

## Galectin-9

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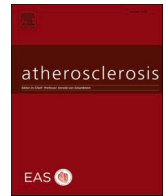
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## Galectin-9: A novel promoter of atherosclerosis progression

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### ABSTRACT

**Background and aims:** Atherosclerosis is widely accepted to be an inflammatory disease driven by lipid accumulation and leukocyte recruitment. More recently, galectins, a family of  $\beta$ -galactoside binding proteins, have been shown to play a role in leukocyte recruitment among other immunomodulatory functions. Galectin (Gal) -9, a tandem repeat type galectin expressed by the endothelium in inflammatory environments, has been proposed to promote leukocyte recruitment. However, the role of Gal-9 in the context of monocyte recruitment remains elusive.

**Methods and Results:** Here, we characterise the immunomodulatory role of Gal-9 in context of atherosclerosis. We show that *ApoE*<sup>-/-</sup>*Gal-9*<sup>-/-</sup> mice have a significantly reduced aortic plaque burden compared to their *ApoE*<sup>-/-</sup> littermate controls after 12 weeks of high fat diet. RNA sequencing data from two independent studies reveal *Lgals9* expression in leukocyte clusters isolated from murine atherosclerotic plaques. Additionally, soluble Gal-9 protein induces monocyte activation and a pro-inflammatory phenotype in macrophages. Furthermore, we show that immobilised recombinant Gal-9 acts as capture and adhesion molecule for CD14<sup>+</sup> monocytes in a  $\beta$ 2-integrin and glycan dependent manner, while adhesion of monocytes to stimulated endothelium is reduced when Gal-9 is knocked down. Gal-9 also facilitates enhanced recruitment of leukocytes from peripheral arterial disease (PAD) patients compared to healthy young and aged controls. We further characterise the endothelium as source of circulating Gal-9, which is increased in plasma of PAD patients compared to healthy controls.

**Conclusions:** These results highlight a pathological role for Gal-9 as promoter of monocyte recruitment and atherosclerotic plaque progression, making it a novel target in the prevention of plaque formation and progression.

### 1. Introduction

The inflammatory response protects us from injury and infection and is reliant on the trafficking of leukocytes from the bloodstream to tissue. Once the initiating stimulus has been cleared, removal of infiltrated leukocytes facilitates termination of the inflammatory response through

an active process known as resolution [1,2]. This resolution process fails in chronic inflammatory pathologies, such as atherosclerosis, which is associated with persistent infiltration of leukocytes, particularly monocytes, into the arterial intima where they develop into pro-inflammatory macrophages and lipid-laden foam cells [3,4]. Whilst primary adhesion molecules and chemokines that direct leukocytes from circulation across

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the endothelium have been identified [5], many parts of the leukocyte trafficking cascade remain elusive, particularly with regards to the persistence of leukocyte infiltrates in chronic inflammation.

A class of lectins, galectins (Gal), play roles in inflammation and autoimmune pathologies [6–13], where they act at key stages of the inflammatory response, including mast cell degranulation [14] and platelet activation [15]. They also modulate leukocyte trafficking into tissue and clearance of the inflammatory infiltrate [6,7], suggesting critical roles, both positive and negative, in the transition to chronic inflammation. Gal-9 belongs to the tandem-repeat subset of the galectin family, which contains two distinct carbohydrate recognition domains (CRDs) at the C- and N-terminus, connected by a flexible linker region [16]. A series of studies have reported increased serum or plasma levels of Gal-9 in patients with various inflammatory diseases, such as systemic lupus erythematosus (SLE) [11], autoimmune hepatitis [10], systemic sclerosis [17], rheumatoid arthritis (RA) [18,19] and large artery atherosclerotic stroke [20], while a recent review suggested that circulating Gal-9 levels correlate with disease severity [21]. Gal-9 was first characterised as an eosinophil chemoattractant [22] and has since been shown to play roles in immune regulation and cellular differentiation: it has been reported to inhibit  $T_H1$  responses and induce apoptosis in  $T_H17$  [23,24] cells, but also induce pro-inflammatory cytokine production in T helper cells, dendritic cells and myeloid-derived suppressor cells [23, 25]. Furthermore, it has been found to play a pro-inflammatory role in pathological conditions, such as RA, by activating granulocytes, generating citrullinated autoantigens through increased expression of peptidyl arginine deiminase 4 and protecting synovial fibroblasts from apoptosis [26,27]. Additionally, Gal-9 treatment of primary T- and B-cells, neutrophils, eosinophils and monocytes increased their adhesion to endothelial cells [28] and more recently our group has unveiled a novel role for Gal-9 as a capture and adhesion molecule for neutrophils [29].

It is clear Gal-9 can regulate various inflammatory mechanisms. Studies have shown that Gal-9 can expand  $T_{reg}$  populations in models of autoimmune arthritis while suppressing the differentiation into  $T_H17$  cells [30], which are both crucial for the resolution of atherosclerosis [31].

These findings suggest that Gal-9 could have an atheroprotective role, as was suggested in a recent review [32]. However, the role of Gal-9 in the development of atheroma, particularly in the context of monocytes, has not yet been investigated. It is also unclear which cells express endogenous Gal-9 and whether Gal-9 can regulate monocyte recruitment and macrophage activation, which are key to this pathology. In this current study we therefore address these knowledge gaps and investigated the role of Gal-9 as a potential promoter of monocyte recruitment and atherosclerotic progression.

## 2. Materials and methods

### 2.1. Stable form of Gal-9

A stable form of Gal-9 from was obtained from GalPharma (Takamatsu, Japan) [33]. Gal-9 is highly susceptible to proteolytic degradation at the linker region. The stable form of Gal-9 is a mutant of native Gal-9 with a truncated linker region, which prevents proteolytic degradation whilst retaining all known activities of native Gal-9.

### 2.2. Mouse models

All animal studies were performed in accordance with UK laws [Animal (Scientific Procedures) Act 1986] with approval of the local ethical committee and UK Home Office approval.

Collection of the tissues used in the RNA sequencing analyses was performed at the NYU School of Medicine under a protocol approved by the Institutional Animal Care and Use Committee (IACUC), following the guidelines of the National Institutes of Health (US).

Mice were sacrificed by administering an overdose of ketamine and xylazine and conformation by cervical dislocation.

#### 2.2.1. Atherosclerosis model

Apolipoprotein E deficient ( $ApoE^{-/-}$ ) mice on a C57BL/6J background were obtained from Charles River. Galectin-9 knockout mice B6 (FVB)- $Lgals9^{tm1.1Cjg}/Mmucd$ , RRID:MMRRC.031,952-UCD, were obtained from the Mutant Mouse Resource and Research Center (MMRRC) at University of California at Davis, an NIH-funded strain repository, and was donated to the MMRRC by Jim Paulson, Ph.D., The Scripps Research Institute.  $ApoE^{-/-}$  and  $Gal-9^{-/-}$  mice were crossed in house to generate double deficient offspring ( $ApoE^{-/-} Gal-9^{-/-}$ ). 10-week old male mice were placed on high fat diet (HFD) (21.4% cocoa butter [w/w] and 0.2% cholesterol [w/w]; Special Diet Services, UK) for 12 weeks before they were sacrificed. Blood was collected for serum analysis and whole aorta and heart were excised for histological analysis.

#### 2.2.2. Zymosan induced peritonitis

Zymosan (0.2 mg, Sigma-Aldrich) was injected i. p. 1 h after injection with PBS with or without Gal-9 (10 mg, sGal9) i. p. to WT C57BL/6J mice. Mice were sacrificed at either 2, 4 or 16 h and the peritoneal cavities were lavaged with ice-cold PBS containing 2 mM EDTA. Quantification of cell types was done by flow cytometry with the following antibodies: F4/80 APC (clone BM8, ThermoFisher Scientific), Ly6G PE (clone 1A8, BD Biosciences), 7/4 FITC (clone 7/4, Abcam). Cells were fixed in 1% paraformaldehyde solution (PFA) after antibody incubations and analysed using BD FACSCalibur™ (BD Biosciences) and evaluated using FlowJo. Cell free lavage fluid was retained for analysis of inflammatory mediator levels by Luminex (Labospace Milan).

