Bisecting GlcNAc expression by bone marrow stromal cells modulates TGF-β1-driven macrophage polarization in myeloid leukemias

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Abstract

There has been growing evidence highlighting the critical role of tumor-associated macrophages (TAM) in promoting immune evasion and disease progression in acute myeloid leukemia (AML) and myelodysplastic syndromes (MDS). Combined single-cell RNA sequencing, flow cytometry, and immunohistochemistry studies of the innate immune compartment in bone marrow of MDS/AML reveal a shift toward a tumor-supportive M2-polarized macrophage as well as the expression of programmed cell death-ligand 1 (PD-L1) in this cell lineage. We found leukemic stroma cells with a high level of TGFβ1 secretion can determine TAM toward M2-polarized subtype. Further mechanistic investigations revealed that bone marrow (BM) stromal cells with specific glycans, reduced bisecting N-acetylglucosamine (GlcNAc) levels, in MDS/AML promoted M2-polarized subtype through the secretion of TGFβ1, which elevated PD-L1 expression and thereby impaired $CD8^+$ T-cell function. Our study provides insights into the mechanisms of selectively modifying specific glycans in BM stroma cells and how these may contribute to targeting strategies aimed at the tumor microenvironment.

Introduction

Tumor-associated macrophages (TAM) represent a large fraction of infiltrating immune cells within the tumor microenvironment (TME) in human cancers. They are considered a potentially effective therapeutic target since they drive tumor progression, metastasis, and recurrence via multiple mechanisms. A common strategy for characterizing macrophage function in tumors has been the "M1" versus "M2" classification, originally based on *in vitro* polarization using Th1 and Th2 cytokines.[1] Although widely used M1 (pro-inflammatory) and M2 (anti-inflammatory) nomenclature does not capture the heterogeneity and dynamic functional complexity of TAM within the TME,[2-5] increasing efforts are being directed toward understanding TME-induced macrophage recruitment and polarization.[6] This is essential for advancing our understanding of TAM-mediated pro-tumor outcomes and for exploring the development of novel therapeutic strategies.[7]

Tumors have evolved multiple mechanisms to evade immune surveillance, including the blockade of antigen presentation and the recruitment of immunosuppressive cells.[8] One of the most critical immune checkpoint pathways is the programmed cell death protein 1/programmed cell death-ligand 1 (PD-1/PD-L1) axis, which restrains the hyperactivation of immune cells, making it a potential therapeutic target in hematologic malignancy including myelodysplastic syndromes (MDS) and acute myeloid leukemia (AML).[9] Notably, PD-L1 expression on TAM amplifies immune suppression, thereby facilitating tumor progression and underscoring the importance of targeting this pathway in these malignancies.[10,11] Thus, investigating the crosstalk and modulating immune cells within TME could provide effective strategies for immunotherapy.

Previous studies indicated M2-tumor supportive macrophages are highly enriched and promote disease progression in MDS and AML. Mussai *et al*. identified that increased Arginase 2 activity released from AML blast cells promotes TAM to polarize towards M2 phenotype, inhibiting T-cell proliferation.[12] Yu *et al*. found higher fractions

of M2 TAM in MDS patients with inferior survival.[13] In AML murine models, increased infiltration of TAM with pro-leukemogenic functions was observed and the transcriptional repressor Gfi1 was identified as a regulator of M2 polarization.[14] Further studies demonstrated that AML-derived TAM exhibit immunosuppressive and pro-leukemogenic functions.[15] These findings suggest that TAM can suppress local immunity and contribute to tumor immune escape and progression. However, the mechanisms by which TAM are educated in the leukemic TME remain elusive.

There is growing evidence to suggest that bone marrow (BM) stroma cells contribute to progression of myeloid malignancies by remodeling TME, which may promote cell malignancy, and inhibit the proliferation or activation of immune cells, including B cells, T cells and macrophages.[16,17] A previous study has shown that stroma cell-derived CXCL12 promotes TAM to M2 polarization, thus affecting their function in tumorigenesis.[18] Donor stromal cells have been found to reprogram host macrophages, restoring the BM microenvironment and inhibiting leukemia development.[19] However, the precise role of stromal cells in TAM polarization is not well elucidated, and the specific mechanisms by which the modulation of stromal cells can influence immune responses remain insufficiently understood.

