Galectin-9 mediates neutrophil capture and adhesion in a CD44 and β2 integrin-dependent manner

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Abstract
Neutrophil trafficking is a key component of the inflammatory response. Here, we have investigated the role of the immunomodulatory lectin Galectin-9 (Gal-9) on neutrophil recruitment. Our data indicate that Gal-9 is upregulated in the inflamed vasculature of RA synovial biopsies and report the release of Gal-9 into the extracellular environment following endothelial cell activation. siRNA knockdown of endothelial Gal-9 resulted in reduced neutrophil adhesion and neutrophil recruitment was significantly reduced in Gal-9 knockout mice in a model of zymosan-induced peritonitis. We also provide evidence for Gal-9 binding sites on human neutrophils; Gal-9 binding induced neutrophil activation (increased expression of β2 integrins and reduced expression of CD62L). Intra-vital microscopy confirmed a pro-recruitment role for Gal-9, with increased numbers of transmigrated neutrophils following Gal-9 administration. We studied the role of both soluble and immobilized Gal-9 on human neutrophil recruitment. Soluble Gal-9 significantly strengthened the interaction between neutrophils and the endothelium and inhibited neutrophil crawling on ICAM-1. When immobilized, Gal-9 functioned as an adhesion molecule and captured neutrophils from the flow. Neutrophils adherent to Gal-9 exhibited a spread/activated phenotype.
**INTRODUCTION**

Neutrophils form the first line of defense during inflammation and their migration into the tissue is critical for pathogen elimination and tissue repair. To exit the vasculature, neutrophils follow directional cues that facilitate interaction with the endothelium and migration through the vessel wall.\(^1,2\)

Members of the galectin (Gal) family have been ascribed roles in immune cell trafficking with both anti- and pro-adhesive functions described for Gal-1 and -3.\(^3-8\) Here, we focus on Gal-9, a tandem repeat galectin with two distinct carbohydrate recognition domains joined by a short linker peptide that is susceptible to cleavage.\(^9,10\)

Originally identified in lymphoid tissues from Hodgkin’s disease patients,\(^11\) Gal-9 was subsequently described as an eosinophil chemoattractant\(^12\) that promotes eosinophil adhesion, and T cell transmigration.\(^13,14\) More recently, its function has been extended to include B cell adhesion.\(^15\)

There is limited evidence of a role for Gal-9 in neutrophil trafficking. Gal-9 induces neutrophil degranulation, priming, and enhanced phagocytosis of bacteria, as well as prolonging lifespan,\(^16-18\) and administration of Gal-9 in an emphysema model reduced neutrophil infiltration into bronchoalveolar lavage fluid and inhibited chemotaxis toward keratinocyte-derived chemokine, the murine orthologue of interleukin-8 (IL-8).\(^19\)

Whether Gal-9 functions as a soluble mediator or as a molecule presented by the activated vascular endothelium is not clear. Increased levels of soluble Gal-9 have been reported in the serum of patients with chronic inflammatory conditions such as rheumatoid arthritis,\(^17,20\) type 2 diabetes,\(^21\) and chronic liver disease,\(^22\) while endothelial expression is increased in inflamed tissue\(^23\) and can be up-regulated by interferon-gamma (IFN-\(\gamma\))\(^14,24\) and double-stranded RNA (dsRNA) in vitro.\(^25\)

In this study, we have identified a hitherto unknown role for Gal-9 as a bona fide adhesion molecule that is capable of capturing neutrophils under flow and strengthening the adhesive interaction between neutrophils and the vascular endothelium.

**MATERIALS AND METHODS**

**2.1 Stable form of Gal-9**

A stable linker-less form of recombinant human (rh) Gal-9 (GalPharma) was used throughout as previously described.\(^5,26\) Removal of the linker region increases the stability of the protein as the linker peptide is susceptible to proteolysis. The length of the linker region does not appear to confer differences in the ability of Gal-9 to induce eosinophil chemoattraction, either between different native forms\(^27\) or in comparison to the mutant form.\(^9\) We cannot exclude, however, that in the absence of the linker, the orientation and rotational flexibility of the protein might be altered, which might impact the accessibility of binding sites.

**2.2 Neutrophil isolation**

Blood was taken according to local research ethics committee approval (QMERC2014/61). Informed consent was provided according to the Declaration of Helsinki. Neutrophils were isolated using a double density histopaque gradient and hypotonic lysis as described previously.\(^4\)

**2.3 Human umbilical vein endothelial cells (HUVEC) isolation**

HUVEC were prepared according to a protocol approved by the East London & The City Local Research Ethics Committee (Ref. 05/Q0603/34) and cultured in M119 media supplemented with 20% human serum. All experiments were performed on cells between passages 1–3.

**2.4 Gal-9 knockdown**

Commercially sourced HUVEC (Promocell) were used for transfection with siRNA against Gal-9 (Qiagen; FlexiTube
GeneSolution GS3965 for LGALS9, Cat# 1027416) and scrambled control (Negative control siRNA, Qiagen, Cat# 1027310) at 25 nM in Opti-MEM medium and RNAi lipofectamine (Lipofectamin™ RNAiMAX, Invitrogen, Cat# 56531). siRNAs and lipofectamine were incubated with HUVECs for 4 h at 37°C. Post-transfection, HUVEC were washed and incubated at 37°C for 24 h in an endothelial cell growth medium (ECGM, PromoCell). HUVEC monolayers were detached using Accutase and seeded in μ-Slide VI0.4 channel slides (Ibidi). Four hours after seeding, HUVEC were stimulated with Poly I:C (20 µg/ml; Tocris Cat# 4287) for 24 h at 37°C. Gal-9 knockdown was evaluated by flow cytometry. Briefly, HUVEC were detached using Accutase, and stained with polyclonal anti-Gal-9 antibody (R&D Systems Cat# AF2045; RRID:AB_2137232) followed by anti-goat FITC conjugated secondary antibody or anti-ICAM-1 APC conjugated antibody (BD Biosciences Cat# 551146, RRID:AB_394073) in PBS for 20 min at 4°C. The cells were fixed in 4% PFA in PBS and analyzed on a CyAn ADP flow cytometer (Beckman Coulter).