### 2.3. RNA sequencing data analysis

Macrophage data sets: Data from two independent studies were used in this analysis. Both isolated  $CD45^+$  selected cells from aorta of mice with atherosclerotic plaques. Lin et al. used  $Cx3cr1^{CreERT2-EYFP/+} R26^{tdTomato/+}$  injected with AAVmPCSK9 and placed on Western Diet for 18 weeks, while Kim et al. used  $Ldlr^{-/-}$  mice on a 12 week high fat diet. The data from Lin et al. (GSE123587) [34] and Kim et al. (GSE116240) [35] were merged and assessed for  $Lgals9$  expression. First, cell types were assigned using SingleR [36] with the meta-analysis from Zernecke et al. [37] as the reference dataset. Cell type annotations were checked using the predominant marker genes in Zernecke et al. [37]  $Lgals9$  expression was visualized on the merged dataset using the FeaturePlot and VlnPlot functions from Seurat v 4.0.1[38].

Endothelial cell data set: Single cell data generated by Zhao et al. (GSE169332) [39] was analysed using Seurat, v4.0 R package. Data were firstly merged, then filtered by removing genes expressed in fewer than 5 cells, cells expressing <200 or >3000 genes and cells containing >10% mitochondrial genes. Data were integrated then scaled and principal component analysis (PCA) was used for data reduction. The top 15 PCs were retained for UMAP projection and clustering analysis with resolution set to 0.7. Subsequently, any non-endothelial cell clusters were removed (using markers defined by Zhao et al.) and the remaining cells assessed for Gal-9 expression via violin or feature plots. The script used for this analysis can be found here: <https://github.com/JuliaManning/Single-cell-data-zhao>.

### 2.4. Patients and control cohorts

Informed consent was provided according to the Declaration of Helsinki. Blood from patients with clinically diagnosed peripheral arterial disease (PAD, average age  $70.71 \pm 9.85$ ) as well as healthy young (HY, average age  $28.00 \pm 3.56$ ) and healthy aged (HA, average age  $68.16 \pm 6.76$ ) was used in this study (Supplementary Fig. 1). Blood was taken according to local research ethics committee approval (PAD patients: HBRC 11–056; Healthy young: ERN 18–0382 The healthy aged

volunteers are part of the 1000 elders cohort).

## 2.5. Flow based adhesion assay

$\mu$ -Slide VI<sup>0.4</sup> channel slides (Ibidi) were coated with Gal-9 for 1 h followed by blocking with 1.5% BSA for at least 1 h at 37 °C or seeded with HUVEC the day prior to flow experiments. In some experiments Gal-9 coated channels were additionally treated with lactose, (25 mM, Sigma Aldrich) or sucrose (25 mM, Sigma Aldrich) for 20 min at 37 °C directly prior to the flow-based assay.

In some experiments, CD14<sup>+</sup> monocytes were incubated with FcR blocking agent for 20 min on ice followed by incubation with anti-human CD18 antibody (10  $\mu$ g/ml, clone IB4, Calbiochem) or isotype control (IgG2a; 10  $\mu$ g/ml, BD Pharmingen) for 20 min on ice prior to perfusion.

$1 \times 10^6$  PBMC or CD14<sup>+</sup> monocytes per ml in pre-warmed PBS (with Ca<sup>2+</sup> and Mg<sup>2+</sup>) containing 0.15% BSA were perfused over immobilised Gal-9 at a wall shear stress of 0.1 Pa or over HUVEC monolayers at a wall shear stress of 0.05 Pa at 37 °C for 4 min followed by a 1 min wash out period. 7 random areas of the channel were imaged for quantification of adhered, spread or transmigrated cells. Adhered cells were considered the sum of all stationary cells (Phase bright and Phase dark). On recombinant protein, Phase dark cells were classed as spread/activated cells, while on endothelial cell monolayers, they were classed as transmigrated cells.

## 2.6. Additional methods

Section staining, cell isolation, culture and treatment and quantification of protein expression is detailed in the supplementary methods.

## 2.7. Statistical analysis

For all statistical analysis, GraphPad Prism software (GraphPad Software) was used. Normal distribution was tested using Shapiro-Wilks test. Differences were analysed using t-test to analyse differences between two groups. One and two way ANOVA with appropriate *post hoc* testing were applied to compare data comprising of more than two groups. A *p* value  $\leq 0.05$  was considered significant.

## 3. Results

### 3.1. Absence of Gal-9 reduces atherosclerosis progression

To test whether Gal-9 deficiency impacts atherogenesis, we studied *ApoE*<sup>-/-</sup>/*Gal9*<sup>-/-</sup> double knockout and *ApoE*<sup>-/-</sup> knockout control mice. Following 12 weeks of a “western-type” high fat diet (HFD), “*en face*” analysis of excised aortas revealed a significant reduction of ~70% and 20% in plaque burden and number, respectively, in *ApoE*<sup>-/-</sup>/*Gal9*<sup>-/-</sup> compared to *ApoE*<sup>-/-</sup> mice (Fig. 1A, Supplementary Figs. 2A and B). Examination of specific aortic regions identified a significant reduction of approximately 68% in plaque burden in the abdominal aorta of *ApoE*<sup>-/-</sup>/*Gal9*<sup>-/-</sup> mice compared to *ApoE*<sup>-/-</sup>, but not the aortic arch or thoracic aorta (Fig. 1A). Quantification of total and average plaque area (Fig. 1C and D), collagen (Fig. 1E) and macrophage (Fig. 1F) content in aortic roots was also performed and representative images are shown in Fig. 1B. Statistically significant reductions in plaque size and area as well as collagen and macrophage content were observed in *ApoE*<sup>-/-</sup>/*Gal9*<sup>-/-</sup> compared to *ApoE*<sup>-/-</sup> mice. Importantly, no significant differences in weight gain, total plasma cholesterol and triglyceride levels were observed between cohorts (Supplementary Figs. 2C–E). Furthermore, we show here that global Gal-9 deficiency in mice does not significantly affect circulating leukocyte subset levels (Supplementary Figs. 2F–I). Collectively, these data indicate a pro-atherogenic role for endogenous Gal-9.

### 3.2. Gal-9 is expressed by macrophage subsets in progressing plaques

We and others have previously reported an upregulation of Gal-9 expression in macrophages in various inflammatory conditions [9]. Here we also show significantly increased *Lgals9* mRNA in aorta of *ApoE*<sup>-/-</sup> mice on high fat diet compared to *ApoE*<sup>-/-</sup> mice on chow diet (Fig. 1G). More specifically, we demonstrate, that Gal-9 is present in atherosclerotic plaques of *Ldlr*<sup>-/-</sup> mice on a 16 week high fat diet (Fig. 1H). This led us to interrogate the expression profile of Gal-9 in CD45<sup>+</sup> cells of progressing plaques using previously published RNA sequencing data sets [34,35]. RNA sequencing data sets from CD45<sup>+</sup> cells, isolated from aortas of *Ldlr*<sup>-/-</sup> mice (Kim.lldlr) or Cx3cr1<sup>CreERT2-EYFP/+</sup> R26<sup>tdTomato/+</sup> (Ioke.prog) with progressing atherosclerotic plaques, were clustered using Zernecke et al. as a reference data set [37] (Supplementary Fig. 3A). The analysis revealed that most leukocyte clusters in progressing plaques, specifically the macrophage clusters, were indeed expressing *Lgals9* mRNA (Fig. 1I, Supplementary Fig. 3A). Interestingly, all macrophage clusters expressed *Lgals9* in both data sets, while monocytes and T cells only in the Kim.lldlr data set expressed *Lgals9* (Fig. 1I). These data, together with our previously published *in vitro* findings of *Lgals9* mRNA and Gal-9 protein expression by macrophages [9] further indicate an inflammation-specific expression of Gal-9.

