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ORIGINAL ARTICLE

Loss of MGAT3 Amplifies TGF-β Signaling to Promote Bronchiolitis Obliterans Progression

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ABSTRACT

Background: **Bronchiolitis obliterans syndrome (BOS), a severe complication following allogeneic hematopoietic stem cell transplantation (allo-HSCT), presents as chronic graft-versus-host disease characterized by inflammation and fibrosis of the small airway epithelium. This progressive fibrosis obstructs bronchiolar airways, leading to respiratory distress in patients. Due to the lack of effective treatments, a deeper understanding of the underlying mechanisms is crucial. This study explored the molecular mechanisms underlying the abnormal glycosylation regulation in BOS, aiming to provide new insights for early diagnosis and treatment of this disease.**

Methods: **Bronchoalveolar lavage fluid (BALF) was collected from patients with and without BOS following allo-HSCT. N-glycans from the BALF were enriched using a solid-phase extraction method. Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) was used to detect N-glycosylation characteristics in the BALF of BOS patients. This phenomenon was validated using a graft-versus-host disease (GVHD) mouse model. The impact of MGAT3 knockdown on the biological functions of airway epithelial cells (BEAS-2B) was then examined using lentivirus transfection. The molecular mechanism by which the loss of MGAT3 enhances TGF-β signaling was explored using western blotting, enzyme-linked immunosorbent assay (ELISA), and immunofluorescence (IF).**

Results: **Our study investigated N-glycosylation changes in BALF of BOS patients and identified a strong correlation between the loss of bisecting-GlcNAc N-glycans and BOS progression. We found a novel mechanism where MGAT3, the enzyme synthesizing bisecting-GlcNAc structures, critically regulates TGF-β signaling. MGAT3 and bisecting-GlcNAc deficiency significantly enhance TGF-β signaling by increasing TGF-β storage through upregulation of latent TGF-β binding protein 1 (LTBP1) and by increasing the availability of TGF-β receptors.**

Conclusions: **Our discovery highlights the crucial role of aberrant glycosylation in BOS progression and holds significant promise for developing novel biomarkers for early diagnosis, identifying new therapeutic targets, and ultimately improving the quality of life for BOS patients.**

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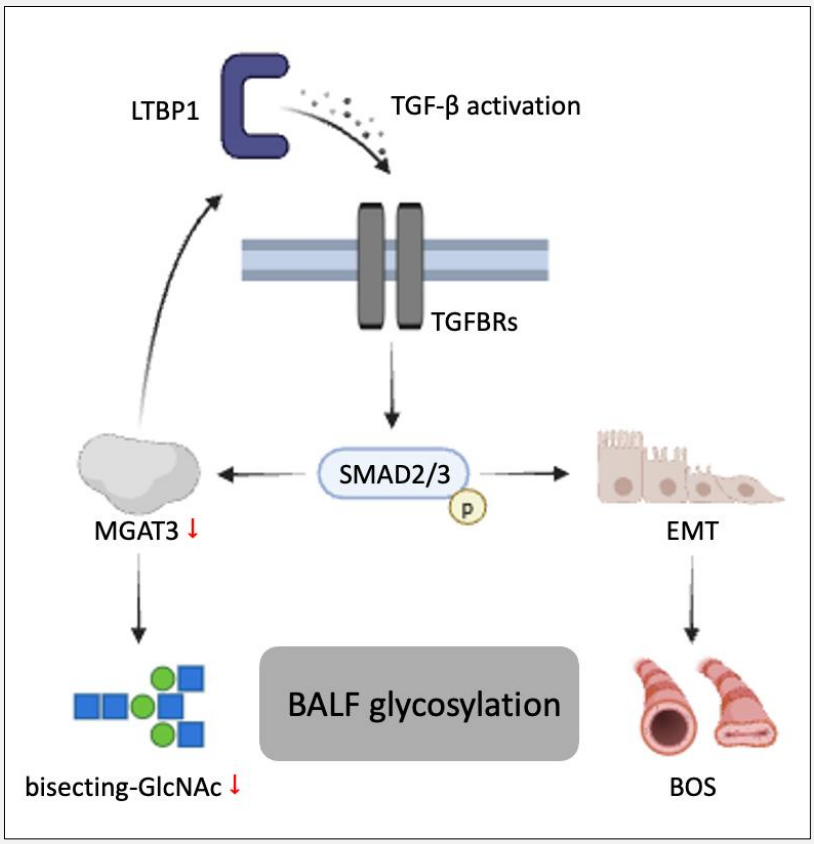
Graphic Abstract



KEYWORDS

BOS, cGVHD, BALF, Bisecting-GlcNAc, MGAT3

INTRODUCTION

Allogeneic hematopoietic stem cell transplantation (allo-HSCT) provides a crucial treatment option for numerous patients with blood and bone marrow disorders, including acute and chronic leukemia, lymphoma, myeloma, aplastic anemia, and myelodysplastic syndromes (MDS) [1]. Despite its efficacy in combating various hematological malignancies, allo-HSCT is a complex procedure associated with significant potential complications, such as graft-versus-host disease (GVHD), infections, and graft failure [2]. Bronchiolitis obliterans syndrome (BOS), a severe pulmonary complication of chronic GVHD (cGVHD), poses a substantial threat to the quality of life and survival of affected patients [3]. BOS is characterized by the scarring and narrowing of small airways (bronchioles), resulting in significant breathing difficulties, particularly during exertion [4]. While the precise mechanisms underlying the development of BOS remain elusive, it is widely believed to originate from abnormal immune responses following transplantation [5]. Elucidating these mechanisms is critical for the development of novel, targeted therapies that offer renewed hope for individuals suffering from this debilitating condition.

Abnormal immune responses in BOS development may be associated with altered glycosylation of proteins, such as p-glycoprotein [6]. Glycosylation, a common post-translational modification (PTM) involving the attachment of glycans to proteins, lipids, and RNAs, plays a crucial role in various biological functions [7-10]. Numerous studies have demonstrated that alterations in glycosylation patterns are associated with diverse diseases. For example, the presence of up to six fucose molecules on haptoglobin can effectively differentiate hepatocellular carcinoma from liver cirrhosis [11], while the increased expression of the Tn epitope on the MUC1 protein serves as a valuable biomarker for both breast and gastric cancers [12]. These findings strongly

suggest that aberrant glycan modifications may hold significant potential for both the diagnosis of BOS and the development of targeted therapies.

Glycosylation analysis of body fluids, including serum, urine, and saliva, has emerged as a promising approach for the discovery of novel disease biomarkers [13-16]. Bronchoalveolar lavage fluid (BALF), obtained through bronchoscopy, represents a particularly valuable source for investigating lung diseases such as lung cancer and BOS. Beyond its utility in cytological diagnosis, BALF contains a diverse array of proteins, RNAs, and cytokines that are directly relevant to disease pathogenesis [17,18]. For instance, previous studies have successfully identified eight potential lung cancer biomarkers by analyzing N-glycoproteins in BALF [19]. Notably, the glycosylation patterns of BOS BALF remain largely unexplored, presenting a promising objective for future research.

BALF samples were collected from patients with and without BOS after allo-HSCT. A comparison was then made to determine the differences in their N-glycosylation patterns. Our initial findings revealed significantly reduced levels of bisecting-GlcNAc N-glycans in the BALF of patients with BOS. This observation prompted us to investigate the role of bisecting-GlcNAc N-glycans and MGAT3 (the enzyme responsible for their synthesis) in the development of BOS. Utilizing GVHD-mouse models, we observed a significant downregulation in the expression levels of both bisecting-GlcNAc N-glycans and MGAT3 in the context of BOS. Furthermore, we found that the development of GVHD is accompanied by an increase in the expression of TGF-β1, a potent pro-fibrotic cytokine [20].

Notably, TGF-β1, while inducing epithelial-mesenchymal transition (EMT), also downregulates MGAT3 expression, providing a potential explanation for the observed decrease in bisecting-GlcNAc N-glycans in BOS BALF. Intriguingly, our investigation uncovered a novel pathway wherein the loss of MGAT3 amplifies TGF-β signaling, thereby promoting the development of airway fibrosis characteristic of BOS.