2.5 | Flow assays

Flow assays were performed using μ-Slides VI0.4 (Ibidi) and neutrophil adhesion, spreading, and migration were quantified offline. Chambers were coated with Gal-9 or rhuICAM-1-Fc (10 µg/ml; R&D Systems Cat# 720-IC) for 2 h followed by blocking with 1.5% BSA for 1 h at 37°C or seeded with HUVEC (isolated in-house) the day prior to flow experiments. HUVEC were stimulated with tumor necrosis factor-alpha (TNF-α; 10 ng/ml, 4 h; Sigma–Aldrich Cat# T0157). Neutrophils were perfused over immobilized proteins at a shear wall stress of 0.1 Pa at 37°C. In some experiments, neutrophils were preincubated with Gal-9 for 10 min at 37°C prior to commencing flow. In some experiments, the following blocking antibodies were incubated with neutrophils for 10 min prior to the flow: 10 µg/ml anti-human CD18 mouse antibody (Clone IB4; Calbiochem Cat# 217660; RRID:AB_10682792) or isotype control (IgG2a; BD Biosciences Cat# 555571, RRID:AB_395950) or anti-CD44 (Clone Hermes-1; Thermo Fisher Scientific Cat# MA4400; RRID:AB_223517). Six distinct areas of the channel were imaged and the number of adhered and spread cells were quantified.

2.6 | Crawling assays

Ibidi chamber slides were coated with recombinant proteins as described above. Neutrophils were allowed to enter the chamber and then the flow was suspended for 5 min to allow adhesion of cells to ICAM-1-Fc. The flow was then re-established at 0.1 Pa and a field of view was recorded for 30 min with frame acquisition every 10 s. Crawling analysis on at least 20 cells per field was performed in Fiji using the manual tracking plugin and further analyses were performed using the Ibidi chemotaxis tool.

For analysis of the effects of soluble proteins, experiments were performed as described above with the following modifications: The flow was re-established at 0.1 Pa with either human recombinant IL-8 (30 ng/ml; Peprotech Cat# 200-08), Gal-9 (30 nM) or both proteins added to the flow buffer. The proteins entered the chamber after 10 min of flow. Migration analyses were performed at minutes 5–10 (pre-soluble proteins entering the chamber) and minutes 20–25 (post-soluble proteins entering the chamber).

2.7 | Detachment assay

HUVECs isolated in-house were plated and stimulated as above. Neutrophils were preincubated with Gal-9 for 10 min at 37°C and then allowed to flow over HUVEC for 2 min. The flow was halted for 6 min and then re-established at a wall shear stress of 0.05 Pa. The flow rate was subsequently increased twofold every 30 s up to a maximum shear stress of 3.2 Pa. The percentage detachment was quantified at each flow rate along with the number of rolling or stationary adherent cells.

2.8 | Modified Boyden chamber chemotaxis assay

Neutrophil chemotaxis was assessed using ChemoTx® 96-well plate with 3-µm pores (Neuroprobe Cat# 101-3). IL-8 (30 ng/ml), Vehicle (RPMI), and Gal-9 (1–30 nM) were added to the bottom wells in triplicate. Neutrophils were added to the top wells at 4 x 10⁶/ml and the plate was incubated at 37°C for 60 min. Cells that migrated to the bottom well were quantified using Alamar Blue (Thermofisher Cat# DAL1025). Cell count was estimated by fluorescence read at 560–590 nm, using a standard curve of known neutrophil concentrations.

2.9 | Flow cytometry

2.9.1 | Assessment of neutrophil activation

Neutrophils were incubated with or without Gal-9 (1–30 nM), PAF (1 nM, C-16; Cayman Cat# 60900) or fMLP (1 µM, Sigma Cat# F3506) for 20 min at 37°C. Neutrophils
were then labelled for analysis by flow cytometry with the following antibodies: CD62L (1 µg/ml; DREG56, Thermo Fisher Scientific Cat# 15-0629-42, RRID:AB_10669698), CD11b (1 µg/ml, ICRF44, Thermo Fisher Scientific Cat# 17-0118-73, RRID:AB_1210558), CD11b (activation epitope; 5 µl/test; CBRM1/5, Thermo Fisher Scientific Cat# 12-0113-42; RRID:AB_10717076) and CD18 (5 µl/test; mAb24, Hycult Biotech Cat# HM2183; RRID:AB_10129817). 10 000 events were acquired on a FacsCaliber (BD Biosciences) and analyzed with FlowJo 7.6 (FlowJo LLC).

2.9.2 | Competition assays for Gal-9 receptors

Freshly isolated neutrophils were resuspended in RPMI 1640 (Sigma–Aldrich, Cat# R8758) at 1 x 10^6 cells/ml and stimulated with Gal-9 at 37°C for 20 min. Cells were labelled for analysis by flow cytometry with the following antibodies: FITC anti-CD43 (1 µl/test, BD Biosciences Cat# 555475, RRID:AB_395867), rat anti-CD44 (2.5 µg/ml, Clone IM7; Thermo Fisher Scientific Cat# 14-0441-85, RRID:AB_467247) and PE anti-CD45 (0.5 µg/ml, Thermo Fisher Scientific Cat# 12-9459-42, RRID:AB_10718238) and incubated on ice for 1h. Cells were then washed and incubated with an AF647 Chicken-anti-Rat antibody (4 µg/ml, Thermo Fisher Scientific Cat# A-21472, RRID:AB_2535875) on ice for 30 min. Samples were acquired to 20 000 single cell events on an LSRFortessa (BD Biosciences) and analyzed with FlowJo 7.6 (FlowJo LLC).

