Activated CD4+ T Cells Target Mesangial Antigens and Initiate Glomerulonephritis

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Key Words

Lupus nephritis · Glomerulus · T cells · Targeted delivery

Abstract

Aims: The role of kidney infiltrating T cells in the pathology of lupus nephritis is unclear. This study was undertaken to investigate whether CD4+ T cell responses to a surrogate mesangial antigen can initiate glomerulonephritis. **Methods:** Ovalbumin (OVA) was deposited in the glomerular mesangium of C57BL/6 (B6) mice using anti- α 8-integrin immunoliposomes (α 8ILs). This was followed by injection of activated OVA-reactive CD4+ transgenic OT2 T cells. Trafficking of antigen-specific OT2 T cells to kidneys and lymph nodes was studied by flow cytometry. Glomerular pathology and immune cell infiltration was characterized by immunostaining. Role of CCR2 deficiency on T cell-mediated glomerulonephritis was investigated using B6.ccr2^{-/-} mice. **Results:** α 8ILs delivered OVA specifically to the renal glomeruli. Adoptively transferred OT2 T cells preferentially accumulated in renal lymph nodes and in the renal cortex. Kidneys showed glomerular inflammation with recruitment of endogenous T cells, dendritic cells and macrophages. T cell-mediated inflammation induced mesangial cell activation

and an increase in glomerular MCP1 and fibronectin. The formation of inflammatory foci was driven by Ly6C monocytes and was CCR2 dependent. **Conclusions:** The findings from this study show that T cells reactive with antigens in the mesangium are sufficient to initiate glomerular pathology. Antigen-specific CD4 T cells act by inducing glomerular MCP1 production which mediates recruitment of inflammatory monocytes resulting in glomerulonephritis. Thus, downmodulation of T cell responses within the kidneys of lupus patients will be a beneficial therapeutic approach.

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Introduction

CD4+ T cells are important mediators of systemic lupus erythematosus [1]. In addition to helping autoreactive B cells to produce autoantibodies, CD4+ T cells are directly involved in the renal pathology of lupus. Our studies in New Zealand Mixed 2328 mice, a spontaneous mouse model of systemic lupus erythematosus, showed that mesangial immune complex deposition was accompanied by CD4+ T cell activation in the renal lymph nodes and glomerular T cell infiltration [2]. T cell recep-

tor analyses indicated a local antigen-driven T cell response. A direct role for T cells in pathogenesis of lupus nephritis is also supported by the finding that inhibiting T cell costimulation increases the life span of nephritic mice. [3]. Yet, whether CD4⁺ T cells mediate the initiation of renal injury is unclear.

In this study, we tested the hypothesis that T cells responding to antigens in the mesangium can induce glomerulonephritis. Since the identity of antigens recognized by pathogenic T cells in lupus is not known, we used ovalbumin (OVA) as a surrogate mesangial antigen. We have previously described anti- α 8-integrin immunoliposomes (α 8ILs), a unique methodology for targeted delivery to the glomerular mesangium [4, 5]. Liposomes sized to approximately 100 nm in diameter enter the mesangial space through the fenestrated glomerular endothelium following intravenous injection. The anti- α 8-integrin antibody conjugated to the liposomes reacts with α 8-integrin on the mesangial cell surface. This allows accumulation of liposomes in the glomerular mesangium and leads to delivery of the liposomal contents at the site. Thus, in the present study, OVA was deposited in the mesangium using α 8ILs and the ability of anti-OVA CD4⁺ T cells responses to initiate glomerulonephritis was investigated.

Methods

Preparation of Liposomes

Anti- α 8ILs were prepared as previously described [4]. To incorporate OVA, the lipid mixture was hydrated either with chicken OVA (Sigma Aldrich Corporation, St. Louis, Mo., USA) or fluorescein isothiocyanate-conjugated OVA (FITC-OVA) (Invitrogen, Carlsbad, Calif., USA) in water, lyophilized and rehydrated in 0.025 M Tris, pH 8.0. After repeated washing, the liposomal OVA content was estimated by modified BCA assay (Peirce Biotechnology, Rockford, Ill., USA). Liposomes were then conjugated to either a rabbit anti- α 8 integrin antibody (OVA- α 8ILs) or to rabbit IgG (control OVA-ILs).

