N-acetylglucosaminyltransferase V negatively regulates integrin α5β1-mediated monocyte adhesion and transmigration through vascular endothelium

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Abstract. Changes in the expression of glycosyltransferases that branch N-linked glycans are associated with many physiological and pathological events, such as cell adhesion, migration, proliferation and tumor cell malignancy. Here, the altered levels of N-acetylglucosaminyltransferase V (GnT-V) and its product β(1,6)-linked GlcNAc in monocytes were observed during inflammation. The effects of GnT-V and aberrant N-linked β(1,6) branching on monocyte adhesion through vascular endothelium and transmigration were investigated. During IFN-γ-induced inflammation, adhesion and transendothelial migration of THP-1 monocytes were enhanced, and the levels of GnT-V and β(1,6)-linked GlcNAc in THP-1 monocytes were significantly decreased. Expression of the GnT-V shRNA vector in THP-1 cells reversed the abnormal IFN-γ-induced characteristics, indicating direct involvement of N-glycosylation in these biological effects. The enhanced adhesion and transendothelial migration were significantly inhibited by functional blockade with antibodies against integrin α5 or β1 in IFN-γ-induced and GnT-V knockdown THP-1 cells, demonstrating the involvement of integrin α5β1 in the monocyte-endothelium interaction. However, IFN-γ treatment and GnT-V knockdown in THP-1 cells lowered expression of N-linked β(1,6) branching on integrin α5 and β1, without affecting the total protein expression of the subunits. Decreased GnT-V expression caused marked enchancement of integrin-induced phosphorylation of focal adhesion kinase (FAK). The augmented FAK-mediated ERK phosphorylation and activation were observed in IFN-γ-induced THP-1 cells. Furthermore, ERK inhibitor pre-treatment nearly abrogated the highly elevated IFN-γ-induced monocyte adhesion and transmigration, concomitant with reversal of the decrease in GnT-V and β(1,6) branching. Our results demonstrate for the first time that decreased GnT-V activity due to inflammatory cytokine induction in human monocytes resulted in enchancement of integrin α5β1-dependent monocyte-vascular endothelium adhesion and transmigration. Consequently, the activation of integrin caused elevation of FAK phosphorylation. These effects promoted FAK-mediated downstream signaling, including the ERK pathway, and indicate that GnT-V may be a potential therapeutic target for vascular inflammatory conditions.

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Introduction

Recruitment of monocytes from the blood circulation to inflamed tissues is critical to the initiation and subsequent progression of inflammatory reactions (1,2). Migration of monocytes from the blood stream to inflamed tissues must pass through the vascular endothelium, which provides a physical barrier to pathogens, peripheral blood leukocytes and tumor cells (3). Monocyte diapedesis depends on multi-step events that are sequentially regulated by numerous adhesion molecules and signaling pathways (4,5). The initial rolling of monocytes along the endothelium is mediated by E- and P-selectins and results in reversible adherence to the endothelial cell monolayers (6). The firm adhesion is mainly mediated by integrins. Subsequently, monocytes spread out on the endothelial surface and transmigrate to the inflamed tissues (7). Past studies have suggested that leukocyte diapedesis is associated with increased expression of adhesion molecules and a variety of chemotactic factors (8,9).

Integrins are α, β heterodimeric adhesion receptors (10). In eukaryotes, there are 18α and 8β subunits assembled into 24 integrins (11). The integrin family is involved in a variety of physiological and pathological events, including cell adhesion, cell migration, activation of intracellular signaling pathways and regulation of cytoskeletal formation (12-14). Integrins are also involved in inside-out signaling, in which intracellular signals received by integrins or other receptors activate their extracellular domains and subsequently enhance assembly of

the extracellular matrix (ECM) (15). Integrin α5β1 is known as a fibronectin (FN) receptor. The interaction between integrin α5β1 and FN is essential for cell-ECM adhesion, cell migration and viability (16). Integrin α5β1 contains 14 and 12 putative N-glycosylation sites on the α5 and β1 subunits, respectively. An increasing body of evidence suggests that the presence of these N-glycans on integrin α5β1 is required for α, β heterodimer formation and proper integrin-matrix interactions (17,18). It has been shown that treatment of purified integrin α5β1 with N-glycosidase F, which cleaves N-linked glycoproteins between the innermost N-acetylglucosamine (GlcNAc) and asparagine N-glycan residues, blocks α5β1 binding to FN and prevents the inherent association between subunits (19).

The production of glycoprotein glycans is catalyzed by various glycosyltransferases. N-acetylglucosaminyltransferase III (GnT-III) catalyzes the addition of β1,4-linked GlcNAc to mannose, producing a unique sugar chain structure-bisecting GlcNAc. N-acetylglucosaminyltransferase V (GnT-V) catalyzes the formation of β1,6 GlcNAc branching structures. Increased expression of bisecting GlcNAc on integrin α5 by overexpression of GnT-III results in attenuated ligand binding and downregulation of cell adhesion and migration (20,21). Contrary to the functions of GnT-III, increased branched sugar chains, catalyzed by overexpression of GnT-V, inhibit the clustering of integrin α5β1 and reduce cell adhesion to FN but promote tumor cell migration and invasion (22,23). These observations clearly support that alteration of the N-glycan structure affects the biological functions of integrin α5β1.

Numerous studies have suggested that β(1,6)-GlcNAc branching, synthesized by GnT-V, is associated with malignant transformation, including tumor invasion and metastasis (24-26). Aberrant N-glycosylation on several types of cell surface receptors, including integrins and cadherins, is associated with altered cell phenotypes, cellular behavior and dysfunctional intracellular signaling (27-29). During inflammation, leukocyte diapedesis is mediated by various cell surface receptors and intracellular cytoskeletal protein (30). Integrin α5β1 is an N-glycosylated (13) cell surface receptor involved in the process of monocyte diapedesis (7). However, the relationship between aberrant N-glycosylation on integrin α5β1 and monocyte diapedesis during inflammation remains unclear. In the present study, we show for the first time that during inflammation, GnT-V expression was downregulated, and the decreased aberrant N-glycosylation on integrin α5β1 resulted in enhancement of monocyte adhesion and transmigration through the vascular endothelium.