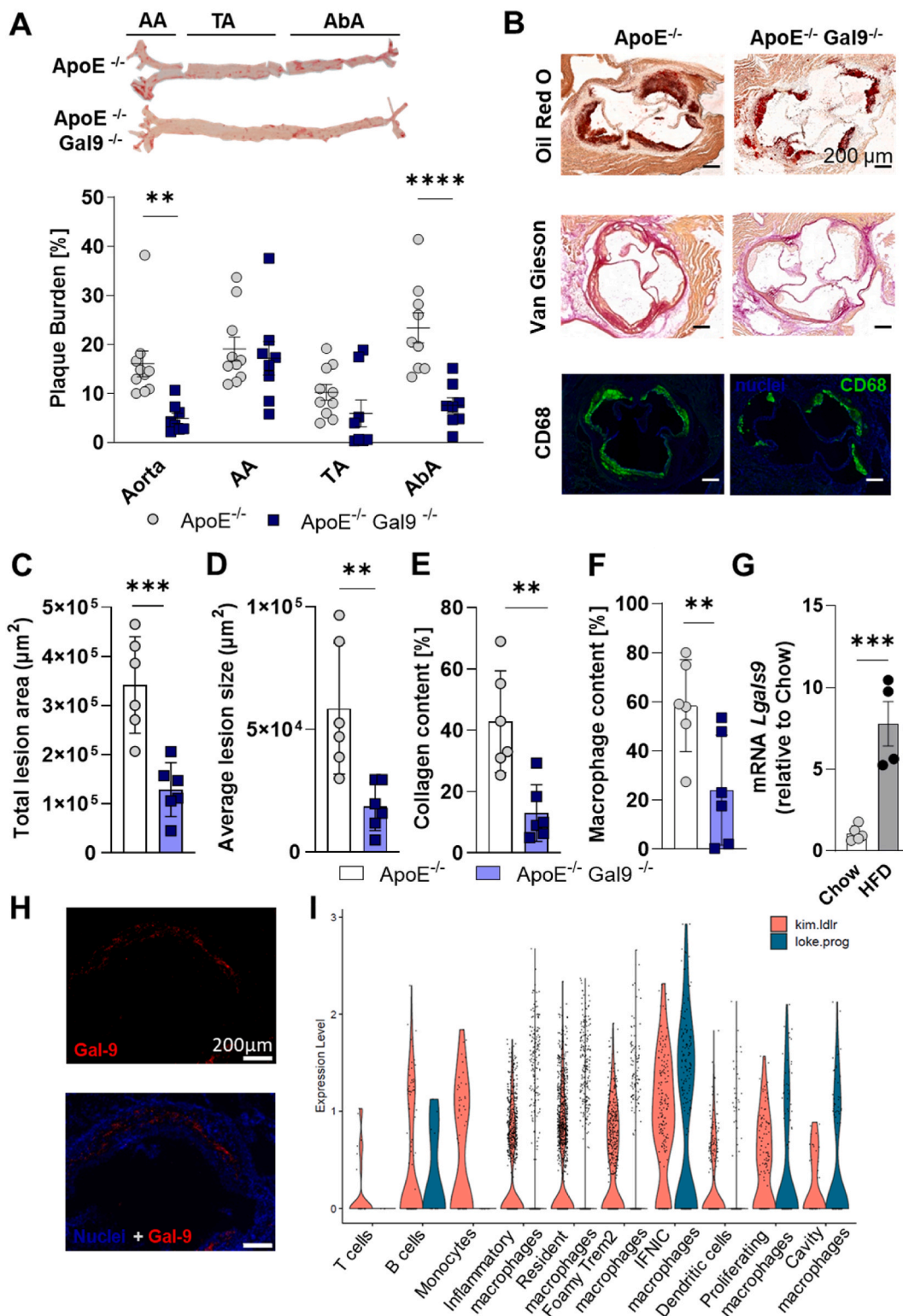
### 3.3. Exogenous Gal-9 induces pro-inflammatory phenotype in macrophages *in vitro*

Given the elevated expression of endogenous Gal-9 associated with macrophages *in situ*, we next evaluated the action of exogenous Gal-9 on human macrophage activation *in vitro*. CD14<sup>+</sup> monocyte-derived macrophages were stimulated with either LPS (100 ng/ml)/IFN $\gamma$  (20 ng/ml), to skew towards an M1 pro-inflammatory phenotype, or IL-4 (20 ng/ml), to skew towards an M2 anti-inflammatory/pro-resolving phenotype, in the absence or presence of 100 nM Gal-9. Remarkably, treatment with exogenous Gal-9 alone skewed macrophages towards a pro-inflammatory phenotype, similar to that observed with LPS/IFN $\gamma$  treatment, with increased TNF $\alpha$ , IL-8 and IL-6 released into culture supernatants (Fig. 2A, representative images in Supplementary Fig. 3B). In fact, stimulation with IL-4 in the presence of Gal-9 reverted macrophages towards an inflammatory phenotype when compared to IL-4 treatment alone (Fig. 2A, Supplementary Fig. 3B). Further analysis of the pro-inflammatory pathways induced by Gal-9 in macrophages revealed NF $\kappa$ B transcription factor and its phosphorylated form were significantly increased in murine macrophage J774A.1 cells following treatment with 100 nM Gal-9 or LPS (100 ng/ml) for 1, 4 and 16 h (Fig. 2B, Supplementary Figs. 3C–E). No differences were observed for STAT-3 and STAT-6 expression (data not shown). Interestingly, the absence of Gal-9 in plaques resulted in a significant reduction of monocyte chemoattractant CCL2, a protein which is regulated by NF $\kappa$ B, within the plaque, further indicating a pro-inflammatory role of Gal-9 (Fig. 2C).

Taken together these data indicate that endogenous Gal-9 is expressed particularly in macrophages in progressing lesions and soluble Gal-9 potentially has the capacity to promote local plaque inflammation via macrophage activation through NF $\kappa$ B dependent pathway.

