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Galectin-3 Functions as an Adhesion Molecule to Support Eosinophil Rolling and Adhesion under Conditions of Flow[1]

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Allergic inflammation involves the mobilization and trafficking of eosinophils to sites of inflammation. Galectin-3 (Gal-3) has been shown to play a critical role in eosinophil recruitment and airway allergic inflammation in vivo. The role played by Gal-3 in human eosinophil trafficking was investigated. Eosinophils from allergic donors expressed elevated levels of Gal-3 and demonstrated significantly increased rolling and firm adhesion on immobilized VCAM-1 and, more surprisingly, on Gal-3 under conditions of flow. Inhibition studies with specific mAbs as well as lactose demonstrated that: 1) eosinophil-expressed Gal-3 mediates rolling and adhesion on VCAM-1; 2) α_4 integrin mediates eosinophil rolling on immobilized Gal-3; and 3) eosinophil-expressed Gal-3 interacts with immobilized Gal-3 through the carbohydrate recognition domain of Gal-3 during eosinophil trafficking. These findings were further confirmed using inflamed endothelial cells. Interestingly, Gal-3 was found to bind to α_4 integrin by ELISA, and the two molecules exhibited colocalized expression on the cell surface of eosinophils from allergic donors. These findings suggest that Gal-3 functions as a cell surface adhesion molecule to support eosinophil rolling and adhesion under conditions of flow. ***The Journal of Immunology,*** **2007, 179: 7800–7807.**

Allergic inflammation is a complex disease that involves the mobilization and trafficking of multiple leukocytes, including eosinophils and T cells, to sites of inflammation. Eosinophils play a prominent proinflammatory role in airway allergic inflammation, including the pathogenesis of asthma (1–3). Several studies, including ours, have demonstrated that eosinophil trafficking under conditions of flow involves a multistep paradigm that includes initial rolling followed by activation-dependent firm adhesion and chemoattractant-induced transmigration into extravascular sites of inflammation (4–10). Rolling is the first and rate-limiting step of the multistep eosinophil adhesion cascade and is supported by both L-selectin and VLA-4 (α_4) (11). On the endothelial side, in addition to the α_4 ligand VCAM-1 (12), eosinophil rolling in inflamed postcapillary venules is also mediated by P-selectin (13) but not E-selectin (5). Subsequently, activation-dependent stable adhesion of eosinophils is mediated by α_4/VCAM-1 and β_2/ICAM-1 interactions (12, 14–18). Although these studies suggest an important role for integrins and selectins in mediating the rolling and adhesion of eosinophils, studies of eosinophil recruitment in selectin and other adhesion molecule knockout mice as well as Ab blockade studies have alluded to the functional engagement of additional known or yet unidentified molecules.

Recent studies have demonstrated a role for galectin-3 (Gal-3),[3] a member of a family of β-galactoside-binding animal lectins, in eosinophil recruitment and airway allergic inflammation in a murine model of allergen-induced asthma using mice lacking Gal-3 (19). This protein is expressed in a variety of tissues and cell types including epithelial cells, dendritic cells, endothelial cells (EC), and inflammatory cells such as mast cells, neutrophils, monocytes/macrophages, and eosinophils (20–23). Although it is mainly found in the cytoplasm and does not contain a classical signal sequence or a transmembrane domain (24, 25), Gal-3 is secreted from cells by a novel and incompletely understood mechanism that is independent of the classical secretory pathway through the endoplasmic reticulum/Golgi network (22). Depending on cell type and proliferative state, this lectin can also be detected in the nucleus, on the cell surface, or in the extracellular environment. Because of its ability to recognize extracellular matrix (ECM) proteins such as laminin, elastin (20), and fibronectin (26), Gal-3 is able to promote cell-ECM attachment (26). Recent studies from our laboratory have demonstrated that Gal-3 can function as an adhesion molecule and mediate the attachment of highly metastatic breast cancer cells to EC by undergoing rapid intracellular redistribution to sites of intercellular contact and participate in homotypic and heterotypic adhesive interactions under conditions of flow (27). Given the fact that this molecule is involved in eosinophil migration to the airways during allergic inflammation (19), we have examined the role played by Gal-3 in mediating eosinophil-EC interactions under conditions of flow in vitro, which are critical in regulating eosinophil trafficking to sites of inflammation.

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[3] Abbreviations used in this paper: Gal-3, galectin-3; CRD, carbohydrate recognition domain; EC, endothelial cell; ECM, extracellular matrix; hpf, high power field; rh, recombinant human.

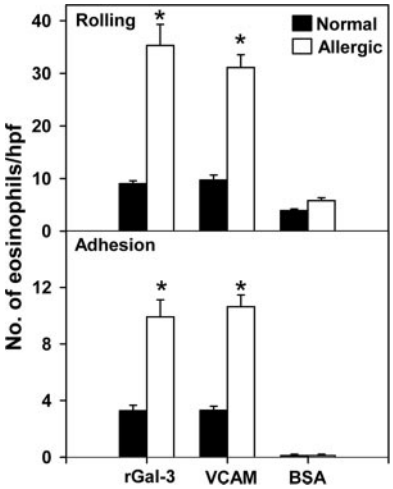

FIGURE 1. Eosinophils from allergic donors exhibit increased rolling and adhesion compared with eosinophils from normal donors under conditions of flow. The ability of eosinophils to roll on vascular adhesion molecules was assessed using the parallel plate laminar flow chamber. Single cell suspensions of eosinophils (2×10^5 cells) from allergic and normal donors were infused into a flow chamber containing coverslips coated with rhGal-3 ($n = 4$ normal and 9 allergic), rhVCAM-1 ($n = 3$ normal and 8 allergic), or BSA ($n = 3$ normal and 4 allergic) at a flow rate of ≈1 ml/min for 5 min, and the interactions of the injected cells with the coated coverslips were recorded. The results shown represent the combined data of all donors for each group expressed as the mean ± SE of the number of rolling or adherent cells per hpf. *, $p < 0.05$.