Materials and methods

Cell lines and materials. THP-1 cells were obtained from the American Type Culture Collection (ATCC). EA.hy926 cells were a gift from the Laboratory of Internal Medicine of the First Hospital, West China University of Medical Sciences (Chengdu, China). RPMI-1640 medium and fetal bovine serum (FBS) were supplied by Gibco (Grand Island, NY, USA). Recombinant human IFN-γ was purchased from PeproTech, Inc. (Rocky Hill, NJ, USA). Takara Taq™ and PrimeScript® RT reagent Kit (Perfect Real Time) were purchased from Takara Biotechnology (Dalian) Co. Ltd (Dalian, China). Lipofectamine™ 2000 reagent was from Invitrogen (Carlsbad, CA, USA). Rabbit anti-integrin α5, mouse anti-integrin β1, mouse anti-ERK1/2 and Super Signal-enhanced chemiluminescence (ECL) reagents were from Millipore Corporation (Billerica, MA, USA). Goat anti-phospho-ERK1/2 (p-ERK1/2), rabbit anti-p-PI3K, rabbit anti-PI3K, rabbit anti-p-AKT and rabbit anti-AKT were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit anti-β-actin, rabbit anti-p-FAK, rabbit anti-FAK and RIPA buffer were from Cell Signaling Technology (Danvers, MA, USA). Rabbit anti-mgat5 (GnT-V) was obtained from Abcam (Cambridge, MA, USA). Biotinylated L4-PHA was from Vector Laboratories, Inc. (Burlingame, CA, USA). Peroxidase-conjugated AffiniPure goat anti-rabbit IgG (H+L), rabbit anti-goat IgG (H+L), goat anti-mouse IgG (H+L) and horseradish peroxidase streptavidin were purchased from Zhongshan GoldenBridge Biotechnology Co. Ltd (Beijing, China).

Cell culture and transfection. Vascular endothelial EA.hy926 cells were grown in RPMI-1640 medium containing 10% FBS, 2% HAT (final concentrations of hypoxanthine, aminopterin and thymidine were 100 μM, 0.4 μM and 16 μM, respectively), 50 U/ml penicillin, 50 μg/ml streptomycin and 2 mM/l-glutamine at 37˚C under 5% CO_2 and 95% air. THP-1 monocytes were grown at 37˚C in 5% CO_2 in RPMI-1640 containing 20% FBS and 2 mM/l-glutamine. Cell transfections were performed with Lipofectamine™ 2000 according to the manufacturer's instructions using 4 μg or 8 μg of recombinant plasmids expressing negative control RNAs or GnT-V small hairpin RNAs (shRNAs) in a 6-well plate or 50 ml flask. Cells were incubated for 48 or 72 h after transfection and used for the indicated experiments.

Small hairpin RNA. Human GnT-V (NM_002410.3) specific oligonucleotides, containing two complementary 19-nucleotide sequences corresponding to positions 1864-1882 downstream of the transcription start site (GCCACATGTGGACTGTTGA) and separated by a 7-nucleotide non-complementary spacer (TCAAGAG), were chosen for targeted suppression of GnT-V. A negative control shRNA vector expressing an oligonucleotide containing a 20-nucleotide sequence not targeting GnT-V and separated by a 9-nucleotide non-complementary spacer (CAAGAGATT) from the reverse complement of the same 20-nucleotide sequence was used. The sequences of all vectors targeting GnT-V are presented in Table I.

Quantitative real-time reverse transcription-PCR (qRT-PCR) analysis. RNAiso Plus (Takara) was used to isolate total RNA. Reverse transcription reactions were performed using the PrimeScript RT reagent Kit Perfect Real Time (Takara). Primers used in the qRT-PCR analysis were as follows: human GnT-V forward (5'-GCTCCCCTGGAAGCTATCG-3') and reverse (5'-GCTTTTGGGTGGGTTGAACTT-3'); human integrin α5 forward (5'-GCACCAACAAGAGAGCCAAAGT-3') and reverse (5'-GCTCCCGCTGCAAGAAAGT-3'); human integrin β1 forward (5'-GTGCCGTAACAACTGTGGTC AA-3') and reverse (5'-ATTCAGTGTTGTGGGATTTGCA-3'). Real-time reactions were performed using the iQ™ SYBR-Green Supermix (Bio-Rad, Hercules, CA) under the following conditions: 3 min at 95˚C for 1 cycle, 10 sec at 95˚C, 30 sec at 60˚C for 39 cycles, 95˚C for 5 sec.

Table I. I shRNA vectors targeting GnT-V.

Name of carrier	shRNA Template sequence	Target sequence
pGPH1/GFP/Neo-shNC	S 5'-CACCGTTCTCCGAACGTGTCACGTCAAGAGATTACGTGACACGTTCGGAGAATTTTTTG-3' A 5'-GATCCAAAAAAATTCTCCGAACGTGTCAVGTAATCTCTTGACGTGACACGTTCGGAGAAC-3'	GTTCTCCGAACGTGTCACGT
HI/GFP/Neo-MGAT5-homo-165	S 5'-CACCGGAAGTTGTCCTCTCAGAAGCTTCAAGAGAGCTTCTGAGAGGACAACTTCCTTTTTTG-3' A 5'-GATCCAAAAAAGGAAGTTGTCCTCTCAGAAGCTCTCTTGAAGCTTCTGAGAGGACAACTTCC-3'	GGAAGTTGTCCTCTCAGAAGC
HI/GFP/Neo-MGAT5-homo-365	S 5'-CACCGCTGGAGTCATGACAGCTTATTTCAAGAGAATAAGCTGTCATGACTCCAGCTTTTTTG-3' A 5'-CATCCAAAAAAGCTGGAGTCATGACAGCTTATTCTCTTGAAATAAGCTGTCATGACTCCAGC-3'	GCTGGAGTCATGACAGCTTAT
HI/GFP/Neo-MGAT5-homo-1245	S 5'-CACCGGGTTCATTACCAGTGCATGCTTCAAGAGAGCATGCACTGGTAATGAACCCTTTTTTG-3' A 5'-GATCCAAAAAAGGGTTCATTACCAGTGCATGCTCTCTTGAAGCATGCACTGGTAATGAACCC-3'	GGGTTCATTACCAGTGCATGC
HI/GFP/Neo-MGAT5-homo-1864	S 5'-CACCGCCACATGTGTGGACTGTTGATTCAAGAGATCAACAGTCCACACATGTGGCTTTTTTG-3' A 5'-GATCCAAAAAAGCCACATGTGTGGACTGTTGATCTCTTGAATCAACAGTCCACACATGTGGC-3'	GCCACATGTGTGGACTGTTGA