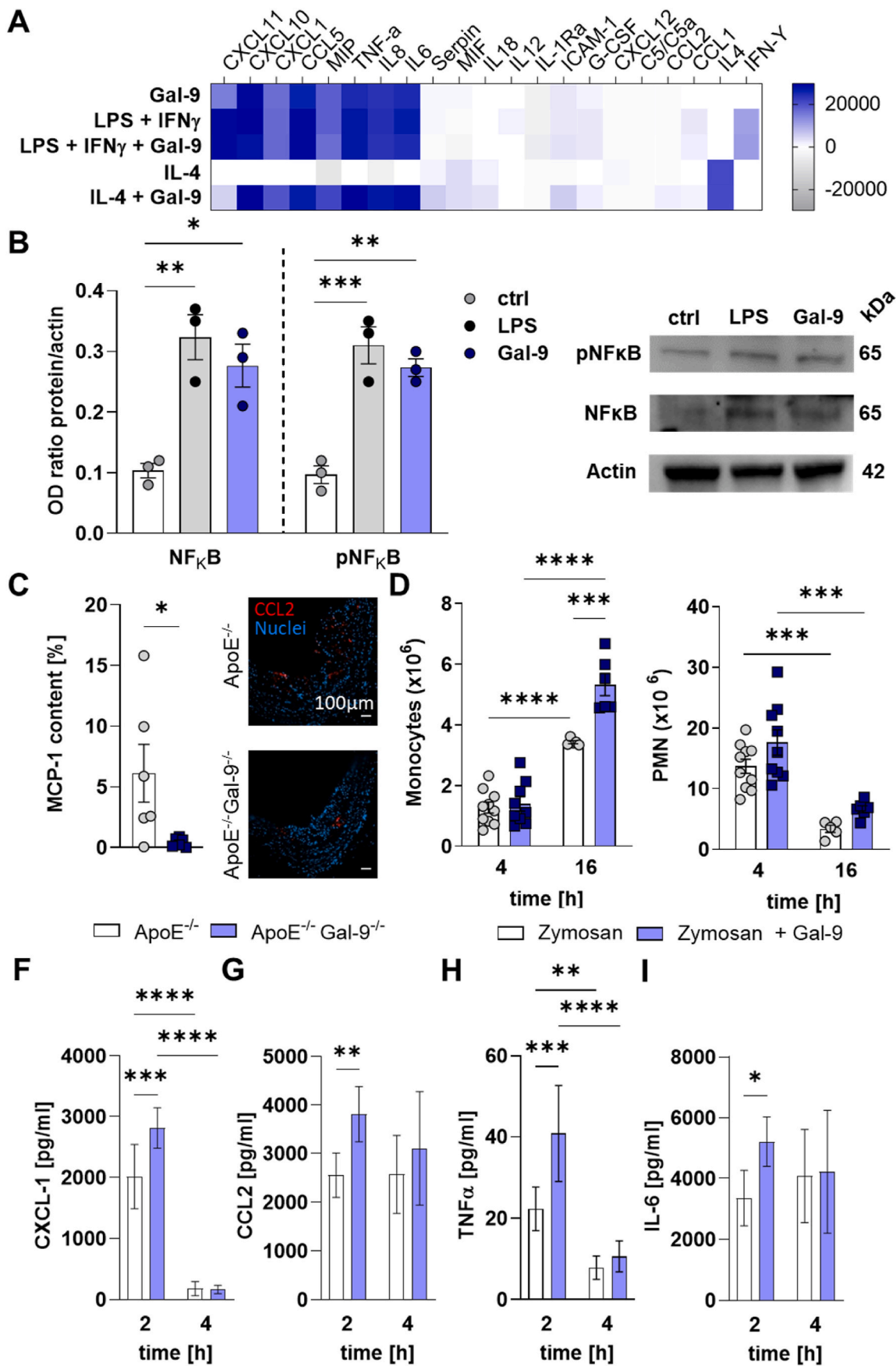
### 3.4. Gal-9 contributes to increase in leukocyte numbers in ZIP model

As Gal-9 levels are increased in the aortas of *ApoE*<sup>-/-</sup> mice fed a high fat diet and given that studies from our group and others have reported a role for Gal-9 in leukocyte recruitment and activation *in vivo*, we aimed to investigate whether Gal-9 plays a role in monocyte recruitment. We have previously investigated the effect of Gal-9 in a well-established model of leukocyte recruitment: zymosan-induced peritonitis (ZIP) and reported significant reductions in monocyte and neutrophil numbers in *Gal9*<sup>-/-</sup> mice compared to wild type mice [29]. Here we



**Fig. 1.** Absence of Gal-9 protects from atherosclerotic plaque progression.

*ApoE*<sup>-/-</sup> and *ApoE*<sup>-/-</sup> *Gal9*<sup>-/-</sup> mice were fed a high fat diet for 12 weeks. Plaque burden in the aorta of these mice were quantified using Oil Red O staining (A). The plaque burden in the whole aorta as well as the aortic arch (AA), thoracic aorta (TA) and abdominal aorta (AbA) was determined (A). Oil Red O and van Gieson staining as well as CD68 antibodies were used to assess the plaque morphology of aortic root plaques (B). Oil Red O<sup>+</sup> areas were used to measure the total plaque area (C) and average lesion size (D). Collagen content was expressed as van Gieson<sup>+</sup> area as percentage of total plaque area (E). CD68 antibodies were used to stain CD68<sup>+</sup> macrophages (F). The macrophage content was expressed as percentage of total plaque area. *Lgals9* mRNA levels of aortas of *ApoE*<sup>-/-</sup> mice fed either a Chow or high fat diet (HFD) for 14 weeks was determined using qPCR (G). Gal-9 protein was detected in aortic root sections of *Ldlr*<sup>-/-</sup> mice on high fat diet for 16 weeks using Gal-9 antibodies (H). RNAseq data from two independent studies of CD45<sup>+</sup> cells from progressing atherosclerotic plaques [34,35] was clustered using Zernecke et al. as reference [37]. *Lgals9* expression levels of these macrophage clusters were blotted individually for each data set (I). N = 4–10 Normality was determined using Shapiro-Wilk test and statistical significance was tested using t-test. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.005, \*\*\*\**p* < 0.001.



(caption on next page)

**Fig. 2.** Exogenous Gal-9 contributes to pro-inflammatory environment.

Human CD14<sup>+</sup> monocyte derived macrophages were treated with either LPS and IFN $\gamma$ , to generate M1 macrophages, or IL-4, to generate M2 macrophages, in the absence of presence of 100 nM Gal-9. After 24 h, the supernatant was collected and analysed using a Proteome Profiler Human Cytokine Array Kit. The cytokine levels were expressed relative to cytokine levels released by untreated macrophages (A). Total NF $\kappa$ B, phosphorylated NF $\kappa$ B (pNF $\kappa$ B) and actin protein expression levels in whole cell lysates of J774A.1 murine macrophages treated with 100  $\mu$ g/ml LPS or 100 nM Gal-9 for 4 h were analysed using western blot (B). Sections of atherosclerotic plaques in aortic arches of *ApoE*<sup>-/-</sup> or *ApoE*<sup>-/-</sup> *Gal-9*<sup>-/-</sup> mice after 12 weeks of high fat diet were stained for CCL2 and the CCL2 content per plaque area was expressed as percentage (C). C57BL6/6J mice were injected i.p. with PBS with or without 10 mg Gal-9 1 h prior to injection with 0.2 mg Zymosan. The mice were sacrificed 2, 4 or 16 post injection and the peritoneal lavage was analysed for monocyte (D) and PMN (E) numbers as well as cytokine contents (F–I). N = 3–10. Normality was determined using Shapiro-Wilk test and statistical analysis was performed using *t*-test or two-way ANOVA with Tukey's *post hoc* test. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.005; \*\*\*\**p* < 0.001.

extend these findings by investigating the role of exogenous, soluble Gal-9 in leukocyte migration during ZIP. Administration of Gal-9 (10  $\mu$ g; i. p.) 1 h prior to zymosan (0.2 mg; i. p.) led to a significant increase in monocytes, but not neutrophils in the peritoneal cavity at 16 h post-zymosan (Fig. 2D and E). An enhanced inflammatory response in the presence of Gal-9 was supported by the detection of significantly higher levels of CXCL-1, CCL2, TNF- $\alpha$  and IL-6 in peritoneal fluid, 2 h post-zymosan (Fig. 2F–I).

### 3.5. Hyperresponsiveness to Gal-9 of PBMCs from peripheral arterial disease (PAD) patients

Given the positive effects of Gal-9 observed on leukocyte recruitment *in vivo* we next sought to validate its role on PBMC recruitment from PAD patients, age-matched healthy controls (HA) and younger donors (HY) using flow-based assays. PBMC were isolated from whole blood and perfused over 100  $\mu$ g/ml immobilised Gal-9 at a wall shear stress of 0.1 Pa for 4 min. Interestingly, we did not observe any rolling, rather immediate capture and adhesion in all three cohorts (representative images Supplementary Fig. 4A). Both, recruitment (number of adhered PBMCs; Fig. 3A) and spreading (percentage of Phase Dark cells; Fig. 3B) on immobilised Gal-9 were significantly elevated in PBMCs from PAD patients compared to HA and HY controls (Fig. 3A and B and representative images Supplementary Fig. 4A), indicating a disease dependent, but age independent profile. Furthermore, significantly elevated levels of Gal-9 were also detectable in the serum of PAD patients compared to both HA and HY controls (Fig. 3C).

