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Immunoregulatory Siglec ligands are abundant in human and mouse aorta and are up-regulated by high glucose

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ABSTRACT

Aim: Inflammation is a driving force in development of atherosclerosis, and hyperglycemia is a significant risk factor for angiopathy. Siglec-9, expressed on human neutrophils and macrophages, engages specific glycan ligands on tissues to diminish ongoing inflammation.

Materials and method: Siglec-9 ligands on human aorta were characterized and the effects of high glucose exposure on the expression of ligands for Siglec-9 on human umbilical vein endothelial cells (HUV-EC-C) in vitro and ligands for the comparable siglec (Siglec-E) on mouse aorta in vivo were studied.

Key findings: Siglec-9 ligands were expressed broadly on human aorta, as well as on HUV-EC-C. Siglec-9 ligands on HUV-EC-C were sharply up-regulated under high glucose exposure in vitro, as were Siglec-E ligands on the aortas of hyperglycemic mice. Exposure of HUV-EC-C to high-glucose resulted in consistent inhibitory changes in co-cultured macrophages including increased apoptosis and decreased phagocytosis. Control of Siglec-9 ligand expression on HUV-EC-C was downstream of changes in an enzyme involved in their biosynthesis, UDP-galactose-4-epimerase (GALE) and increased cellular *N*-acetylgalactosamine. The alteration of GALE was associated with the regulatory microRNA hsa-let-7f.

Significance: We conclude that exposure to high-glucose results in up-regulation of immune inhibitory Siglec-9 sialoglycan ligands on aorta and HUV-EC-C cells downstream of altered GALE and GalNAc expression, resulting in up-regulation of apoptosis and decrease of phagocytic activity of macrophages. Changes in Siglec-9 sialoglycan ligand expression on vascular endothelial cells may be a natural response to the initial steps of atherosclerosis and might be a potential target to regulate inflammation in diabetic angiopathy.

1. Introduction

Inflammation contributes to angiopathy in atherosclerosis, for which diabetes is a significant risk factor [1,2]. Among the molecules that regulate the level of tissue inflammation are members of the siglec family (sialic acid-binding immunoglobulin-type lectins), cell surface molecules on leukocytes that bind to sialylated glycans and translate glycan binding into changes in immune cell function. There are fourteen human siglecs, most of which are expressed on specific subsets of cells in the innate and adaptive immune systems. Siglecs are single-pass membrane proteins containing multiple extracellular immunoglobulin-like domains that mediate glycan ligand binding and short cytoplasmic

Non-standard abbreviations and acronyms: benzyl-α-GalNAc, benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside; ECL, enhanced chemiluminescence; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; LPS, bacterial lipopolysaccharide; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; PBS, Dulbecco's phosphate-buffered saline; PBSTr, PBS supplemented with 0.1% Triton X-100; BCA, bicinchoninic acid protein assay; PBSTw, PBS supplemented with 0.1% Tween 20; PVDF, polyvinylidene fluoride

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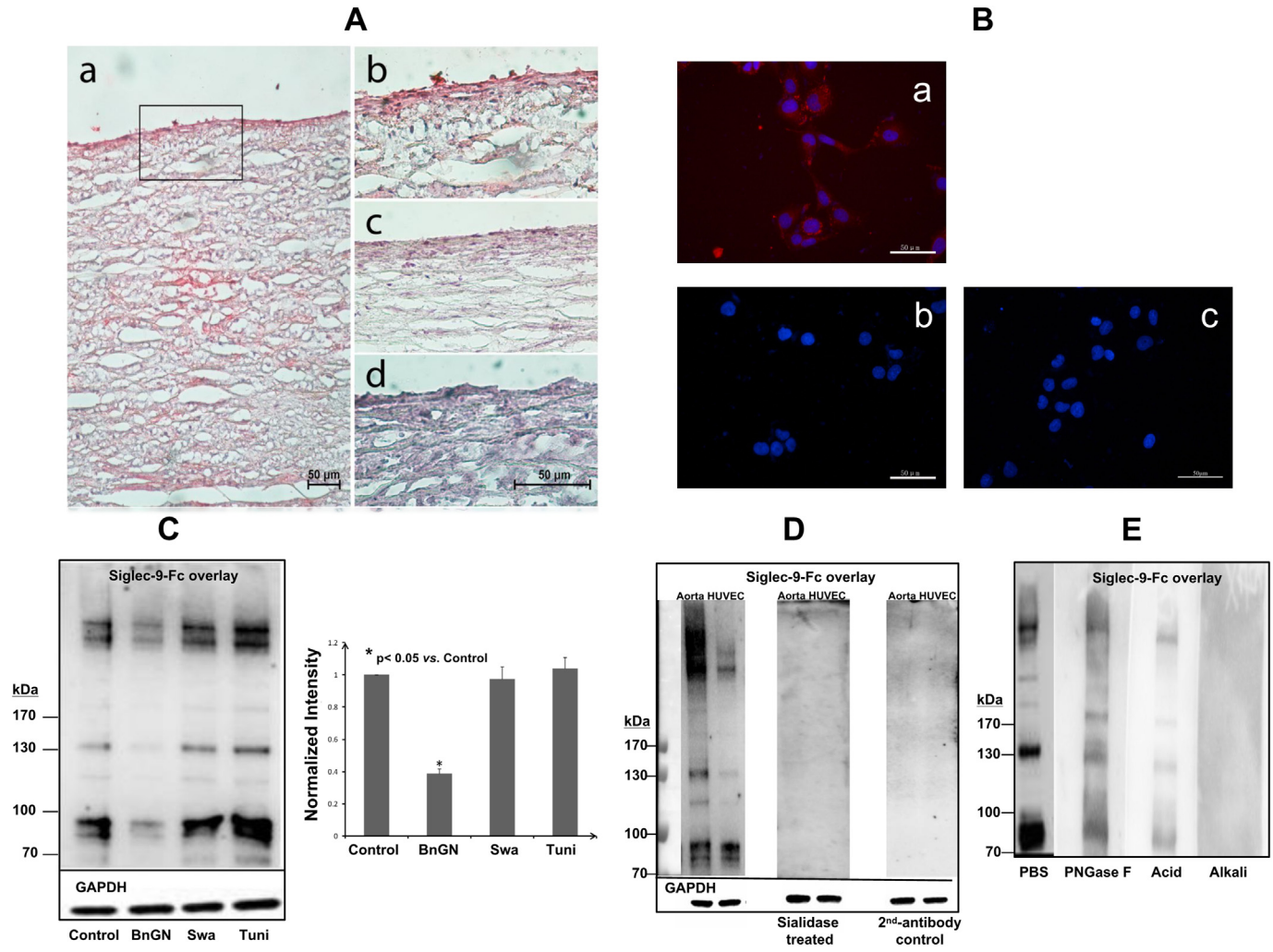

Fig. 1. Siglec-9 ligands on aorta tissue and HUV-EC-C cells. Serial tissue sections of normal human aorta (A) (a–c: Siglec-9 Fc overlay; c: sialidase pretreatment; d: 2nd antibody only, representative of triplicates) and HUV-EC-C cells (B) (a–b: Siglec-9 Fc overlay; b: sialidase pretreatment; c: 2nd antibody only, representative of 6 replicates) were stained with Siglec-9-Fc, control sections were treated with only secondary antibody or pretreated with sialidase (bars = 50 μm). HUV-EC-C cells cultures were treated 12 h with benzyl-α-GalNAc (BnGN), swainsonine (Swa) or tunicamycin (Tuni) as indicated. Below: Total Siglec-9-Fc band (all of the visable bands represented the ligands which could be recognized by Siglec-9 Fc) densities relative to GAPDH (C) (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$). Protein samples from normal human aorta and HUV-EC-C cells were resolved by gel electrophoresis and stained with Siglec-9-Fc, control sections were treated with only secondary antibody or pretreated with sialidase (D) (representative of triplicates). Samples which had been electro-blotted onto PVDF membranes were pretreated with PNGase F, acid (pH = 1.0) or alkali in the presence of 1 M $NaBH_3CN$, then were stained with Siglec-9-Fc (E) (representative of triplicates).

tails, most of which express immunoreceptor tyrosine-based inhibitory motifs (ITIMs) that inhibit the inflammatory activity of the immune cells on which they are expressed. Siglec-9 is expressed on neutrophils, monocytes, macrophages, dendritic cells and natural killer cells. Its ligation on the immune cells commonly results in their inhibition and/or apoptotic death [3]. As a result of its regulatory activity, Siglec-9 plays an important role in many disease processes and has been recognized as a therapeutic target for diseases [4,5].