Cell adhesion assay. The monocyte adhesion assay was performed as previously described (49). In brief, EA.hy926 cells were seeded in 96-well culture plates at $3x10^4$ cells/well for 48 h before the experiments. THP-1 monocytes were pre-treated with or without the ERK inhibitor (PD98059, 25 μM) for 1 h and then treated with or without specified concentration(s) of IFN-γ for the specified time. The cells were then labeled with the fluorescent dye BCECF-AM at 10 μM final concentration in RPMI-1640 medium containing 1% FBS at 37˚C for 1 h, after which the labeled cells were washed three times with PBS and resuspended in medium. The labeled cells were added to EA.hy926 cells in 96-well culture plates at $3x10^4$ cells/well and incubated at 37˚C for 1 h. After the co-incubation, the suspension cells were removed and the adherent cells were gently washed three times with PBS. Fluorescent images were obtained using a Leica DMRX microscope (Wetzlar, Germany), and the fluorescence intensity of each sample was measured with the Image-Pro Plus 6.0 software (Media Cybernetics, Bethesda, MA, USA). Data are expressed as the mean values of cells in five fields based on three independent experiments.

Transendothelial migration. Motility assays were performed using 24-well Transwell units with 8-μm pore size polycarbonate inserts. Prior to the migration assay, EA.hy926 cell monolayers ($3x10^4$ cells/per well) were cultured on the upper compartment of the Transwell units. THP-1 cells ($10x10^4$/per well), pre-treated with or without the ERK inhibitor (PD98059, 25 μM) for 1 h, were exposed to specified concentrations of IFN-γ for 24 h, suspended in 200 μl RPMI-1640 containing 1% FBS and then added to the upper chamber of the Transwell inserts. RPMI-1640 (600 μl) containing 20% FBS (as a chemotactic factor) was placed in the bottom chamber. Cells were allowed to migrate for 24 h at 37˚C in a humidified atmosphere containing 5% CO_2. THP-1 cells from the top chamber of the Transwell unit that had migrated to the bottom chamber were collected, centrifuged and then incubated in 100 μl BCECF-AM at 10 μM final concentration for 1 h. After removing the dye solution, THP-1 cells were washed three times with PBS and then resuspended in 100 μl PBS in a 96-well plate. Fluorescent images were obtained using a Leica DMRX microscope, and the fluorescent intensity of each sample was measured with the Image-Pro Plus 6.0 software. Data are expressed as the mean values of cells in five fields based on three independent experiments.

Immunoprecipitation, western blot and lectin blot. THP-1 cells were harvested and lysed in 1XRIPA (CST) lysis buffer containing 1 mM PMSF, 5 mM Na fluoride and 1 mM Na orthovanadate protease inhibitors. Proteins from total cell lysates (20-80 μg), measured using the BCA protein assay (Pierce Chemical Co., Rockford, IL), were boiled in 5X SDS-sample buffer for 10 min, electrophoresed on an 8-12% polyacrylamide minigel and then transferred onto a PVDF membrane. After blocking with 5% non-fat milk or BSA in TBS, the membrane was incubated with primary antibodies or biotinylated lectins in TBS buffer containing 0.1% Tween-20 (TBST) overnight at 4˚C. The membrane