Materials and Methods

Isolation of eosinophils

Eosinophils were isolated from allergic or normal human blood donors. Allergic donors included subjects with allergies to known allergens such as house dust, ragweed, dust mites, or unknown allergens as well as subjects who had elevated eosinophil counts due to unknown etiology. Normal donors included subjects with no known allergies. All donors provided informed consent for a phlebotomy at the time of blood draw. Only donors who were not being treated with any anti-inflammatory drugs (steroidal and nonsteroidal), aspirin, or antihistamines were selected. Eosinophils were purified from the peripheral blood of normal or allergic donors by gravity sedimentation through hetastarch followed by Percoll gradient centrifugation and negative selection of CD16-positive cells using a magnetic bead separation technique as described in detail in our previous studies (28). The viability and purity of the final negatively selected eosinophil preparations were assessed by staining with trypan blue and Diff-Quik (Baxter Healthcare), respectively. Viability and purity were routinely >98%.

Flow cytometry

Cell surface expression of Gal-3 by human eosinophils (nonpermeabilized) from normal and allergic donors or by HUVEC that were stimulated with IL-1β stimulation (50 ng/ml for 4 h at 37°C) to create a proinflammatory state was assessed using murine mAb against human Gal-3 (A3A12) (29) at 10 μg/ml, followed by FITC- or PE-conjugated anti-mouse IgG in the presence of mouse IgG1 as the isotype control (both from Sigma-Aldrich) on a BD LSR II flow cytometer equipped with BD FACSDiva-based software for data acquisition (BD Biosciences). Results are expressed as mean ± SE of the percentage of Gal-3-positive cells after subtracting isotype.

In vitro laminar flow assay

The interaction of eosinophils with recombinant human (rh) Gal-3 (30), rhVCAM-1 (12), rhICAM-1 (31), or BSA (Sigma-Aldrich) was assessed in an in vitro parallel plate laminar flow chamber (100-μm thickness). Glass coverslips either coated overnight at 4°C or for 1 h at 37°C with rhGal-3, rhVCAM-1, rhICAM-1 or BSA (10 μg/ml, 200 μl/coverslip) were placed in the bottom of the flow chamber and exposed to flow conditions (1 ml/min; wall shear stress, 1.0–2.0 dynes/cm^2) by perfusing warm medium (RPMI 1640 containing 0.75 mM $Ca2^+$ and $Mg2^+$ and 0.2% human serum albumin) through a constant infusion syringe pump (Harvard Apparatus).

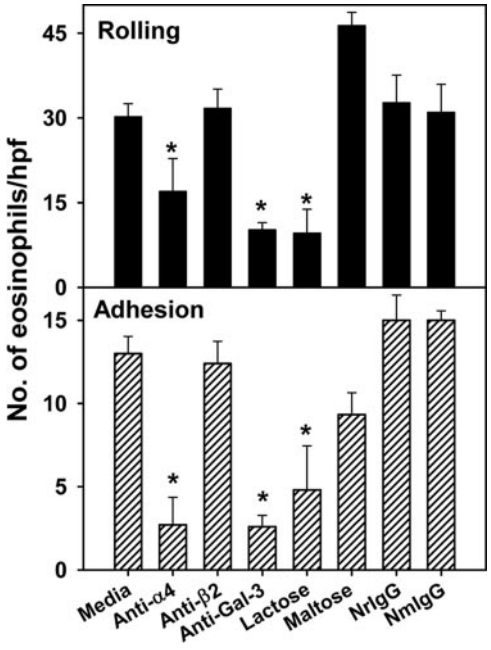

FIGURE 2. Eosinophil-expressed Gal-3 mediates eosinophil rolling and adhesion to VCAM-1. Eosinophils from allergic donors were preincubated with medium alone ($n = 16$), mAbs against Gal-3 ($n = 3$), α_4 ($n = 10$), or β_2 ($n = 10$), normal rat IgG (NrIgG; $n = 3$), or normal mouse IgG (NmIgG; $n = 3$) at 10 μg/ml for 20 min and infused into the flow chamber containing rhVCAM-1-coated coverslips. The interactions of the injected cells with the VCAM-1-coated coverslips were recorded. In some experiments, eosinophils were preincubated with lactose ($n = 5$) or maltose as a control ($n = 3$) at 3 mM before infusion. Results shown represent combined data for each treatment expressed as mean ± SE of the number of rolling or adherent cells per hpf. *, $p < 0.05$.

In certain experiments, confluent monolayers of IL-1β-stimulated HUVEC (Clonetics) cultured on poly-L-lysine-coated coverslips (32) were used instead of rhGal-3 and rhVCAM-1. The flow chamber was next perfused with a single cell suspension of eosinophils (2×10^5 cells) for a period of 5 min. The interaction of the injected cells with the adhesion molecule-coated coverslips was observed using a Leitz Wetzlar inverted microscope as previously described (12, 27). The images were video recorded for subsequent offline video analysis to manually determine the number of interacting cells. Rolling cells demonstrate multiple discrete interruptions and flow slowly, whereas adherent cells remain stationary at a given point for extended periods of time (>30 s). Results are expressed as the number of rolling or adherent cells per high power field (hpf). In some experiments, eosinophils were preincubated with mAbs against murine Gal-3 prepared from a rat hybridoma (M3/38.1.2.8 HL.2; catalog no. TIB-166 from American Type Culture Collection) that is known to specifically recognize human Gal-3 (33), anti-human α_4 mAb P4G9 (Immunotech), anti-human β_2 mAb 60.3 (34), normal rat IgG (Santa Cruz Biotechnology), normal mouse IgG (BD Pharmingen), or a combination of anti-α_4 and anti-Gal-3 for 20 min before infusion into the flow chamber. All Abs were used at a concentration of 10 μg/ml. Because the biological activities of Gal-3 are often mediated via its carbohydrate recognition domain (CRD), it has a high affinity for lactose (35), which, in turn, has been used as an inhibitor of its biological activity (36). In the present study, the ability of lactose or maltose as a control (Sigma-Aldrich) to inhibit the rolling and adhesion of eosinophils was tested at a concentration of 3 mM.

To investigate the role of EC-expressed Gal-3 in eosinophil trafficking, coverslips containing IL-1β-stimulated HUVEC were treated with mAbs against Gal-3 or rhVCAM-1 (Chemicon International) at 10 μg/ml for 20 min before the perfusion of eosinophils into the flow chamber. Cells were treated with normal mouse IgG as a control. All reagents were tested for endotoxin before flow chamber studies and, if necessary, treated appropriately to ensure that there was minimal or no endotoxin contamination (<0.1 endotoxin units/ml).