Mice

Mice were maintained in a specific pathogen-free mouse facility at the University of Virginia. All experimental procedures were approved by the Institutional Animal Care and Use Committee. Breeders for B6.Cg-Tg(*Tcr α Tcr β*) 425Cbn/J (OT2) and B6.129S4-*Ccr2*^{tm1lf/J} (B6.*ccr2*^{-/-}) mice were obtained from Jackson Laboratories (Bar Harbor, Me., USA). C57BL/6 (B6) mice were purchased from National Cancer Institute (Frederick, Md., USA). OT2 transgenic mice express CD4⁺ T cell receptor transgene (V α 2, V β 5) reactive with OVA₃₂₃₋₃₃₉ peptide and were used as donors for antigen-specific T cells [6]. B6 and B6.*ccr2*^{-/-} female mice were used as recipients.

Cell Transfers

OT2 T cells were activated with OVA₃₂₃₋₃₃₉ peptide (Ana Spec, Fremont, Calif., USA) for 72 h using standard protocols [7]; live cells were harvested over a Ficoll gradient and injected intravenously into recipient mice. For some experiments, CD4⁺ T cells were purified by negative selection (Miltenyi Biotec, Auburn, Calif., USA), activated with OVA₃₂₃₋₃₃₉ peptide and irradiated syngenic spleen cells. This method of stimulation yielded Th1-deviated OT2 cells, and this was confirmed by detection of IFN γ in the culture supernatants at 24 and 48 h after stimulation (data not shown). To investigate activation of OT2 cells *in vivo*, naïve OT2 T cells were isolated and injected intravenously into recipient mice. In all experiments, mice received 12–14 million OT2 T cells.

Flow Cytometry

Cell suspensions from spleens, lymph nodes and kidneys were studied by flow cytometry using standard methods [2]. Antibodies conjugated to fluorochromes were obtained from eBiosciences (San Diego, Calif., USA) or BD Biosciences (San Jose, Calif., USA) except as indicated. Cells were incubated with anti-CD16/32 (Clone 93) and OT2 T cell frequencies were analyzed by 4-color staining for CD45 (30-F11), CD4 (L3T4), T cell receptor V α 2 (B20.1) and V β 5 (MR9-4). Kidney-infiltrating macrophages and dendritic cells were studied by 6-color flow cytometry using antibodies to CD45 (30-F11), F4/80 (BM8), CD11b (M1/70), CD11c (HL3) and Ly6C (HK1.4, Abcam). Bromodeoxyuridine (BrdU) incorporation was measured by intracellular staining using anti-BrdU antibody (BU20A) followed by FITC-conjugated F(ab)₂ anti-mouse IgG. Stained cells were acquired on 8-color BD FACS Calibur using Cytex dxP8 software (Cytex Technologies, Fremont, Calif., USA). Data were analyzed using FlowJo Software 9.1 (Tree Star Inc., Ashland, Oreg., USA).

Immunofluorescence Staining of Isolated Glomeruli and Kidney

For detection of FITC-OVA, glomeruli were isolated and stained as previously described [4]. Nonspecific staining was blocked with normal rabbit serum and incubated with goat anti-FITC antibody (Vector Laboratories, Burlingame, Calif., USA). Bound antibody was detected with fluoresceinated rabbit anti-goat IgG (Vector Laboratories).

In the kidney, FITC-OVA was detected in frozen sections, fixed in cold acetone and observed under a fluorescence microscope. Infiltrating cells were characterized by immunostaining with antibodies to CD4 (GK1.5), CD11c (HL3), CD68 (AbD serotec, Raleigh, N.C., USA) and MHC II (M5/114.15.2) as previously described [2, 8]. MCP1 was detected using rabbit anti-MCP1 antibody (Peprotech, Rocky Hill, N.J., USA) followed by PE-conjugated anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa., USA). Glomerular fibronectin was detected with rabbit anti-fibronectin antibody (Millipore Corporation, Sunnyvale, Calif., USA) [8]. All fluorescence images were captured on a Zeiss Confocal Microscope or Zeiss Apotome fluorescence microscope, and the images were analyzed using LSM5 and Axiovision 4.7 software (Carl Zeiss MicroImaging LLC, Thornwood, N.Y., USA), respectively.