2.10 | Gal-9 expression by HUVEC

Commercially sourced HUVEC were incubated in starvation media (M199 (Gibco), 2% FCS, 100 U/ml Penicillin-Streptomycin and 2.5 µg/ml amphotericin B) containing either TNF-α (100 U/ml, R&D systems Cat# 210-TA), IFN-γ (20 ng/ml, Peprotech Cat# 300-02), a combination of TNFα and IFNγ, or Poly (I:C) (20 µg/ml) for 24 h. After 24 h, supernatants were harvested to quantify Gal-9 levels using Human Galectin-9 Quantikine ELISA Kit (R&D Systems Cat# DGAL90). Cells were lifted using StemPro™ Accutase™ Cell Dissociation Reagent (Gibco) and stained with APC anti-ICAM-1 (1 µl/test, Clone HA58, BD Biosciences Cat# 559771, RRID:AB_398667), FITC anti-CD106 (2 µl/test, Clone 51-10C9, BD Biosciences Cat# 551146, RRID:AB_394073) or unconjugated anti-hGalectin-9 (2 µl/test, R&D Systems Cat# AF2045; RRID:AB_2137232). Following primary antibody incubation, cells were washed and incubated with AF488 Donkey-anti-Goat (0.5 µl/test, Molecular Probes Cat# A-11055, RRID:AB_2534102) before fixation with 4% PFA. Corresponding isotype or secondary only controls were also prepared. The samples were analyzed by flow cytometry on a CyAn™ ADP Analyser (Beckman Coulter) and evaluated using FlowJo (FlowJo LLC).

2.11 | Lectin binding assay

Following fixation neutrophils were incubated with a panel of biotinylated plant lectins with different binding specificities; SNA (0.156 µg/ml; Vector Labs Cat# B-1305-2; binds α2,6-linked sialic acid residues), LEL (0.625 µg/ml; Vector Labs Cat# B-1175-1; binds poly-LacNAc residues with more than 3 repeats), and PHA-L (20 µg/ml; Vector Labs Cat# B-1115-2; binds complex tri- and tetra-antennary N-glycans). Neutrophils were then incubated with a streptavidin-PE (0.12 µg/ml; Thermo Fisher Scientific Cat# 12-4317-87) and analyzed by Flow Cytometry using a BD FACSCalibur.

2.12 | Western blotting

Human neutrophils (2 x 10^6) were stimulated with Gal-9 (30 nM) at 37°C. To test the carbohydrate dependency, cells were incubated with Gal-9 in the presence of lactose (30 mM). Cells were lysed in 95°C NuPAGE LDS sample buffer (Thermo Fisher Scientific Cat# NP0007) containing 1-mM DTT.

Samples were run on a 10% w/v sodium dodecyl sulfate polyacrylamide tris-glycine gel (BioRad Cat# 4561035) and analyzed by immunoblotting with the following antibodies: pAkt-Ser473 (Cell Signaling Technology Cat# 9271, RRID:AB_329825), pERK-Thr202/204 (Cell Signaling Technology Cat# 9101, RRID:AB_331646), GAPDH(Thermo Fisher Scientific Cat# AM4300, RRID:AB_2536381).

2.13 | Zymosan-induced peritonitis

Galectin-9 knockout mice (B6(FVB)-Lgal9tm1.1Cfg/Mmucd) on a C57BL/6J background were obtained from the Mutant Mouse Resource and Research Centers, USA (originally deposited by J Paulson, The Scripps Research Institute, USA). C57BL/6J mice were obtained from Charles River, UK. Zymosan (0.1 mg; Sigma–Aldrich Cat# Z4250) was administered i.p. to C57BL/6J or Gal-9 knockout mice. Mice were sacrificed at the indicated time points and peritoneal cavities were lavaged with ice-cold DPBS containing 2-mM EDTA. Total cell numbers in the peritoneum were quantified by cell counts using Turk’s Solution. Immune cells were labeled with the following panel of fluorescently conjugated antibodies: F4/80 APC
(clone BM8, Thermo Fisher Scientific Cat# 17-4801-82, RRID:AB_2784648), Ly6G PE (clone 1A8, BD Biosciences Cat# 551461, RRID:AB_394208), 7/4 FITC (clone 7/4, Abcam Cat# ab53453, RRID:AB_881408). Following antibody incubations, cells were washed twice with FACS buffer before fixation in 1% paraformaldehyde (PFA) solution. Acquisition of cells was performed with CyAn™ ADP Analyzer (Beckman Coulter) and evaluated using FlowJo (FlowJo LLC).

2.14 | Intra-vital microscopy

The cremaster was inflamed via intrascrotal administration of (500 ng) Gal-9. To visualize neutrophil emigration into the cremaster, a PE-conjugated anti-mouse Ly6G (clone 1A8; BD Biosciences Cat# 551461, RRID:AB_394208) was administered intravenously. Briefly, mice were anesthetized with a mixture of xylazine (7.5 mg/kg) and ketamine (150 mg/kg); the cremaster was then dissected free of skin and fascia, opened longitudinally, and pinned against the viewing platform of a plexiglass stage. To avoid drying out, the cremaster muscle was superfused with bicarbonate-buffered saline (pH 7.4, 37°C, gassed with 5% CO2/95% N2) at a rate of 2 ml/min. Following a 30 min stabilization period, images were acquired using Slidebook software for subsequent off-line analysis. Total leukocyte adhesion and emigration were quantified using bright-field illumination. The number of Ly6G positive neutrophils were quantified in the same vessel segment using the same parameters. In each animal, responses from several vessel segments (three to five) and multiple vessels (three to five) were quantified.