### 3.6. Gal-9 induces CD14<sup>+</sup> monocyte activation and spreading in a glycan- and CD18-dependent manner

To further characterise the behaviour of monocytes, we quantified capture and adhesion/spreading of purified CD14<sup>+</sup> monocytes from HY donors to increasing concentrations of immobilised Gal-9. Consistent with our previous observation we saw no rolling. Monocyte capture was observed at all concentrations but interestingly peaked at 20  $\mu$ g/ml of Gal-9 and decreased again with increasing concentration (Fig. 3D and E). However, no differences in the percentage of spread cells between the various Gal-9 concentrations was observed (Fig. 3D and F). Further investigation into the capture mechanism revealed the process was indeed glycan-dependent, since the presence of lactose, a  $\beta$ -galactose containing sugar, significantly decreased monocyte adhesion (Fig. 3G and Supplementary Fig. 4B). Sucrose was used as a non-specific sugar control and had no impact on monocyte adhesion to Gal-9 (Fig. 3G).

Since no rolling of PBMCs or monocytes was observed, we used an anti-CD18 blocking antibody to determine if Gal-9 mediated monocyte capture/adhesion was integrin-dependent. We found that pre-incubation of monocytes with an anti-CD18 blocking antibody significantly reduced CD14<sup>+</sup> monocyte adhesion to Gal-9 by ~90% (Fig. 3H and Supplementary Fig. 4C). Collectively, our data supports the view that Gal-9 acts as a direct capture and adhesion molecule for PBMC, which is significantly enhanced in disease, and is mediated via glycan and  $\beta$ 2-integrin interactions.

Given that our data indicated that immobilised Gal-9 binds and activates monocytes in a CD18-dependent manner, we examined this

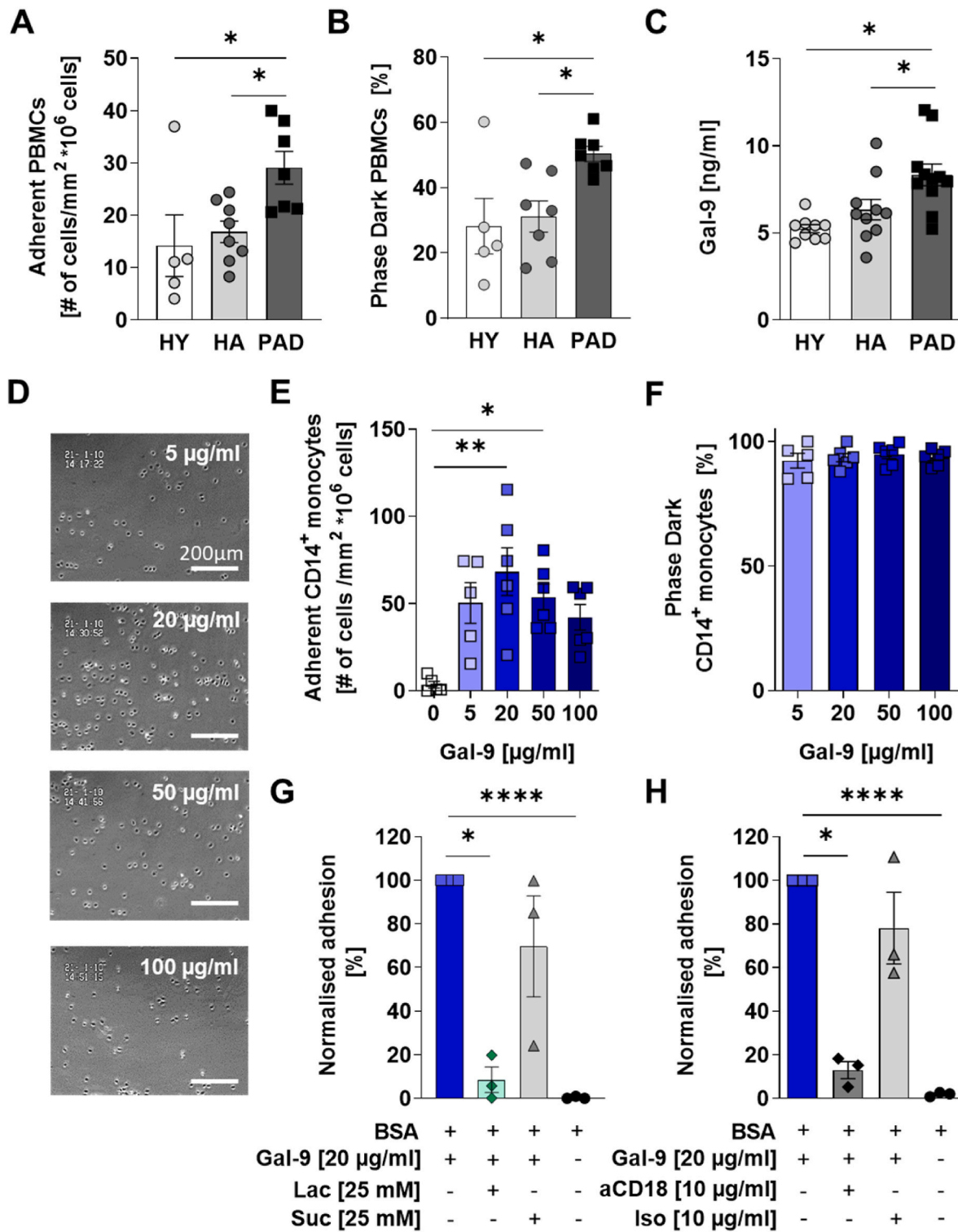
interaction further using activation assays with flow cytometry. We found that soluble Gal-9 binds to monocytes in suspension and that this binding could be inhibited in the presence of lactose but not sucrose (Fig. 4A). While not inducing CD62L shedding to the same extent as fMLP treatment, Gal-9 binding to monocytes caused a decrease in CD62L surface levels, a marker of activation, particularly compared to lactose treated monocytes (Fig. 4B). Interestingly, levels of CD11b and the active conformation of CD18, further indications of monocyte activation, were not affected by any of the treatments (Fig. 4C and D) However, in the presence of Gal-9 a trend towards decreasing CD18 levels was observed, which was rescued in the presence of lactose, while fMLP treatment caused a slight increase in the active conformation of CD18 (Fig. 4D). This Gal-9 induced reduction in the MFI of active CD18 was apparent in all three cohorts (HY, HA and PAD), while fMLP increased levels in all three (Fig. 4E). CD62L was shed in all three cohorts in the presence of both, Gal-9 or fMLP; however, the basal levels of CD62L were lower in PAD patients compared to the healthy cohorts (Fig. 4F), indicating a more activated monocyte phenotype in PAD patients.