MGAT3 deficiency leads to increased expression of latent TGF-β binding protein 1 (LTBP1), which in turn causes greater storage of TGF-β, a key cytokine that promotes fibrosis [20]. Concurrently, knocking down MGAT3 enhances the availability of TGF-β receptors (TGFBR1 and TGFBR2). This synergistic effect significantly amplifies TGF-β signaling and promotes EMT. Collectively, our research findings innovatively unveil a novel mechanism by which aberrant glycosylation regulates the progression of BOS. This discovery fills a critical gap in our understanding of BOS pathogenesis and provides a promising new target for the development of therapeutic interventions aimed at delaying BOS progression, ultimately improving patient prognosis.

MATERIALS AND METHODS

Chemicals and reagents

AminoLink™ Plus resins (beads), PNGase F, anhydrous methanol, and anhydrous ethanol were purchased from Thermo Scientific (Waltham, MA, USA), New England BioLabs (Ipswich, MA, USA), and Aladdin Chemical (Shanghai, China), respectively. Additional reagents, including sodium cyanoborohydride ($NaCNBH_3$), ammonium bicarbonate (NH_4HCO_3), p-toluidine (pT), N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide (EDC), 1-hydroxybenzotriazole (HOBt), formic acid (FA), acetonitrile (ACN) and bovine serum albumin (BSA) were obtained from Sigma-Aldrich (St. Louis, MO, USA), Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China), Macklin Biochemical Co., Ltd. (Shanghai, China), Tedia (Fairfield, OH, USA), Solarbio Science and Technology Co. Ltd. (Beijing, China) and Yamei Biotechnology Co. Ltd. (Shanghai, China), respectively. Finally, Beyotime Biotechnology Co., Ltd. (Shanghai, China) supplied phosphate-buffered saline (PBS), Tris-HCl, BCA and CCK-8 kits, SDS-PAGE loading buffer, FITC-streptavidin, goat serum, and ECL reagents.

BALF sample collection and protein extraction

This study was conducted with the approval of the Institutional Review Board (IRB) at the Fourth Affiliated Hospital of Soochow University, ensuring adherence to all relevant ethical guidelines. Informed consent was obtained in writing from all participants prior to their participation. BALF samples were collected from the Department of Respiratory Medicine: Five patients confirmed to have bronchiolar occlusion through bronchoscopy (BOS patients) and 5 patients who had not yet developed BOS following allo-HSCT. BOS patients included in the study met four specific diagnostic criteria: first, pulmonary function tests showed an FEV1/FVC ratio less than 0.7 and an FEV1 less than 75% of the predicted value, with a post-salbutamol increase in FEV1 of less than 200 mL; second, sputum cultures or BALF samples showed no pathogenic bacteria; third, bronchoscopy revealed grade 8 - 10 airway obstruction in the bronchioles; and finally, the BALF was collected to test for subsequent experiments. To collect the BALF, a bronchoscope was used to access the obstructed airways, and 5 - 10 mL of physiological saline was instilled and then suctioned out. All collected samples were free from blood and immediately frozen at -80°C for later analysis. Protein was precipitated from each individual BALF sample by adding six volumes of ice-cooled ethanol (100%, v/v) and incubating overnight at -20°C. The samples were then centrifuged at 13,000 x g for 15 minutes at 4°C. The resulting protein pellets were resuspended in 200 µL of 1x PBS. Prior to glycan enrichment, BALF samples from BOS patients were pooled separately from those of non-BOS patients to create two distinct sample pools.

Enzyme release of sialic acid-derivatized N-glycans

For N-glycan analysis, 500 μg of BALF-extracted protein was initially pre-treated with 500 μL of 1 x binding buffer (10 mM sodium citrate and 5 mM sodium carbonate) and then incubated with 200 μL of AminoLink Plus resin for 4 hours at room temperature (RT) to allow protein binding [21,22]. Subsequently, 50 mM $NaCNBH_3$ was added to complete reductive amination, followed by washing with 500 μL of 1 x PBS and further incubation in 1 x PBS with 50 mM $NaCNBH_3$ for 4 hours at RT. Unreacted aldehydes on the resin were then blocked using 1 M Tris-HCl (pH 7.4). To derivatize sialic acids, 2,6-linked sialic acids were modified with a mixture of 0.5 M EDC (200 μL) and 0.5 M HOBt (200 μL) in ethanol for one hour at 37°C [22]. Following washes with deionized water, 2,3-linked sialic acids were further derivatized with 500 μL of 1 M p-toluidine (pT). Finally, PNGase F used was employed to cleave and release N-glycans from the glycoproteins following the solid-phase derivatization of sialic acids [23].

MALDI-TOF/TOF-MS identification of N-glycans

For mass spectrometry analysis, 1 μL of the enzymatically released N-glycan solution was mixed with 1 μL of DHB MALDI matrix and spotted onto a MALDI plate. The mixture was then allowed to air-dry at room temperature. Mass spectrometry data were acquired using a Bruker Autoflex MALDI-TOF/TOF-MS instrument within a mass range of m/z 1,000 - 6,000. FlexAnalysis software was utilized to visualize the mass spectrometry signals and identify the various N-glycan types present. Subsequently, GlycoWorkBench software was employed to predict the specific composition of each identified N-glycan [24]. Finally, the relative abundance of different N-glycan types was determined by analyzing the peak areas in the mass spectra [25].

GVHD BALB/c mouse model establishment

Female C57BL/6 and BALB/c mice aged 4 - 12 weeks were obtained from Gempharmatech Co., Ltd (Nanjing, China) and housed in a specific pathogen-free (SPF) facility under the supervision of the Soochow University Animal Care and Use Committee. One week prior to the commencement of the experiment, kanamycin was administered to eliminate intestinal bacteria. For the GVHD model, male SPF-grade C57BL/6 (H-2b) mice served as donors, while male BALB/c (H-2d) mice were recipients. Recipient mice received a myeloablative dose of 650 cGy radiation. Bone marrow (BM) and splenocytes (SP) were harvested from deceased donor mice through aseptic dissection of the spleen, spine, and femur under a laminar flow hood using disinfected ophthalmic scissors and forceps. The harvested cells were prepared by grinding in RPMI-1640 medium, filtering through a nylon mesh, lysing red blood cells (RBC) using 1 x RBC lysis solution, and purifying them via centrifugation (1,300 rpm, 10 minutes, 4°C) with a single 1 x PBS wash. Finally, cell concentrations were adjusted prior to injection. To establish acute GVHD (aGVHD), recipient mice received 1×10^7 C57BL/6 BM cells and 5×10^6 whole splenocytes. Chronic GVHD (cGVHD) was induced with 2.5×10^6 C57BL/6 BM cells and 1×10^6 splenocytes. Mouse growth, body weight, and GVHD symptoms were monitored daily and every 3 days, respectively. Following the establishment of the GVHD model, experimental mice were euthanized, and lung tissues were collected. A portion of the lung tissue was allocated for western Blot (WB) analysis, while the remaining lung tissue was fixed in 10% formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin (H&E) for histological evaluation of GVHD.

Identification of glycosyltransferases with abnormal expression in BOS

Expression profile data of BOS-related genes were obtained from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). Due to the lack of data for BOS after HSCT, the database we selected was derived from BOS-mouse models after lung transplantation. The dataset (GSE137169) was retrieved, containing gene expression profiles of BOS mouse lung. We screened glycosyltransferases involved in human glycosylation pathways and their matrix data were converted into $\log_2$ values. The R software package (version 4.2.2) was used for data normalization and differential expression analysis. The screening criteria for differentially expressed glycosylation genes (glyco-DEGs) in BOS were $|\log_2(\text{fold change})| > 1$ and $p < 0.05$.