Renal Histopathology

Kidneys were collected in formalin and processed for paraffin embedding. Sections (3 μ m thickness) were stained with hema-

toxylin and eosin and evaluated for pathologic changes as previously described [2].

Statistical Analysis

Statistical analyses were done by Student's *t* test or ANOVA with Bonferroni posttest using GraphPad Prism 5.0 (GraphPad Software Inc., La Jolla, Calif., USA).

Results

Deposition of OVA as a Surrogate Self-Antigen in the Glomerular Mesangium

To study the kinetics of glomerular delivery, FITC-OVA-loaded α 8ILs were injected intravenously into B6 mice. FITC-OVA was rapidly deposited in the glomeruli and could be detected by indirect immunofluorescence 6 h after injection (fig. 1a). The amount of FITC-OVA in the glomeruli increased by day 5 (fig. 1b). In the kidney, FITC-OVA was restricted to glomeruli and showed a typical mesangial pattern of distribution (fig. 1c). FITC-OVA loaded liposomes conjugated to rabbit IgG (control OVA-ILs) did not traffic to the renal glomeruli.

Activated OT2 T Cells Target Mesangial OVA and Induce Glomerulonephritis

Mice were given two intravenous injections of OVA- α 8ILs or control liposomes, 12 h apart followed by a single injection of activated OT2 splenocytes as shown in figure 2. Kidneys studied on day 8 after transfer showed inflammation in the renal cortex predominantly in glomeruli and periglomerular regions (fig. 3a). These results were further confirmed by adoptive transfer of purified and activated OT2 T cells (94% purity, 99% V α 2 V β 5-positive). Kidneys were harvested on days 3, 8 and 14 after cell transfer, and infiltrating cells analyzed by flow cytometry showed an increased frequency of OT2 cells on days 8 and 14 (fig. 3b). This was accompanied by an increase in the CD4⁺ T cells infiltrating the kidney. Interestingly, OT2 cells were only 3–8% of the infiltrating CD4⁺ cells. Thus, trafficking of OT2 cells into the kidney led to the recruitment of endogenous, non-OT2, CD4⁺ T cells.

Immunostaining showed aggregates of CD4⁺ T cells and MHCII antigen presenting cells in the renal cortex on day 3; with significant intraglomerular and periglomerular infiltrates on days 8 and 14. The CD11c⁺ dendritic cells (fig. 4a) and CD68 and F4/80 positive macrophages (fig. 4b) in the infiltrates were the predominant MHCII⁺ cells, suggesting that they are the antigen-presenting cells (insets to fig. 4a, b, respectively). Inflammatory foci were not seen in control mice.

Fig. 1. Accumulation of OVA in the kidney is rapid, specific to the glomeruli and has a mesangial distribution. FITC-OVA- α 8ILs or control FITC-OVA-ILs were injected into B6 mice and delivery of FITC-OVA was studied at different time points. **a** Representative photomicrographs show accumulation of FITC-OVA (arrows) in glomerular isolates at 6 h after injection. **b** Frozen sections of the kidney on day 5 after transfer show FITC-OVA restricted to the glomeruli within the kidney (g). **c** Higher magnification shows a typical mesangial distribution of FITC-OVA. Scale bar = 50 μ m.

To evaluate the pathogenic effect of glomerular inflammation, kidneys were stained for fibronectin, a component of the extracellular matrix and an early marker for mesangial cell activation [9]. On day 8, glomerular fibronectin was increased in mice given OVA- α 8ILs and OT2 cells (fig. 4c). At these time points, the kidney function was normal. Thus, activated CD4⁺ T cells reacting to mesangial antigens can initiate mesangial injury and glomerulonephritis.

Fig. 2. Experimental design. Female mice were injected with OVA- α 8ILs in the tail vein on day 1. The OVA- α 8ILs traffic to the renal glomeruli and deposit OVA in the glomerular mesangium. This was followed by i.v. injection of activated OT2 cells on day 0. Mice were euthanized on days 3, 8 or 14 after OT2 cell injection and kidneys and lymph nodes were studied. Mice injected with only α 8ILs (lacking OVA), only OVA- α 8ILs or only activated OT2 T cells were used as controls as indicated in each experiment.

