Treatment with the Hyaluronic Acid Synthesis Inhibitor 4-Methylumbelliferone Suppresses LPS-Induced Lung Inflammation

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Abstract—Exposure to bacterial endotoxins, such as lipopolysaccharide (LPS), can lead to the induction of acute lung injury/acute respiratory distress syndrome (ALI/ARDS). To date, there are no known effective treatments for LPS-induced inflammation. In the current study, we investigated the potential use of the hyaluronic acid (HA) synthesis inhibitor 4-methylumbelliferone (4-MU) on LPS-induced acute lung inflammation. Culturing LPS-activated immune cells with 4-MU led to reduced proliferation, reduced cytokine production, and an increase in apoptosis when compared to untreated cells. Treatment of mice with 4-MU led to protection from LPS-induced lung injury. Specifically, 4-MU treatment led to a reduction in LPS-induced hyaluronic acid synthase (HAS) messenger RNA (mRNA) levels, reduction in lung permeability, and reduction in proinflammatory cytokine production. Taken together, these results suggest that use of 4-MU to target HA production may be an effective treatment for the inflammatory response following exposure to LPS.

KEY WORDS: acute lung inflammation; LPS; hyaluronic acid; extracellular matrix.

INTRODUCTION

Exposure to lipopolysaccharide resulting from infection with gram-negative bacteria can lead to life-threatening complications due in large part to overactivation of the inflammatory response. This exaggerated response leads to accumulation of inflammatory cells and the production of a number of proinflammatory cytokines including IL-1β, IL-6, interferon gamma (IFN-γ), and tumor necrosis alpha (TNF-α) systemically and in the lungs of infected patients [1]. Ultimately, the immune response initiated by lipopolysaccharide (LPS) can lead to endothelial cell injury, acute lung injury (ALI), acute respiratory distress syndrome (ARDS), and vascular collapse (shock) [1]. The development of ALI and ARDS can result in multiple organ failure and death of patients. The mortality rate in patients that develop ARDS is 35–40% [2, 3]. In the USA alone, approximately 75,000 people die from ALI and it is estimated that in the next 25 years, the number of deaths due to ALI in the USA will rise to close to 150,000 per year [2]. Unfortunately, the current treatments are mostly ineffective. Therefore, new therapies designed to treat the underlying cause of the injury while limiting any additional damage to the various affected organs are needed [4].

Modulation of the extracellular matrix can play an important role in the regulation of the inflammatory response. For example, a number of reports demonstrate that elevated levels of hyaluronic acid are associated with various inflammatory conditions [5–7]. Hyaluronic acid can exist in units ranging from a low-molecular-weight form (LMW-HA) of approximately $10^5$ Da to a high-molecular-weight form (HMW-HA) of up to $10^7$ Da. Substantial evidence suggests that increased levels of lower-molecular-weight HA plays an important role in elevation and maintenance of the inflammatory response [8–10]. Under normal noninflammatory conditions, hyaluronic acid exists primarily in its HMW-HA. However, under inflammatory condition, LMW-HA accumulates [11]. Additional evidence suggests that LMW-HA has proinflammatory activity while HMW-HA has anti-inflammatory properties. For example, LMW-HA led to increased production...
of cytosolic phospholipase A2α, eicosanoids, and IL-1β in monocytes and macrophage, while HMW-HA led to reduced IL-1β production in human monocyte/macrophage [12, 13]. In addition, HMW-HA was shown to increase the activity of regulatory T cells [14]. Interestingly, LPS exposure has been shown to stimulate HA production [15]. Furthermore, it has been shown that administration of HMW-HA can protect mice from LPS-induced lung injury and lead to suppression of the development of autoimmunity in MRL-lpr/lpr mice through increased IL-10 production and reduced chemokine production [16, 17].