### 3.7. Endothelial-derived Gal-9 promotes monocyte adhesion under physiological flow

While our data show an interaction between recombinant Gal-9 and monocytes, we wanted to explore the physiological interaction of Gal-9 expressed by endothelium and monocytes. To address this, we initially characterised Gal-9 expression levels in HUVECs. We confirmed HUVEC activation based on increased E-selectin, VCAM-1 and ICAM-1 mRNA and protein expression (Supplementary Figs. 5A–E) and found that *LGALS9* mRNA was upregulated 24 h post treatment with IFN $\gamma$ , TNF $\alpha$ /IFN $\gamma$  or Poly I:C (Fig. 5A). Endothelial surface Gal-9 levels were increased only in HUVECs treated with TNF $\alpha$ /IFN $\gamma$  or Poly I:C between 16 and 48 h, but not with IFN $\gamma$  treatment alone (Fig. 5B). Soluble Gal-9 was also released into supernatants at 24 h following treatment IFN $\gamma$ , TNF $\alpha$ /IFN $\gamma$  or Poly I:C (Fig. 5C). Based on the results obtained we used Poly I:C stimulation for 24 h in all subsequent flow assays. Moreover, analysis of a published data set from Zhao et al. [39] also confirmed Gal-9 expression in arterial endothelial cells in atherosclerotic lesions (Fig. 5D and Supplementary Figs. 6A and B). Interestingly, endothelial cells from non-atherosclerotic, non-diabetic, chow fed mice also express Gal-9 and no significant differences in Gal-9 expression levels to aortic endothelial cells from atherosclerotic, diabetic mice were detected.

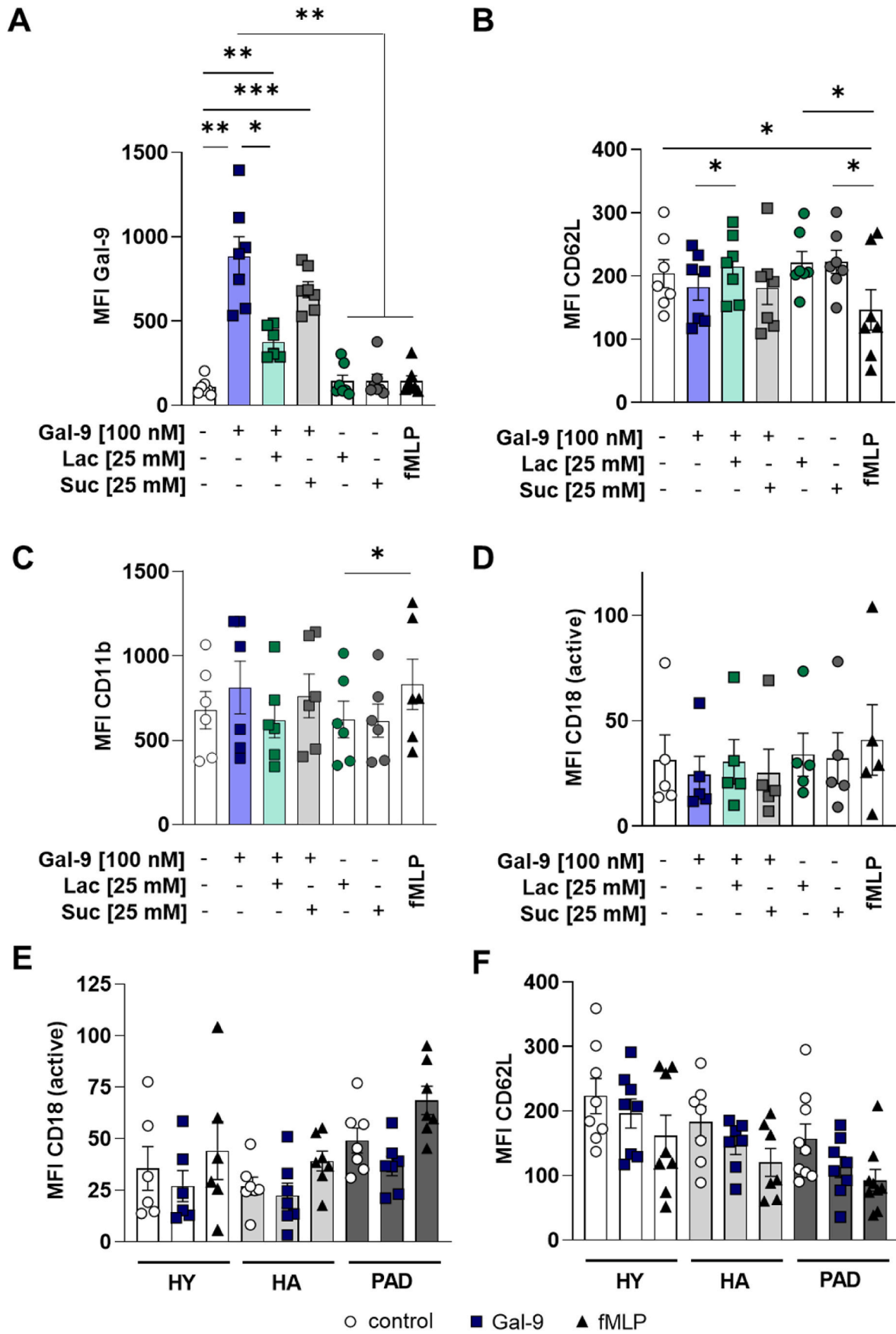
To investigate the role of endothelial Gal-9 in the recruitment of monocytes, we utilised siRNA to knock down (KD) Gal-9 in activated HUVEC. KD efficiency was confirmed using flow cytometry, revealing a 25% extracellular and 75% intracellular reduction in Gal-9 levels, while ICAM-1 levels were unaffected compared to control siRNA treatment following stimulation with Poly I:C (Supplementary Figs. 6C–E). Flow based assays with purified CD14<sup>+</sup> monocytes revealed a significant reduction in monocyte adhesion on Gal-9 KD HUVECs (Fig. 5E and F) compared to control siRNA treatment while transmigration remained unaffected (Fig. 5G).

These results highlight a novel role for endothelial expressed Gal-9 as a capture/adhesion molecule for circulating monocytes during inflammation.



**Fig. 3.** Gal-9 acts as capture and adhesion molecule for monocytes in physiological flow conditions. PBMCs from whole blood of healthy young (HY), healthy aged (HA) and peripheral arterial disease patients (PAD) were perfused over 100 μg/ml immobilised Gal-9 at a wall shear stress of 0.1 Pa (A, B). The total number of adherent cells was assessed (A) and cellular spreading/activation was assessed by determining Phase Dark cells as percentage of total adherent cells (B). Gal-9 levels in plasma of HY, HA and PAD were determined using ELISA (C). CD14<sup>+</sup> monocytes were isolated from whole blood of HY and perfused over various concentrations of immobilised Gal-9 (D). Total adhesion (E) and activated/spread cells (F) were quantified. CD14<sup>+</sup> monocyte adhesion was blocked by incubating immobilised Gal-9 with lactose (G) or by incubating CD14<sup>+</sup> monocytes with CD18 antibodies (H) prior to perfusion. Sucrose and an isotype control were used respectively as unspecific controls. N = 3–8.10 Normality was determined using Shapiro-Wilk test and statistical analysis was performed using one-way ANOVA with Tukey's *post hoc* test. \**p* < 0.05; \*\**p* < 0.01; \*\*\*\**p* < 0.001.





(caption on next page)

**Fig. 4.** Soluble Gal-9 binds to monocytes and induces CD62L shedding.

Levels of Gal-9 (A), CD62L (B), CD11b (C) and the active conformation of CD18 (D) on monocytes were analysed via flow cytometry after incubation of PBMCs with Gal-9 in the presence of PBS (+/+). Lactose was used to establish a glycan dependent effect of Gal-9 binding. Antibodies against CD14 and CD16 were used to identify monocytes. fMLP treatment was used as positive control for monocyte activation. The levels of the active conformation of CD18 (E) and CD62L (F) were measured on monocytes from healthy young (HY), healthy aged (HA) and peripheral arterial disease patients (PAD) after treatment with soluble Gal-9 or fMLP. The median fluorescent intensity (MFI) value of each sample was blotted. N = 5–9.10 Normality was determined using Shapiro-Wilk test and statistical analysis was performed using One Way ANOVA with Tukey's *post hoc* tests. \**p* < 0.05; \*\**p* < 0.01; \*\*\**p* < 0.005.