Fig. 3. OVA delivered to glomeruli by OVA- α 8ILs is a target for activated antigen-specific T cells. **a** OVA- α 8ILs injection followed by transfer of OT2 cells induced glomerulonephritis with inflammatory cell infiltrates (arrows) on day 8 after cell transfer. This was not seen in control mice injected with control OVA-ILs and OT2 cells. **b** Frequencies of OT2 cells (of CD4 gate) and total CD4+ T cells (of CD45 gate) infiltrating the kidneys were analyzed by flow cytometry on days 3, 8 and 14, and show increased accumulation in mice injected with OVA- α 8ILs and OT2 T cells (●) compared to OT2-injected control mice (○). **c** Mice injected with activated OT2 cells developed infiltration of CD4+ T cells

and inflammation following glomerular OVA deposition. Representative photomicrographs of kidneys from B6 mice, injected with OVA- α 8ILs followed by OT2 cells and sacrificed 3 days or 14 days later are shown. Sections were stained for CD4+ T cells (red) and MHCII antigen-presenting cells (green). Inflammatory foci (arrows) with clusters of CD4+ T cell and MHCII+ antigen-presenting cells were seen in the renal cortex on day 3 with periglomerular inflammation on day 14. A higher magnification photomicrograph of CD4+ T cells infiltrating into the glomeruli is also shown. Scale bar = 50 μ m. Control mice injected with OT2 cells alone did not develop inflammatory infiltrates.

Fig. 4. Inflammatory cells and glomerular responses in OT2 T cell-mediated glomerulonephritis. Representative photomicrographs of renal cortex from mice treated with OT2 T cells or OVA- α 8ILs and OT2 T cells on day 8 after cell transfer stained for dendritic cells (**a**), macrophages (**b**) and fibronectin (**c**). **a** Arrows indicate clusters of CD11c (red) cells coexpressing MHCII (green). Inset shows intraglomerular infiltration of dendritic cells. **b** Macrophages detected by CD68 (red) and F4/80 (green) were increased and are indicated with arrows. Inset B shows that intraglomerular CD68 (red) macrophages infiltrating the glomeruli were activated and express MHC II (green). Representative photomicrographs from control mice injected with OT2 T cells alone did not show inflammatory infiltrates. Results are representative of two independent experiments. **c** Kidney sections stained for fibronectin and images captured from four different areas of the renal cortex in each mouse at 20 $\times$ magnification and the intensity of fluorescent staining in glomeruli was evaluated. Representative images show that fibronectin deposition in glomeruli (arrows) was increased in mice injected with OVA- α 8ILs and OT2 T cells compared to controls given OT2 cells alone. Scale bars = 50 μ m.

Mesangial Antigens Are Presented to Activated T Cells in Renal Lymph Nodes

Lymph nodes and spleens were harvested from mice to study trafficking of OT2 T cells (fig. 5). On day 8 after transfer, mice with mesangial OVA consistently showed an increased frequency of OT2 cells in the renal lymph nodes ($p < 0.01$) compared to controls (fig. 5b). Furthermore, the enrichment of OT2 cells was specific to renal lymph nodes compared to the nonrenal lymph nodes ($p < 0.001$). This enrichment was not seen in control mice given activated OT2 cells alone. In the spleen, frequencies of OT2 cells in mice injected with OVA- α 8ILs and activated OT2 cells were 11.6 ± 1.7 (absolute numbers $1.06 \pm 0.15 \times 10^6$; mean $\pm$ SEM) and were not significantly higher than mice injected with activated OT2 cells alone with an average frequency of 8.3 ± 0.5 (absolute numbers $0.85 \pm 0.15 \times 10^6$). These data suggest that the presentation of OVA was occurring predominantly in the kidney and local lymph nodes and provides additional evidence that delivery by OVA- α 8ILs was specific to the kidney.