Confocal microscopy

Cell surface expression and the cellular distribution of Gal-3 were analyzed as described in our previous studies (27) with minor modifications. Eosinophils

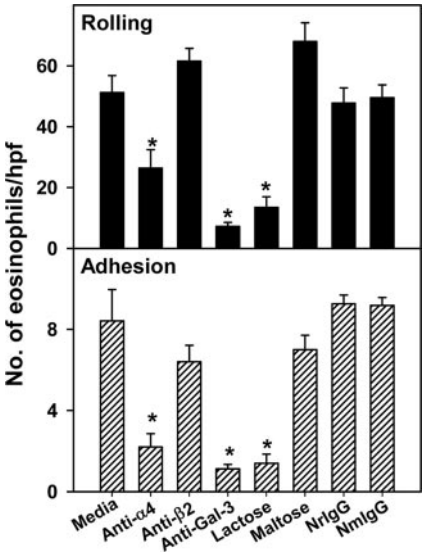

FIGURE 3. Eosinophil-expressed α_4 integrins and Gal-3 mediate eosinophil rolling and adhesion to Gal-3. Eosinophils from allergic donors were preincubated with medium alone ($n = 10$), mAbs against Gal-3 ($n = 6$), α_4 ($n = 5$) or $\beta 2$ ($n = 5$), normal rat IgG (NrIgG; $n = 3$), or normal mouse IgG (NmIgG; $n = 3$) before infusion into the flow chamber containing rhGal-3-coated coverslips, and the interactions of the injected cells with the coated coverslips were recorded. In some experiments, eosinophils were preincubated with lactose ($n = 9$) or maltose as a control ($n = 4$) before infusion. Results shown represent combined data for each treatment expressed as mean ± SE of the number of rolling or adherent cells per hpf. *, $p < 0.05$.

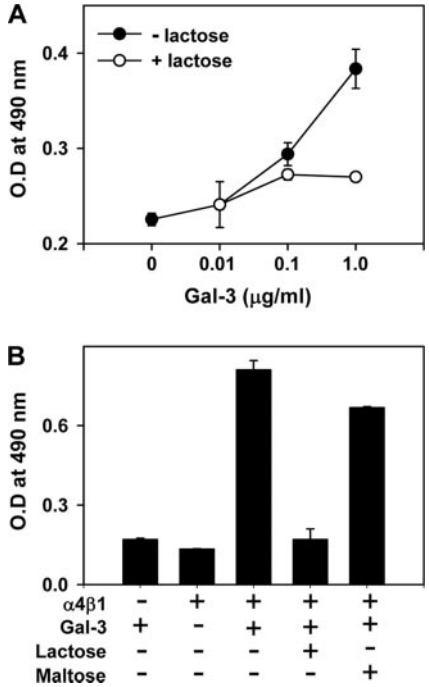

FIGURE 4. $\alpha_4\beta_1$ binds to Gal-3. The ability of $\alpha_4\beta_1$ to directly bind to Gal-3 was determined by ELISA ($n = 4$). *A*, ELISA plates coated overnight with rh$\alpha_4\beta_1$ were exposed to different concentrations of rhGal-3 (0.01–1.0 μg/ml) in the presence or absence of 25 mM lactose. Plates were then incubated with rabbit anti-human Gal-3 followed by HRP-conjugated goat-anti-rabbit Abs, and the bound Ab was detected using α-phenylenediamine-dihydrochloride. *B*, The ability of lactose, but not maltose, to specifically inhibit binding of Gal-3 to α_4 was determined in the above ELISA ($n = 4$). Data shown are representative of a single experiment. Results are expressed as mean ± SD of OD measured at 490 nm.

(1×10^3 cells/ml; 200 μl/coverslip) were adhered to rhVCAM-1-coated (described above) or uncoated coverslips for 1 h at 37°C in a CO_2 incubator. Nonadherent cells were removed by gentle washing with PBS containing 0.02% NaN_3, and the adhered nonpermeabilized eosinophils were incubated with FITC-conjugated mouse anti-human Gal-3 (1/20) or FITC-conjugated normal mouse IgG as a control (both from BD Pharmingen) in a 0.15 M saline solution buffered with 10 mM HEPES containing 2.5% BSA (pH 7.3). For the dual staining studies to localize α_4 and Gal-3 expression, coverslips with adhered eosinophils were first exposed to the mAb P4G9 (10 μg/ml), gently washed to remove unbound Abs, and then incubated with rhodamine-conjugated goat anti-mouse IgG (1/20; Upstate Biotechnology). Appropriate controls were included. The coverslips were washed once again and then exposed to FITC-conjugated anti-human Gal-3 as described above. All incubations with primary and secondary Abs were conducted for 30 min on ice. The cells were fixed and the coverslips were finally washed with PBS-NaN_3 and mounted on glass slides for observation by confocal microscopy at the ambient temperature using a FluoView FV1000 confocal laser scanning biological microscope equipped with UPlanApo lenses (20×0.7 and 60×1.4 numerical aperture (oil)) and FluoView 1000 software for image acquisition (model no. FV10-ASW; Olympus).

$\alpha_4\beta_1$-Gal-3 binding ELISA

Nunc MaxiSorp high protein-binding capacity ELISA plates were coated with rh$\alpha_4\beta_1$ (at 1 μg/ml; provided by Dr. Ma. Humphries, University of Manchester, Manchester, U.K.) (37) in HBSS containing Ca^{2+} and Mg^{2+} (Invitrogen Life Technologies) overnight at 4°C. After washing, wells were blocked with blocking buffer (1% BSA in Ca^{2+}-Mg^{2+} HBSS containing 1 mM Mn^{2+}) for 1 h at room temperature. rhGal-3 (0.01–1.0 μg/ml) in binding buffer (blocking buffer containing 0.05% Tween 20) was then added to the blocked wells and incubated overnight at 4°C. In some experiments, rhGal-3 was added to rh$\alpha_4\beta_1$-coated wells in the presence of lactose or maltose at a concentration of 25 mM as described in previous studies (38). Wells to which lactose or maltose in binding buffer were added served as controls for these latter experiments. Additionally, to rule out nonspecific binding, rhGal-3 was added to blocked wells coated with HBSS alone (without rh$\alpha_4\beta_1$) and binding buffer alone (without rhGal-3) was added to rh$\alpha_4\beta_1$-coated and blocked wells. After washing, plates were incubated with rabbit anti-human Gal-3 (39) at 2 μg/ml in binding buffer (100 μl/well) for 2 h at room temperature. The bound Abs were detected using HRP-conjugated goat-anti-rabbit IgG (Zymed Laboratories/Invitrogen Life Technologies) at a dilution of 1/2000 (100 μl/well) for 1 h followed by α-phenylenediamine-dihydrochloride for 30 min at room temperature. The reaction was stopped with 4 N sulfuric acid (25 μl/well) and plates were read at 490 nm using a microplate reader (Molecular Devices). Results are expressed as mean ± SD of the OD measured at 490 nm.