Lectin fluorescence and immunofluorescence

Cell climbing slides were prepared by placing 14 mm cover glasses within 24-well plates and directly culturing cells on them. Following cell culture, the slides were washed three times with 1 x PBS and fixed with 4% paraformaldehyde for 15 minutes. After three additional 5-minute washes with 1 x PBS, the cells were blocked with 10% goat serum for 60 minutes. For lectin staining, cells were incubated with biotinylated PHA-E (1:500 dilution) for 1 hour at room temperature. This was followed by three washes with 1 x PBS and subsequent incubation with FITC-conjugated streptavidin (1:500 dilution) for another hour at room temperature. After three final washes with 1 x PBS, DAPI staining solution was added for 5 minutes, followed by a final PBS wash. Images were then captured using an inverted fluorescence microscope (TS2R-FL, Nikon). For immunofluorescence staining, following the blocking step, cells were incubated overnight at 4°C with primary antibodies. Subsequently, Cy3-labeled goat anti-rabbit/mouse IgG (H+L) was added and incubated for 1 hour at room temperature. After three 5-minute washes with 1 x PBS, images were collected.

Lentiviral transfection

A lentiviral transfection kit was purchased from Genechem (Shanghai, China). shRNA targeting MGAT3 was

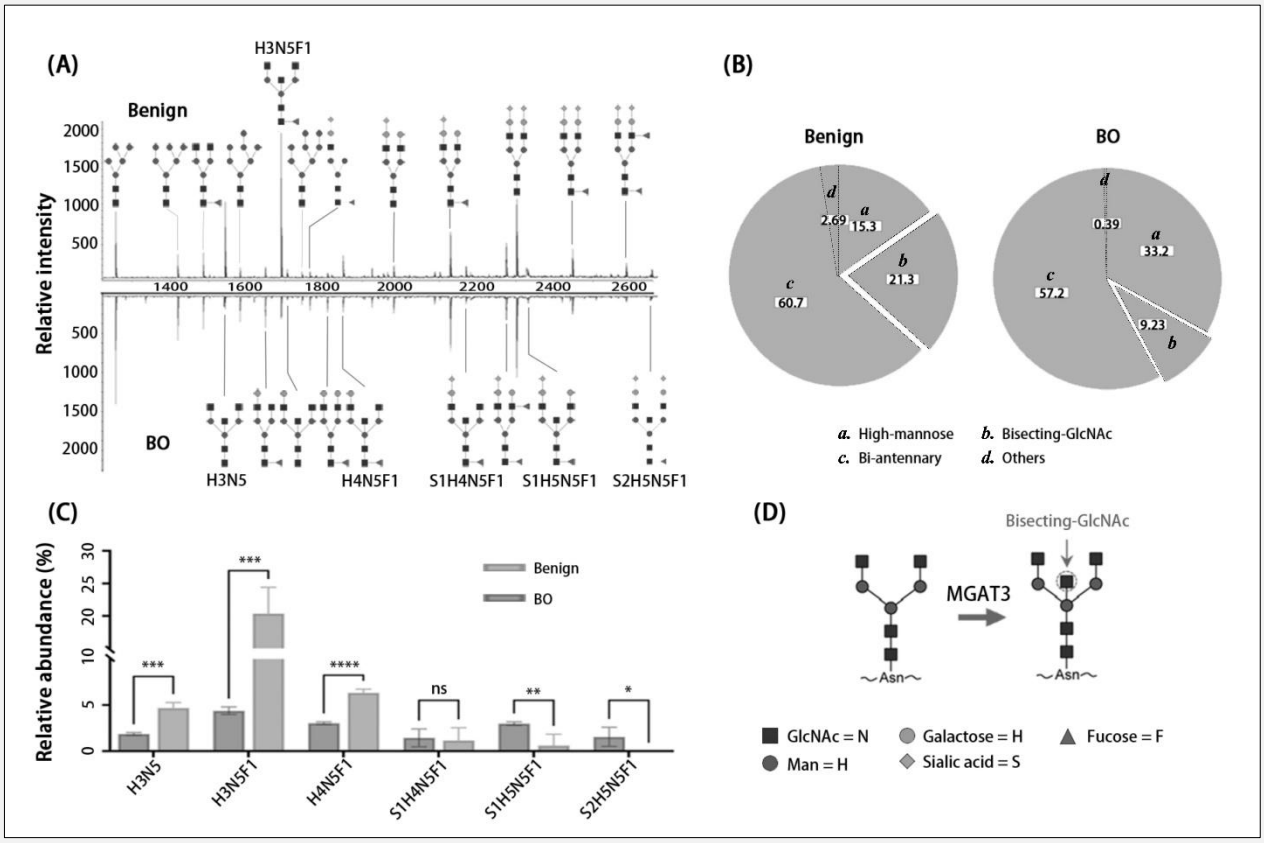


Figure 1. Quantitative analysis of N-glycans in BALF of BOS patients by MALDI-TOF/TOF-MS.

A: The expression patterns of N-glycans in the BALF of non-BOS and BOS patients, revealing similarities with notable differences, particularly a lower expression of bisecting-GlcNAc N-glycans in BOS BALF. B: The relative abundance of different N-glycan types, including high-mannose, bi-antennary, and bisecting-GlcNAc N-glycans, in non-BOS and BOS BALF. The relative abundance of bisecting-GlcNAc N-glycans is significantly reduced in BOS BALF. C: Quantitative analysis demonstrating a significant downregulation of several bisecting-GlcNAc N-glycans (H3N5, H3N5F1, H4N5F1) in BOS BALF. D: Enzymatic catalysis of bisecting-GlcNAc structure by MGAT3 (H = Hexose, N = HexNAc, F = Fucose, S = Sialic acid).

inserted into the lentiviral vector GV493 (hU6-MCS-CBh-gcGFP-IRES-puromycin). BEAS-2B cells were seeded at a density of 5×10^4 cells/mL and cultured until they reached approximately 50% confluence. The culture medium was then replaced with 1 mL of DMEM medium containing 7 μL of well-coated lentivirus reagent and 40 μL of HstransG P infection enhancer. The lentivirus stock had a titer of 1×10^9 transducing units (TU)/mL. Forty-eight hours post-transfection, the transfection efficiency was assessed by observing green fluorescence protein (GFP) expression. The same procedure was performed using an empty viral expression vector as a negative control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software Inc.). For comparisons between two groups, Student's t-tests were employed. Spearman's rank correlation coefficient (ρ) was used to assess correlations. A p-value of less than 0.05 was considered statistically significant, with significance levels denoted by asterisks: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. For a comprehensive understanding of our findings, detailed descriptions of additional experiments are provided in the Supplementary Information. These experiments include cell culture assays, wound healing assays, cell proliferation assays using the cell counting kit-8 (CCK-8), cell adhesion and invasion assays, WB analysis, lectin blotting, enzyme-linked immunosorbent assays (ELISA), gene correlation analysis, and predic-