In the aorta, Siglec-9 may be involved in the regulation of inflammation in atherosclerosis, a well-known chronic inflammatory blood vessel disease [6], which involves activated immune cells. Knowing the molecular characteristics of human Siglec-9 ligands and regulating their expression in human aorta during pathological process may provide additional insight into this pathway of immune system regulation in health and disease. We report here the tissue distribution and molecular characteristics of Siglec-9 ligands in normal human and mice aorta, as well as cultured endothelial cells. We also explore the effects of high glucose, an important risk factor of atherogenesis [7], in correlation with Siglec-9 ligand expression.

2. Materials and methods

2.1. Patients and biopsy specimens

Aorta tissue samples were obtained after informed consent and with approval of the Clinical Research Ethics Committee of Daping Hospital, Third Military Medical University (Chongqing, China). This study was performed conform the declaration of Helsinki. Patients with aortic aneurysm were recruited from the Department of Cardiovascular Surgery of the Daping Hospital. The samples were obtained from the aneurysmectomy of thoracic aorta in patients with aortic aneurysm.

2.2. Siglec-9 ligands histochemistry and immunoblotting

Siglec-9 ligands were detected by Siglec-9 chimeras consisting of the complete extracellular domain of Siglec-9 fused to human Fc [8]. Aorta tissue and cultured cells were detected with the same protocol which presented in supplementary file.

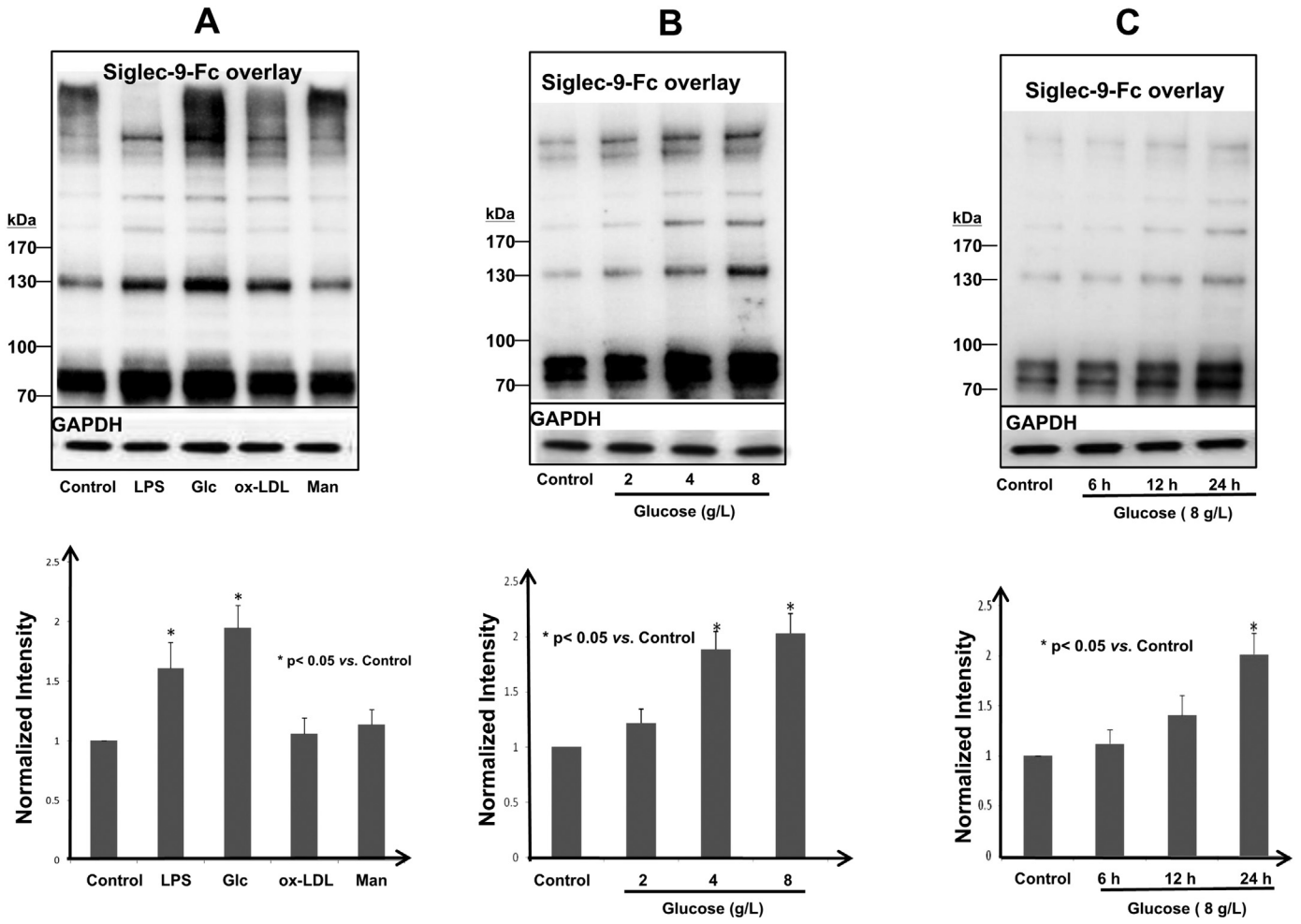

Fig. 2. High glucose increases Siglec ligands in vitro and in vivo. Treatments of high glucose (8 g/L) and LPS (100 μg/L) significantly increased the expression of total Siglec-9 ligands (A) & (D) (a: control; b: mannose; c: high glucose; d: LPS). The up-regulation of Siglec-9 ligands induced by high glucose occurred in a dose - (B) and time-dependent (C) manner. Lower panels (A–C): Total Siglec-9-Fc band densities relative to GAPDH (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$). Hyperglycemia occurred in mice fed a high-glucose diet (F) (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$). Compared with control, expression of Siglec-E ligands on mouse aorta was uniformly intense in mice in the high-glucose diet group (E). Siglec-E-Fc blotting of extracted proteins revealed significantly increased ligands in aortas of high-glucose (Glc) fed mice, while expression of GALE was decreased (G); right panel: Siglec-E-Fc band densities relative to GAPDH (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$).

2.3. Cell culture and treatments

HUV-EC-C cells (CRL-1730, ATCC, Manassas, VA) were maintained on plastic tissue culture dishes in F-12K Medium (30-2004, ATCC). For experiments, cells were treated with different factors. For details, see supplementary file.

2.4. Mice and treatments

Twelve male BALB/C mice aged 10 weeks (22–25 g) underwent a common 1-week acclimatization period and were maintained on a 12 h light-dark cycle at the local animal facility at the Animal Center of the Third Military Medical University, under specific pathogen-free conditions. The mice were randomly divided into two groups: high glucose group and control group ($n = 6$). All procedures performed conform to the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978) and the Animal Management Rules of the Ministry of Health of the People's Republic of China (No. 55, 2001), the protocol was previously approved by the Institutional Ethics Committee for Use of Animals at Third Military Medical University (Chonqing, China). For details, see supplementary file.