Recruitment of Ly6C⁺ Monocytes Is Critical for Glomerular Inflammation

Monocyte chemoattractant protein 1 (MCP1) in the glomerulus is an early indicator of inflammation [10]. MCP1 mediates macrophage recruitment through its receptor, chemokine CC motif receptor 2 (CCR2) [11]. To study the role of macrophages and the MCP1-CCR2 axis, OVA- α 8ILs were injected into *ccr2*^{+/+}-sufficient (wild-type) and *ccr2*^{-/-} mice, and followed by adoptive transfer of activated OT2 cells. MCP1 expression measured by qPCR was increased in the kidneys from both *ccr2*^{+/+} (2.8 ± 1.0 ; mean $\pm$ SD, $n = 5$) and *ccr2*^{-/-} mice (6.0 ± 2.5 ; mean $\pm$ SD, $n = 5$) fold over controls injected with activated OT2 cells alone. These results were confirmed by immunostaining for MCP1 protein in the glomeruli (fig. 6). Control mice did not show glomerular MCP1 expression. Flow cytometry analyses showed that CD4⁺ T cell infiltration was comparable between wild-type and *ccr2*^{-/-} mice (fig. 7a, left panel) and there was no significant difference in the recruitment of antigen-specific OT2 cells (data not shown) into the kidney. Further, the frequencies of CD11b macrophages and CD11c dendritic cells were comparable (data not shown). However, there was a small though consistent, statistically significant reduction in the frequency of Ly6C inflammatory monocytes in *ccr2*^{-/-} mice ($n = 5$ /group; $p = 0.0096$; fig. 7a, right panel). This was reproduced in an additional experiment; $p = 0.019$; $n = 4$ /group, data not shown). Representative gates used for analyses of Ly6C cells are shown (fig. 7b).

Fig. 5. OT2 T cells traffic preferentially to renal lymph nodes in mice with mesangial OVA. **a** Trafficking of OT2 T cells was studied by flow cytometry in renal and nonrenal lymph nodes. Gates for CD4 and OT2 T cells (V α 2 V β 5) are shown. **b** OT2 frequencies in renal and nonrenal lymph nodes from mice injected with OVA- α 8ILs and OT2 cells or with OT2 cells alone. Data are from 1 of 5 different experiments. Each data point indicates 1 mouse. p values calculated by ANOVA with Bonferroni's post test.

Surprisingly, despite comparable OT2 and endogenous CD4⁺ T cell recruitment, *ccr2*^{-/-} mouse kidney showed a complete lack of inflammatory foci (fig. 7c). Thus, trafficking of antigen-specific and endogenous T cells into the kidney was not affected by CCR2 deficiency. However, recruitment of Ly6C cells was important for the development of glomerulonephritis.

Mesangial Antigens Traffic to the Renal Lymph Nodes and Activate Antigen-Specific T Cells

The results presented above show that mesangial antigens are presented to activated CD4⁺ T cells in the kidney leading to recruitment of bystander T cells and

inflammatory macrophages inducing glomerular inflammation. This is associated with activation of antigen-specific T cells in the renal lymph nodes. To investigate whether mesangial antigens drain to the renal lymph nodes, naïve OT2 cells were transferred into mice injected with OVA- α 8ILs. Control mice received only naïve OT2 cells. The mice were given a single injection of BrdU intraperitoneally (2 mg/mouse), 12 h prior to sacrifice on day 3 after transfer (fig. 8a). Accumulation of naïve OT2 cells in the renal lymph nodes was accompanied by increased proliferation in mice injected with OVA- α 8ILs compared to controls (fig. 8b). At this point, few OT2 cells were detected in the kidneys of either group and

Fig. 6. Activated OT2 cells induce MCP1 expression in glomeruli of mice injected with OVA- α 8ILs. Increased expression of glomerular MCP1 (red) was seen in B6.*ccr2*^{+/+} [wild-type (WT)] and *ccr2*^{-/-}. MCP1 was not detected in glomeruli of WT mice given OT2 cells alone. Note that the MCP1 expression is predominantly in the glomeruli.

Fig. 7. Ly6C⁺ monocyte recruitment is required for glomerular inflammation. B6.*ccr2*^{+/+} (WT) and *ccr2*^{-/-} mice were injected with OVA- α 8ILs followed by activated OT2 cells, and kidneys were studied on day 8 after cell transfer. **a** In the CD45 gates, frequencies of CD4⁺ T cells (left panel) were comparable between the two groups. However, Ly6C⁺ cells (right panel) were reduced in *ccr2*^{-/-} mice. **b** Representative gates for analysis of CD45⁺ and Ly6C⁺ cells infiltrating the kidney are shown. **c** Immunostaining for CD4 (red) and MHC II (green) cells in the kidney is shown in inflammatory foci (arrows) in B6 *ccr2*^{+/+} mice. These foci were absent in *ccr2*^{-/-} mice, although CD4⁺ T cells (red) were seen scattered in the renal cortex. Scale bar = 50 μ m.