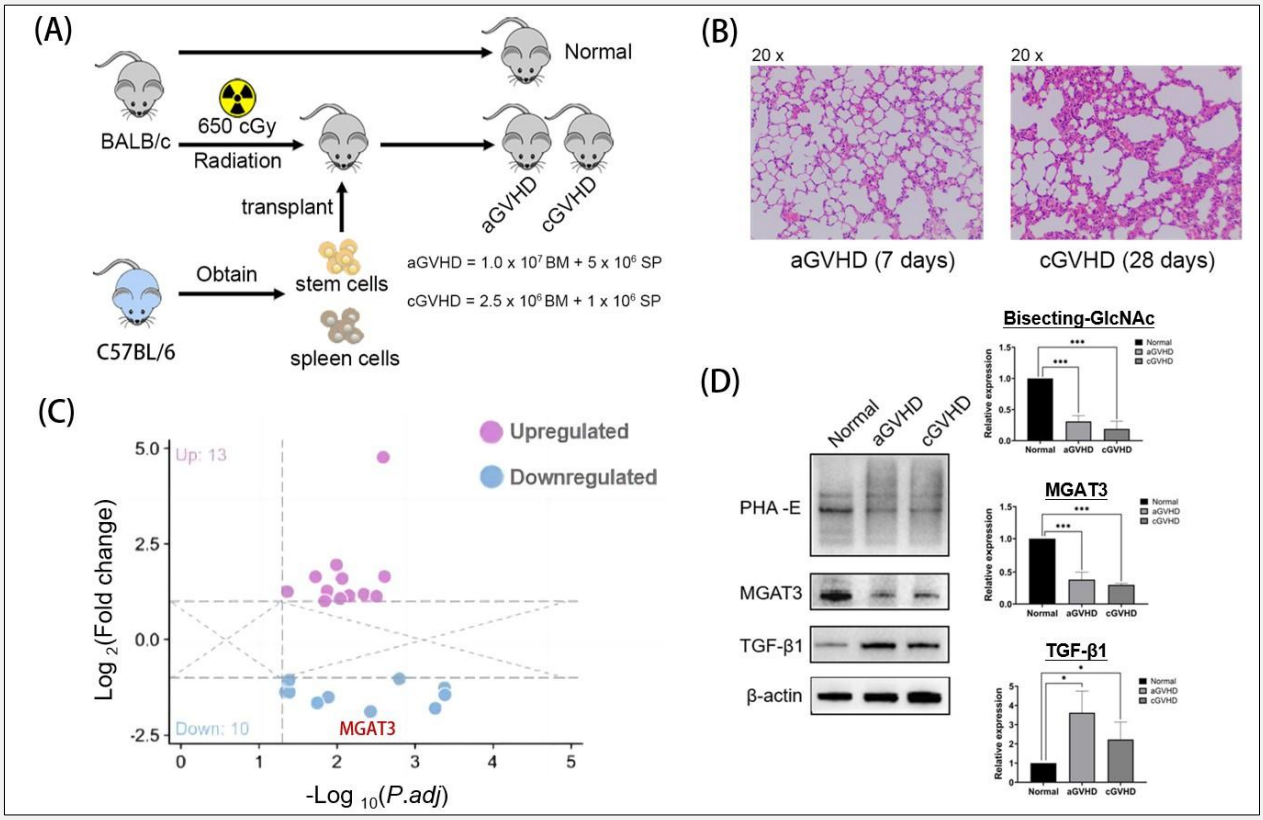


Figure 2. Downregulation of MGAT3 and bisecting-GlcNAc N-glycans in GVHD mice.

A: Induction of GVHD models: BALB/c mice were lethally irradiated with X-rays (650 cGy). Bone marrow (BM) cells and splenocytes (SP) were harvested from deceased C57BL/6 donor mice. To establish an acute GVHD (aGVHD) model, 1×10^7 C57BL/6 BM cells and 5×10^6 splenocytes were injected into BALB/c recipient mice. To generate chronic GVHD (cGVHD) models, 2.5×10^6 C57BL/6 BM cells and 1×10^6 splenocytes were injected into BALB/c recipient mice. B: Twenty-eight days post-transplantation, cGVHD model mice showed significant interstitial proliferation and infiltration of inflammatory cells in the small bronchi. C: Compared with non-BOS mice, 13 glycosyltransferases were upregulated and 10 glycosyltransferases were downregulated in the lung tissue of BOS mice after lung transplantation. D: Western blot (lectin blot) showed a significant decrease in MGAT3 and bisecting-GlcNAc N-glycans in lung tissues of aGVHD and cGVHD mice, while TGF-β1 content increased. The statistical significance: * $p < 0.05$, *** $p < 0.0001$.

tions of N-glycosylation sites. For the western blot and lectin blot experiments, three biological replicates were performed for each sample, including the controls. The error bars were generated from the results of these experiments.

RESULTS

Our initial findings demonstrated a significant reduction in bisecting-GlcNAc N-glycans (e.g., H3N5, H3N5F1, and H4N5F1) within the BALF of BOS patients. This observation led us to hypothesize that MGAT3, the enzyme responsible for the synthesis of bisecting-GlcNAc N-glycans, is downregulated during the progression of GVHD. This hypothesis was subsequently confirmed using GVHD mouse models. Previous research has demonstrated that TGF-β signaling plays a critical role in promoting fibrosis and cancer metastasis, and can lead to decreased MGAT3 expression in certain cancer cell types [26]. Based on these findings, we investigated the potential involvement of TGF-β signaling in the progression of GVHD. Indeed, we observed that GVHD mouse lungs exhibited a concomitant decrease in MGAT3 expression and an increase in TGF-β1 expression, validating our hypothesis. Intriguingly, we discovered that MGAT3 loss can significantly enhance TGF-β signaling. Mechanistically, we found that the absence of MGAT3 promotes the storage of latent TGF-β and increases the expression of TGF-β receptors, thereby amplifying the availability of active TGF-β. These combined effects ultimately amplify TGF-β signaling, pro-

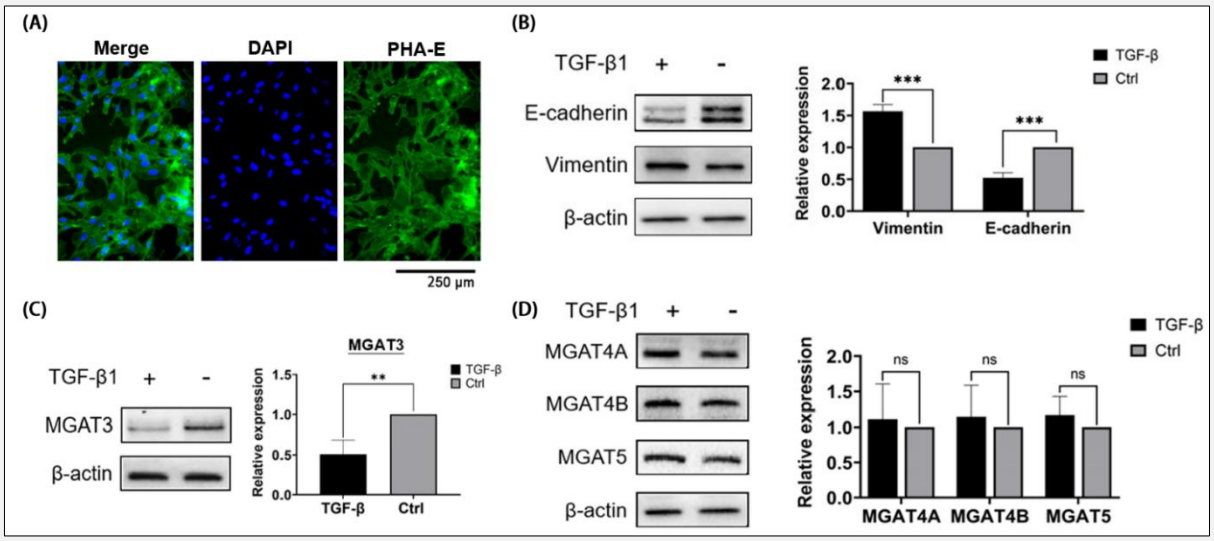


Figure 3. TGF-β1-induced EMT downregulates the expression of MGAT3 but MGAT4 and MGAT5.

A: Immunofluorescence staining with PHA-E lectin was performed to confirm the presence of bisecting GlcNAc N-glycans in BEAS-2B cells. Scale bar = 250 μm. B: Treatment with TGF-β1 (5 ng/mL for 48 hours) induced changes in the expression of E-cadherin and Vimentin, two EMT-related markers. Specifically, Vimentin, a marker of mesenchymal cells, was upregulated, while E-cadherin, an epithelial marker, was downregulated. C: Conversely, TGF-β1 treatment downregulated MGAT3 expression during EMT. D: No significant changes were observed in the expression levels of MGAT4A, MGAT4B, and MGAT5 during TGF-β1-driven EMT. The statistical significance: ** p < 0.001, *** p < 0.0001, ns = not significant.

