and presentation within these organelles. The cytosolic localization of the mutant p40^{phox} R105Q and lack of PI3P binding (3) may explain its inability to completely restore exogenous Ag presentation in p40^{phox}-deficient B cells. In B cells, the oxidase may regulate BCR signaling (28) and, as revealed in this study, MHC II Ag presentation. Constitutive and inducible intracellular ROS production was higher in B cells expressing functional p40^{phox}, supporting a direct role for p40^{phox} in regulating B cell intracellular ROS generation. Interestingly, p40^{phox}-deficient B cells were capable of presenting epitopes derived from endogenous membrane-resident proteins, suggesting that p40^{phox} may modulate the peptide repertoire displayed by MHC II on B cells and, subsequently, CD4$^+$ T cell activation. Alterations in the function of the oxidase in B cells may therefore contribute to genetic predisposition to autoimmunity in some CGD patients. Increased incidences of rheumatoid arthritis, inflammatory bowel disease, as well as discoid lupus have been associated with oxidase subunit mutations and CGD (29).

Disclosures

The authors have no financial conflicts of interest.

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