In recent work examining a possible role of HA in staphylococcal enterotoxin B (SEB)-induced vascular damage, we revealed that following SEB exposure, there was an increase in the level of HA in the lungs and that treatment with a HA blocking peptide led to a significant reduction in SEB-induced lung injury [18]. Furthermore, we demonstrated that treatment with the hyaluronic acid synthesis inhibitor 4-methylumbelliferone (4-MU) led to significant protection from SEB-induced lung injury [19]. In the current study, we expanded our work to examine whether 4-MU had protective effects following exposure to the bacterial endotoxin LPS. Specifically, we tested the hypothesis that inhibition of hyaluronic acid production by 4-MU will lead to a reduction in lung inflammation following exposure to LPS. To test this hypothesis, we examined the effects of 4-MU on SEB-induced acute lung inflammation (ALI). Knowledge gained from this study will advance our understanding of the role of HA in LPS-mediated vascular damage and may ultimately lead to significantly improved treatment of symptoms associated with LPS exposure.

**MATERIALS AND METHODS**

**Reagents**

4-Methylumbelliferone was purchased from Alfa Aesar (Ward Mill, MA).

**In Vitro Proliferation Assay**

The splenocytes (5 × 10⁵ in 100 μL/well) were cultured in 96-well flat-bottomed plates in the presence of various concentrations of 4-MU and either left unstimulated or stimulated with 2 μg/mL LPS for 48 h. The relative number of viable cells was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay following the manufacturer’s (Trevigen, Gaithersburg, MD, USA) instructions. Briefly, 10 μL of MTT reagent was added to each well and the plates were incubated at 37 °C for 4 h. Next, 100 μL of detergent was added to each well and the plates were incubated in the dark for 4 h, after which the absorbance at 570 nm was determined using a microplate reader.

**Analysis of Cytokine Protein Levels**

Cytokine assessment was carried out using the BD Cytometric Bead Array (CBA) (BD Bioscience, San Jose, CA, USA) or ELISA (IL-1). CBA was used for simultaneous detection of multiple cytokines (IL-6, IFN-γ, and TNF-α) in cell supernatants and bronchoalveolar lavage fluid (BALF) of phosphate-buffered saline (PBS)-or LPS-exposed mice. Briefly, test samples and PE detection antibody were incubated with capture beads for 2 h, in the dark, at room temperature. After which, all unbound antibodies were washed and the beads were resuspended in 300 μL wash buffer. The cytokine levels were analyzed using a BD FACSAria II cell sorter. Cytokine levels were calculated using the FCAP Array Software v3.0 (BD Bioscience, San Jose, CA, USA). IL-1 levels were determined using an ELISA kit (DuoSet, R&D Systems, Minneapolis, MN).

**Quantification of Apoptosis**

Spleen cells (5.0 × 10⁶ cells/well) were cultured in 24-well plates in the presence of various concentrations of 4-MU (0.1, 0.5, or 1.0 mM) or vehicle control and then stimulated for 48 h with LPS (2 μg/mL) and analyzed for the induction of apoptosis using the TdT-mediated dUTP-biotin nick end labeling (TUNEL) method. Briefly, cells were washed twice with PBS and fixed with 4 % P-formaldehyde for 30 min on ice. The cells were then washed with PBS, permeabilized by adding 70 % EtOH for 20 min, and incubated with FITC-dUTP and TdT (Promega, Madison WI) for 1 h at 37 °C and 5 % CO₂. The samples were analyzed using a flow cytometer (FACS Aria II, BD Biosciences, San Jose, CA, USA). In all experiments, 10,000 cells were analyzed using forward/side scatter gating.
RNA Isolation and Real-Time RT-PCR Analysis

Total RNA was isolated from a single-cell suspension of splenocytes or from whole lung tissue using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). RNA concentration and integrity were determined spectrophotometrically. Complementary DNA (cDNA) was synthesized by reverse transcription of 50 ng total RNA using the High Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was performed using a SYBR Green PCR kit (Applied Biosystems). Amplifications were performed and monitored using an ABI 7300 real-time PCR system (Applied Biosystems). The gene-specific primers for β-actin have been previously described (17). In addition, the following primers were used: IL-1β primers 5′-CTGGAATGCTCTCATAAGACA-3′ and 5′-ATGGATGCATTACCAAATGGAT-3′; IL-6 primers 5′-CTGGGAAGCTGTCTT-3′ and 5′-AAGCAAAGGAGGCAACA-3′; hyaluronic acid synthase (HAS)-1 primers 5′-ACCTCAACACCCGAAATGCTT-3′ and 5′-GAAGGAAGGAGGGCG-3′; HAS-2 primers 5′-TGATCAAAAGAGGATGTCT-3′ and 5′-ATTGTCAGGGTGTGTTTGTTTCC-3′; HAS-3 primers 5′-CTACTTTGTAGCTGCCCAGAATCTG-3′ and 5′-GAGTACAAAAAACAGCACCGGAAT-3′. The threshold cycle (CT) method was used for relative quantification of gene transcription in relation to expression of the internal standard β-actin. Fold changes of messenger RNA (mRNA) levels in LPS-stimulated immune cells relative to unstimulated cells were determined using the 2−ΔΔCt method [20].