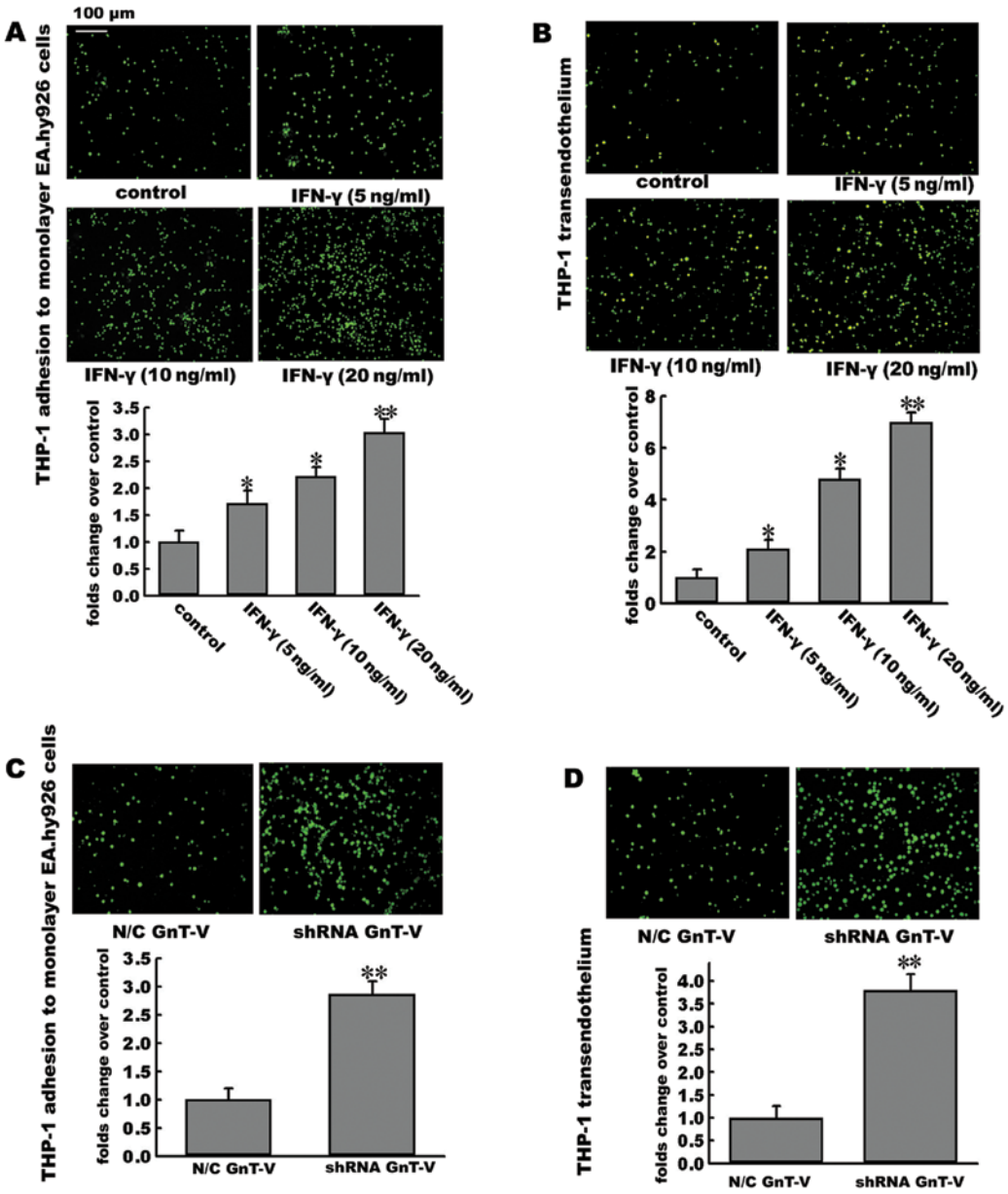

Figure 1. Effects of IFN-γ treatment and GnT-V expression on monocyte-endothelium adherence and transmigration. THP-1 monocytes were treated with IFN-γ at various concentrations (5, 10, 20 ng/ml) for 24 h and then added to a monolayer of EA.hy926 vascular endothelial cells. (A) THP-1 cell adhesion to the EA.hy926 cell monolayer after 1 h in 96 wells. (B) THP-1 cell transmigration through the EA.hy926 cell monolayer for 24 h in Transwells. THP-1 cells were transiently transfected with GnT-V shRNA (targeting the 1864 site) for 72 h and then added to monolayers of EA.hy926 vascular endothelial cells to observe the (C) adhesion and (D) transmigration. *P<0.05, IFN-γ-treated group vs. control group. **P<0.01, IFN-γ-treated group vs. control group or GnT-V shRNA group vs. N/C GnT-V group. Bars, 100 μm.

was washed with TBS and probed with HRP-conjugated anti-rabbit or anti-mouse IgG (1:3000) or HRP-conjugated streptavidin (1:500) for 1 h at room temperature. After the membrane was washed with TBST, protein bands were developed with ECL reagents and exposed to X-ray film. Images of immunoblots and lectin blots were quantitated using a Fluor-S (Bio-Rad) instrument.

Antibody-mediated functional blockade of integrin α5β1. THP-1 cells were concentrated and then pre-incubated with diluted primary antibodies to integrin α5 (10 μg/ml), integrin β1 (10 μg/ml) for 30 min at 37˚C. After the pre-incubation, THP-1 cells were treated with or without IFN-γ before being added to monolayers of vascular endothelial EA.hy926 cells for adhesion and transmigration assays.

Results

IFN-γ-induced inflammation reduces the level of GnT-V and β(1,6) branching of N-linked oligosaccharides (β1,6- GLcNAc) in monocytes. During inflammation, leukocyte diapedesis is increased by the introduction of various inflammatory cytokines and cell surface adhesion molecules (31). IFN-γ is a cytokine that can initiate inflammatory reactions (32). In order to establish the inflammation model, we used IFN-γ to treat THP-1 monocytes and then observed their adhesion to and

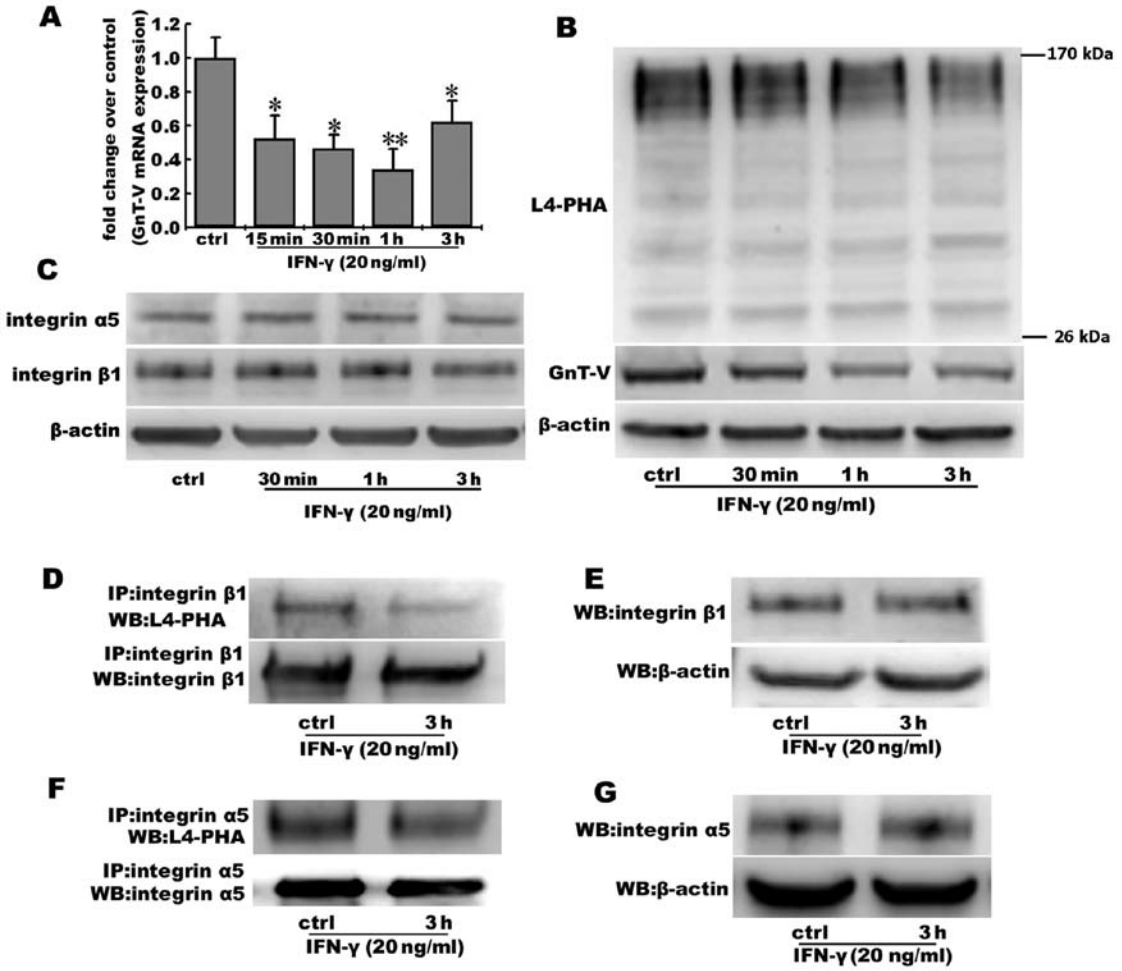

Figure 2. Effects of IFN-γ treatment on the expression of GnT-V, β(1,6)-GlcNAc and integrin α5β1. THP-1 monocytes were treated with 20 ng/ml IFN-γ for 30 min, 1 or 3 h and then lysed for RNA and protein extraction. (A) GnT-V mRNA expression was detected by qRT-PCR. (B) GnT-V and β(1,6)-GlcNAc protein expression in total cell lysates was observed by western blot analysis. Integrin α5β1 expression and β(1,6)-GlcNAc modification on integrin α5β1 were observed by (C,E,G) western blot analysis and (D,F) immunoprecipitation. Data are presented as the means ± SEM (n=3). *P<0.05, **P<0.01, IFN-γ-treated group vs. control group.