2.5. UDP-galactose-4-epimerase (GALE) siRNA and CRISPR activation plasmid transfection

To investigate the relationship of Siglec-9 ligands and GALE expression on HUV-EC-C cells, siRNA and CRISPR Activation Plasmid of GALE were used to regulate GALE expression. GALE siRNA (sc-78950, Santa Cruz), GALE CRISPR Activation Plasmid (sc-408127-ACT, Santa Cruz), non-targeting (control) siRNA-A (sc-37007, Santa Cruz) or Control CRISPR Activation Plasmid (sc-437275, Santa Cruz), each at 1 mg/L, was transfected into HVUEC with UltraCruz Transfection Reagent (sc-395739, Santa Cruz). Transfected cells were cultured for 48 h prior to further reagent administrations.

2.6. Apoptosis and phagocytosis of co-cultured THP-1 derived macrophages

HUV-EC-C cells were grown to 60% confluence in 6-well plate, then were treated different factors. Then HUV-EC-C cells and macrophages derived from THP-1 were co-cultured in a 10:1 ratio for 24 h. The cells were collected for apoptosis and phagocytosis detections. For details, see supplementary file.

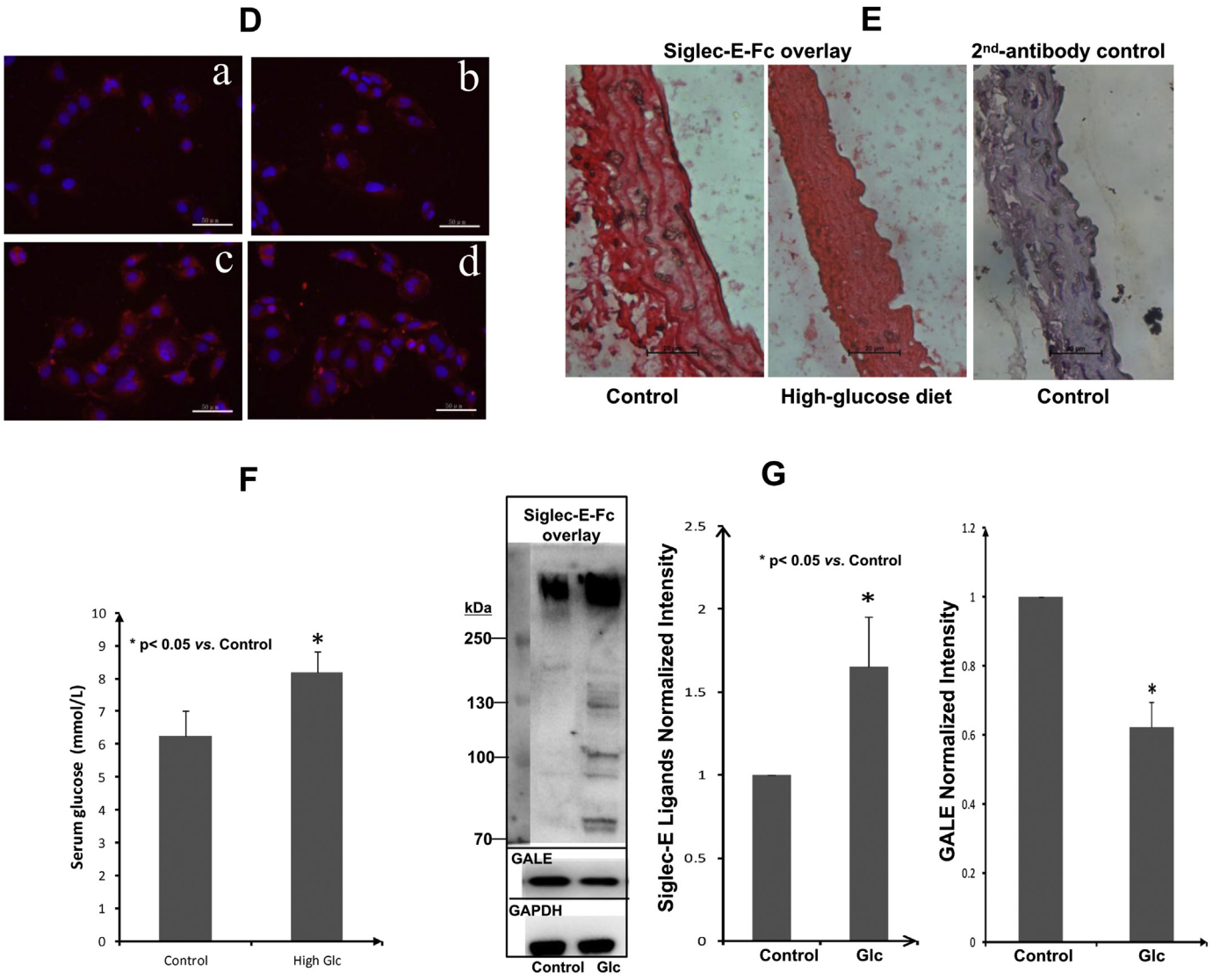

Fig. 2. (*continued*)

2.7. Cellular GalNAc and GlcNAc level [9]

Fluorophore-assisted carbohydrate electrophoresis (FACE) was used for cellular GalNAc and GlcNAc level detection. For details, see supplementary file.

2.8. DNA methylation of GALE and miRNA analysis

There are three transcription start sites (TSS) of GALE gene, named Pair 1 (NM_001127621), Pair 2 (NM_001008216) and Pair 3 (NM_000403). A BSAS (bisulfite amplicon sequencing) method was used to detect the methylation level of these three TSS of the GALE gene. Total miRNA was extracted from samples with the miRNA Isolation Kit (DP501, Tiangen), then the levels of hsa-let-7a-5p, hsa-let-7b-5p, hsa-let-7c-5p, hsa-let-7d-5p, hsa-let-7e-5p, hsa-let-7f-5p, hsa-let-7g-5p, hsa-let-7i-5p and hsa-miR-98-5p were detected. For details, see supplementary file.

2.9. Statistics

Data are expressed as mean ± SEM. Statistical significance was tested using paired or unpaired Student's *t*-test or ANOVA with Tukey post hoc analysis as indicated.

3. Results

3.1. Siglec-9 ligands on aorta tissue and HUV-EC-C

Siglec-9 on the surfaces of inflammatory cells encounters its ligands on target tissues to regulate ongoing inflammation. To determine the histological distribution of Siglec-9 ligands, sections from normal human aorta and HUV-EC-C cells were stained with recombinant human Fc-tagged form of Siglec-9.

Siglec-9 ligands were broadly distributed on intima and media of aorta tissue, and prominent Siglec-9 ligand bands were all > 70 kDa (all of the visable bands represented the ligands which could be recognized by Siglec-9 Fc) (Fig. 1A, C). Binding of Siglec-9 was sharply reduced by sialidase treatment of histological sections (Fig. 1C) and completely absent after sialidase treatment of extracted proteins (Fig. 1D), indicating that binding of the tagged siglec-9 was to sialoglycan ligands. Similar results were found on HUV-EC-C cells (Fig. 1B).