Results

Eosinophils from allergic donors exhibit increased rolling and adhesion on VCAM-1 and Gal-3 in comparison to normal eosinophils under conditions of flow

Because initial rolling followed by firm adhesion are critical early steps during eosinophil trafficking under conditions of flow, we compared the ability of human eosinophils isolated from allergic and normal donors to roll and adhere on surfaces coated with VCAM-1, an endothelial adhesion molecule that supports eosinophil rolling and adhesion (12). Further, because studies from our laboratory have shown that EC-expressed Gal-3 participates in mediating adhesive interactions with tumor cells (27), the ability of eosinophils to roll or adhere on rhGal-3 was also determined (Fig. 1). Eosinophils from allergic donors exhibited significantly increased rolling and adhesion on VCAM-1 as well as Gal-3 compared with cells isolated from normal donors. Negligible rolling and adhesion was observed on BSA-coated control coverslips. These data further demonstrate that immobilized Gal-3 can support eosinophil rolling and adhesion under conditions of flow in addition to VCAM-1. Eosinophils from allergic donors were also found to adhere to Gal-3- and VCAM-1-coated coverslips under static conditions (51.7 ± 3.03 and 42.7 ± 2.86 cells/field, respectively, vs 25.3 ± 1.98 cells/field with BSA-coated coverslips; $n = 2$

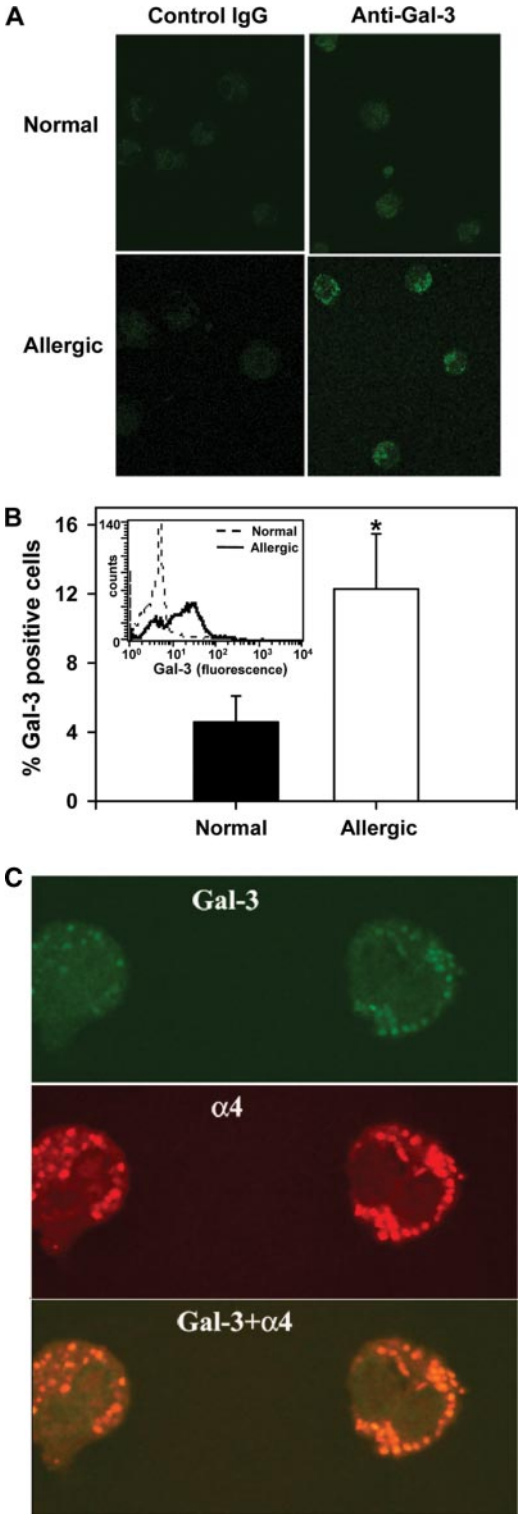

FIGURE 5. Gal-3 expression on eosinophils from allergic donors is elevated and is colocalized with α_4 expression. *A*, Eosinophils (nonpermeabilized) from normal ($n = 3$) and allergic donors ($n = 4$) were adhered to VCAM-1-coated coverslips and incubated with FITC-conjugated anti-human Gal-3 or FITC-conjugated mouse IgG (control). The cells were fixed, mounted on glass slides, and observed by confocal microscopy. Original magnification was ×20. *B*, Gal-3 expression on eosinophils from normal ($n = 3$) and allergic ($n = 5$) donors was analyzed by flow cytometry using mAb A3A12 against human Gal-3. The percentage of Gal-3 positive cells (mean ± SE) after subtracting the isotype is shown. *, $p < 0.05$. The *inset* shows a histogram of an overlay of Gal-3 expression by a representative allergic donor vs a normal donor. *C*, Eosinophils from allergic donors ($n = 3$) were first exposed to anti-α_4 mAb P4G9 (10 μg/ml) followed by rhodamine-conjugated goat anti-mouse IgG (1/20) and then exposed to FITC-conjugated anti-human Gal-3. Original magnification was ×60. Images shown in *A* and *C* are representative of a single donor.

experiments in duplicate). In all additional experiments, eosinophils from allergic donors were used.