transmigration through a monolayer of vascular endothelium EA.hy926 cells as described in the Materials and methods. As shown in Fig. 1A and B, the adhesion and transendothelial migration of IFN-γ-treated THP-1 cells were both enhanced compared to the control. Meanwhile, the effects on adhesion and transmigration of the THP-1 cells through the EA.hy926 cell monolayer were IFN-γ dose-dependent and maximal at the concentration of 20 ng/ml. The results suggest that the model of IFN-γ-induced inflammation was feasible, and that during the inflammation, adherence and transmigration of THP-1 cells to monolayers of vascular endothelial cells were significantly strengthened.

GnT-V is reported to be closely associated with various cell biological phenomena, including cell adhesion and migration (24,27), tumor formation, metastasis and poor prognosis (25,33,34). To determine the expression level of GnT-V in THP-1 monocytes during inflammation, total cell lysates were analyzed by SDS-PAGE and western blotting. L4-PHA lectin blot analysis, which preferentially recognizes the GlcNAc residues on β(1,6)-GlcNAc branches of tri- or tetra-antennary sugar chains, was also performed to detect the activity of GnT-V. The results showed that both the protein levels of GnT-V and N-linked β(1,6)-branched glycans modified on total proteins in THP-1 cells were significantly decreased and maximal after IFN-γ treatment for 3 h (Fig. 2B). Further analysis of IFN-γ-treated THP-1 cells revealed that GnT-V mRNA expression, as detected by qPCR, was decreased (Fig. 2A), consistent with its protein level.

Knockdown of GnT-V by shRNA vectors causes positive alterations in monocyte adhesion and transmigration through vascular endothelium. To examine the involvement of GnT-V and its specific substrate β(1,6)-GlcNAc-branched N-glycans in the adhesion of monocytes to the vasculur endothelium and subsequent transmigration, shRNA vectors targeting GnT-V at various sites were constructed (Table. I) and transiently transfected into THP-1 cells; GnT-V expression was reduced by at least 70% in the group transfected with shRNA directed at site 1864 (Fig. 3A and B), accompanied by a 50% decrease in the levels of its β(1,6)-branched products compared to the mock-transfected group (Fig. 3E). Compared with mock-transfected cells, monocyte adhesion to the EA.hy926 vascular endothelium and subsequent transmigration were significantly increased in the GnT-V knockdown THP-1 cells

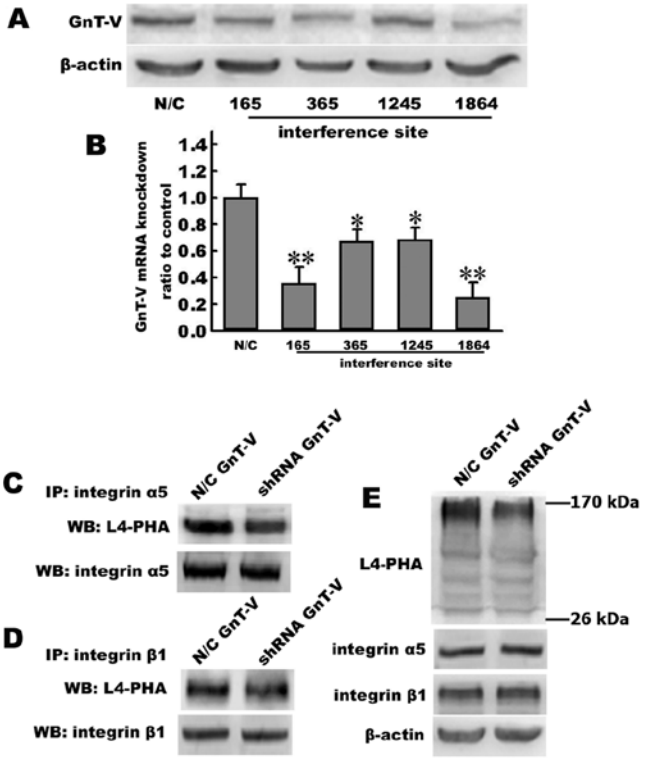

Figure 3. THP-1 monocytes were transiently transfected with shRNA GnT-V vectors. THP-1 cells in the logarithmic phase were collected and transiently transfected with GnT-V shRNA vectors using Lipofectamine™ 2000 for 48 h. The shRNA vectors targeted five interference sites in the GnT-V sequence. Western blot analysis was performed to observe the protein expression of GnT-V (A), β(1,6)-GLcNAc and integrin α5β1 (E), qPCR was used to observe the mRNA expression of GnT-V, and immunoprecipitation was employed for the level of β(1,6)-GLcNAc modification on integrin α5β1 (C,D). Data are presented as the means ± SEM (n=3). *P<0.05, **P<0.01, GnT-V shRNA group vs. N/C GnT-V group.

(Fig. 4C and D). These findings demonstrate that reduced expression levels of GnT-V and N-Linked β(1,6) branching in THP-1 monocytes positively regulated the monocyte-endothelium interaction, which was consistent with our observations in the IFN-γ-induced inflammation model.

Functional blockade of integrin α5β1 inhibits the GnT-V-mediated reduction in monocyte adhesion and transmigration through vascular endothelium. Leukocyte-endothelial cell adhesion is thought to be mediated predominantly by interaction of integrins with their ligands (35). Integrin α5β1 is one of the integrin family members that plays a role in cell adhesion and tumor metastasis (13,36). It has been reported that integrin α5β1 can activate intracellular signaling pathways and cytoskeletal formation (outside-in signaling), as well as its extracellular domain and contribute to the assembly of the ECM through intracellular signals received by integrin or other receptors (inside-out signaling) (12,15).