In this study, we observed a significantly higher proportion of M2 TAM in the BM of MDS/AML compared to that in healthy donors (HD). This increase in M2 TAM was accompanied by elevated PD-L1 expression, which subsequently impaired the function of $CD8^+$ T cells. This phenomenon is associated with the aberrant glycosylated levels in leukemic stromal cells. Furthermore, we demonstrate the mechanisms by which stromal cells acclimate M2 TAM to facilitate tumor immune escape through increased secretion of TGFβ1, highlighting a promising target for MDS/AML immunotherapy.

Methods

Bone marrow sample processing and cell identification

Unless otherwise specified, BM stroma in the study refers to BM stroma derived from AML/MDS patients or HD. Briefly, BM samples were obtained via BM puncture using anticoagulant tubes, and mononuclear cells were separated by Ficoll Hypaque gradient centrifugation. The samples were stained with the following antibodies: CD14-FITC (cat. #11014941), CD68-PE (cat. #12068941), CD163-PEcy7 (cat. #25163942), CD45-APC (cat. #MHCD45054), CD3-PE (cat. #12003842), CD8-FITC (cat. #12003842), and isotype control antibodies (#31903/#31235, eBiosciences, San Diego, CA, USA). The stained cells were analyzed by flow cytometry (FACS; ACEA Biosciences, San Diego, CA, USA). M2 TAM were identified as $CD14^+CD68^+CD163^+$ cells, while $CD8^+$ T cells were defined as $CD45^+CD3^+CD8^+$ cells. Written informed consent was obtained from all patients in accordance with the principles of the Declaration of Helsinki. The study was approved by the Medical Ethics Committee of Northwest University (approval number: 230306006, 6 March 2023).

Mouse model

All animal experiments were randomized and conducted in accordance with the Guide for the Care and Use of Laboratory Animals. Animal experiments were conducted following guidelines under the Animal Care and Use Committee at Northwest University (approval number: NWU-AWC-20231202M, 2 December 2023). The generation of conditional $MGAT3^{loxP/loxP}$ mice has been previously described.[20] For *in vivo* experiments, clodronate liposomes (250 μL) were infused intravenously to eliminate macrophages in the BM of 6-8-week-old C57BL/6 mice.[21] A total of $2x10^6$ RAW264.7 cells, either pre-treated with 10 ng/μL TGFβ1 recombinant protein (rTGFβ1) for 48 hours (hr), or pre-treated with conditioned medium from mNBM stroma or mNBM stroma-shM3 cells for 48 hr, along with $6x10^6$ M1-GFP cells, were co-transplanted via tail vein.

One-week post injection, mice were administered intraperitoneally with 7.5 mg/kg anti-mouse PD-L1 antibody (BioXcell, West Lebanon, NH, USA) or rat IgG isotype control on days 0, 3, 6, 9, 12, and 15. Peripheral blood was analyzed on days 10, 17, and 24 to determine the proportion of GFP-labeled M-1 (M-1-GFP) cells by flow cytometry. Subsequently, mice were euthanized, and the spleen and BM were collected.

Statistical analysis

The GraphPad Prism V.8.0 software (GraphPad Software; La Jolla, CA, USA) was used for statistical analysis. Data between two groups were compared using Student *t* test for normally distributed variables (mean ± standard error mean) or the Mann-Whitney U test for non-normally distributed variables (median and interquartile range [IQR)]. $P<0.05$ was considered statistically significant. All experiments were performed in triplicate.