Fig. 8. Proliferation of naïve OT2 cells in renal lymph nodes in response to mesangial antigens. **a** Female mice were injected with OVA- α 8ILs in the tail vein followed by i.v. injection of naïve OT2 (nOT2) cells on day 0. Mice injected with only naïve OT2 T cells were used as controls. Mice were injected with BrdU 12 h before sacrifice on day 3 and kidneys and lymph nodes were studied. **b** Frequencies of OT2 cells incorporating BrdU in kidney lymph

nodes from mice injected with nOT2 cells alone or OVA- α 8ILs and nOT2 cells ($n = 5$ mice/group; $p = 0.016$). Data are from 1 of 2 independent experiments. **c** Histogram showing BrdU incorporation in OT2 T cells in one representative mouse from each group. Pooled lymph node cells stained with isotype control antibody for BrdU staining is also shown.

none of them showed BrdU uptake (data not shown). These results suggest the glomerular antigens drain into the renal lymph nodes or are carried there by antigen-presenting cells and are presented to antigen-specific CD4 T cells.

Discussion

Patient and experimental mouse studies suggest that pathology in lupus glomerulonephritis initiates in the mesangium [12, 13] and CD4⁺ T cells participate in the pathogenesis of lupus nephritis [2, 3]. However, the lack of information on target antigens in lupus nephritis is a major barrier in investigating mechanisms of T cells in initiating disease. In this study, we demonstrate that activated antigen-specific CD4⁺ T cells, directed against a surrogate mesangial antigen, are sufficient to initiate the typical inflammatory lesions consisting of periglomerular macrophage, dendritic cell and T cell infiltrates. This induces mesangial cell activation and extracellular matrix production. These changes represent early events and at this time point, are not associated with loss of kidney function. This pattern of glomerulo-

nephritis mimics the patterns reported in some lupus patients with CD4⁺ T cell infiltrates forming focal or circumferential caps around glomeruli in the renal cortex [14]. The novelty of this study lies in being able to target antigens that are in the mesangium with antigen-specific T cells in immune sufficient mice, mimicking the location and pathology of spontaneous glomerulonephritis. This will allow us to dissect the individual contributions of inflammatory mediators in mesangio-proliferative glomerulonephritis.

Use of antigen-specific T cells in the induction of glomerulonephritis have been previously described. Mesangial deposition of OVA polymers followed by injection of OVA-reactive T cell lines in SCID mice showed that Th1 T cells could traffic into the kidney leading to glomerulonephritis [15]. Transgenic mice expressing OVA and hen egg lysozyme under the nephrin promoter in podocytes (NOH) mice have been recently used to investigate glomerulonephritis [16]. Repeated transfers of CD8⁺ OT1 T cells coinjected with activated CD4⁺ OT2 T cells in NOH mice resulted in glomerulonephritis. However, injection of either cell type alone failed to cause disease. These results are different than our study, where activated CD4⁺ OT2 cells were sufficient for glomerulonephritis. Other

studies have proposed glomerular basement membrane proteins like collagen [17] as potential targets for autoreactive T cells using rats primed with pertussis toxin to facilitate trafficking of T cells into nonlymphoid tissues, or antigens trapped on the glomerular basement membrane using Rag-deficient recipient mice facilitating homeostatic expansion of autoreactive T cells [18]. In our experiments, glomerular inflammation was comparable in mice with or without priming with pertussis toxin (data not shown) and was seen in normal B6 recipient mice. Although the role of podocyte antigens in spontaneous glomerulonephritis is unclear [19], together these studies show that the glomerular location of the target antigen clearly influences CD4 T cell-mediated pathology.

This study focuses on the early events in initiation of glomerulonephritis. Whether this sequence of events occurs with each renal flare would be of clinical rele-

vance and would provide the rationale for preventing local T cell responses and macrophage trafficking into the kidney as important strategies for therapeutic intervention.

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Disclosure Statement

The authors have no financial conflicts of interest to disclose.

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