2.15 | Immunofluorescence

Paraffin-embedded rheumatoid synovial biopsies (obtained from patients undergoing total knee/hip replacement collected after informed consent [LREC07/ Q0605/29 granted by the East London & The City Research Ethics Committee 3]) were deparaffinized and rehydrated prior to epitope retrieval in target retrieval solution (Agilent Dako Cat# S236984-2). Following blocking (serum protein block; Agilent Dako Cat# X090930-2), sections were incubated with the following primary Abs; Polyconal goat anti-iGal-9 (1:50-100 dilution, R and D Systems Cat# AF2045, RRID:AB_2137232), monoclonal mouse anti-alpha-smooth muscle actin (SMA) (dilution 1:50, IgG2a, clone 1A4, Sigma-Aldrich Cat# C6198, RRID:AB_476856), monoclonal mouse anti-human Von Willebrand Factor (VWF; dilution 1:50, IgG1k, Agilent Cat# M0616, RRID:AB_2216702), or monoclonal rat anti-human/mouse peripheral node addressing (PNAd; 1:100 dilution, IgM, clone MECA-79, BD Biosciences Cat# 553863, RRID:AB_395099).

After washing, sections were incubated with an appropriate secondary Ab for 1 h at RT Alexa 488-conjugated chicken anti-goat (Molecular Probes Cat# A-21467, RRID:AB_141893); Alexa 488/555-conjugated rabbit antimouse (Thermo Fisher Scientific Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488, Cat A-11059, RRID AB_2534106; Thermo Fisher Scientific Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 555, Cat A-21427, RRID AB_2535848). For Gal-9, a biotinylated secondary Ab was used (Donkey anti-goat, IgG, Jackson ImmunoResearch, RRID:AB_2340396), followed by a third amplification step with Streptavidin linked to either Alexa 488 or 555 (Thermo Fisher Scientific Streptavidin- Alexa 488 Cat# S11223; Streptavidin-Alexa555 Cat# S32355). Sections were counterstained with 4′,6-Diamidino-2-phenylindole (DAPI) and mounted with fluorescent mounting medium (Agilent Dako Cat# S302380-2). Images were taken with an Olympus BX51 fluorescence microscope connected to a digital camera Olympus C-3030. Image capture was carried out using Camedia Master 2.0 software.

For analysis of cells by confocal microscopy, flow assays were performed and neutrophils were fixed with 1% paraformaldehyde (PFA) followed by permeabilization with 0.01% Triton X-100. Cells were incubated with a mouse anti-human vinculin antibody (Sigma-Aldrich Cat# V9131, RRID:AB_477629), followed by an Alexa-594-conjugated secondary antibody (Molecular Probes Cat# A-21215, RRID:AB_141593). Cells were then stained with Alexa-488-conjugated phalloidin (Thermo Fisher Scientific Cat# A12379) and mounted with fluoroshield mounting medium with DAPI (Abcam Cat# ab104139) and images were obtained on a Zeiss LSM800 microscope incorporating a x63 oil Plan apochromat objective (N.A. 1.4) and using Zen blue 2.6. Images were deconvolved with Huygens Professional version 19.10 (Scientific Volume Imaging) using the classic maximum likelihood estimation algorithm. For detection of active CD18, cells were incubated for 5 min with monoclonal antibody 24 (Hycult Biotech Cat# HM2183; RRID:AB_10129817) at the end of the flow assays. Cells were then fixed with 1% PFA and subsequently stained with an Alexa-488-conjugated secondary antibody (Thermo Fisher Scientific Cat# A-11001, RRID:AB_2534069). Cells were mounted with fluoroshield mounting medium with DAPI (Abcam) and imaged as above.

2.16 | Statistics

Data are mean ± SEM. Multiple group comparisons were made using one-way or two-way ANOVA followed by Dunnnett’s or Bonferroni’s post hoc analysis. Where two
conditions were compared using a Student’s *t* test. *p* < .05 was considered significant.

### 3 | RESULTS

#### 3.1 Activated endothelial cells express and release Gal-9

We first sought to confirm whether endothelial Gal-9 expression is increased in the vasculature of chronically inflamed human tissue and to this end examined expression in rheumatoid synovial biopsies by immunofluorescence. Gal-9 was detected in the endothelium of VWF vessels (Figure 1A). This expression did not co-localize with SMA indicating the absence of Gal-9 in smooth muscle cells (Figure 1B). Gal-9 was also expressed in PNAD-positive endothelial cells of high endothelial venules in line with recently reported findings (Figure 1C). Having confirmed the endothelial expression of Gal-9, we wanted to determine whether Gal-9 is expressed on the endothelial surface, where it would be available to interact with cells within the vasculature, and also whether it is released in a soluble form into the extracellular environment. Previous studies have detected Gal-9 expression in endothelial cells in response to IFN-γ and the synthetic analog of dsRNA Poly:IC, but secretion by the endothelium has not, to the best of our knowledge, been reported. IFN-γ alone did not activate the endothelium, as determined by assessing ICAM-1 and VCAM-1 expression or significantly alter Gal-9 expression or release. However, when combined with TNF-α, the ICAM-1 expression was significantly increased (Figure 1D) and there was a trend toward an increased VCAM-1 expression (Figure 1E). Poly:IC had a similar effect and significantly induced ICAM-1 and VCAM-1 expression (Figure 1D,E). Treatment with TNF-α and IFN-γ combined, as well as Poly:IC, resulted in a trend toward increased surface expression of Gal-9, which correlates with what has been shown previously (Figure 1F). Treatment of endothelial cells with TNF-α and IFN-γ also caused significant levels of Gal-9 to be released into the extracellular environment (Figure 1G).