#### 4. Discussion

In this study, we have shown that Gal-9 contributes to accelerated, diet-induced atherogenesis, as indicated by reduced plaque burden and number as well as collagen and macrophage content in Gal-9 deficient *ApoE*<sup>-/-</sup> mice.

The observed differences in plaque burden in the different areas of the aorta have previously been described in the progression of atherosclerosis in *ApoE*<sup>-/-</sup> mice. Tomita et al., suggest differences in plaque formation occur due to differences in hemodynamics in different sections [40]. Whether Gal-9 expression is affected by changes in shear stress requires further investigation.

Clinical studies such as the CANTOS and LoDoCo trials have highlighted the important role of inflammation in atherosclerotic plaque progression [41,42]. More specifically, a large body of evidence has shown that macrophages play a critical role in driving these atherogenic mechanisms. We have previously shown that *Lgals9* and Gal-9 protein are upregulated in M1 compared to M2 macrophages *in vitro* [9]. Here we show that *Lgals9* is also expressed particularly in aortic macrophage clusters from two independent RNA sequencing data sets (Fig. 1I). Similar results were reported in pre-clinical RA models: Gal-9 levels in T-cells of patients with severe disease were elevated, interestingly the study also reported decreased levels after treatment [18]. These data further indicate a disease/pro-inflammatory dependent expression of Gal-9 in various leukocyte subsets. Interestingly, several studies have demonstrated that increased expression of Gal-9 itself strengthens pro-inflammatory mechanisms. Gal-9 has been reported to regulate genes of inflammatory cytokines such as IL-1 $\alpha$  and -1 $\beta$  in THP-1 cells, a monocyte cell line, via direct interaction with NF-IL6 [43]. Similar findings were reported in previous studies, which showed that Gal-9 treatment of dendritic cells led to the release of cytokines, known to be regulated through NF $\kappa$ B mediated pathways, such as TNF $\alpha$  [23], IL-1 $\beta$  and IL-6 production in myeloid derived suppressor cells [25]. We extend these findings here and show that exogenous Gal-9 induces the release of pro-inflammatory cytokines and chemokines by macrophages through an NF $\kappa$ B dependent mechanism (Fig. 2B, Supplementary Figs. 3C–E). Interestingly, while we show that Gal-9 protein *in vitro* can override anti-inflammatory IL-4 signalling and induce a pro-inflammatory cytokine and chemokine profile associated with M1 phenotype [44], the absence of Gal-9 *in vivo* resulted in reduced CCL2 expression in atherosclerotic plaques. This suggests that not only intracellular Gal-9 [43] but also exogenous Gal-9 can act in a pro-inflammatory manner on myeloid cells *in vitro* and *in vivo*. Whether the disease/pro-inflammatory upregulation and release of Gal-9 in macrophages can act in an autocrine or paracrine manner to induce cytokine release and whether the presence of Gal-9 in plaques can prevent IL-4 signalling necessary for plaque regression [45] or whether its downregulation is required for resolution of inflammation and regression of plaques requires further validation. Furthermore, the role of Gal-9 in the expression of the monocyte chemoattractant CCL2 requires further investigation, particularly in the context of monocyte recruitment to atherosclerotic plaques, as well as the effect of exogenous Gal-9 on other cell types within the atherosclerotic plaque such as endothelial cells or smooth muscle cells.

*In vivo* studies have expanded our understanding of the role of exogenous Gal-9 in various disease models, for example, injection of

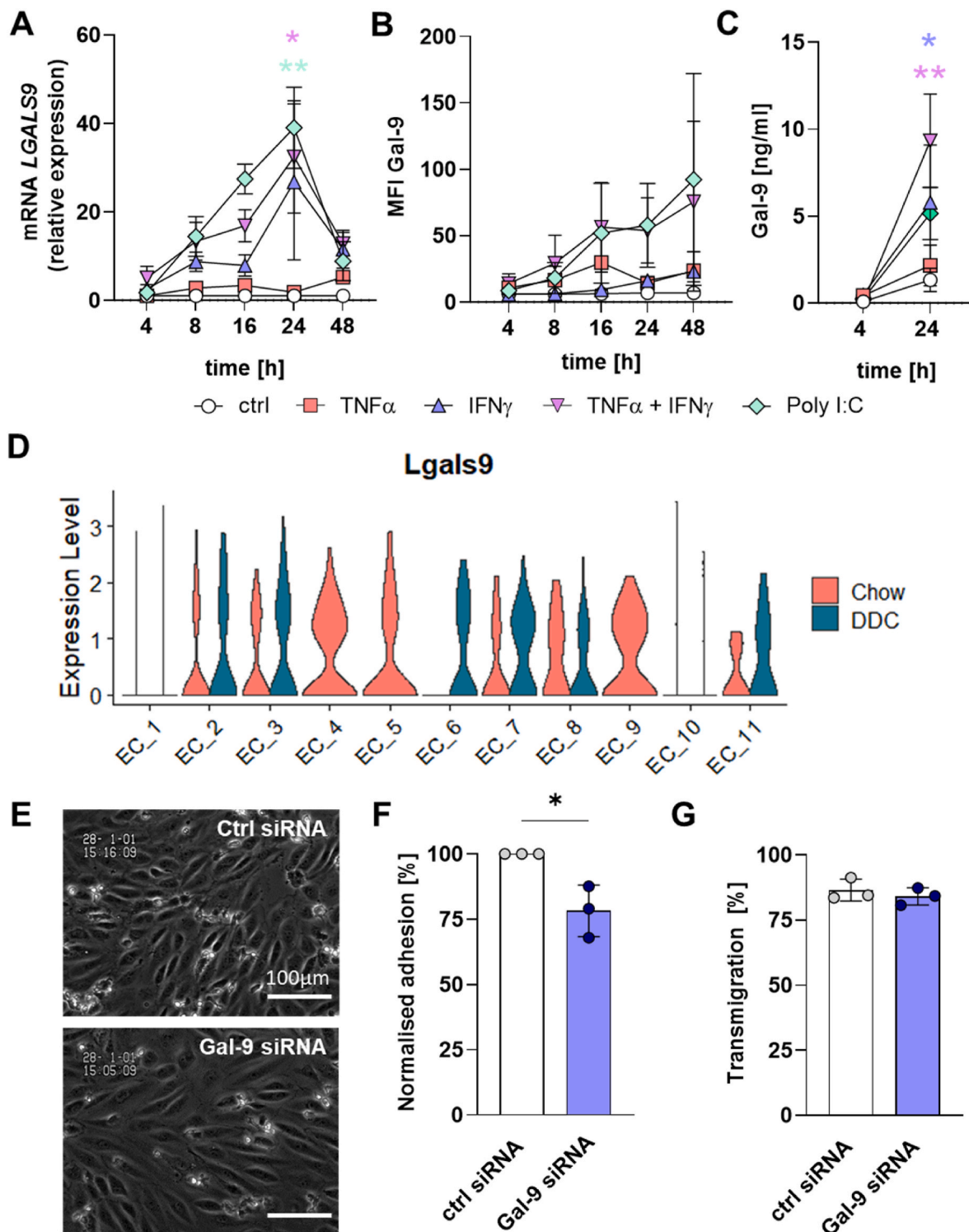
Gal-9 into mouse knees increased their circumference and infiltration with F4/80<sup>+</sup> cells [6]. In line with these findings, we show here that Gal-9, administered *i. p.*, increases the inflammatory response induced by ZIP (Fig. 2D–I), indicated by increased cytokine levels in the peritoneal lavage as well as increased infiltration of monocytes into the peritoneal cavity 16 h post injection. However, studies using other murine models of inflammation have reported anti-inflammatory properties of exogenous Gal-9 *in vivo*: administration of Gal-9 induced apoptosis of Th1-cells and in turn improved SLE-associated arthritis and proteinuria [12] as well as diabetes in NOD mice [23].