To investigate the role of integrin α5β1 in the interaction between monocytes and the vascular endothelium, functional blockade of integrin α5β1 by specific antibodies was performed as described in Materials and methods. Blocking of the integrin α5 as well as the β1 subunit severely inhibited adhesion and transmigration of THP-1 cells in the monolayer of EA.hy926 cells, compared to the high levels of the cell behaviors induced by IFN-γ (Fig. 4A and B). Furthermore, antibody-mediated blockade of either the α5 or β1 subunit of integrin basically reversed the enhancement of adhesion and migration by GnT-V knockdown in THP-1 cells (Fig. 4C and D). These results suggest that integrin α5β1 is considerably involved in the GnT-V-mediated reduced adhesion of THP-1 monocytes to the EA.hy926 vascular endothelial cell monolayer and concomitant transmigration.

Reduced expression levels of GnT-V and N-Linked β(1,6) branching enhances tyrosine phosphorylation of focal adhesion kinases (FAK). As the importance of integrin α5β1 in the adhesion and migration of monocytes to vascular endothelium both during inflammation and in GnT-V knockdown experiments was confirmed, it was necessary to determine if total levels of integrin α5β1 were altered in those conditions. In fact, IFN-γ-induced inflammation had no effect on the protein expression of either the α5 or β1 subunit (Fig. 2C). Furthermore, compared to the control group, significantly lower levels of β(1,6)-GlcNAc modified α5 and β1 subunits were pulled down from THP-1 cell lysates in IFN-γ-stimulated THP-1 cell lysates but without change in the total expression of the two subunits (Fig. 2D-G). The same results were observed in GnT-V knockdown THP-1 cells (Fig. 3C-E), demonstrating that reduced expression of GnT-V caused decreased levels of β(1,6)-branched glycans on integrin α5β1.

A growing body of evidence indicates that the overexpression of GnT-V results in the inhibition of integrin α5β1 clustering and concomitant focal adhesion formation (integrin activation) (27). On the other hand, phosphorylation of FAK leads to integrin activation and significantly affects integrin-mediated cell adhesion and cell motility by eliciting focal adhesion formation and modulation of integrin receptor turnover (37,38). In this study, FAK was shown to be significantly activated by phosphorylation at Tyr397 in IFN-γ-stimulated THP-1 cells (Fig. 5A). Taken together these results demonstrate that reduction of GnT-V and its specific product β(1,6)-GlcNAc increase the monocyte-endothelium interaction, not through changing the expression of integrin α5β1, but through FAK Tyr397 phosphorylation-induced activation of integrin α5β1.

Extracellular signal-regulated kinase (ERK) inhibitor protects against the decrease of GnT-V and β(1,6)-GlcNAc during inflammation. FAK, a 125-kDa protein-tyrosine kinase, is an important mediator of signal transduction initiated by integrins in cell adhesion and migration. Recently, FAK was reported to mediate inflammatory responses through the activation of the ERK pathways (39,40), and it is an important component of the PI3K/Akt pathway as well (41). To further understand the mechanism of the effect of GnT-V on the integrin α5β1-mediated interaction between monocytes and vascular endothelia, the FAK signaling pathway was investigated. Activation of FAK by phosphorylation amplified the activity of downstream signaling molecules. ERK phosphorylation was significantly elevated during IFN-γ-induced inflammation, demonstrating involvement of the FAK-ERK pathway (Fig. 5A). However, the FAK-PI3K/Akt pathway was not activated, as no change in the levels of phosphorylated PI3K and Akt was observed (Fig. 5B).