To determine whether endothelial Gal-9 has a functional effect on neutrophil recruitment, we used siRNA to knockdown Gal-9 expression in endothelial cells prior to stimulation with poly:IC (Figure 1H–K), which resulted in a significant reduction in neutrophil adhesion under flow (Figure 1J). No effect on transmigration was observed (Figure 1K). Finally, we used a model of zymosan-induced peritonitis in Gal-9 knockout mice to confirm a role for Gal-9 in neutrophil trafficking in vivo. As shown in Figure 1L, neutrophil recruitment into the peritoneal cavity was significantly reduced in Gal-9 knockout mice compared to wild-type (WT) controls at the 4-h time-point. In contrast, monocyte recruitment at this early timepoint was not impacted by the absence of Gal-9 (Figure 1M). Neutrophil clearance was not negatively affected by the absence of Gal-9 as indicated by comparable numbers of cells in the peritoneal cavity at the 16 h time-point (Figure 1L). A significant reduction in monocyte numbers was observed in Gal-9 knockout mice at 16 h, which may be a responsible to the reduced neutrophil recruitment observed in these mice (Figure 1M).

#### 3.2 Soluble Gal-9 binds to and activates human neutrophils to promote adhesion to the endothelium

Neutrophils isolated from healthy volunteers express some Gal-9 on their surface (Figure 2A), but further binding sites for Gal-9 are available as shown by significant binding of rhGal-9 to the neutrophil surface (Figure 2B). The majority of the Gal-9 interaction is carbohydrate-dependent, as it is significantly diminished in the presence of lactose (Figure 2B). Peripheral blood neutrophils are known to express Gal-9 receptors on their surface but Gal-9 binding was not modulated by short-term exposure to pro-inflammatory stimuli (Figure 2C).
activated neutrophils in a concentration-dependent manner inducing the active conformation of both CD11b and CD18 as well as causing a reduction in CD62L expression to a similar degree as fMLP (Figure 2D). Again, lactose inhibited the actions of Gal-9 on adhesion molecule activation/expression. Integrin (CD18) activation was confirmed by immunofluorescence; a low level of expression was observed on neutrophils adherent to the β2 ligand ICAM-1 and this was enhanced in the presence of Gal-9 (Figure 2E). To determine whether modulation of neutrophil adhesion molecule expression translated into a functional effect, flow chamber assays were performed whereby neutrophils were pre-treated with Gal-9 prior to flow over TNF-α-activated endothelial cells. At the
highest concentration tested (10 nM), Gal-9 significantly reduced the number of rolling neutrophils, through a conversion to firm adhesion, culminating in increased levels of transmigration (Figure 2F).

A pro-recruitment role for Gal-9 was confirmed by intravital-microscopy of the cremaster muscle following intrascrotal administration of Gal-9 (500 ng). Increased numbers of leukocytes were observed to be adherent within...
post-capillary venules with significantly higher numbers of emigrated cells compared to control mice (Figure S1A–C). Through the use of an anti-Ly6G antibody, it was apparent that both the emigration of neutrophils and other leukocyte subtypes were significantly enhanced.

### 3.3 Soluble Gal-9 strengthens neutrophil-endothelial interactions

To determine whether the observed pro-adhesive effects of Gal-9 were solely due to integrin activation, we performed flow experiments in DPBS lacking divalent cations (calcium and magnesium) required for integrin function. Neutrophil rolling on activated HUVECs was not impacted under these flow conditions, presumably due to sufficient residual calcium levels to facilitate selectin function, whereas adhesion was significantly abrogated (Figure 3A,B). Gal-9 (30 nM) sustained neutrophil adhesion indicating that calcium and magnesium and by extension integrins are not solely required for Gal-9 pro-adhesive effects. To further characterize the actions of Gal-9, we next performed flow assays under increasing wall shear stresses. In the absence of Gal-9, neutrophils were retained on the endothelium up to a shear stress of 0.4 Pa at which point significant numbers detached, this coincided with an increased conversion to rolling of those neutrophils still interacting with the endothelium (Figure 3C). In the presence of Gal-9, neutrophil detachment and conversion to rolling were significantly reduced, even at the highest shear stress tested (1.6 Pa; Figure 3D), indicating that Gal-9 functions to strengthen the interaction between neutrophils and endothelial cells, even at high shear stress.

**FIGURE 3** Gal-9 strengthens neutrophil-endothelial cell interactions in an integrin-independent manner. (A, B) Human neutrophils were pre-incubated with Gal-9 (10 min, 37°C) prior to flow over TNF-α stimulated endothelial monolayers at a wall shear stress of 0.1 Pa for 8 min. Both calcium and magnesium were absent from the flow buffer. Neutrophil (A) rolling and (B) adhesion were quantified. Results are mean ± SEM, n = 4 donors, *p < .05 vs. Con (w/o Ca²⁺ and Mg²⁺); one-way ANOVA followed by Bonferroni’s post hoc test. (C, D) Neutrophils were allowed to flow over a HUVEC monolayer for 2 min and then flow was stopped for 6 min. At the end of the 6 min, the flow was re-established at a shear rate of 0.05 Pa. The flow rate was increased twofold every 30 s up to a maximum shear rate of 1.6 Pa. The number of adherent and rolling cells was quantified at each flow rate in order to assess (C) cell detachment and (D) rolling interactions. Results are mean ± SEM, n = 5 donors, *p < .05, **p < .01, ***p < .001 vs. Con; one-way ANOVA followed by Bonferroni’s post hoc test.
3.4 Soluble Gal-9 inhibits neutrophil crawling

Once adherent in the vasculature, neutrophils typically crawl on ICAM-1. Our data indicate that Gal-9 strengthens the interaction between neutrophils and the endothelium, so we next questioned whether this would impact neutrophil crawling, especially in light of the impact on β2 integrin activation, which are known to mediate crawling on ICAM-1.28 In our assays, neutrophils crawled on ICAM-1 in a random manner as expected (pre-flow; Figure 4A), however, the addition of Gal-9 into the flow chamber caused crawling to halt and neutrophils appeared tethered to the spot (post-flow; Figure 4A and Video S1). We compared the action of Gal-9 to another neutrophil activator, IL-8, demonstrated to induce stationary adhesion under conditions of flow as a result of activation of β2 integrins.26 In contrast to Gal-9, IL-8 did not significantly modify neutrophil crawling (Figure 4B and Video S2). When both IL-8 and Gal-9 were added to the flow buffer together, crawling velocity and Euclidean distance traveled were significantly reduced indicating a dominance of Gal-9 in these conditions (Figure 4C and Video S3). Examination of actin polymerization and the cytoskeletal protein vinculin indicated Gal-9 induced a spread/non-polarized morphology with evident focal adhesions (Figure 4D). In contrast, cells exposed to IL-8 were highly polarized with evident F-actin (Figure 4E). Neutrophils treated with both Gal-9 and IL-8 lacked clear polarization and cortical F-actin (Figure 4F).