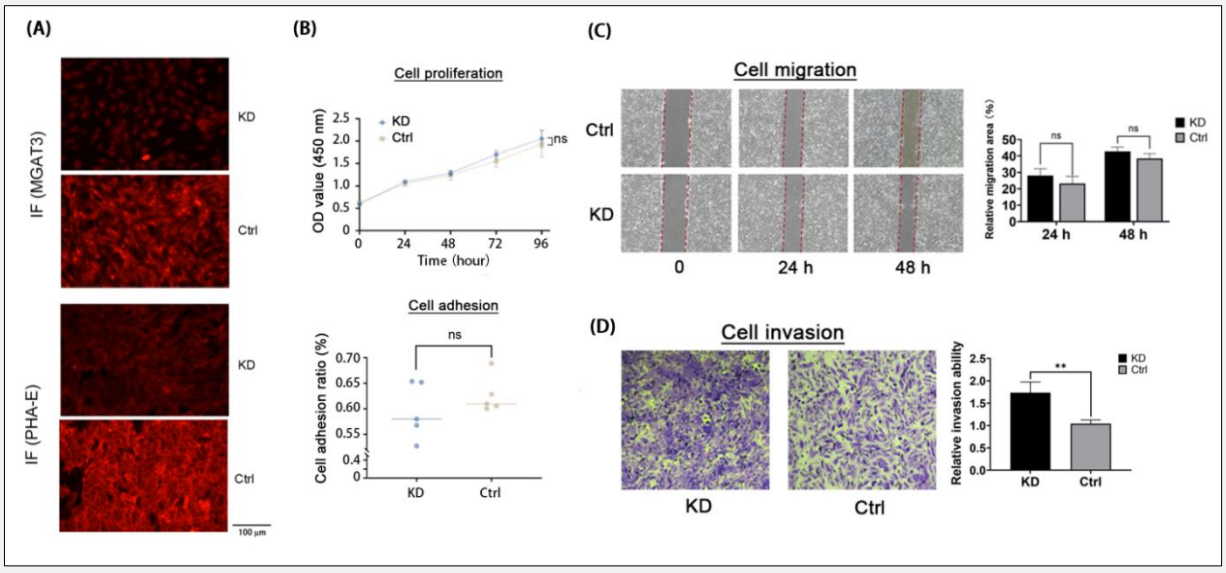


Figure 4. MGAT3 knockdown leading to EMT-related alterations in cells.

A: Fluorescence staining confirmed a decrease in both MGAT3 protein levels and bisecting-GlcNAc N-glycan expression following MGAT3 knockdown in BEAS-2B cells. B: MGAT3 knockdown did not significantly influence the proliferation or adhesion of BEAS-2B cells. C: MGAT3 knockdown displayed a modest stimulatory effect on cell migration. D: A significant increase in the invasive potential of BEAS-2B cells was observed upon MGAT3 knockdown. The statistical significance: ** p < 0.001, ns = not significant.

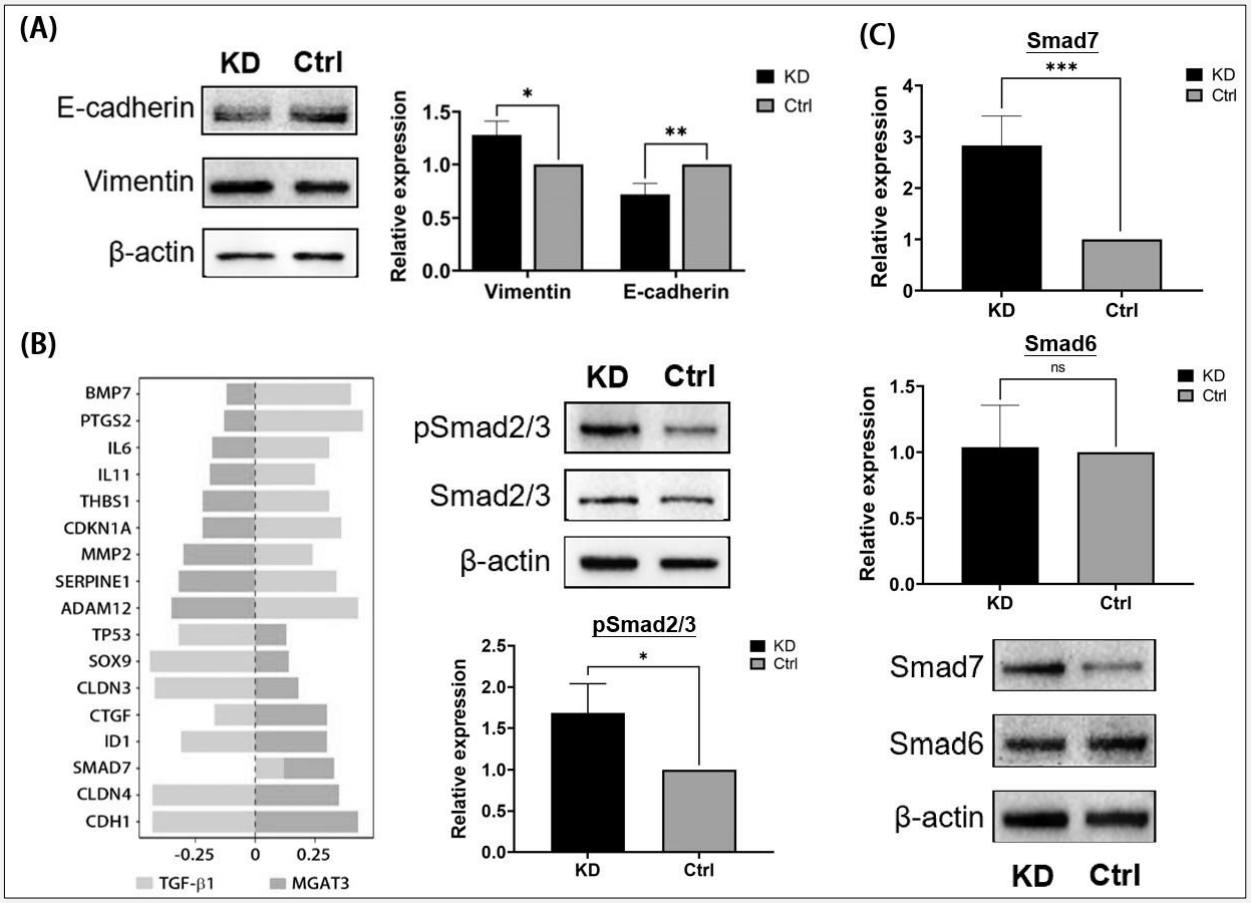


Figure 5. Knocking down MGAT3 leading to activation of TGF-β signaling.

A: MGAT3 knockdown (KD) decreased E-cadherin expression and increased Vimentin expression. B: Upon MGAT3 KD, phosphorylation of Smad2/3 was increased. Gene correlation analysis identified 16 genes exhibiting an opposing correlation with MGAT3 compared to their correlation with TGF-β1. Among these, the top nine genes displayed a positive correlation with TGF-β1 expression but a negative correlation with MGAT3 expression. The remaining eight genes showed a positive correlation with MGAT3 and a negative correlation with TGF-β1. Additionally, MGAT3 KD led to increased phosphorylation of SMAD2/3. C: Knocking down MGAT3 induced an increase in Smad7 expression in cells, but not Smad6. The statistical significance: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, ns = not significant.

moting the development of airway fibrosis (Figure S1).

Downregulated bisecting-GlcNAc N-glycans in BALF of BOS patients

N-glycosylation analysis of BALF revealed a significant decrease in tri-antennary N-glycans in patients with BOS compared to control groups (Figure 1A). These N-glycans consist of three major types: β1,4-bisecting-GlcNAc N-glycans synthesized by MGAT3, β1,4-branching-GlcNAc N-glycans synthesized by MGAT4, and β1,6-branching-GlcNAc N-glycans directed by MGAT5 (Figure S2). Notably, the two most abundant tri-antennary N-glycans (H3N5 and H3N5F1) in non-BOS BALF lacked terminal modifications, suggesting that they are likely bisecting-GlcNAc type N-glycans. This is because the formation of the bisecting-GlcNAc structure inhibits subsequent glycosylation processes [27]. The proportion of these bisecting-GlcNAc N-glycans was significantly reduced in BALF from BOS patients (Figures 1B and 1C). This reduction suggests a potential downregulation of MGAT3 activity during the development of BOS.