Eosinophil-expressed Gal-3 mediates eosinophil rolling and adhesion to VCAM-1

Gal-3 is expressed by a wide variety of cell types including inflammatory cells such as mast cells, neutrophils, monocytes/macrophages, and eosinophils (20–23). To confirm that eosinophils used in the present study were a cellular source of Gal-3, eosinophil lysates ($n = 4$ donors) were assessed by ELISA as previously described (39) and found to express 0.4–2.7 ng/ml Gal-3. To examine whether eosinophil-expressed Gal-3 mediates rolling and adhesion, eosinophils preincubated with medium alone or with mAbs against human Gal-3 were evaluated for their ability to roll and adhere on rhVCAM-1-coated coverslips under conditions of flow. Eosinophils treated with mAbs against α_4 served as a positive control, whereas cells treated with mAbs against β_2 integrin were considered as a negative control (Fig. 2). Treatment with mAbs against Gal-3 significantly inhibited rolling (47.58 ± 3.38%, $p < 0.05$) and adhesion (81.66 ± 3.46%, $p < 0.001$) of eosinophils on VCAM-1-coated coverslips. As expected, anti-α_4 also significantly inhibited eosinophil rolling and adhesion (50.86 ± 11.83%, $p < 0.021$ and 75.07 ± 12.86%, $p < 0.001$, respectively), while mAbs against β_2 had no effect. These findings suggest that eosinophil-expressed Gal-3 can mediate eosinophil rolling and adhesion on endothelial adhesion molecules such as VCAM-1 under conditions of flow. Preincubation of eosinophils with a combination of mAbs against α_4 and Gal-3 did not further inhibit rolling or adhesion (data not shown). Additionally, eosinophils preincubated with lactose (3 mM), a Gal-3-binding sugar used as an inhibitor of its biological activity (36), significantly inhibited eosinophil rolling (62.04 ± 16.73%, $p < 0.001$) and adhesion (53.58 ± 27.39%, $p < 0.05$) to VCAM-1, indicating that the CRD of Gal-3 is involved in mediating these interactions. Maltose, a non-galactose disaccharide that was used as a negative control, had no effect when tested at a similar concentration.

Eosinophil-expressed α_4 integrins and Gal-3 mediate eosinophil rolling and adhesion to immobilized Gal-3

Studies described above suggest that immobilized VCAM-1 and Gal-3 support eosinophil rolling and adhesion (Fig. 1) and that eosinophil-expressed Gal-3 and α_4 participate in rolling and adhesion on VCAM-1 (Fig. 2). To identify the eosinophil-expressed adhesion molecule(s) that are likely to mediate rolling and adhesion on immobilized Gal-3, eosinophils preincubated with medium alone, anti-α_4, or anti-β_2 were infused into the flow chamber containing rhGal-3-coated coverslips (Fig. 3). Because eosinophil-expressed Gal-3 appears to mediate rolling and adhesion on VCAM-1 (Fig. 2), cells treated with mAbs against Gal-3 were also evaluated. Surprisingly, mAbs against α_4 significantly inhibited rolling (60.27 ± 10.07%, $p < 0.01$) and adhesion (62.9 ± 13.26%, $p < 0.02$) of eosinophils to rhGal-3-coated coverslips, suggesting that eosinophil-expressed α_4 integrins can mediate rolling and adhesion via interaction with immobilized Gal-3 in addition to VCAM-1. More interestingly, even greater inhibition of eosinophil rolling and adhesion (85.37 ± 3.2%, $p < 0.01$ and 81.9 ± 4.47%, $p < 0.01$, respectively) was observed when cells were pretreated with anti-Gal-3 mAbs. Treatment with mAbs against β_2 had no effect on eosinophil rolling or adhesion. These findings suggest

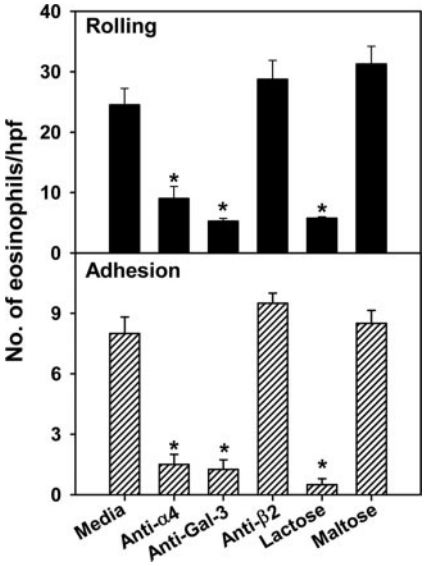

FIGURE 6. Eosinophil-expressed $\alpha_4\beta_1$ and Gal-3 mediate eosinophil rolling and adhesion on endothelium. Eosinophils from allergic donors ($n = 2$) were preincubated with medium alone, mAbs against Gal-3, α_4, or $\beta 2$, and lactose or maltose before infusion into the flow chamber containing IL-1β-stimulated HUVEC-coated coverslips. The interactions of the injected cells with HUVEC were observed under an inverted microscope. The results shown represent combined data for each treatment expressed as mean ± SE of the number of rolling or adherent cells per hpf. *, $p < 0.05$.

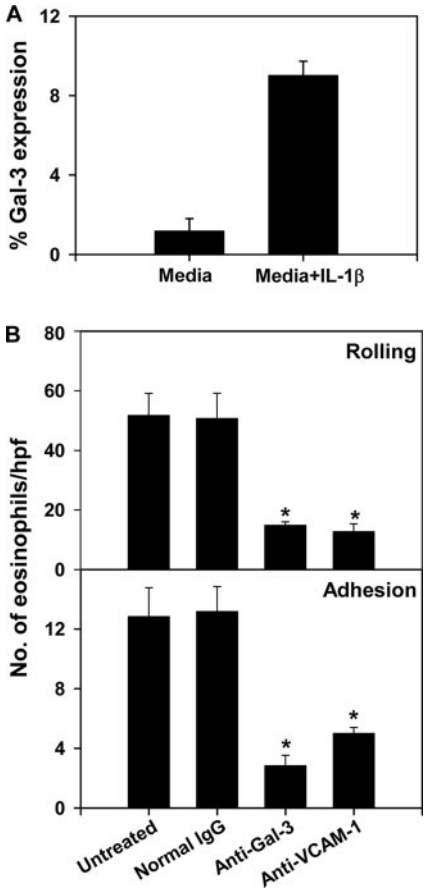

FIGURE 7. Gal-3 expression on inflamed EC is elevated and involved in mediating eosinophil rolling and adhesion. *A*, Gal-3 expression on HUVEC stimulated with IL-1β at 50 ng/ml was evaluated by flow cytometry using mAb A3A12. The percentage of Gal-3 expression (mean ± SE) after subtracting the isotype is shown. Combined data of two experiments performed in duplicate are shown. *B*, Eosinophils from allergic donors ($n = 2$) were infused into the flow chamber containing IL-1β-stimulated HUVEC that were pretreated with mAbs against Gal-3 and VCAM-1 or with control IgG in duplicate, and the interactions of the injected cells with HUVEC were observed under an inverted microscope. Results shown represent combined data for each treatment expressed as mean ± SE of the number of rolling or adherent cells per hpf. *, $p < 0.05$.

that eosinophil-expressed Gal-3 can mediate eosinophil trafficking on immobilized Gal-3 in addition to its ability to support eosinophil rolling and adhesion on VCAM-1 as described in Fig. 2. Treatment of eosinophils with a combination of mAbs against α_4 and Gal-3 did not further inhibit rolling or adhesion on Gal-3 (data not shown). Eosinophil rolling and adhesion on immobilized Gal-3 was also significantly inhibited (72.9 ± 10.8% and 81.9 ± 5.11%, $p < 0.01$, respectively) by lactose (3 mM) but not by maltose, demonstrating the involvement of the CRD in these interactions.