Recent evidence suggests that galectins may impede chemokine binding to their cognate receptors and, therefore, elicit inhibitory effects on chemotaxis.5 We hypothesized that Gal-9 might, therefore, act in a similar manner. In support of this hypothesis, migration toward IL-8 was inhibited in a concentration-dependent manner in a chemotaxis assay (Figure 4G), with levels of chemotaxis reduced to baseline in the presence of 30 nM Gal-9. This inhibition was only observed when Gal-9 was added to the bottom well of the plate together with the IL-8 suggesting that Gal-9 is able to inhibit and/or override the effects of IL-8.

3.5 Gal-9 functions as an adhesion molecule

As our initial studies demonstrated that knockdown of Gal-9 in endothelial cells led to reduced neutrophil capture and adhesion and that Gal-9 expression is increased on the surface of activated endothelial cells, we next investigated whether plate-bound human recombinant Gal-9 was able to capture neutrophils under flow. We compared the properties of Gal-9 in these assays to ICAM-1 as the major adhesion molecule that mediates neutrophil adhesion in the inflamed vasculature. As expected, neutrophils were not captured by ICAM-1 under flow conditions; in contrast, Gal-9 was able to capture flowing neutrophils in a concentration-dependent manner (Figure 5A,B). In contrast to capture mediated by selectins, neutrophils did not roll on Gal-9, rather they instantly formed firm attachments, and a significant proportion were observed to transition from being phase light (adherent) to phase dark (spreading), indicating activation/polarization (Figure 5A,B and Video S4). Captured by Gal-9 was not supported at wall shear stresses higher than 0.4 Pa. The inclusion of lactose significantly reduced the number of neutrophils captured by Gal-9 as well as their activation (Figure 5C and Video S5) again indicating the carbohydrate-dependent nature of Gal-9 mediated effects.

3.6 Gal-9 induces neutrophil activation/spreading in an integrin-dependent manner

As Gal-9 was both capturing and activating neutrophils, and in light of the ability of Gal-9 to induce integrin activation, we next investigated whether adhesion to immobilized Gal-9 was dependent on β2 integrins. In the absence of divalent cations, there was a trend toward reduced neutrophil spreading (Figure 5D). In support of a role on neutrophil activation, pre-incubation of neutrophils with a CD18 neutralizing antibody significantly reduced neutrophil transition to an activated (spread) phenotype (Figure 5E).

3.7 Neutrophils fail to crawl on Gal-9

Neutrophils crawl within the vasculature as a result of interactions between integrins (particularly Mac-128) and members of the Ig superfamily such as ICAM-1. As expected, neutrophils crawled readily on immobilized ICAM-1, while neutrophils adherent to Gal-9 exhibited frustrated migration in a similar manner to when neutrophils on ICAM-1 were exposed to Gal-9 in solution (Figure 5F and Videos S6 and S7). Although neutrophils appeared to spread and become phase dark, their crawling behavior was significantly impeded, and crawling distance and velocity (Figure 5G) were dramatically reduced. Interestingly, when Gal-9 and ICAM-1 were co-immobilized, neutrophils were still unable to crawl and exhibited similar behavior to when adherent to Gal-9 alone (Figure 5F,G and Video S8). As neutrophil polarization is a key component of crawling behavior, actin
polymerization (F-actin), and vinculin localization were assessed (Figure 5H). Neutrophils adherent to ICAM-1 exhibited F-actin polarization and change in morphology. In contrast, neutrophils adherent to Gal-9 lacked distinct polarization and displayed a rounded morphology with cortical F-actin apparent (Figure 5H). Vinculin localization was similar for each substrate. Given the effectiveness of the CD18 neutralizing antibody on reducing neutrophil spreading on ICAM-1 we stained cells adherent to ICAM-1-Fc, Gal-9, or a combination of...
both proteins for active CD18 and observed expression by confocal microscopy. Cells adherent to Gal-9 had detectable active CD18 at a level similar to that observed in cells adherent to ICAM-1-Fc. When neutrophils were adherent to both proteins, increased levels of active CD18 were apparent, which displayed a cortical location (Figure 5I).

3.8 | Gal-9 functions in a CD44-dependent manner

Galectins are relatively promiscuous in nature, binding a range of glycoproteins on the cell surface with CD44, Tim-3, CD45, and PDI all identified as Gal-9 receptors. We have been unable to detect Tim-3 on neutrophils from...
healthy volunteers. PDI is detectable on neutrophils but in T cells Gal-9 mediated its effects on cell migration by retaining PDI at the cell surface, an effect, which we could not detect in neutrophils. CD44 in contrast is readily detectable on neutrophils (Figure 6A); furthermore, pre-incubation of neutrophils with Gal-9 significantly reduced binding of an anti-CD44 Ab suggesting it is a possible Gal-9 receptor on neutrophils. This was in contrast to the binding of antibodies to CD45, and the known Gal-1 receptor CD43, which showed a marginal reduction with 30 nM Gal-9 (Figure 6B,C). Flow assays were, therefore, repeated in the presence of a CD44 neutralizing antibody. Neutrophil spreading on immobilized Gal-9 (20 µg), but not adhesion, was significantly reduced by blocking CD44 (Figure 6D).