Quantification of Vascular Permeability

Vascular leakiness was studied by measuring the extravasation of Evan’s blue, which when given i.v. binds to plasma proteins, particularly albumin, and following extravasation can be detected in various organs as described previously [21]. Vascular leak was induced by injection of LPS. Briefly, groups of five mice were injected intranasally (i.n.) with LPS (5 μg/50 μL PBS) or PBS (50 μL). The mice were exposed to LPS for 6–24 h. Two hours prior to harvesting the lungs, the mice were injected i.v. with 0.1 mL of 1% Evan’s blue in PBS. After 2 h, the mice were exsanguinated under anesthesia, and the heart was perfused with heparin in PBS as described previously. The lungs were harvested and placed in formamide at 37 °C for 16 h. The Evan’s blue in the organs was quantified by measuring the absorbance of the supernatant at 650 nm with a spectrophotometer. In experiments examining the effect of 4-MU on LPS-induced vascular permeability, mice were treated 1 day prior to, and on the day of, LPS exposure with vehicle or 4-MU (450 mg/kg, intraperitoneal i.p.)). The dose of 4-MU was based on previous studies examining the effect of 4-MU on tumor growth and our study examining the effect of 4-MU on SEB-induced inflammation which showed that doses up to 450 mg/kg were therapeutically effective [22–24]. The vascular permeability seen in LPS-exposed mice was expressed as percent increase in extravasation when compared with that in PBS-treated controls and was calculated as follows: [optical density of dye in the lungs of LPS-exposed mice]−[optical density of dye in the lungs of PBS-treated controls])/[optical density of dye in the lungs of PBS-treated control]×100. Each mouse was individually analyzed for vascular permeability, and data were expressed as mean±SEM percent increase in vascular permeability in LPS-exposed mice when compared to that seen in PBS-treated controls [18, 20, 23].

Statistical Analysis

Student’s t test or ANOVA was used to determine statistical significance, and p<0.05 was considered to be statistically significant.

RESULTS

4-MU Suppresses LPS-Induced Splenocyte Proliferation In Vitro

Exposure to LPS leads to activation of the immune response. One measure of activation is the proliferative response. Initial experiments were conducted to examine the effect of 4-MU on spleen cell proliferation following stimulation. To this end, spleen cells were treated with various concentrations of 4-MU (0.1, 0.5, or 1 mM) or vehicle control and then stimulated with LPS. The choice of 4-MU concentrations was based on our previous studies examining the effects of 4-MU on cancer cell viability and on our previous study examining the effect of 4-MU on SEB-induced inflammation [23, 25–27]. The proliferative response was determined 48 h later by MTT assay (Fig. 1). The results demonstrated that treatment of spleen cells with 4-MU at concentrations as low as 0.5 mM led to significant suppression of the proliferative response, suggesting that 4-MU may be effective at preventing the immune cell response to LPS.
4-MU Suppresses LPS-Induced Cytokine Production

Cytokine production following exposure to LPS plays a critical role in the development of ALI/ARDS. Experiments were conducted to examine the effect of 4-MU treatment on LPS-induced cytokine production in spleen cell cultures in vitro. Spleen cells were treated with 4-MU (1 mM) or vehicle control. The cells were then stimulated with LPS, and cytokine mRNA levels were determined 3–24 h later by real-time PCR (Fig. 2). The results demonstrated that exposure to LPS led to a significant increase in IL-1, IL-6, and TNF-α gene expression as early as 3 h following stimulation with LPS. Treatment with 4-MU at concentration of 1 mM led to a significant reduction in the levels of IL-6 and TNF-α mRNA at 3 h and a significant reduction of IL-1 at 6 h. The mRNA levels of IL-1 and IL-6 remained suppressed in the 4-MU-treated groups through 48 h. In contrast, the levels of TNF-α mRNA rebounded in the 4-MU-treated group following 24 h of culture and was elevated at 48 h compared to vehicle-treated cells. Together, these results suggest that 4-MU may be effective at modulating cytokine production associated with LPS exposure.