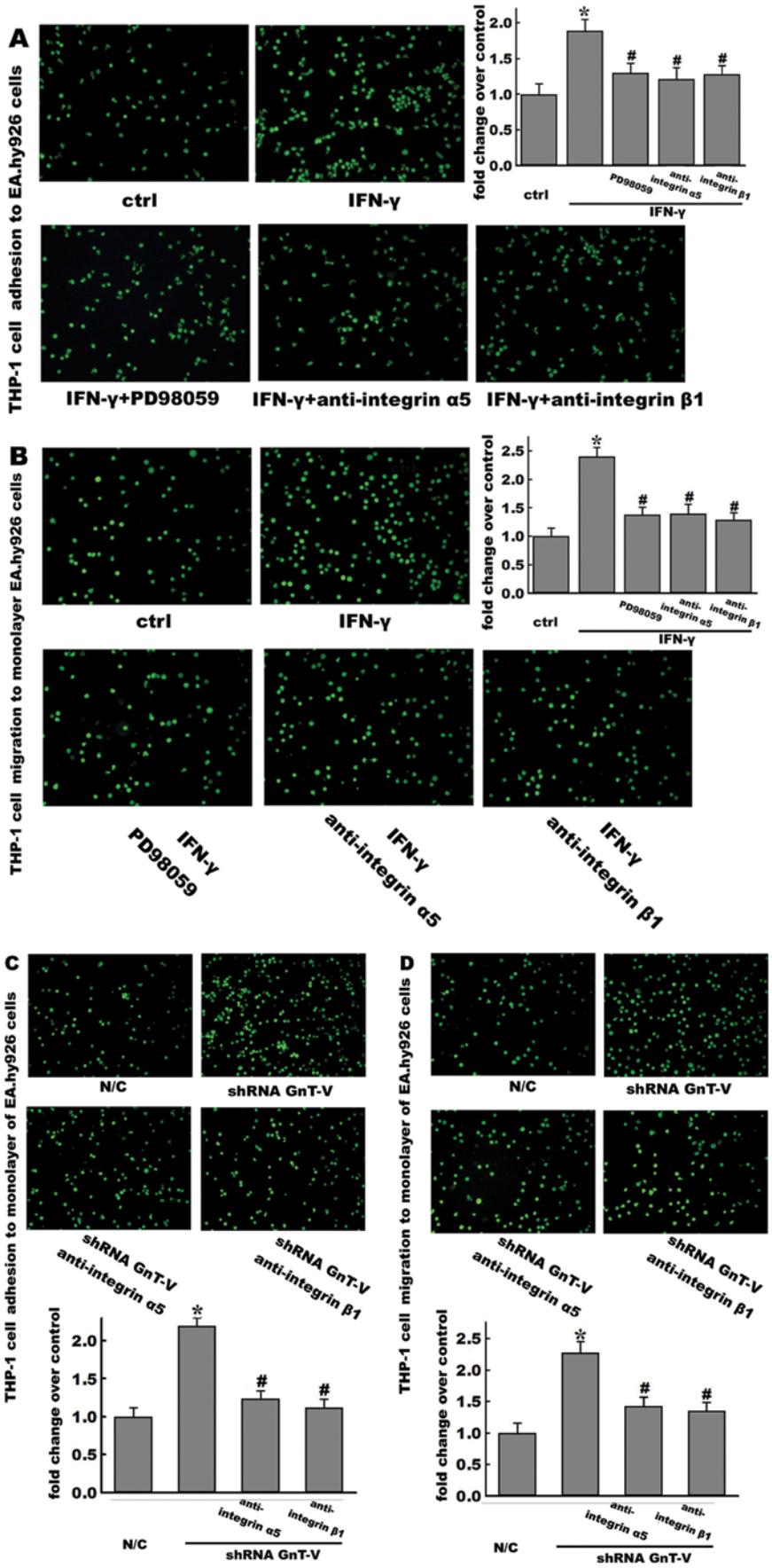

Figure 4. Effect of functional antibody blockade of integrin α5β1 or PD98059 on monocyte adherence and transmigration. IFN-γ-induced THP-1 cells were pre-treated with the ERK inhibitor PD98059 for 1 h or after treatment with antibodies to block function of integrin α5/β1 subunits for 30 min. The cells were then added to monolayers of EA.hy926 cells to observe (A) adhesion and (B) transmigration. After knockdown of GnT-V, THP-1 cells were treated with antibodies to block the function of integrin α5/β1 and then added to a monolayer of EA.hy926 cells to observe (C) adhesion and (D) transmigration. Bars, 100 μm. Data are presented as the means ± SEM (n=3). *P<0.05, IFN-γ-treated group vs. control group or GnT-V shRNA group vs. N/C GnT-V group; #P<0.05, IFN-γ+PD98059/anti-integrin α5/anti-integrin β1 vs. IFN-γ-treated group or GnT-V shRNA+anti-integrin α5/ anti-integrin β1 group vs. GnT-V shRNA group.

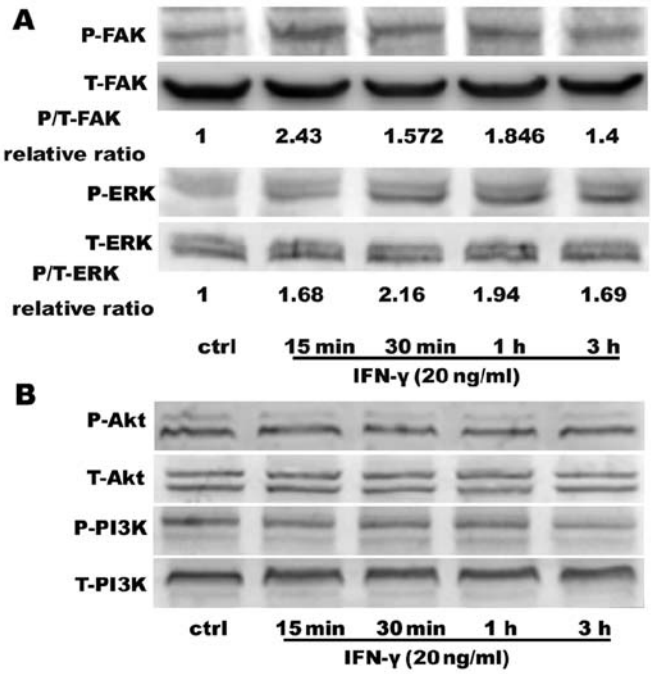

Figure 5. Effects of IFN-γ treatment on the phosphorylation of FAK, ERK, Akt and PI3K. THP-1 monocytes were treated with 20 ng/ml IFN-γ for 30 min, 1 or 3 h and then lysed to extract proteins. Phosphorylation levels of (A) FAK and ERK (B) Akt and PI3K were detected by western blot analysis. Relative ratios represent expression of proteins in the IFN-γ group vs. control group.

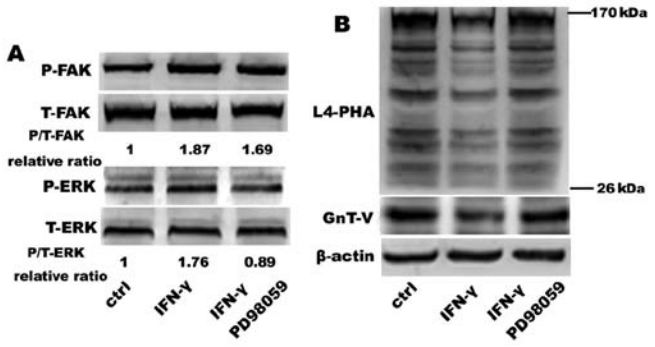

Figure 6. PD98059 treatment reverses the IFN-γ-induced decrease in GnT-V and β(1,6)-GLcNAc expression. IFN-γ-induced THP-1 cells were pre-treated with the ERK inhibitor PD98059 for 1 h and lysed to extract proteins for western blot analysis. (A) Phosphorylation levels of FAK and ERK. (B) Protein expression of GnT-V and β(1,6)-GLcNAc. Relative ratios represent expression of proteins in the experimental group vs. control group.

To further examine the role of the FAK-ERK signaling pathway in the changes in expression of GnT-V and β(1,6)-GlcNAc during IFN-γ-induced inflammation, THP-1 cells were pre-treated with the ERK inhibitor PD98059 before exposure to IFN-γ. Western blotting confirmed that PD98059 considerably inhibited ERK phosphorylation without affecting FAK phosphorylation at Tyr397 (Fig. 6A), indicating that ERK activity was downstream of FAK. The PD98059 pre-treatment also reversed the IFN-γ-induced decrease in the expression of GnT-V and its specific substrate β(1,6)-GlcNAc in THP-1 cells (Fig. 6B). Furthermore, the enhancement of THP-1 monocyte adhesion to the monolayer of endothelial EA.hy926 cells and transmigration in the IFN-γ-induced inflammation group were significantly eliminated by PD98059 pre-treatment (Fig. 4A and B). These results suggest that inhibition of the FAK-ERK signal pathway could reverse the positive regulation of monocyte-endothelium adhesion and transmigration by reduction in GnT-V and β(1,6)-GlcNAc.