To evaluate Siglec-9 ligand glycan biosynthesis, HUV-EC-C cells were treated with three different glycosylation pathway inhibitors: swainsonine, α-benzyl *N*-acetylgalactosamine (benzyl-α-GalNAc) and tunicamycin. Among the inhibitors, benzyl-α-GalNAc significantly reduced Siglec-9 ligands expression, whereas swainsonine and tunicamycin had no effect (Fig. 1C). Treatment of the blot with alkali erased

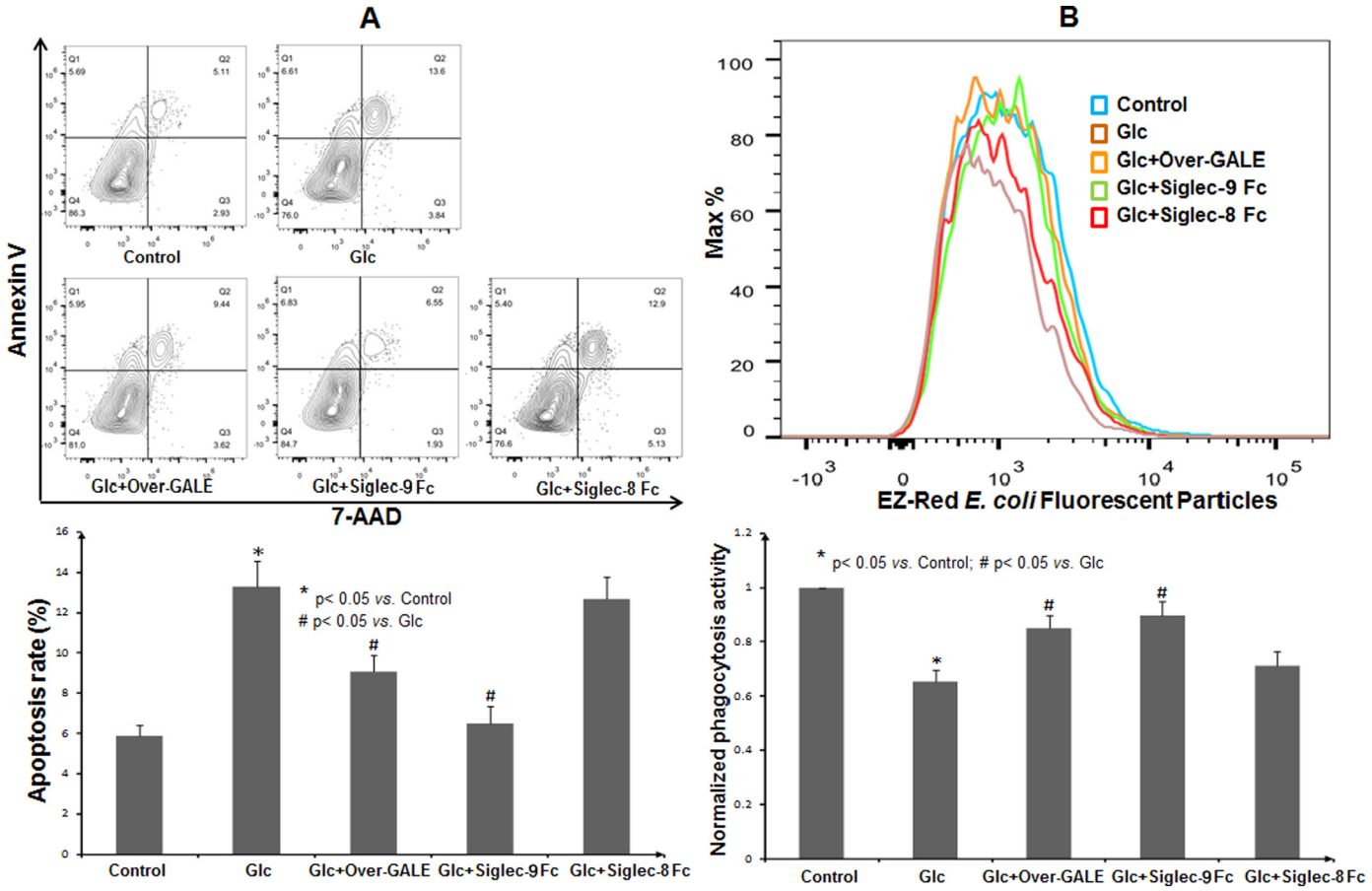

Fig. 3. Increased expression of Siglec-9 ligands on HUV-EC-C cells reduced the activity of co-cultured THP-1 derived macrophages. The increased expression of Siglec-9 ligands on HUV-EC-C cells caused by growth in high glucose (Glc, 8 g/L) significantly up-regulated macrophage apoptosis (A) (mean ± SEM; *, $p < 0.05$ vs Control; #, $p < 0.05$ vs Glc, $n = 6$) and down-regulated their phagocytosis (B) (mean ± SEM; *, $p < 0.05$ vs Control; #, $p < 0.05$ vs Glc, $n = 6$) of THP-1 derived macrophages. Up-regulation of GALE expression by activation plasmid reversed these effects (Glc + Over-GALE), as did addition of Siglec-9-Fc Chimera Protein. Siglec-8 Fc was used as a control protein.

Siglec-9-Fc binding, treatment with acid reduced binding, and treatment with PNGase F had little effect (Fig. 1E). These data indicate that the Siglec-9 ligands of HUV-EC-C cells are primarily *O*-linked sialoglycoproteins.

3.2. Expression of Siglec-9 ligands is up-regulated by high glucose in vitro and in vivo

To provide a ready source of Siglec-9 ligand-expressing cells, a stable human endothelial cell line HUV-EC-C (ATCC CRL-1730), was tested for Siglec-9 ligands expression. Siglec-9-Fc overlay binding studies revealed that these cells, when grown as submerged cultures, express Siglec-9 ligands, which are similar to the ligands in aorta tissue (compare Fig. 1C,D). To test whether expression of Siglec-9 ligands is modulated by the risk factors of atherosclerosis, cells were treated with glucose (8 g/L), ox-LDL (100 mg/L) or LPS (100 μg/L), mannose (8 g/L) was used as a control for glucose. Treatment with glucose and LPS significantly increased Siglec-9 ligands expression, while ox-LDL and mannose did not (Fig. 2A, D). The increase of Siglec-9 ligands induced by glucose was dose- and time-dependent manner (Fig. 2B, C). The results of high glucose in HUV-EC-C cells were corroborated in vivo by the alteration of Siglec-E (a homolog of Siglec-9) ligands in mice fed with a high-glucose diet. The glucose concentration in mice of high-glucose diet was significantly increased (Fig. 2F). Expression of Siglec-E ligands on aorta was significantly increased in the high-glucose diet group compared with control (Fig. 2E), while the GALE expression (see below) was significantly decreased (Fig. 2G).

3.3. Increased Silgec-9 ligands on HUV-EC-C reduced the activity of co-cultured macrophages

To explore the possible biological roles of high glucose-induced changes in the expression of Silgec-9 ligands, apoptosis and phagocytosis of co-cultured macrophages were measured. The increased expression of Siglec-9 ligands on HUV-EC-C cells correlated with up-regulation of macrophage apoptosis (Fig. 3A) and down-regulation of their phagocytosis (Fig. 3B). Up-regulation of GALE expression (see below) using an activation plasmid reversed the effects of growth in high glucose, as did addition of a competitive blocker, Siglec-9-Fc Chimera Protein (Fig. 3A, B).