Treatment with 4-MU Leads to Increased Apoptosis in LPS-Exposed Leukocytes In Vitro

Downregulation of the immune response through activation-induced cell death (AICD) through the induction of apoptosis plays an important role in controlling the inflammatory response. Previous results from our laboratory demonstrated that SEB exposure leads to increased production of HA in the lungs, and in separate studies, we demonstrated an important role of CD44 in lymphocyte AICD, suggesting the possibility that SEB-induced HA production may protect immune cells from apoptosis through binding CD44 [18, 28]. In a recent study, we demonstrated that treatment of SEB-exposed leukocytes to 4-MU led to increased apoptosis [23]. Therefore, in the current study, we examined whether a similar increase in apoptosis would occur following treatment of LPS-exposed spleen cells to 4-MU. To this end, spleen cells were exposed to LPS and then treated with 4-MU (0.1, 0.5, or 1 mM) or vehicle control. The levels of apoptosis were determined 48 h later by TUNEL assays (Fig. 3). The results demonstrated that treatment with 4-MU at concentrations as low as 0.1 mM led to an increase in the levels of apoptosis, suggesting that 4-MU may act to suppress the immune response to LPS through the induction of apoptosis.

4-MU Treatment Leads to a Reduction in the Expression of HAS mRNA in the Lungs of LPS-Exposed Mice

Previously, we reported that exposure to SEB led to an increase in the levels of soluble HA in the lungs of mice and that treatment with a HA blocking peptide could
protect mice from SEB-induced lung injury and that 4-MU treatment could reduce SEB-induced expression of HAS mRNA and HA in the lungs [18, 23]. In the current study, we examined whether treatment with 4-MU had any effect

Fig. 2. 4-MU inhibits LPS-induced leukocyte cytokine production in vitro. Spleen cells from C57BL/6 mice were treated with 4-MU (0.1, 0.5, and 1.0 mM) or vehicle control and then stimulated with LPS (2 μg/mL). The effect of 4-MU on the cytokine production was determined 3–48 h later by real-time RT-PCR. Asterisks indicate statistically significant difference when compared with vehicle controls, $p \leq 0.05$.

Fig. 3. 4-MU treatment leads to increased apoptosis in LPS-exposed leukocytes in vitro. Spleen cells from C57BL/6 mice were treated with 4-MU (0.1, 0.5, and 1.0 mM) or vehicle control and then stimulated with LPS (2 μg/mL). The effect of 4-MU on LPS-induced apoptosis was determined 48 h later by TUNEL assay.
on the HAS gene expression or soluble HA levels in the lungs of LPS-exposed mice. Experiments were conducted to examine the effects of 4-MU treatment on the expression of HAS mRNA in the lungs of LPS-exposed mice. Three isoforms of HAS (HAS-1, HAS-2, and HAS-3) have been identified [29]. LPS exposure resulted in an increase in the expression of all three isoforms. This increase in mRNA expression was significantly inhibited by 4-MU treatment (Fig. 4). The results showed that LPS exposure led to significantly elevated levels of HAS in the lungs and that this increase was significantly inhibited by 4-MU treatment.

4-MU Treatment Is Effective at Preventing LPS-Induced Lung Injury In Vivo

LPS exposure can lead to many of the symptoms related to endotoxin-mediated sepsis commonly associated with infections with gram-negative bacteria. The development of lung damage characterized by an increase in vascular permeability is one such life-threatening event. In a previous study, we demonstrated that treatment with 4-MU could protect mice from SEB-induced lung injury. In the current study, we examined whether this protection could be extended to LPS-induced lung injury. To this end, mice were treated 1 day prior to, and on the day of, LPS exposure with 4-MU (450 mg/kg) or vehicle control and then exposed to PBS or LPS (10 μg/50 μL PBS). The effect of 4-MU on the LPS-induced inflammatory response was examined by determining levels of vascular permeability in the lungs 6 and 24 h following LPS exposure (Fig. 5). The results demonstrate that vascular permeability is significantly greater in LPS-exposed mice treated with vehicle control than in corresponding PBS-exposed mice. Treatment with 4-MU led to a significant reduction in the level of vascular permeability at both the 6 and 24 time points.