Discussion

Previous studies have suggested the involvement of GnT-V in regulating cell adhesion, migration and invasion, simply by affecting the N-glycosylation of cell surface adhesion receptors, including integrins and cadherins (27,42,43). In the present study, we investigated the effects of N-glycosylation on integrin α5β1 and its biological functions, such as cell adhesion and migration, and found that the enchanced monocyte-enothelium interaction in an inflammation model was associated with altered GnT-V expression and its β1,6-GLcNAc modification of integrin α5β1. shRNA-mediated knockdown of GnT-V was also used to specifically examine its role in monocyte adhesion and migration. To the best of our knowledge, this is the first report to clearly demonstrate

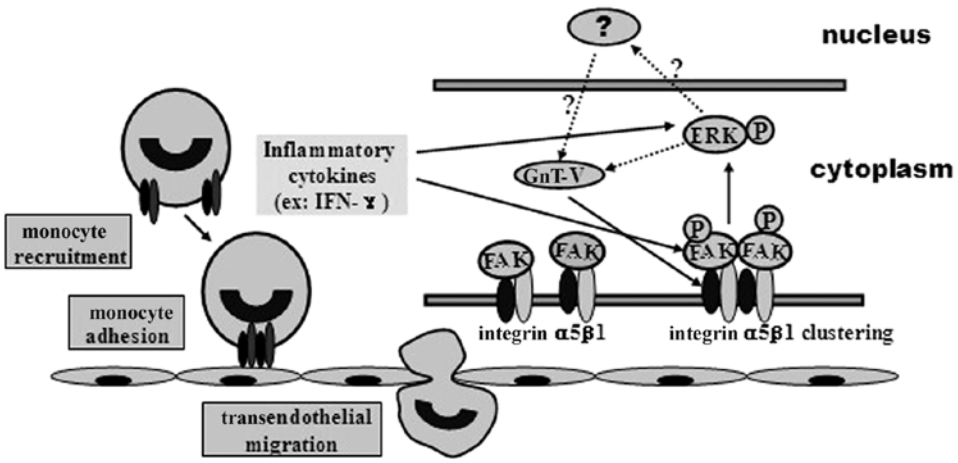

Figure 7. Signal network. The effect of GnT-V in IFN-γ-provoked inflammation.

that during an inflammatory reaction, the levels of GnT-V and its specific substrate β(1,6)-GLcNAc are significantly reduced, which could stimulate the integrin α5β1-dependant monocyte-endothelium interaction.

During inflammation, monocytes migrate through vascular endothelia to the local site of injury to perform functions such as secretion of various cytokines and anti-pathogenic activities. Much evidence exists to indicate that monocyte diapedesis is a multi-step process and is predominantly induced by the cellular surface adhesion molecule integrin (7), although the underlying mechanisms remain to be clarified. To develop an experimental system in which the monocyte-endothelium interactions are strongly enhanced, we used IFN-γ to stimulate THP-1 monocytes and then observed their adhesion to monolayers of endothelial cells and transmigration in 3D-culture. Compared to uninduced THP-1 cells, both the adhesion and transmigration were considerably heightened in the IFN-γ-stimulated cells. To directly prove the hypothesis that changes in GnT-V activity altered monocyte-endothelium adhesion and migration, we detected the levels of GnT-V and β1,6-GLcNAc in IFN-γ-induced THP-1 cells by western blotting and GnT-V mRNA expression by qRT-PCR. Notably, we found that, compared with uninduced THP-1 cells, the levels of GnT-V and β1,6-GLcNAc modification in the total cell lysates were highly reduced in the IFN-γ-induced cells. Furthermore, negative regulation of GnT-V on monocyte adhesion and transmigration through the endothelium was confirmed by an shRNA interference strategy to knockdown GnT-V in the THP-1 cells. These results were consistent with the effect of GnT-V activity in extravillous trophoblast invasion (24), but were differrent from that in tumor invasion (22,26). These conflicting results may be due to the differences in the malignancy of the cells.

To further investigate the potential molecular mechanisms involved in the monocyte-endothelium interaction, we investigated the effect of GnT-V activity on integrin α5β1 function. GnT-V activity had no effect on total expression of integrin α5 or β1, but it was associated with the level of β1,6-GLcNAc modification on both those subunits. Previous studies have demonstrated that increased aberrant branched sugar chains inhibits the clustering of integrin α5β1, composition of focal adhesion and phosphorylation of FAK (27,37,38). Conversely, GnT-V null mouse embryonic fibroblasts (MEF) displays enhanced cell adhesion to FN-coated plates (44). Of note, our results suggest that both monocyte adhesion to the monolayer endothelium and transmigration were markedly increased after transfection of the GnT-V shRNA vector in THP-1 cells. Moreover, these events were most likely mediated by integrin α5β1, which was confirmed by antibody-mediated blockade of its function. These findings provided strong evidence that changes of N-linked glycosylation caused by decreased GnT-V activity has a direct positive effect on the integrin α5β1-mediated adhesion of monocytes to the vascular endothelium and concomitant transmigration.

Aberrant modification of branching N-glycans affects the functions of cell surface receptors and the signaling pathways mediated by these receptors (43,45,46). In general, phosphorylation of FAK is accompanied by the formation of focal adhesion, which is the result of the activation of certain integrins including integrin α5β1 (36). FAK interacts with several different signaling proteins, such as PI3-kinase/Akt, Src-family PTKs, Grb2, ERK and JNK/mitogen-activated protein kinases (47,48). This enables FAK to function as a network of integrin-stimulated signaling pathways, leading to the activation of downstream targets. In our study, we found significant activation of FAK via phosphorylation in IFN-γ-induced THP-1 cells. FAK downstream signaling pathways were also detected, including those of ERK and PI3K/Akt. However, we found that only the ERK signaling pathway was activated. To confirm whether this pathway is involved in the downregulation of GnT-V expression during inflammation, the effect of the ERK specific inhibitor PD98059 on the protein expression of GnT-V and β1,6-GlcNAc was determined. We found that compared to IFN-γ-treated THP-1 cells, the expression levels of GnT-V and its β1,6-GLcNAc modified proteins in total cellular lysate were nearly restored in PD98059 pretreated THP-1 cells, and the adhesion and transmigration of THP-1 monocyte cells were also restored. Based on the findings in this study, we propose a model in which reduced GnT-V expression is regulated by activation of the FAK-ERK signaling pathway during inflammation (Fig. 7), but the exact mechanism requires further investigation.

In summary, the present study specifically focused on aberrant modification of branching sugar chains on integrin α5β1 and its function in monocyte-endothelium interactions during inflammation. Markedly, the negative effects of GnT-V activity on integrin-mediated monocyte function observed here should be regarded as a new anti-inflammatory response with a potential therapeutic role against inflammation-dependent conditions.

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