To gain further mechanistic insight into the effects of soluble Gal-9 on neutrophil behavior, we assessed activation of intracellular signaling pathways known to operate downstream of CD44. Gal-9 typically induced a rapid phosphorylation of Akt that subsided by 15 min (Figure 6E). In comparison, ERK phosphorylation exhibited slower kinetics with peak phosphorylation at 5 min (Figure 6E). Induction of signaling pathways by Gal-9 was susceptible to inhibition by lactose (Figure 6F).

3.9 | Neutrophil glycophenotype is permissive for Gal-9 binding pre-transmigration

Galectin binding is dictated by cellular glycosylation status, which is dynamic according to the extracellular milieu. Gal-9 has a strong affinity for poly-N-acetyllactosamine (poly-LacNacS) on N-glycans (Figure S1D–F). Our preliminary assessment of the neutrophil glycophenotype pre- and post-transmigration indicated that upon transmigration, the glycosylation profile of neutrophils is significantly altered with a reduction in molecules bearing N-glycans suggesting that Gal-9 is more likely to exert effects on neutrophils in the circulation rather than following transmigration (Figure S1G–L).

4 | DISCUSSION

We have identified novel functions for Gal-9 as an adhesion molecule that captures and immobilizes neutrophils under physiological levels of flow. Importantly, we have verified that Gal-9 is expressed on the surface of human endothelial cells and released into the extracellular environment by activated endothelial cells. The increased surface expression of Gal-9 in response to IFN-γ and Poly:IC, but not TNF-α, may indicate that Gal-9 plays a role in viral infections or immune-mediated inflammatory diseases such as rheumatoid arthritis as indicated by the increased vascular expression of Gal-9 in rheumatoid synovial biopsies. IFN-γ induces Gal-9 expression in endothelial cells in a histone deacetylase 3 (HDAC3)-dependent manner and histone deacetylases are known to be dysregulated in immune cells of RA patients, whether this dysregulation extends to the vascular endothelium is not known. Our knockdown data indicate that endothelial Gal-9 has a role in mediating neutrophil capture and adhesion specifically and importantly, a role in neutrophil trafficking was also observed in vivo in Gal-9 knockout mice, confirming a role for the endogenous molecule.

Overall our data indicate that Gal-9 functions as both a molecule expressed on the endothelial surface as well as in solution and we have clearly shown that neutrophils are a target for both soluble and bound Gal-9. We have identified CD44 as a Gal-9 receptor on neutrophils with a significant component of neutrophil activation upon adhesion to Gal-9 under flow being mediated by CD44. CD44 facilitates neutrophil trafficking through interactions with hyaluronan and E-selectin. Interestingly, murine neutrophils were not captured by hyaluronan under flow in vitro, suggesting distinct differences in the interaction with Gal-9. CD44-hyaluronan interactions support neutrophil recruitment specifically in liver sinusoids, with no effect on trafficking observed in post-sinusoidal venules of CD44 null mice. These findings underscore not only organ-specific mechanisms but also vascular bed-specific
recruitment. Neutrophils recruited within sinusoids do not tend to roll but rather tether and adhere immediately in a similar manner to the behavior observed with Gal-9. The cross-linking of CD44 can activate LFA-1, promote cell spreading and F-actin polymerization in colorectal cancer cells and neutrophil rolling on E-selectin induces activation of β2 integrins in a CD44-dependent manner, actions in line with our data indicating that Gal-9 induces integrin

**FIGURE 6** CD44 is a potential ligand for Gal-9 that promotes neutrophil adhesion. Human neutrophils were incubated with vehicle (DPBS; Control) or Gal-9 (3–30 nM), for 15 min and binding of antibodies against potential surface-expressed receptors was assessed by flow cytometry: (A) CD43, (B) CD44, and (C) CD45 was assessed by flow cytometry. (D) Human neutrophils were pretreated with anti-CD44 (10 µg/ml) blocking antibody or isotype control for 20 min prior to flow over Gal-9 (20 µg/ml). Neutrophil adhesion was quantified. (E) Neutrophils were treated with Gal-9 (30 nM) for the times indicated, prior to lysis and analysis by Western blotting for phosphorylated ERK and Akt. (F) Neutrophils were treated with Gal-9 (30 nM) for 5 min with/without the addition of lactose (30 mM), prior to lysis, and analysis by Western blotting for phosphorylated ERK and Akt. Membranes were stripped and reprobed for GAPDH as a loading control. Results are expressed as mean ± SEM, n = 3–5 donors, *p < .05, **p < .01; one-way ANOVA followed by Bonferroni’s post hoc test.
activation.\textsuperscript{38,39} In addition, CD44\textsuperscript{−/−} neutrophils exhibit defective migration in β2 integrin-dependent models in vitro providing further evidence of integrin activation downstream of CD44.\textsuperscript{34} Our lectin binding assays suggest that Gal-9 binding may be reduced on neutrophils outside the vasculature. Expression of CD44, which is heavily glycosylated, is also reduced on transmigrated neutrophils.\textsuperscript{40,41}