3.4. Alteration of Siglec-9 ligands by glucose is associated with UDP-galactose-4-epimerase (GALE) level

GALE regulates UDP-glucose/UDP-galactose and UDP-GlcNAc/UDP-GalNAc balance, and is a key enzyme in galactose metabolism in the Leloir pathway. To test whether changes in this balance may be involved in high-glucose regulation of Siglec-9 ligands, GALE levels were tested in response to glucose exposure of human vascular endothelial cells. High glucose exposure reduced the expression of GALE significantly, while ox-LDL and LPS did not (Fig. 4A). Down-regulation of GALE expression with siRNA further increased high glucose-induced Siglec-9 ligands, while up-regulation of GALE with CRISPR activation plasmid largely reversed the high-glucose mediated Siglec-9 ligand increases (Fig. 4B). A prior study showed that increased Siglec-9 ligands

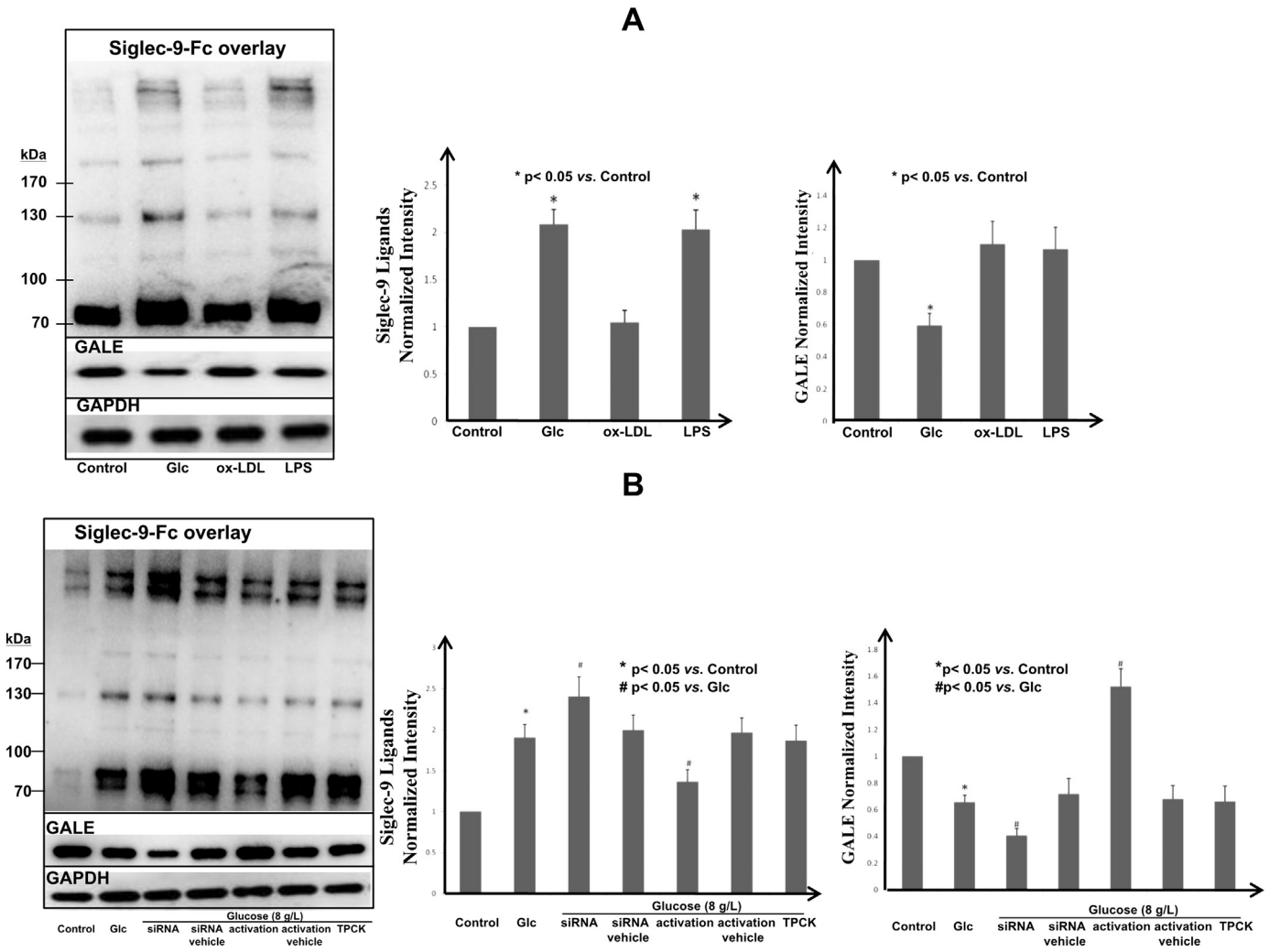

Fig. 4. GALE is involved in the alteration of Siglec-9 ligands on HUV-EC-C cells. High glucose (8 g/L) decreased the expression of GALE protein, while LPS did not (A) (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$). In the presence of high glucose, GALE reduction via siRNA further increased Siglec-9 ligands, while CRISPR activation plasmid of GALE attenuated the effects of high glucose. Treatment with TPCK did not affect the expression of Siglec-9 ligands (B) (mean ± SEM; *, $p < 0.05$ vs Control; #, $p < 0.05$ vs Glc, $n = 6$). In the absence of high glucose, treatment with GALE siRNA up-regulated Siglec-9 ligands on HUV-EC-C cells (C) (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$), whereas GALE activation had no effect on Siglec-9 ligands (C). In the presence of high glucose, galactose (2 g/L) and GalNAc (2 g/L) partially reversed the effect of GALE activation plasmid without altering GALE expression (D) (mean ± SEM; *, $p < 0.05$ vs Control; #, $p < 0.05$ vs Glc; &, $p < 0.05$ vs activation, $n = 6$). Cellular GalNAc levels in HUV-EC-C cells were determined by a FACE. High glucose (but not mannose) significantly increased cellular GalNAc, GlcNAc and GalNAc/GlcNAc ratio, and the activation of GALE expression partially reversed the glucose-induced increase of GalNAc and GalNAc/GlcNAc ratio, but not GlcNAc (E). (mean ± SEM; *, $p < 0.05$ vs Control; #, $p < 0.05$ vs Glc, $n = 6$).

in Calu-3 (human airway submucosal) cells treated with LPS occurred via NF-κB activation. However, in HUV-EC-C cells treated with high glucose neither Siglec-9 ligands nor GALE expression were altered by TPCK, an inhibitor of the NF-κB pathway (Fig. 4B). In the absence of high glucose, GALE siRNA reduced GALE expression and increased the expression of Siglec-9 ligands on HUV-EC-C cells, whereas there was no significant effect when GALE expression was increased using a CRISPR activation plasmid (Fig. 4C). In the presence of high glucose, addition of galactose or GalNAc to the culture medium reversed the effect of GALE activation plasmid on the expression of Siglec-9 ligands without reducing GALE expression (Fig. 4D). The cellular GlcNAc and GalNAc levels were significantly elevated by growth in high glucose (but not mannose), and activation of GALE expression partially reversed the increase of GalNAc, but not GlcNAc. The activation of GALE expression decreased GalNAc/GlcNAc ratio significantly, compared with Glc group (Fig. 4E).

3.5. High glucose-mediated reduction of GALE expression is at the transcriptional level

The mRNA level of GALE was significantly decreased by growth in high glucose, whereas there was no significant difference of GALE mRNA levels due to treatment with LPS or ox-LDL (Fig. 5A). The alterations of GALE and Siglec-9 ligands caused by growth in high glucose were not reversed by Decitabine (DNA methyltransferase inhibitor), Thiamet G (UDP-GlcNAc: polypeptide *O*-ß-*N*-acetylglucosaminyl-transferase (OGT) inhibitor) or TPCK (inhibitor of NF-κB) (Fig. 5B). There was no significant difference of GALE DNA methylation levels among the groups treated with different factors (Fig. 5C).