The ability of α_4 to interact with Gal-3 is further supported by the observation that $\alpha_4\beta_1$ directly binds to Gal-3 by ELISA (Fig. 4, *A* and *B*). In these studies, rhGal-3 was found to specifically bind to rh$\alpha_4\beta_1$-coated wells in a dose-dependent manner that was selectively inhibited by lactose (90.36 ± 10.35%) but not by maltose. Collectively, these findings suggest that eosinophil-expressed Gal-3 and α_4 integrins mediate eosinophil trafficking via interaction with endothelial adhesion molecules such as VCAM-1 and Gal-3.

Cell surface expression of Gal-3 on eosinophils

Because Gal-3 appears to be involved in mediating the rolling and adhesion of eosinophils from allergic donors on VCAM-1 and Gal-3, confocal microscopy and flow cytometry studies were conducted to examine the expression of Gal-3 on nonpermeabilized eosinophils. Eosinophils from allergic donors ($n = 4$) were found to express higher levels of Gal-3 compared with normal eosinophils ($n = 3$) by confocal microscopy (Fig. 5*A*). This was further substantiated by evaluating the cell surface expression of Gal-3 on eosinophils from allergic and normal donors ($n = 5$ allergic and 3 normal) using flow cytometry (Fig. 5*B*). The data not only show an increased number of Gal-3-positive eosinophils in allergic donors, but a histogram (*inset* to Fig. 5*B*) of an overlay of Gal-3 expression by a representative allergic donor vs a normal donor also demonstrates increased surface expression of Gal-3 by eosinophils from allergic donors. Because both anti-Gal-3 and anti-α_4 mAbs inhibit eosinophil rolling and adhesion on VCAM and Gal-3 (Figs. 2 and 3), dual staining confocal microscopy was conducted to examine the pattern of expression of these two receptors on the surface of eosinophils. Interestingly, Gal-3 expression on eosinophils from allergic donors was colocalized with the expression of α_4 (Fig. 5*C*). Overall, the increased cell surface expression of Gal-3 by eosinophils from allergic donors demonstrated by these studies further supports the enhanced adhesive interactions of these cells with VCAM-1 and Gal-3 compared with eosinophils from normal donors (Fig. 1).

Eosinophil-expressed Gal-3 and α_4 mediate eosinophil rolling and adhesion to EC

The ability of eosinophil-expressed Gal-3 and α_4 to mediate eosinophil trafficking via interactions with endothelial adhesion molecules such as VCAM-1 and Gal-3 was further validated by using intact EC as opposed to recombinant adhesion molecules (VCAM-1 or Gal-3) adhered to coverslips (Fig. 6). Eosinophils from allergic donors exhibited substantial rolling and adhesion on IL-1β-stimulated HUVECs. However, pretreatment of eosinophils with anti-α_4 and anti-Gal-3 significantly inhibited eosinophil rolling (68.8 ± 4.3 and 77.5 ± 3.8%, respectively, $p < 0.01$) and

adhesion (79.1 ± 4.1 and 43.3 ± 4.0%, respectively, $p < 0.05$) on theses cells. Further, pretreatment of eosinophils with lactose also significantly inhibited eosinophil rolling (75.5 ± 3.35%, $p < 0.02$) and adhesion (94.3 ± 3.2%, $p < 0.02$) on HUVEC. In these experiments, anti-β_2 mAbs and maltose used as negative controls had no effect.

Endothelial-expressed Gal-3 mediates eosinophil rolling and adhesion

To corroborate the finding that the endothelial adhesion molecules VCAM-1 and Gal-3 support eosinophil rolling and adhesion in intact cells, IL-1β-stimulated HUVEC were used. IL-1β is known to up-regulate adhesion molecule (ICAM-1, E-selectin, and VCAM-1) expression on endothelial cells (40). To determine whether Gal-3 expression by HUVEC is also induced under proinflammatory conditions, cells were treated with IL-1β and assessed for Gal-3 expression by flow cytometry. Treatment with IL-1β was found to induce a 4-fold increase in Gal-3 expression by HUVEC (Fig. 7*A*). IL-1β-stimulated HUVEC were then treated with mAbs against Gal-3, VCAM-1, or normal mouse or rat IgG (as controls) before exposure to eosinophils in the flow chamber (Fig. 7*B*). Treatment of HUVEC with mAbs against Gal-3 and VCAM-1 significantly inhibited eosinophil rolling (65.82 ± 7.71 and 79.55 ± 4.46, respectively, $p < 0.01$) and adhesion (68.19 ± 13.32 and 68.15 ± 2.34, respectively, $p < 0.01$) on these cells, whereas control IgG had no effect.

Discussion

Airway allergic inflammation, including asthma, is characterized by a drastic increase in the number of eosinophils in the bronchial mucosa as a result of an increase in selective eosinophil migration to the airways. Eosinophils play a prominent proinflammatory role, and the rolling and adhesion of these cells to the vascular endothelium preceding chemokine-directed selective chemotaxis are critical early steps in the overall pathogenesis of airway allergic inflammation. The cross-talk between eosinophils and EC under conditions of flow is mediated by a plethora of molecules on the two cell types and in their environment (9, 10). Studies using flow chambers and intravital video microscopy, as well as cytokine- and adhesion molecule-deficient mice, have provided important insights into the role played by key adhesion molecules such as selectins and α_4 integrins in eosinophil trafficking (11, 12) and have led to an understanding of the mechanisms underlying the eosinophil-endothelial interactions leading to trafficking within inflamed blood vessels and into tissues in vivo (10). However, leukocyte-endothelial interactions mediated by molecules other than selectins or integrins, such as CD44 for example, are also known to occur (41).