Neutrophil firm adhesion occurs as a result of LFA-1/Mac-1 interactions with ICAM-1, which mediate adhesion and crawling, respectively,\textsuperscript{28} although other binding partners that support adhesion have been identified including ICAM-2, JAM-A, JAM-C, and RAGE.\textsuperscript{42–45} Unlike Gal-9, while these molecules support neutrophil arrest and migration, they are not able to capture from flow, which is typically selectin-dependent.\textsuperscript{46,47} Neutrophil tethering and adhesion can occur in a VLA-4-VCAM-1-dependent manner at low shear stresses; however, neutrophils require prior activation to induce VLA-4 expression.\textsuperscript{48} Typical β2 ligands induce cell spreading and polarization.\textsuperscript{49} Gal-9, in contrast, induces cell spreading, but not elongation. Our data with the CD18 blocking antibody suggests that while at least a component of this response is β2 integrin-dependent there is also an integrin-independent component and investigations are required to understand why shape change and migration of neutrophils is absent on Gal-9. Mac-1 bonds dominate over LFA-1 bonds for neutrophil polarization and in some circumstances blocking LFA-1 increases polarization due to increased signals through Mac-1.\textsuperscript{44} We found that Gal-9 induced activation of CD11b and CD18; however, it is currently unknown whether there is direct interaction between Gal-9 and either integrin. Our data suggest that Gal-9 may inhibit Mac-1-induced polarization as indicated by the reduced shape change on co-immobilized ICAM-1/Gal-9 or on ICAM-1 in the presence of soluble Gal-9.

CD44 also plays a role in neutrophil polarization as a binding partner for ezrin, radixin, and moesin, which are involved in association of actin filaments.\textsuperscript{49} During cell migration, CD44 is a reliable marker of cell polarity and in its absence, neutrophil polarization, and motility is significantly reduced.\textsuperscript{50,51} It is feasible that Gal-9 crosslinks but prevents redistribution of CD44 to negatively regulate polarization as shown for inhibition of CD43 relocation to the T cell uropod by Gal-1.\textsuperscript{52} HA binding to CD44 induces ERK phosphorylation and focal adhesion formation and promotes adhesion in CD44 transfected COS-7 cells; in fact, HA was identified as the first extracellular ligand to regulate focal adhesion turnover.\textsuperscript{53,54} ERK phosphorylation in neutrophils functions as a stop signal with sustained ERK activation associated with cessation of migration.\textsuperscript{55} Our findings that Gal-9-induced ERK phosphorylation that was at least partially inhibited by a blocking CD44 antibody suggests that Gal-9 may be working via a similar mechanism to promote and strengthen neutrophil adhesion. Vinculin, a major component of focal adhesions, links the actin cytoskeleton with integrins. Gal-9 was the only condition that induced the formation of vinculin-containing focal adhesions in our study, similar to the TNF-α treatment of neutrophils.\textsuperscript{56} Vinculin assumes a peripheral distribution in neutrophils in areas of strong adhesion\textsuperscript{57} and neutrophils lacking vinculin exhibit lower levels of adhesion to ICAM-1 and CXCL1 as well as a reduced ability to spread.\textsuperscript{58} It is feasible that the ability of Gal-9 to induce focal adhesions underpins its effects on the strengthening of neutrophil adhesion.

Lack of polarization correlates with the ability of Gal-9 to inhibit neutrophil crawling. These effects are similar to the role identified for CD99, whereby co-immobilization of CD99 Fc with ICAM-1 and P-selectin enhances shear-resistant adhesion of neutrophils.\textsuperscript{59} However, in contrast to CD99, we found that Gal-9 is able to support neutrophil adhesion in the absence of ICAM-1.

The pro-adhesive effects of Gal-9 observed herein, correlate with actions observed in cancer models, whereby Gal-9 demonstrates therapeutic potential through its ability to reduce metastasis by impairing detachment of tumor cells from the primary site.\textsuperscript{60} In terms of auto-immune/inflammatory pathologies, Gal-9 has been described as a biomarker due to increased serum levels as reported in systemic lupus erythematosus\textsuperscript{51,62} type 2 diabetes,\textsuperscript{25} chronic liver disease,\textsuperscript{22} and Sjogren’s syndrome, where levels were found to correlate with disease activity.\textsuperscript{62} Of relevance to our data, increased serum Gal-9 has also been reported in RA patients and found to correlate with disease activity and CRP levels.\textsuperscript{17,63} Furthermore, Gal-9 has been shown to increase intracellular levels of the enzyme protein arginine deiminase 4, which catalyzes protein citrullination, suggesting a potential role in disease pathogenesis in RA.\textsuperscript{17} Our findings expand current knowledge of Gal-9 function during inflammation by identifying novel functions for Gal-9 as a soluble mediator of neutrophil activation as well as an adhesion molecule in its own right.

Further studies are required to fully understand the role of Gal-9 in neutrophil trafficking and whether its effects extend to other immune cells and different vascular beds. Our data suggest that Gal-9 functions to strengthen the interaction between neutrophils and the vascular endothelium. However, one caveat of our study is the use of HUVEC as a model endothelium. Further investigations are required to determine whether Gal-9 functions similarly in endothelial cells from other tissues. We propose that Gal-9 is a facilitatory molecule, whose actions come to the fore when expression levels are significantly increased as reported in several autoinflammatory diseases. We hypothesize that in these scenarios, excessive Gal-9
functions alongside the canonical leukocyte recruitment cascade to increase neutrophil recruitment within the vasculature; the increased strength of adhesion and the ability of Gal-9 to render neutrophil:endothelial interactions less susceptible to increased shear suggest that Gal-9 may function in arteries as well as in post-capillary venules.

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The authors have stated explicitly that there are no conflicts of interest in connection with this article.

AUTHOR CONTRIBUTIONS
Asif J. Iqbal, Dianne Cooper, Franziska Krautter, Rachael D. Wright, Shani N. Austin-Williams, Mathieu-Benoit Voisin, Mohammed T. Hussain, Isobel A. Blacksell, Hannah L. Law, Lucy V. Norling, Gerard B. Nash performed research and analyzed data. Alok Tiwari and Michele Bombardieri provided clinical samples and helped analyze data. Costantino Pitzalis provided clinical samples. Dianne Cooper and Asif J. Iqbal designed the research. Dianne Cooper and Asif J. Iqbal wrote the paper. All authors provided critical revision of the manuscript.

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