To test post-transcriptional regulation, the program TargetScan (http://targetscan.org) was used to search for potential microRNA regulators, which revealed the hsa-let-7 miRNA family (data not shown). Among these, miRNA hsa-let-7f was significantly increased with treatment of high glucose, compared with control group and other hsa-let-7 miRNA members (Fig. 5D). The inhibitor of hsa-let-7f reversed the decrease of GALE expression caused by high glucose, as well as the

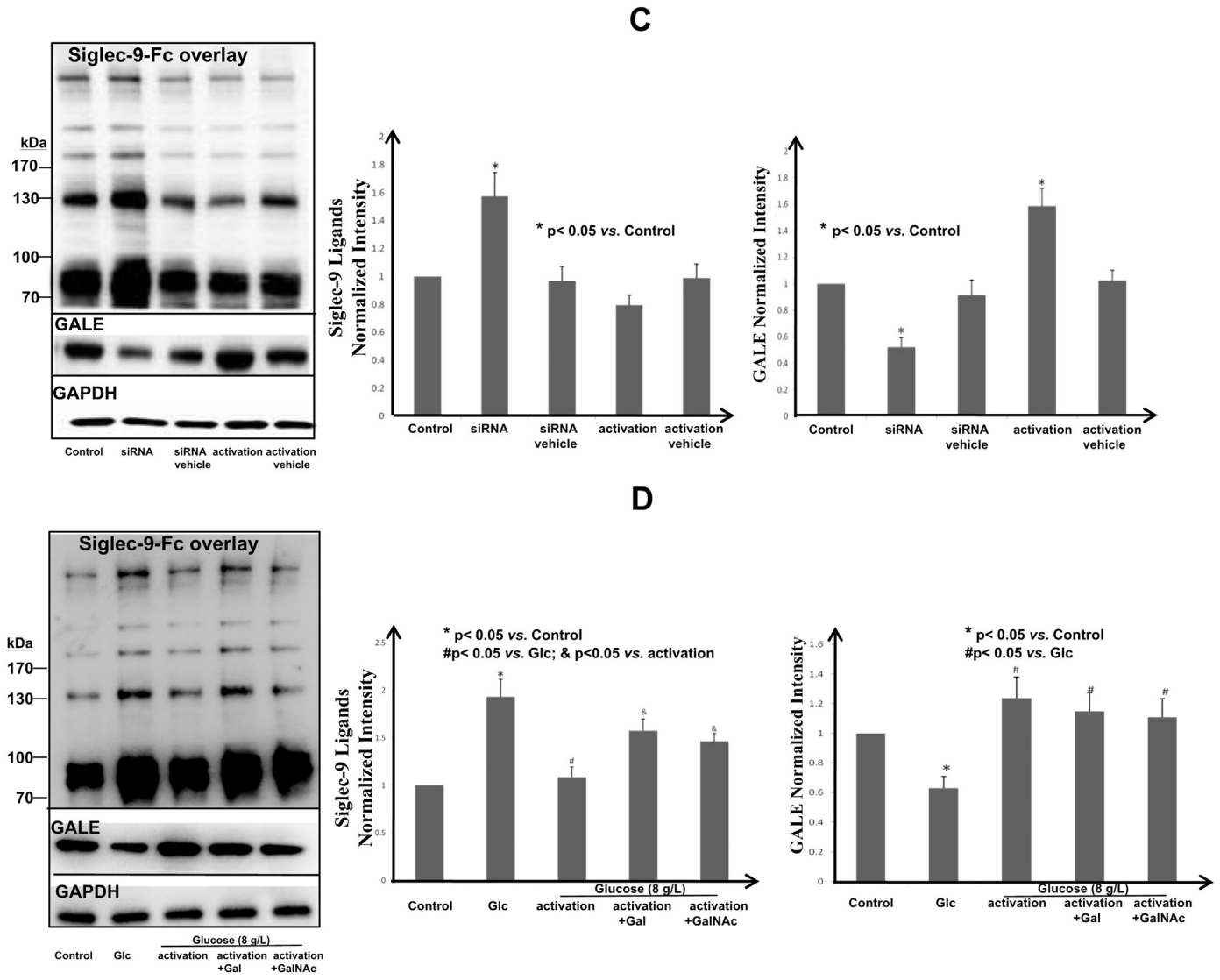

Fig. 4. (*continued*)

increase of Siglec-9 ligands (Fig. 5E). In cells without treatment of high glucose, the mimic or inhibitor of hsa-let-7f could decrease or increase the expression of GALE, respectively (Fig. 5F), with opposite changes in Siglec-9-Fc ligands.

4. Discussion

Inflammation is a driving force in development of atherosclerosis, a disease for which type 2 diabetes is a significant risk factor [10]. Based on the pathophysiological relation between high glucose and angiopathy [11], we studied the role of vascular glycan ligands for immune regulatory siglecs. Expression of specific glycans and their engagement with siglecs on incoming activated inflammatory cells may contribute to proper immune function or immune dysfunction [12]. Although Siglec-1 was considered to be associated with diabetes [13], whether high glucose contributes to Siglec-9 immune regulatory system has not been well studied. A longer term goal of such studies is to direct the use of synthetic molecules that mimic siglec ligands to therapeutically modulate inflammation [14].

Different siglecs are expressed on overlapping sets of immune cells. Among these, Siglec-9 is expressed on human neutrophils and macrophages, and carries an intracellular ITIM, implicating it in the control of neutrophil- and macrophage-driven inflammation. Evidence supporting this hypothesis comes from studies in mice. Although mice lack Siglec-9, mice engineered to lack a Siglec-9 homolog (Siglec-E) have exacerbated inflammation in inflammatory disease models [15]. Relevant to the current study, our previous study on inflamed human airways in Chronic Rhinosinusitis patient nasal polyp extracts as sample and human airway cells revealed increased expression of Siglec-9 ligands. The specific nature of siglec ligands on target tissues, control of their expression in response to inflammation, and use of that knowledge to therapeutically intervene are areas of active exploration.

We report here that the distribution of Siglec-9 ligands on human and mouse aorta are ubiquitous, with prominent ligand expression on endothelial cells and smooth muscle cells (among others). Extracted glycoproteins resolved by polyacrylamide gel electrophoresis indicate ligands in the molecular range ~90–300 kDa. Siglec-9-Fc binding to ligands on tissues was sharply reduced and on electrophoretic blots was completely lost upon treatment with sialidase, indicating that the ligands under consideration are sialoglycoproteins. Prior studies using human airway and airway cells identified a very large (4 MDa) *O*-linked sialoglycoprotein, MUC5B as an airway ligand for Siglec-9. Whereas Siglec-9 ligands in aorta are also *O*-linked sialoglycoproteins (Fig. 1), they all resolved on 4–12% polyacrylamide gels, indicating significantly lower molecular weights (≤300 kD). This suggests that, consistent with other functional glycan binding proteins, similar glycan ligands are