Collectively, these studies highlight the complexity in the mode of action of galectins in different inflammatory settings and further studies are required to unravel the mechanisms involved in these settings.

While we and others have shown that soluble Gal-9 mediates inflammation and increased Gal-9 in circulation has been proposed as an indicator of disease severity [21], its source remains unknown. Endothelial cells are known to be a major source of galectins [46–48]. Here we confirm an upregulation of Gal-9 in HUVECs treated with Poly I:C or IFN $\gamma$  and TNF $\alpha$  (Fig. 5A–C), which are expressed by Th1 cells within atherosclerotic plaques [49] and elevated in circulation of atherosclerosis patients [50,51]. We further show that HUVECs also release Gal-9 into supernatant. Therefore, we suggest that increased plasma Gal-9 levels detected in PAD patients and patients with other inflammatory diseases [18,20,21] stem from activated vascular endothelial cells. Whether increased levels of IFN $\gamma$  and TNF $\alpha$  in plaques [49] and in circulation [50,51] contribute to endothelial Gal-9 expression and a subsequent increase in circulating Gal-9 levels is subject to further investigation.

There is evidence from several studies including our own to suggest a positive role for Gal-9 in leukocyte trafficking [28,29,48]. For example, using intravital microscopy, we have shown that Gal-9 increases adhesion of both, Ly6G<sup>+</sup> and Ly6G<sup>-</sup> cells to the vessel wall *in vivo* [29]. Here we expanded upon the current understanding of Gal-9 in the context of monocyte recruitment, which is critical in driving atherosclerotic plaque progression and regression [52], particularly since we observed Gal-9 expression in aortic endothelial cells *in vivo* (Fig. 5D) and decreased macrophage content in plaques of *ApoE*<sup>-/-</sup>Gal-9<sup>-/-</sup> mice compared to *ApoE*<sup>-/-</sup> mice. Interestingly, we found that, contrary to *in vitro* observations, Gal-9 is expressed *in vivo* in aortic endothelial cells under basal conditions as well as during inflammation. This suggests that Gal-9 expression may not only be regulated by inflammatory mediators, but potentially also by mechanical shear stress induced by blood flow.

We show that immobilised Gal-9 acts as direct capture molecule under physiological flow and that its expression in endothelial cells is required for monocyte adhesion to endothelium (Fig. 3D–H and Fig. 5E–G). Interestingly, Gal-9 mediated capture peaked at 20  $\mu$ g/ml Gal-9. This is potentially due to decreased availability of the Gal-9 binding site due to more tightly packed Gal-9 molecules on the channel surface. Gal-9 mediates monocyte capture without rolling, which is initiated by selectins [53], suggesting a selectin independent capture mechanism. Instead, based on our results we propose a  $\beta$ 2-integrin mediated capture and adhesion mechanism under flow conditions. Interestingly, another member of the Galectin family, Gal-8 has already been identified as  $\beta$ 2 integrin LFA-1 binding partner which inhibits the interaction of PBMC with immobilised ICAM-1 under static conditions [54]. Furthermore, we show that Gal-9 also induces the second step of the migration cascade:



**Fig. 5.** Endothelial cells express Gal-9 required for monocyte adhesion and are source of soluble Gal-9. Human Umbilical Vein Endothelial Cells (HUVEC) were isolated from umbilical cords, cultured and treated with pro-inflammatory stimulants TNF $\alpha$ , IFN $\gamma$ , TNF $\alpha$  and IFN $\gamma$  or Poly I:C for 4–48 h. *LGALS9* mRNA levels were determined by qPCR (A) and cell surface Gal-9 expression via flow cytometry (B). The mean fluorescence intensity (MFI) was blotted. Gal-9 released into the supernatant after 4 and 24 h was detected using ELISA (C). Gal-9 expression in endothelial cell (EC) clusters of atherosclerotic mice with diabetes (DDC) and healthy control mice (Chow) was analysed in a previously published data set from Zhao et al. [39] (D). By knocking down Gal-9 in HUVECs before activating them with Poly I:C for 24 h, CD14<sup>+</sup> monocytes were perfused over these cells (E) and total adhesion (F) as well as transmigration (G) were assessed. Transmigration is expressed as a percentage of total adherent cells. N = 3.10 Normality was determined using Shapiro-Wilk test and statistical analysis was performed using one way ANOVA with Tukey's *post hoc* test or *t*-test. \**p* < 0.05; \*\**p* < 0.01.

adhesion/spreading of monocytes. To our knowledge, only VCAM-1 and fractalkine have so far been demonstrated to be capable of inducing capture and adhesion/spreading of leukocytes [55,56]. However while fractalkine capture and adhesion are mediated via an integrin-independent mechanism, the capture and adhesion to VCAM-1 is  $\alpha 4$  integrin dependent. Remarkably, Gal-9 also enhances the capture and adhesion/spreading of PBMCs from PAD patients (Fig. 3A and B). Other studies have reported similar findings of increased adhesion of monocytes to IL-1 $\beta$  stimulated endothelium and VCAM-1, in inflammatory conditions such as RA [57] and hypertriglyceridemia [58], respectively, due to increased activation of monocytes. We also show increased basal activation of monocytes in PAD patients, indicated by decreased CD62L levels (Fig. 4F), which confirms findings from previous studies in healthy aged and individuals with hypertriglyceridemia [58, 59]. However, we did not detect increased levels of the active conformation of CD18, another indicator of cellular activation. Nevertheless, several other studies have reported the upregulation of total  $\beta 2$  integrin [58] in monocytes of PAD patients and increased CD11b/CD18 in patients with unstable angina [60], as well as CD11c, Mac-1 and LFA-1 in patients with myocardial infarction [61,62]. These findings could explain the enhanced Gal-9 mediated  $\beta 2$ -integrin dependent capture and adhesion of PBMC from PAD patients. Additionally, inflammatory diseases can alter the glycan profile of cells [63,64]. We have shown that Gal-9 captures monocytes in a glycan-dependent manner, but whether changes in the glycan profile of PBMCs of PAD patients facilitates enhanced capture by Gal-9 remains unknown. Nevertheless, our results propose Gal-9 as novel capture and adhesion molecule for monocytes, making it an attractive therapeutic target in the treatment of progressing atherosclerotic plaques.

In conclusion, our results demonstrate the inflammation-dependent expression and increase of Gal-9 which contributes to atherosclerosis in two ways: i) as soluble protein, inducing the release of inflammatory mediators in macrophages and the activation of monocytes; and ii) by facilitating monocyte recruitment as a direct capture molecule expressed by endothelial cells through glycan- and  $\beta 2$  integrin dependent interaction. Further studies are required to understand the pathways which regulate Gal-9 mediated inflammation, particularly during plaque progression and if decreasing Gal-9 levels could facilitate atherosclerotic regression by reducing inflammation and monocyte influx.

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### CRedit authorship contribution statement

**Franziska Krautter:** Investigation, Writing – original draft, Visualization, Formal analysis. **Mohammed T. Hussain:** Investigation. **Zhaogong Zhi:** Investigation. **Danielle R. Lezama:** Investigation. **Julia E. Manning:** Investigation. **Emily Brown:** Investigation. **Noemi Margigliano:** Investigation. **Federica Raucci:** Investigation. **Carlota Recio:** Investigation. **Myriam Chimen:** Investigation. **Francesco Maione:** Investigation, Validation. **Alok Tiwari:** Resources. **Helen M. McGettrick:** Investigation, Validation. **Dianne Cooper:** Conceptualization, Writing – review & editing. **Edward A. Fisher:** Supervision, Funding acquisition, Writing – review & editing. **Asif J. Iqbal:** Conceptualization, Supervision, Funding acquisition, Project administration, Writing – review & editing.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.atherosclerosis.2022.11.014>.

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