Downregulation of bisecting-GlcNAc N-glycans and MGAT3 in GVHD-mice lung

To investigate whether the decreased bisecting-GlcNAc N-glycans in BOS BALF resulted from MGAT3 deficiency, we established two GVHD mouse models. In

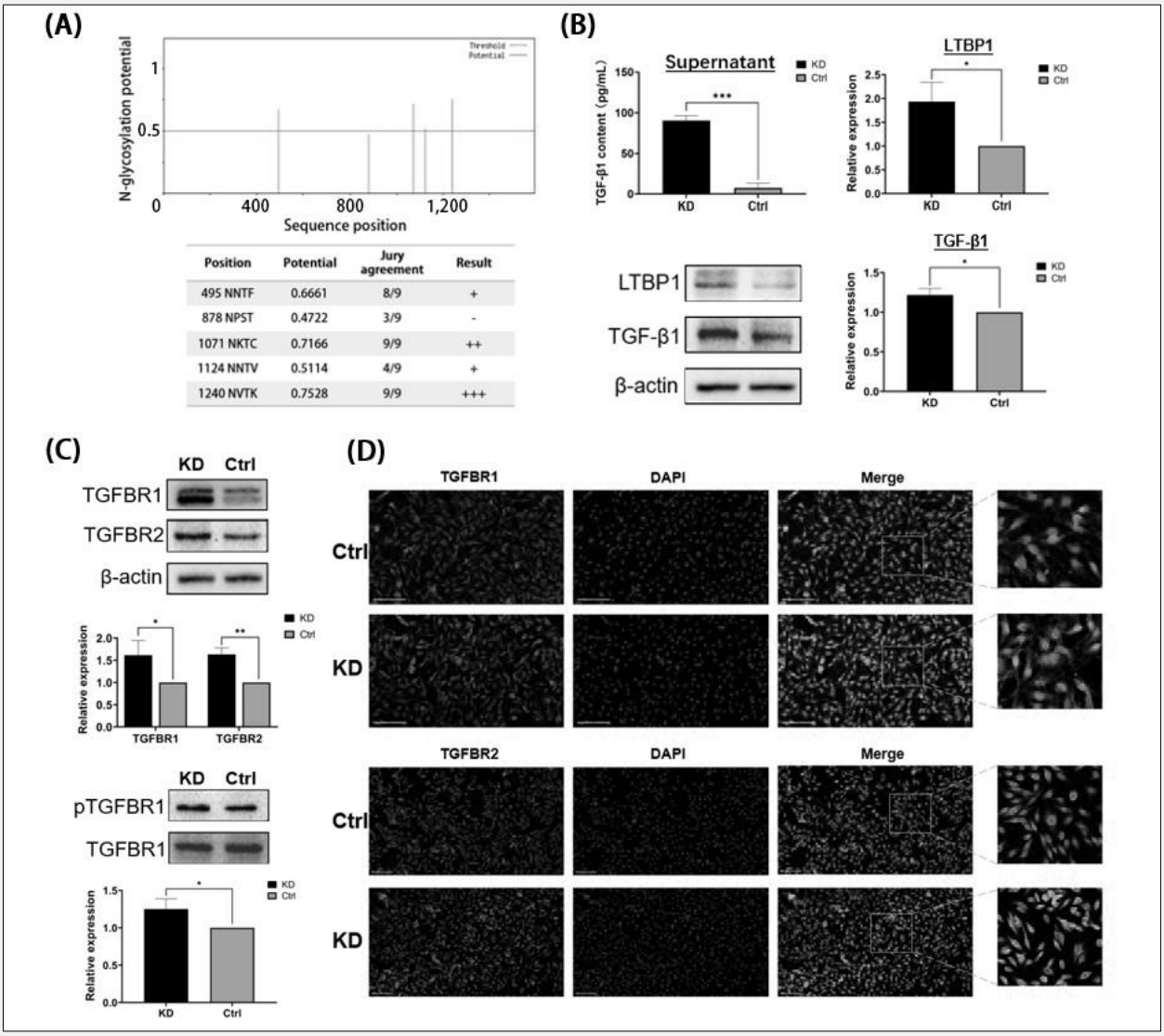


Figure 6. Elucidating the mechanism by which MGAT3 deficiency activates TGF-β signaling.

A: Prediction of N-glycosylation sites on LTBP1: According to the NetNGlyc database, LTBP1 contains multiple potential N-glycosylation sites, including N495, N1071, and N1240. B: Effect of MGAT3 knockdown on TGF-β1 expression and secretion: MGAT3 knockdown increased both intracellular TGF-β1 expression and the secretion of TGF-β1 into the cell supernatant. This may be attributed to increased TGF-β storage and protection, as MGAT3 knockdown also increased the expression of LTBP1, a protein involved in the storage and extracellular matrix deposition of latent TGF-β. C: Effect of MGAT3 knockdown on TGF-β receptor expression and signaling: MGAT3 knockdown increased the expression levels of TGF-β receptors (TGFBR1 and TGFBR2) and enhanced TGF-β signaling, as evidenced by increased phosphorylation of TGFBR1. Immunofluorescence analysis further demonstrated that in the absence of MGAT3, a greater proportion of TGF-β receptors was localized to the cell membrane. The statistical significance: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$, ns = not significant.

the acute GVHD (aGVHD) model, BALB/c recipient mice received 1 x 10^7 C57BL/6 bone marrow (BM) cells and 5 x 10^6 splenocytes (SP), while the chronic GVHD (cGVHD) model utilized 2.5 x 10^6 BM cells and 1 x 10^6 SP (Figure 2A). H&E staining confirmed successful cGVHD development, characterized by interstitial hyperplasia and inflammatory infiltration in lung tissues (Figure 2B). Subsequently, we analyzed the abnormal expression of N- and O-glycosyltransferases in BOS lungs using the Gene Expression Omnibus (GEO) database. Compared to control mice without BOS, 13 glycosyltransferases were upregulated and 10 were