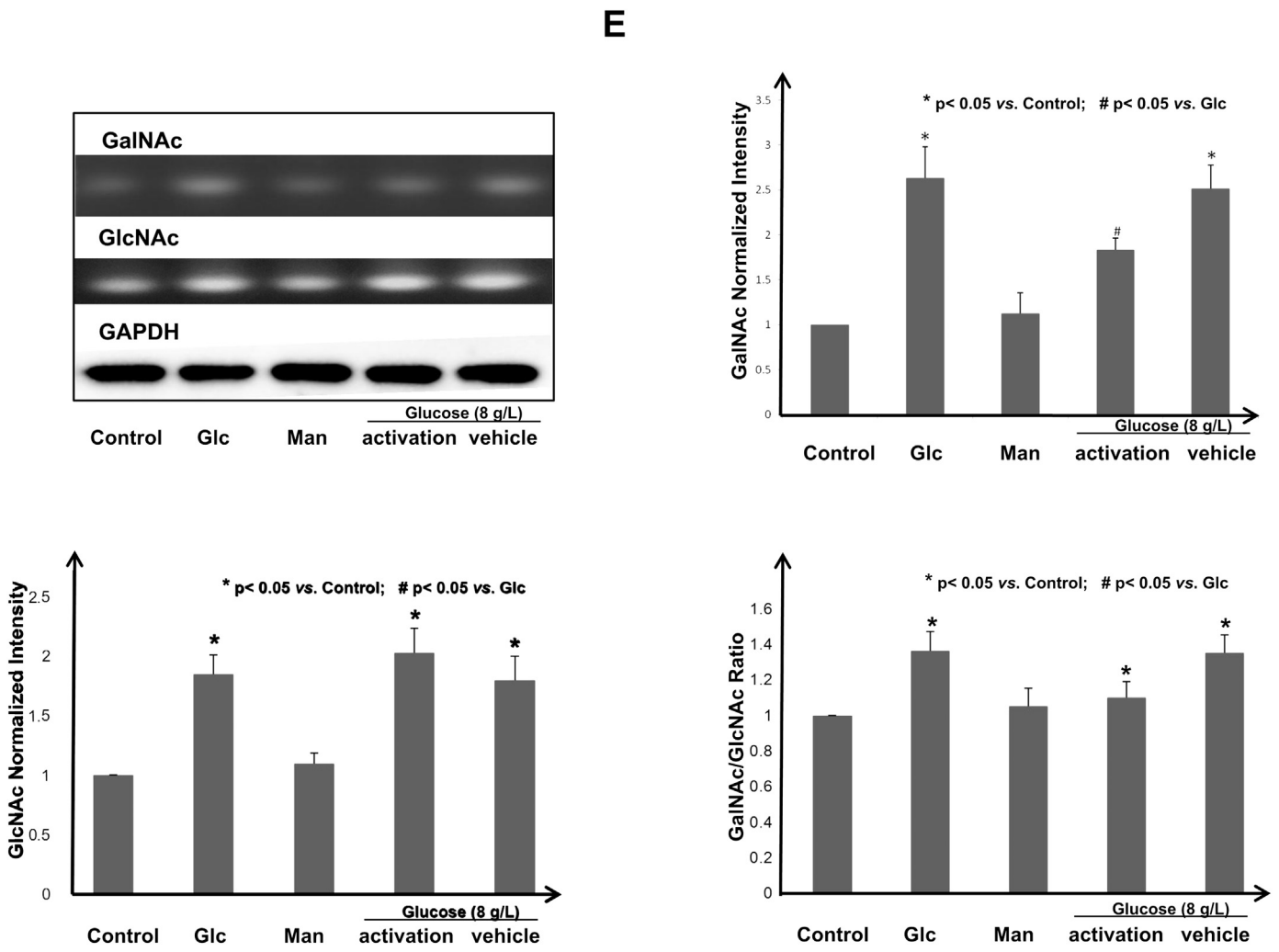

Fig. 4. (*continued*)

expressed on different carriers in different tissues [16–18]. Notably, HUV-EC-C cells expressed Siglec-9 ligands with similar electrophoretic migration pattern and molecular weight bands as on human aorta. Although the carrier proteins and Siglec-9-binding *O*-linked glycans have yet to be identified, regulation of Siglec-9 ligands in response to well-established inflammatory conditions – notably exposure to high glucose and LPS – was determined.

Both high glucose exposure and LPS (but not ox-LDL) up-regulated the expression of Siglec-9 ligands in what may be a general tissue regulatory response to inflammatory stimuli, consistent with findings of inflamed human airways and airway cells. Notably, the data indicate that the signaling mechanisms upstream of the control of Siglec-9 ligand expression varied between exposure to high glucose and LPS. LPS acts via TLR4 and the NF-κB, whereas the effects of high glucose were not altered by inhibiting the NF-κB pathway. Instead, exposure to high glucose, but not LPS, resulted in GALE down-regulation that was on the pathway to increased Siglec-9 ligand expression. In support of this conclusion, direct inhibition of GALE expression by siRNA up-regulated the level of Siglec-9 ligands and over-expression of GALE reversed the effect of high glucose. The finding that cellular GalNAc was negatively associated with GALE expression and positively associated with Siglec-9 ligands indicate that GALE regulation of relative Glc/Gal and/or GlcNAc/GalNAc concentrations has a marked effect on expression of Siglec-9 binding *O*-linked glycans. In light of these data, whether human diseases in the Leloir pathway of galactose/GalNAc metabolism have altered immune [19–21] or inflammatory [22] responses due to changes in siglec ligands is worthy of examination. Zhu's study also revealed a role of hepatic GALE activity in diabetes via whole-body glucose homeostasis [23]. Furthermore, we found that high glucose exposure per se resulted in transcriptional down-regulation of GALE, which may have pleiotropic effects well beyond Siglec-9 ligand expression. To explore the possible of transcriptional change of GALE, we detected the DNA methylation level of GALE gene, because of the well known roles of DNA methylation, a common type of epigenetics, on vascular pathological changes in diabetes [24,25], especially in metabolic memory [26]. But our results did not reveal associations between the change of GALE mRNA levels and GALE gene DNA methylation. Then we analyzed the miRNA levels, and found the hsa-let-7f miRNA was significantly increased by high glucose, which consistent with previous clinical study [27]. Further experiments with the mimic and inhibitor of hsa-let-7f supported the role of hsa-let-7f on the transcriptional regulation of GALE. Therefore, the results indicated that high glucose could down-regulate the expression of GALE via the increase of hsa-let-7f, resulting in up-regulation the expression of Siglec-9 ligands.

Consistent with an increase in Siglec-9 ligands [28,29], endothelial cells treated with high glucose increased macrophage apoptosis. Although HUV-EC-C treated with high glucose could decrease macrophage phagocytosis, the alteration was not as noticeable as apoptosis. The reason might be that the major biological effects of the activation of Siglec-9 was inducing apoptosis, and the survival macrophages still contained some activity of phagocytosis. Whether this change is an