Gal-3, an animal lectin, has recently been shown to play a role in eosinophil recruitment and airway allergic inflammation in a murine model of allergen-induced asthma using mice lacking Gal-3 (19). Although Gal-3 is predominantly located in the cytoplasm, it has also been detected in the nucleus, on the cell surface, or in the extracellular environment, suggesting the multifunctionality of this molecule (22). Although the manner in which Gal-3 gets to the cell surface is not entirely clear, it is thought to be secreted by a mechanism that is independent of the classical secretory pathways, circumventing the endoplasmic reticulum and the Golgi compartment either by accumulating in aggregates under the plasma membrane followed by ectocytosis (42) or by plasma membrane shedding of extracellular vesicles containing high concentrations of the lectin (43). Previous studies have shown that human eosinophils express Gal-3 (also known as Mac-2), and Northern blot analysis with eosinophil RNA hybridized with the human Mac-2 cDNA probes revealed that eosinophils contain a unique transcript of 1.2 kb (44). These studies, further supported by our own demonstration of Gal-3 not only on the cell surface (Fig. 5, *A* and *B*) but also in eosinophil lysates, validate that this Gal-3 is most likely derived from the eosinophils. Eosinophils from allergic donors demonstrated increased surface expression of Gal-3 compared with normal donors (Fig. 5, *A* and *B*). Although the number of Gal-3 expressing eosinophils in allergic donors may be relatively low, it is still significantly higher than that observed in normal donors. Considering that not all eosinophils roll on VCAM, Gal-3, or HUVECs (Figs. 2, 3, and 6), it is likely that only those eosinophils expressing Gal-3 contribute to the overall early adhesive interactions (which also include L-selectin- and α_4-mediated interactions). In addition, because rolling is a transient event in the multistep adhesion cascade, it is unlikely that cells that do interact would necessarily need a high level of expression of Gal-3 to participate in the rolling process. Therefore this molecule can contribute to the initial rolling despite modest surface expression. Although there is little information regarding the effect of cytokines on Gal-3 expression by eosinophils, cytokine priming may play a role in increased Gal-3 expression as well as rolling and adhesion (Fig. 1) on eosinophils from allergic donors. Cytokines such as IL-5, IL-3, IL-25, and GM-CSF, which are important during allergic inflammation, are known to induce ICAM-1, β_2, α_6, and CD44 expression as well as adhesion and chemotaxis by eosinophils (45, 46).

We examined the role played by Gal-3 in eosinophil-EC interactions at a molecular level in the context of allergic asthma. Eosinophils from allergic donors also exhibit increased rolling and adhesion to immobilized VCAM-1 in the classical α_4-dependent manner and, more interestingly, by the engagement of Gal-3 via its CRD under conditions of flow (Figs. 1 and 2). Although the α_4 integrin/VCAM-1 pathway for eosinophil trafficking is well established (11, 12), this is the first report of a role for Gal-3 as an adhesion molecule during eosinophil trafficking. Inhibition studies with mAbs against Gal-3 and with lactose (Fig. 2) suggest that eosinophil-expressed Gal-3 may interact with VCAM-1 via its CRD as described previously in the case of other ligands such as laminin, elastin, fibronectin, and various membrane proteins (22). However, it is not entirely clear from experiments in the present study whether this is a direct interaction between Gal-3 and VCAM-1 or whether eosinophil-expressed Gal-3 participates by binding to α_4 on the eosinophil surface via its CRD, altering the activation state of $\alpha_4\beta_1$ and enabling efficient α_4-mediated rolling and adhesion on VCAM-1. Recent studies have demonstrated that $\alpha_4\beta_1$ has at least four different receptor states, each with independent ligand capture and binding affinity (47). Binding of Gal-3 to $\alpha_4\beta_1$ may modify its receptor state and alter its affinity for VCAM-1, thereby regulating $\alpha_4\beta_1$-dependent rolling. The latter implication is further supported by the finding that rhGal-3 can directly bind to rh$\alpha_4\beta_1$ (Fig. 4) and that the two adhesion molecules exhibit colocalized expression on eosinophils from allergic donors (Fig. 5*C*). Based on the absence of an additive effect on eosinophil rolling and adhesion when preincubated with a combination of mAbs against Gal-3 and α_4, another possibility is that Gal-3 and α_4 integrins may have a putative common binding site on VCAM-1. Although Gal-3 is largely known to mediate cell-ECM protein interactions (22), previous studies have demonstrated its ability to bind to other β_1 integrins such as $\alpha_3\beta_1$ and $\alpha_1\beta_1$ (48) via the CRD in a lactose-dependent manner and thus modulate cell-cell adhesion. Murine Gal-3 has also been shown to bind to the α-subunit of $\alpha_M\beta_2$ (49). In the present study also the binding of

Gal-3 to $\alpha_4\beta_1$ appears to be mediated by the CRD, because eosinophil rolling on Gal-3 (Fig. 3), as well as Gal-3 binding to $\alpha_4\beta_1$ by ELISA (Fig. 4), was inhibited by lactose but not maltose.

The ability of EC-expressed Gal-3 to participate in rolling and adhesion has been described in previous studies from our laboratory (27). In those studies EC-expressed Gal-3 was also found to undergo redistribution to accumulate at points of contact with highly metastatic MDA-MB-435 breast cancer cells. In this study, in addition to the increased rolling and adhesion on VCAM-1, eosinophils from allergic donors also exhibit significantly increased rolling and adhesion to immobilized Gal-3 compared with normal eosinophils (Fig. 1). Inhibition studies with lactose, mAbs against α_4 and Gal-3 individually as well as in combination, and the demonstration of direct binding by ELISA (Figs. 3 and 4) suggest that: 1) eosinophil-expressed α_4 can interact directly with EC-expressed Gal-3; 2) eosinophil-expressed Gal-3 can interact directly with EC-expressed Gal-3; and 3) interaction between eosinophil-expressed Gal-3 and α_4 may be required for efficient α_4-mediated rolling and adhesion on Gal-3. This again is based on the lack of an additive effect on eosinophil rolling and adhesion by a combination of anti-α_4 and anti-Gal-3. Treatment of eosinophils with anti-α_4 can not only inhibit the interaction of eosinophil-expressed α_4 with EC-expressed Gal-3 but also inhibit the binding of eosinophil-expressed α_4 to eosinophil-expressed Gal-3 and thereby inhibit competent α_4-mediated eosinophil-endothelial interactions. The involvement of Gal-3-Gal-3 interactions via the CRD between eosinophils and EC in eosinophil trafficking (Fig. 3) is not unexpected because Gal-3 is known to self-associate, forming multivalent complexes or undergo homodimerization in a concentration-dependent manner (23, 50, 51). Therefore, Gal-3-Gal-3 interactions are likely to constitute an important component of the cross-talk that exists between eosinophils and inflamed EC, especially during airway allergic inflammation when eosinophils are likely to express high levels of Gal-3. Importantly, the findings from flow chamber studies with immobilized vascular adhesion molecules were validated using IL-1β-stimulated HUVEC (Figs. 6 and 7).