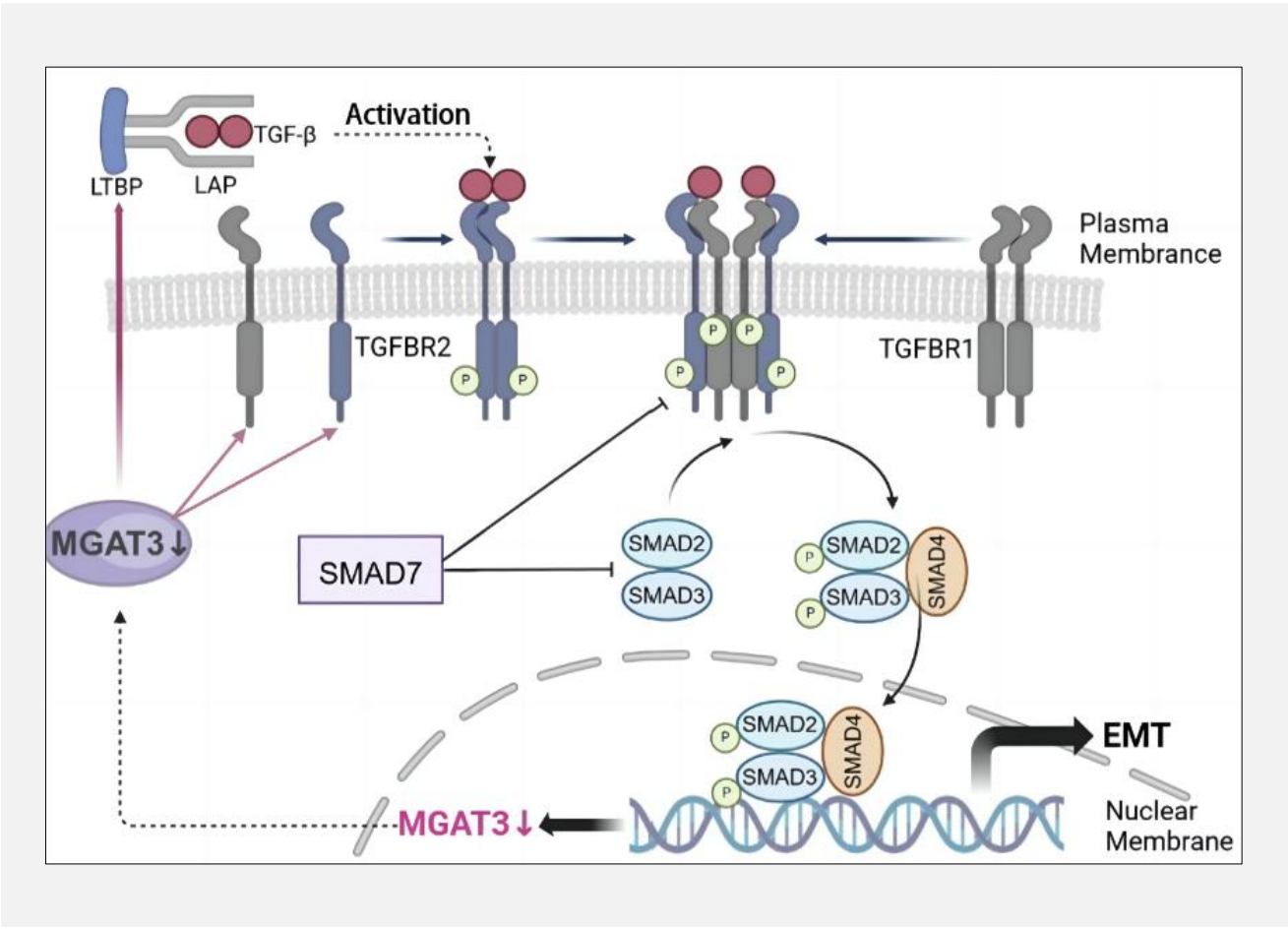


Figure 7. Loss of MGAT3 contributes to TGF-β-driven progression of BOS.

To understand how MGAT3 loss enhances TGF-β signaling, we can explore the specific pathway involved: a) TGF-β storage and activation: During GVHD, increased TGF-β1 expression occurs. Latent TGF-β is covalently bound to LTBP via disulfide bonds. Upon activation, TGF-β is released from LTBP. Free TGF-β binds to TGFBR2, which then phosphorylates itself and recruits and phosphorylates TGFBR1. The phosphorylated TGFBR1 interacts with the TGF-β/TGFBR2 complex, forming a signaling complex. b) Signal transduction: This complex activates SMAD2/3 signaling cascades. Activated SMAD2/3 downregulate MGAT3 expression and promote epithelial-to-mesenchymal transition (EMT). c) Feedback loop and EMT: Loss of MGAT3 increases TGF-β storage in the extracellular matrix (ECM) and enhances the availability of TGF-β receptors, facilitating activation of the TGF-β signaling pathway. This creates a positive feedback loop, further amplifying TGF-β effects and promoting airway fibrosis associated with BOS.

downregulated in BOS (Figure 2C). Among the 10 downregulated genes, MGAT3 exhibited the most significant reduction (Table S1). Given that TGF-β1 promotes fibrosis by activating EMT and that TGF-β signaling can downregulate MGAT3 expression [28,29], we hypothesized that the TGF-β pathway might also be activated during the GVHD process, contributing to BOS-specific abnormal glycosylation. Intriguingly, compared to normal mice, MGAT3 protein expression and bisecting-GlcNAc levels (detected by PHA-E binding) were significantly downregulated in the peripheral lung tissue of GVHD mice, while TGF-β1 expression exhibited an opposite trend (Figure 2D). These findings suggest that the low abundance of bisecting-GlcNAc N-glycans in BOS BALF is likely attributable to activated TGF-β signaling during cGVHD progression.

TGF-β signal activation promotes EMT and downregulates MGAT3 expression in BEAS-2B cells

TGF-β1-induced EMT in BEAS-2B cells, accompanied by a specific downregulation of MGAT3, led to a reduction in bisecting GlcNAc N-glycans. PHA-E staining in Figure 3A confirms the presence of bisecting GlcNAc N-glycans in BEAS-2B cells. Treatment with TGF-β1 for 48 hours induces EMT, as evidenced by the upregulation of the mesenchymal marker (Vimentin) and downregulation of the epithelial marker (E-cadherin) in Figure 3B. Concurrently, TGF-β1 treatment significantly downregulates the expression of MGAT3, an enzyme crucial for bisecting GlcNAc N-glycan synthesis, as shown in Figure 3C. Notably, the expression levels of other branching-glycosyltransferases (MGAT4A, MGA T4B, and MGAT5) remain unchanged during TGF-β1-induced EMT, as depicted in Figure 3D. These findings

suggest that the downregulation of MGAT3 is a specific event associated with TGF-β1-induced EMT in BEAS-2B cells, highlighting the potential role of MGAT3 in regulating glycosylation during BOS-associated EMT.

MGAT3 loss facilitates the EMT process in BEAS-2B cells

MGAT3 has been implicated in various diseases, including its role in suppressing hypoxia-induced EMT in breast cancer [30,31]. Our findings suggest a similar suppressive role for MGAT3 in airway epithelial fibrosis. Using shRNA lentiviral vectors, we successfully reduced MGAT3 expression in BEAS-2B cells, confirmed by decreased bisecting-GlcNAc N-glycans staining with PHA-E fluorescence (Figure 4A). While MGAT3 knockdown had minimal impact on cell proliferation (Figure 4B), it resulted in a modest increase in cell migration and a decrease in cell adhesion (Figure 4C). Notably, MGAT3 knockdown significantly enhanced cell invasion (Figure 4D), suggesting a potential role for MGAT3 in regulating EMT processes, similar to its function in breast cancer [32].

MGAT3 loss activates TGF-β signaling transduction

MGAT3 knockdown (KD) indeed downregulated E-cadherin expression and significantly upregulated Vimentin expression (Figure 5A). However, whether MGAT3 loss can activate TGF-β signaling has not been explored. We conducted a correlation analysis between MGAT3 expression and 27 key genes involved in TGF-β1 signaling (GTEx database), including cell membrane receptors, signaling proteins, and transcription factors. Interestingly, 16 of these genes displayed an opposite correlation with MGAT3 compared to TGF-β1 (Figure 5B and Figure S3). Furthermore, MGAT3 knockdown led to increased phosphorylation of SMAD2/3, a key process during the activation of the TGF-β signaling pathway (Figure 5B). Inhibitory SMADs (I-SMADs), including SMAD6 and SMAD7, are often seen as negative feedback regulators in TGF-β signaling. Smad7 is a broad-spectrum inhibitory protein of the TGF-β family, while Smad6 specifically inhibits BMP signaling [33]. Here, we also investigated the impact of MGAT3 KD on SMAD6 and SMAD7 expression. Notably, MGAT3 KD increased SMAD7 expression (Figure 5C), but not SMAD6, suggesting it indeed activated the TGF-β signaling pathway. These findings highlight its potential as a therapeutic target not only for cancer but also for fibrotic diseases.

MGAT3 deficiency increases latent TGF-β storage and the availability of TGF-β receptors to promote TGF-β-induced EMT

To further understand how MGAT3 regulates TGF-β signaling, we investigated its impact on both TGF-β1 expression and secretion in BEAS-2B cells, a key prerequisite for cytokine function [34]. WB analysis revealed that MGAT3 knockdown (KD) increased cellular TGF-β1 expression, and ELISA results showed a significant increase in secreted TGF-β1 in the cell supernatant (Figure 6). This suggests that MGAT3 loss may increase the availability of TGF-β1 for signal activation. Latent TGF-β binding protein 1 (LTBP1) forms complexes with latent TGF-β, acting as a reservoir for its controlled release upon stimulation and is critical for storing TGF-β in the extracellular matrix (ECM) [35]. We found that the LTBP1 protein has at least three potential N-glycosylation sites (Figure 6A). Figure 6B showed that the expression of LTBP1 in BEAS-2B cells increased when MGAT3 was absent. This suggests that in the absence of MGAT3, more LTBP1 might be available to bind and protect TGF-β from degradation, leading to a larger pool of available TGF-β and further amplifying its EMT-promoting effects [35].