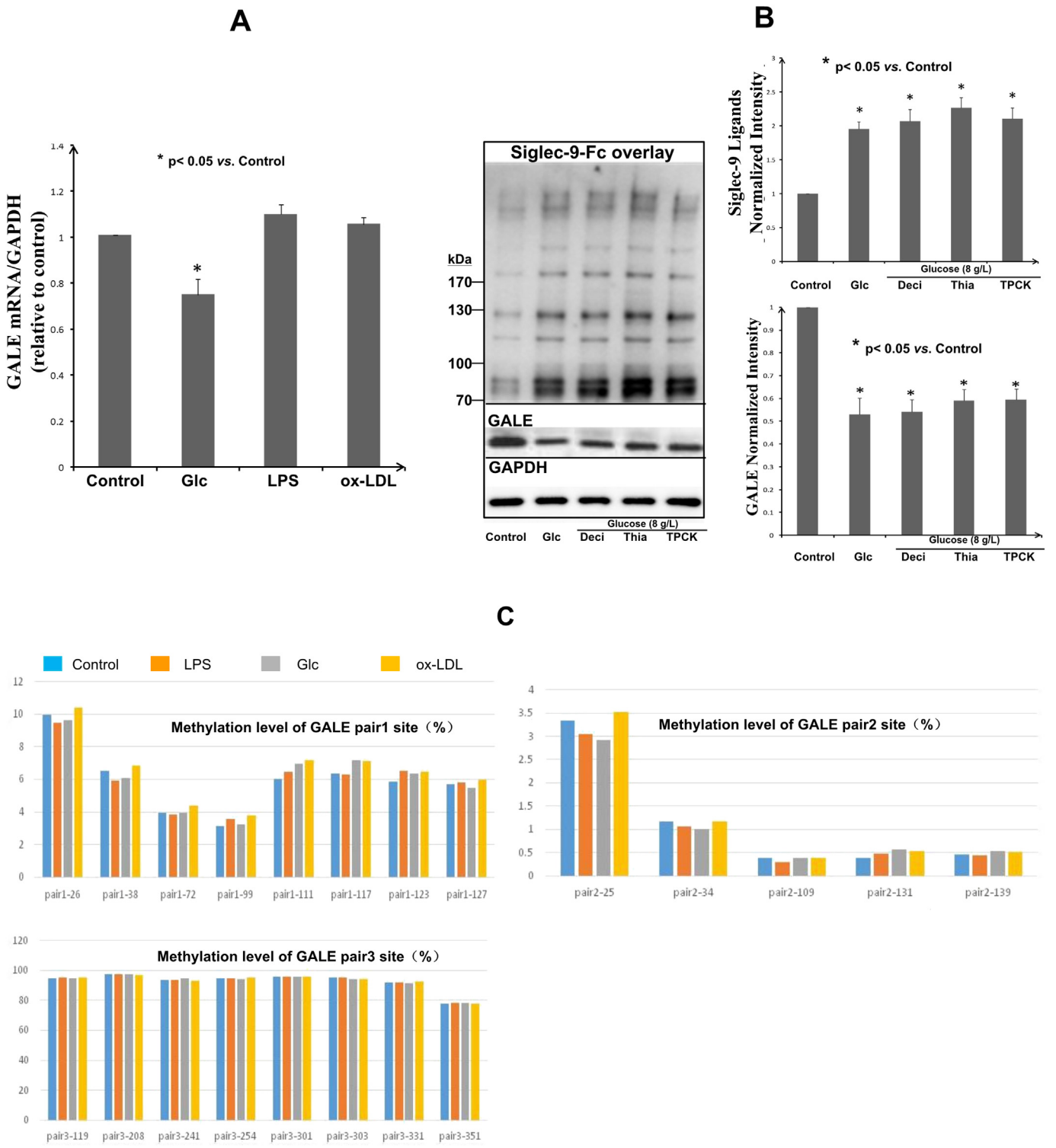

Fig. 5. Alteration of GALE expression caused by high glucose is associated with hsa-let-7f miRNA. Growth in high glucose (8 g/L) significantly decreased the mRNA level of GALE (A) (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$). Concurrent treatments with Decitabine (Deci, 10 μM), Thiamet G (Thia, 20 μM) or TPCK (20 μM) did not alter the effects of high glucose (B) (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$). There was no significant difference of the GALE gene DNA methylation levels among the groups (C (mean ± SEM; $n = 6$)). The level of hsa-let-7f was increased by the treatment of high glucose (D) (mean ± SEM; *, $p < 0.05$ vs Control, $n = 6$). The inhibitor of hsa-let-7f reversed the decrease of GALE expression caused by high glucose, as well as the increase of Siglec-9 ligands (E) (mean ± SEM; *, $p < 0.05$ vs Control; #, $p < 0.05$ vs Glc, $n = 6$). In cells without treatment of high glucose, a mimic or inhibitor of hsa-let-7f decreased or increased the expression of GALE, respectively (F) (mean ± SEM; *, $p < 0.05$ vs Control; #, $p < 0.05$ vs Glc, $n = 6$).

incomplete protective response to inflammation or itself contributes to angiopathy has yet to be determined. Normally, the decrease of macrophage activity can attenuate the inflammatory reaction in aorta. On the other hand, the reduction of macrophage phagocytosis may be associated with the lower clearance of some substance, such as ox-LDL and AGE (advanced glycation end-product) [30], which may promote the formation of atherosclerosis. The overall role of the alteration of Siglec-9 ligands might be dependent on the specific stages of

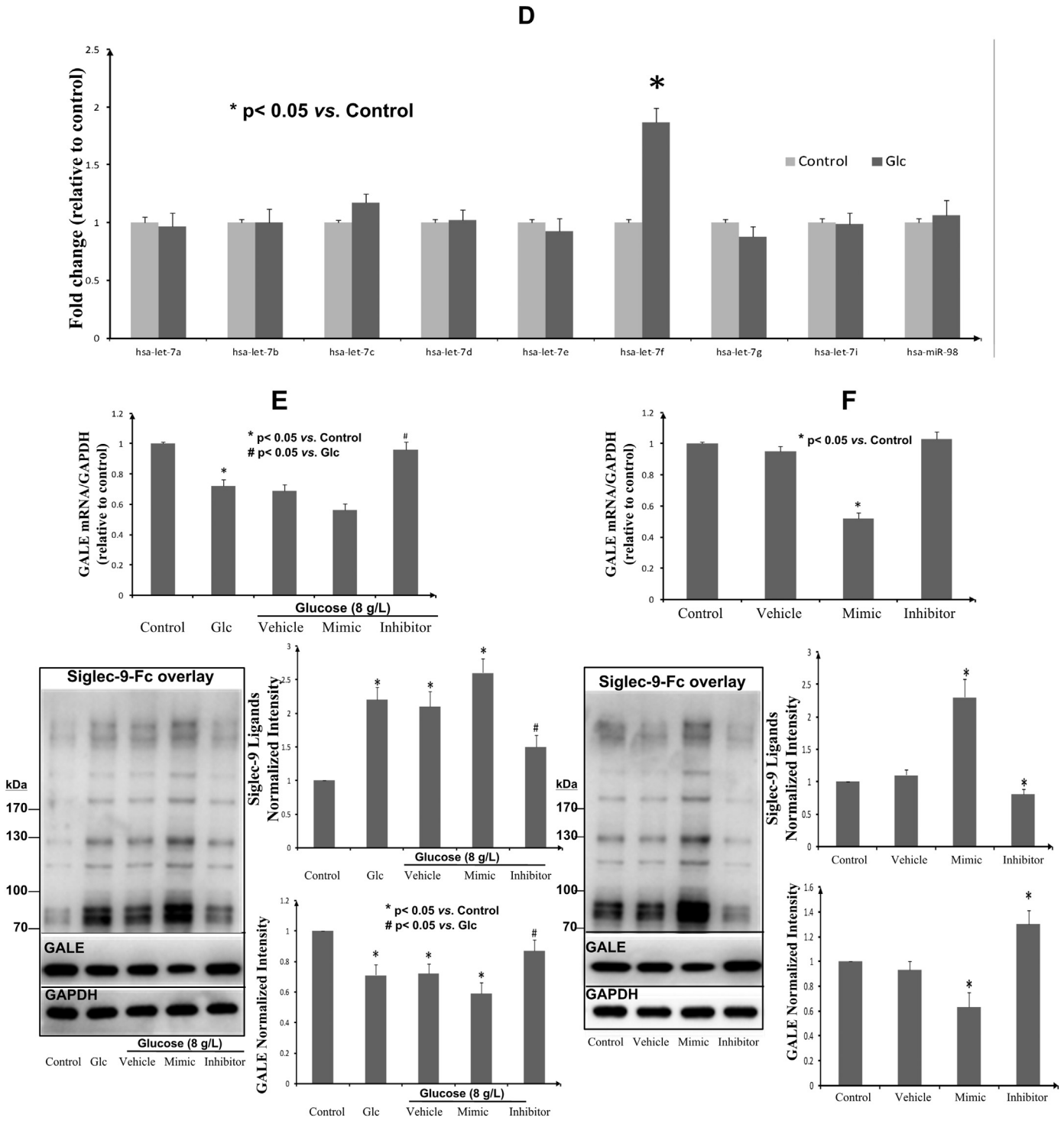

Fig. 5. (*continued*)

atherogenesis.

Our results indicate that, in addition to its other metabolic effects [31,32], high glucose disturbs the metabolism of endothelial galactose/GalNAc, increases expression of Siglec-9 ligands, and alters endothelial cell-cell interactions with inflammatory cells [33]. Whether this previously unanticipated downstream effect of diabetes contributes to formation of atherosclerosis and other vasculopathies is as yet unclear. The potential to better understand the relationship between regulation of inflammation and atherosclerosis, and potentially target siglecs therapeutically as modulators of this disease, requires additional exploration.

Conflict of interest

The authors have declared that no competing interests exist.

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Author contributions

Yi Jia, Ronald Schnaar and Xiaohui Li designed the experiments and wrote the manuscript; Yingxian Zhang performed the cell experiments and miRNA analysis; Yu Zheng performed animal experiment and immunoblotting; Jin Li performed Fluorophore-assisted carbohydrate electrophoresis and tissue histochemistry; Ling Nie performed tissue section and mRNA analysis; Yijie Hu and Qianjin Zhong performed clinical samples collection; Fangjie Wang performed flow cytometry; Hongmei Liu performed siRNA and CRISPR Activation Plasmid transfection; Steve M. Fernandes provided Siglec-9-Fc.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2018.11.049.

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