Previous studies have suggested that the effects of Gal-3 on cell adhesion are either indirect via the modulation of the activity of integrins or by direct interaction. For instance, Gal-3 can increase the avidity of binding indirectly by ligating glycosylated cell surface CD98, an ubiquitously expressed transmembrane glycoprotein (52) that, in turn, can mediate integrin clustering on the surface of cells (49), or it can directly bind to integrins via its CRD to the tri- and tetra-antennary branches of polylactosamine residues on the integrins (35, 48) thus enhancing cell-cell affinity. Our studies suggest that EC-expressed Gal-3 may regulate eosinophil rolling and adhesion by directly binding to eosinophil-expressed α_4 on eosinophils based on its ability to directly bind to $\alpha_4\beta_1$ at a molecular level in a lactose-sensitive manner (Fig. 4). However, it is possible that both phenomena are operative during eosinophil-endothelial interactions, and additional specifically designed studies will have to be performed to determine this. Because Gal-3 does not contain a classical signal sequence or a transmembrane domain (24, 25) and is yet secreted by cells (22, 23), the more distinctive finding is that eosinophils from allergic donors express elevated levels of this molecule on their cell surface and use it as an adhesion molecule during trafficking. Based on our studies, it is likely that eosinophil-derived Gal-3 is expressed on the cell surface anchored to $\alpha_4\beta_1$ via its CRD (based on the blockade of these interactions by lactose) after exiting the cell where it is then able to mediate eosinophil rolling and adhesion on VCAM-1- and Gal-3-coated surfaces. Although Gal-3 can potentially bind to other integrins such as $\alpha_4\beta_7$, another eosinophil-expressed adhesion molecule (53, 54), or other unidentified proteins, the colocalized expression of Gal-3 and α_4 on the surface of eosinophils from allergic donors (Fig. 5*C*) and our direct binding studies (Fig. 4) additionally support its binding to $\alpha_4\beta_1$.

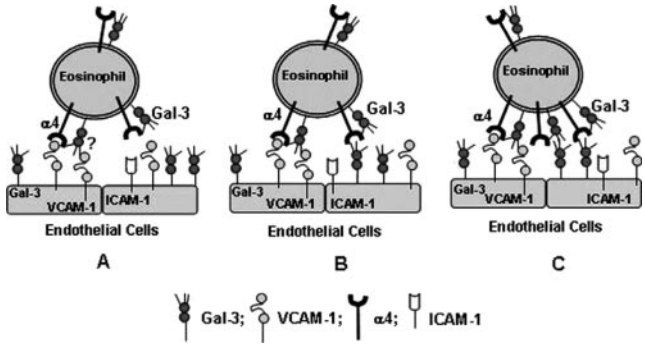

FIGURE 8. Potential interplay between eosinophil and EC adhesion molecules mediating eosinophil trafficking under conditions of flow. The potential interactions likely to occur between eosinophil-expressed $\alpha_4\beta_1$ and Gal-3 and their corresponding counter receptors on EC are illustrated. *A*, Eosinophil-expressed Gal-3 may interact directly with EC-expressed VCAM-1 and/or cluster with eosinophil-expressed $\alpha_4\beta_1$, which, in turn, binds to EC-expressed VCAM-1. *B*, Eosinophil-expressed $\alpha_4\beta_1$ clustered with eosinophil-expressed Gal-3 can bind to EC-expressed Gal-3. *C*, Eosinophil-expressed Gal-3 directly binds to EC-expressed Gal-3. Eosinophil-expressed $\alpha_4\beta_1$ and Gal-3 may initially promote eosinophil rolling and adhesion to the endothelium by binding to EC-expressed VCAM-1, which is followed by the reorganization of EC Gal-3 to the points of contact, making it available to interact with eosinophil-expressed $\alpha_4\beta_1$ and Gal-3 to further augment this binding.

The schematic depicted in Fig. 8 illustrates the potential interplay between eosinophil and EC adhesion molecules that is likely to be involved in mediating eosinophil trafficking under conditions of flow. It is conceivable that in allergic patients $\alpha_4\beta_1$ and Gal-3, that exhibit colocalized expression on eosinophils with Gal-3 potentially anchored to $\alpha_4\beta_1$, first promote eosinophil rolling and adhesion to the endothelium by binding to EC-expressed VCAM-1, which is followed by the reorganization of EC Gal-3 to the points of contact as described in previous studies (27), making it available to interact with additional $\alpha_4\beta_1$ receptors as well as Gal-3 on eosinophils to further enhance this binding. Overall, not only do our studies validate the role of eosinophil-expressed Gal-3 as a novel adhesion molecule that mediates the rolling and adhesion of eosinophils from allergic donors on inflamed EC via interaction with EC-expressed VCAM-1 and Gal-3 directly or indirectly via binding to eosinophil-expressed $\alpha_4\beta_1$ and augments the function of the latter molecule as an adhesion molecule, but they also show that eosinophil-expressed $\alpha_4\beta_1$ can mediate eosinophil-endothelial interactions by binding to EC-expressed Gal-3 in addition to VCAM-1. Thus, Gal-3 expressed on the cell surface of eosinophils from allergic donors and on EC is a significant contributor to eosinophil rolling and adhesion under conditions of flow in vitro and could, either independently or in cooperation with selectins and integrins, participate in or augment the multistep adhesion cascade to promote eosinophil recruitment to sites of allergic inflammation.

Disclosures

The authors have no financial conflict of interest.

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