LPS-Induced Cytokine Production in the Lungs Is Suppressed by 4-MU Treatment

The production of inflammatory cytokines following exposure to LPS is an important mediator of lung damage and is characterized by the production and release of high concentrations of cytokines such as IL-1, IL-6, IFN-γ, and TNF-α. Therefore, effective control and/or prevention of LPS-mediated lung injury requires controlling the levels of inflammatory cytokines. Experiments were set up to explore the ability of 4-MU to reduce the LPS-induced increase in cytokine levels in the lung. To this end, groups of mice were treated with either vehicle control or 4-MU (450 mg/kg i.p.) 1 day prior, and on the day of, LPS exposure. Following 4-MU treatment, the mice were exposed to either PBS or LPS (10 μg/50 μL PBS i.n.). BALF was harvested 6–24 h later. Cytokine protein levels in the BALF were determined by Cytometric Bead Array (CBA) or ELISA (IL-1) (Fig. 6). The results show that treatment with 4-MU led to a significant reduction in the levels of proinflammatory cytokines in LPS-exposed mice.

DISCUSSION

The development of ALI/ARDS in septic patients continues to be a significant health care issue resulting in upward of 75,000 yearly deaths in the USA alone. This number is estimated to increase in the next 25 years to close to 150,000 deaths per year [2]. Unfortunately, the current treatments are mostly ineffective. Therefore, developing a better understanding of the development of ALI/ARDS may lead to new therapies designed to treat the underlying cause of the injury while limiting any additional damage to the various affected organs [4]. In the current study, we demonstrated the potential usefulness of targeting hyaluronic acid production in the treatment of LPS-induced ALI. Specifically, we demonstrated that treatment with 4-MU can lead to protection from LPS-induced ALI in mice through the reduction of HAS and the subsequent
reduction in inflammatory cytokines and reduction in vascular permeability.

In the current study, we used the LPS mouse model of ALI to investigate the potential usefulness of 4-MU treatment. The LPS model is widely used and has been shown to closely mimic the development of ALI in septic patients [30]. A hallmark feature of septic patients with ALI exhibits increased lung permeability. Using the LPS model, we demonstrated a significant increase in lung permeability further establishing the usefulness of this model in investigating ALI. In addition, our data show that use of HA synthesis inhibitors such as 4-MU may provide protection against endotoxin-induced ALI. These data are further supported by our previous studies using the SEB model of ALI. In those studies, we demonstrated similar result in which exposure to SEB led to increased lung permeability which could be inhibited by 4-MU treatment. Together, these studies strongly support the possible development of treatment regimens for ALI that target HA synthesis.

In addition to increased lung permeability, the increased production of proinflammatory cytokines such as IL-1, IL-6, IFN-γ, and TNF-α all has been implicated as playing important roles in the development and pathogenesis of ALI in septic patients [31]. In the current study, we demonstrated that exposure to LPS led to an increase in proinflammatory cytokines both in vitro and in vivo. Furthermore, treatment with 4-MU was able to reduce LPS-induced cytokine production in both the in vitro models as well as in the lungs of LPS-exposed mice. Interestingly, the ability to suppress the expression of TNF-α mRNA levels in the spleen cells cultures in vitro was restricted to early time points up to 3 h. After which, 4-MU was ineffective, and at later time points, 4-MU treatment seemed to increase mRNA levels of TNF-α. The exact mechanism of this remains unclear but may be the result of delaying the inflammatory response to LPS, suggesting that prolonged treatment with HA synthesis inhibitors may be necessary to fully prevent the inflammatory response in the lungs. This delay in response was not seen in mRNA levels for IL-1 or IL-6, as the expression of these cytokines was suppressed at early as well as later time points.