Beyond expression and storage, another critical factor influencing cytokine activity is receptor availability. We investigated the impact of MGAT3 KD on the expression of key TGF-β receptors, TGFBR1 and TGFBR2. MGAT3 loss appeared to increase TGFBR1 and TGFBR2 expression, potentially making more receptors available for TGF-β binding (Figure 6C). The increase in phosphorylation levels of TGFBR1 also indicated the enhancement of TGF-β signaling (Figure 6C). Indeed, research on glycosylation modifications and the TGF-β signaling pathway primarily focuses on receptor localization or expression [33]. Immunofluorescence (IF) indicated that when MGAT3 was absent, the expression of TGF-β receptors increased, suggesting that more receptor proteins are localized to the cell membrane for binding ligands (TGF-βs) to exert their effects.

The reciprocal regulation between TGF-β and MGAT3 contributes to BOS progression

In this study, we innovatively discovered a characteristic N-glycopattern in the BALF of BOS patients: reduced expression of bisecting GlcNAc N-glycans. This finding may be attributed to enhanced TGF-β signaling during graft-versus-host disease (GVHD) progression. To our knowledge, this is the first study to characterize glycosylation patterns in BOS BALF and to investigate the underlying mechanisms driving these alterations. While it is known that TGF-β signaling can downregulate the expression of MGAT3 and subsequently reduce bisecting GlcNAc N-glycans, our study demonstrates that MGAT3 itself can serve as a critical regulator of TGF-β signaling. MGAT3 deficiency triggers a cascade of events in airway epithelial cells, including EMT and ultimately leading to airway fibrosis. Specifically, MGAT3 deficiency exerts multiple effects: a) it increases LTBP1 expression, creating a reservoir for sustained TGF-β storage and release and b) it elevates the availability of TGF-β receptors. These combined effects render epithelial cells more susceptible to TGF-β's influence. Heightened responsiveness to TGF-β fuels a positive feedback loop, further amplifying TGF-β signaling, ultimately driving EMT and fibrosis (Figure 7). Importantly, TGF-β signaling itself downregulates MGAT3 expression, perpetuating this cycle and solidi-

fying the fibrotic cascade, contributing to the aggressive progression of BOS.

DISCUSSION

Bronchiolitis obliterans syndrome (BOS) is a chronic complication following allogeneic hematopoietic stem cell transplantation (allo-HSCT) that significantly reduces patients' quality of life due to severe airway narrowing [36]. Unfortunately, there is no effective treatment to reverse BOS, and existing anti-fibrotic drugs, such as pirfenidone and nintedanib, can only partially slow the progression of fibrosis [37,38]. Therefore, early diagnosis and intervention are essential to reduce the mortality and morbidity of BOS before the disease leads to irreversible structural changes in the lung. Our research demonstrated that bisecting GlcNAc N-glycans are significantly reduced in bronchoalveolar lavage fluid (BALF) from BOS patients compared to non-BOS patients. This downregulation may be a consequence of persistent TGF-β signaling. MGAT3, the glycosyltransferase responsible for the synthesis of bisecting GlcNAc, is known to suppress tumor metastasis [26], and has also been implicated in regulating airway fibrosis associated with BOS. These findings suggest that alterations in pulmonary glycosylation may reflect disease progression. Given that BALF is a fluid sample that closely mirrors the characteristics of the lung epithelium, monitoring its glycan profiles could offer valuable insights into tracking disease progression. However, a notable limitation of our study is the relatively small sample size. Moving forward, we aim to increase the sample size and further investigate the association between specific N-glycan levels and the progression of BOS.

Currently, drugs targeting the TGF-β signaling pathway primarily include antibodies that inhibit the binding of TGF-β to its receptors, TGF-β receptor fusion proteins, and small molecule kinase inhibitors of TGF-β. However, the clinical application of these drugs is limited, mainly due to the potential for serious adverse reactions, which has made it difficult for these candidates to gain market approval. Our study proposes a novel mechanism by which MGAT3 regulates this TGF-β signaling-induced epithelial-to-mesenchymal transition (EMT). The loss of MGAT3 amplifies TGF-β's effects on airway epithelial cells by increasing TGF-β storage and receptor availability. LTBP1 is a key protein that stores latent TGF-β and works with TGF-β receptors to regulate the activation of the TGF-β pathway [39]. In the future, we hope to overcome existing challenges by developing drugs based on glycosylation regulation, which could offer a better approach for drug development.

For example, modulating glycosylation on critical proteins might allow for more specific regulation of their functions while avoiding some unavoidable systemic adverse reactions.

As mentioned earlier, the presence of bisecting-GlcNAc inhibits N-glycan biosynthesis directed by MGAT4 and MGAT5. A reduction in MGAT3 activity would weaken these inhibitory effects, potentially enhancing MGA T5 function. Studies showed that MGAT5 in mouse hepatic stellate cells upregulates galectin-3 expression and inhibits TGF-β receptor sensitivity [40,41]. Research has shown that MGAT3 plays a crucial role in inhibiting EMT, in contrast to MGAT5 [42]. These findings are consistent with our findings. Mechanistically, various glycosyltransferases, including MGAT3, form different polysaccharide complexes that affect the disease process. This suggests that inhibiting their corresponding gene expression is a potential therapeutic approach. The complex interactions between MGAT3 and other glycosyltransferases, however, still require further exploration.

In this study, we dissected a previously undescribed mechanism by which abnormal N-glycosylation regulates the progression of BOS. We confirmed that a significant feature of BALF from BOS patients is the downregulation of bisecting-GlcNAc N-glycans, which is attributed to the downregulation of the key glycosyltransferase, MGAT3. Moreover, the deficiency of MGAT3 further amplifies TGF-β signaling, stimulating the occurrence of EMT (epithelial-mesenchymal transition) and ultimately leading to exacerbated airway fibrosis in BOS patients. This study was primarily based on N-glycosylation analysis. Our future work will also explore the potential role of O-glycosylation in BOS. We'll investigate whether MGAT3-associated TGF-β signaling affects O-glycopatterns in proteins and RNAs. To do this, we will leverage our established methods for solid-phase O-glycosylation analysis using single or multiple O-glycoproteases [22,43,44].

CONCLUSION

This study unveils a novel mechanism regulating BOS progression, emphasizing the intricate interplay between MGAT3 downregulation, altered glycosylation, and TGF-β signaling. Bronchoalveolar lavage fluid (BALF) from BOS patients exhibited a unique glycopattern characterized by significantly reduced bisecting GlcNAc N-glycans, which correlated with downregulated MGAT3 expression. Functional assays further validated MGAT3's role as an EMT suppressor. Importantly, the loss of MGAT3 significantly impacts TGF-β signaling through several intricate mechanisms, including regulating the expression of the key TGF-β binding protein LTBP1 and influencing the availability of TGF-β receptors. These combined effects amplify TGF-β's influence, further promoting EMT and fibrosis associated with BOS. In conclusion, our findings establish MGAT3 as a critical regulator in TGF-β-driven EMT in BOS, highlighting its significant therapeutic potential for this debilitating condition and potentially other fibrotic diseases.

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Supporting Information:
Experimental methods are listed in the supplementary information, including cell culture and cellular EMT establishment, lentiviral transfection, CCK-8, would healing, cell adhesion, cell invasion, Western blotting, ELISA, Human Protein Atlas analysis, GO and KEGG analysis, statistical analysis. Figure S1 - experimental design; Figure S2 - bisecting-GlcNAc biosynthesis; Figure S3 - MGAT3 and TGF-β correlation; Table S1 – Glycosyltransferase expression.

Ethical Approval:
This research study adhered to the ethical guidelines of the Institutional Review Board (IRB) at the Fourth Affiliated Hospital to Soochow University, with approval obtained prior to sample collection. All participants provided written informed consent before participating in the study.

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Availability of Data and Materials:
All data necessary to replicate this study's findings have been included in the paper.

Declaration of Interest:
The authors have no conflicts of interest to declare.

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