In this study, 4-MU treatment led to increased levels of apoptosis in LPS-activated splenocytes. In our previous study examining the effect of 4-MU on SEB-induced ALI, we showed similar effects as 4-MU led to increased apoptosis in activated cells. Besides these two studies, little has been reported on the role of 4-MU inducing apoptosis in activated immune cells. However, there is a growing body of evidence supporting a role of 4-MU in inducing apoptosis in various tumor cells [22, 24–27, 32]. Furthermore, a number of studies demonstrate a potential role of HA in modulating apoptosis in various immune responses. For example, exposure to HMW-HA led to protection from apoptosis in LPS-exposed chondrocytes and in UVB-induced apoptosis in human epithelial corneal cells [33, 34]. Alternatively, treatment of PMA-stimulated CD44+ Jurkat cells with LMW-HA led to increased levels of apoptosis [35]. Together, these studies support an important role of HA in the regulation of apoptosis in immunological responses.

4-MU suppresses HA synthesis by inhibiting HAS activity either by affecting the expression of HAS mRNA or by depletion of the HA precursor (UDP)-glucuronic acid [18, 36]. In the current study, we demonstrated that treatment with 4-MU led to a reduction in LPS-induced HAS
mRNA expression. In this study, the expression of HAS-1, HAS-2, and HAS-3 genes was elevated following LPS exposure and the levels of mRNA of all three HAS isoforms were reduced following 4-MU treatment. This is similar to what we demonstrated in our previous study examining the effects of SEB exposure further supporting a role of 4-MU in suppressing HAS expression. Furthermore, we demonstrated that the increased expression of HAS occurred as early as 1–2 h after LPS exposure and returned to baseline level within 6 h mirroring the rapid progression of LPS-induced ALI. Interestingly, we noticed that the suppression of HAS isoforms by 4-MU treatment, especially HAS-3, lasted for approximately 24 h following 4-MU treatment (data not shown), suggesting that future treatments with 4-MU may have to be prolonged to achieve maximal efficacy.

Hyaluronic acid production takes place at the intracellular face of the plasma membrane and ultimately results in deposition of hyaluronic acid on the cell surface and pericellular space or release into the extracellular matrix. Production of hyaluronic acid is regulated through the activity of HAS. HAS is located within the plasma membrane and acts as a dual glycosyltransferase with the ability to act on both UDP-GlcNAc and UDP-GLcA resulting in the formation of HA without help from any additional proteins [38]. Hyaluronic acid can exist in units ranging from a LMW-HA of approximately $10^5$ Da to a HMW-HA with a molecular weight of up to $10^7$ Da. Evidence for control of HA size and rate of synthesis has been related to the differential activity of the HAS isoforms. To date, three
HAS isoforms, HAS1, HAS2, and HAS3, have been identified. Experimental evidence suggests that HA is produced at a faster rate by HAS2 and HAS3 compared to HAS1 and that smaller forms of HA are produced by HAS1 and HAS3 compared to HAS2 [29]. The precise significance for the differences in rate and size of HA synthesis remains unclear. However, there is a significant body of evidence suggesting that increased levels of HA and lower-molecular-weight HA play an important role in enhancing inflammation while higher-molecular-weight HA seems to be anti-inflammatory [39–43]. In our studies, 4-MU treatment led to the reduction of all three isoforms in activated immune cells. Therefore, identification of compounds that modulate the activity of specific isoforms may lead to effective treatments of various inflammatory conditions.

In conclusion, results from our study show that treatment with 4-MU led to protection from LPS-induced ALI. Specifically, we demonstrated that 4-MU was able to suppress the inflammatory response in vitro by suppressing cytokine production and inducing apoptosis in immune cells. Furthermore, 4-MU treatment was able to protect mice from LPS-induced lung injury through suppression of cytokine production and suppression of HAS gene expression. Taken together, the results from our study suggest that targeting HA synthesis using compounds such as 4-MU to reduce the inflammatory response to LPS but does not examine the effect on bacterial growth and/or clearance. Future studies examining the potential of 4-MU to improve outcomes resulting from bacterial lung infections with E. coli or Staphylococcus aureus would provide important information regarding the effectiveness of targeting HA production in a highly relevant clinical model. The current study examined the ability of 4-MU to reduce the inflammatory response to LPS but does not examine the effect on bacterial growth and/or clearance. In addition, studies exploring the potential role of HAS isoforms may lead to novel targets for treating deleterious inflammatory responses in the lungs. These areas are currently under investigation in our laboratory.

Conflict of Interest. The authors have no conflict of interest.

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