Results

Alteration of tumor-associated macrophages associated immunity in the myelodysplastic syndrome/acute myeloid leukemia microenvironment

As crucial immune cells within the TME, TAM display substantial heterogeneity and complexity in their functional roles and molecular characteristics.[22] We analyzed the scRNA-seq dataset (GSE116256) from BM cells of 16 AML patients and 4 HD and identified 10 distinct cell populations, including AML-cell/normal-progenitor, B cells, $CD8^+$ T cells, $CD4^+$ T cells, dendritic cells, erythrocytes, monocyte-macrophages, natural killer (NK) cells, plasmacytoid dendritic cell, and plasma cells (Figure 1A, *Online Supplementary Figure S1A*). To assess macrophage

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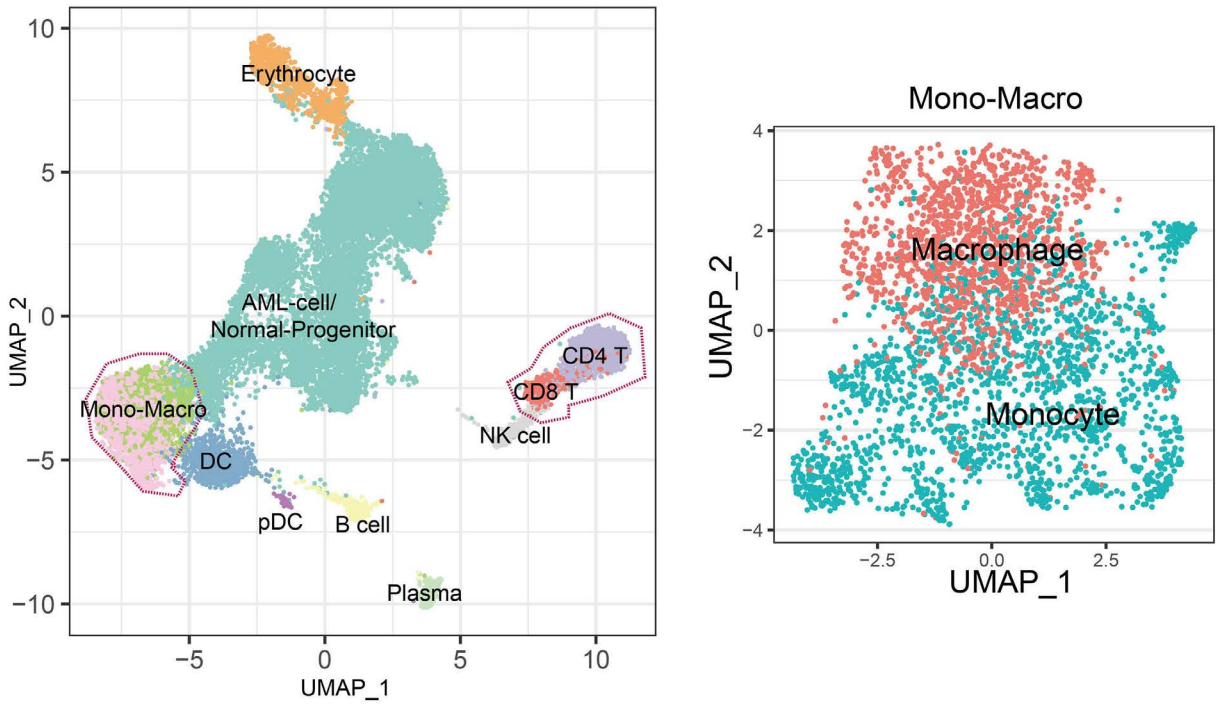

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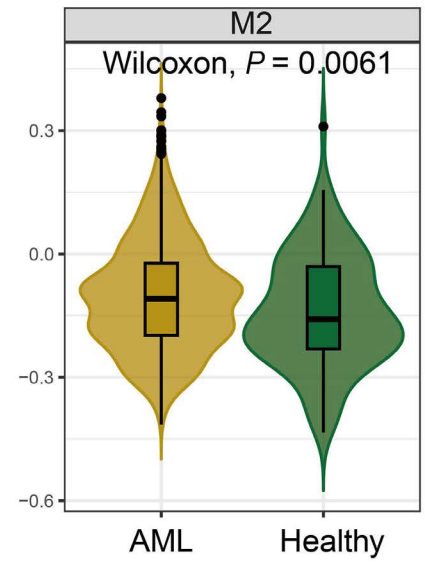

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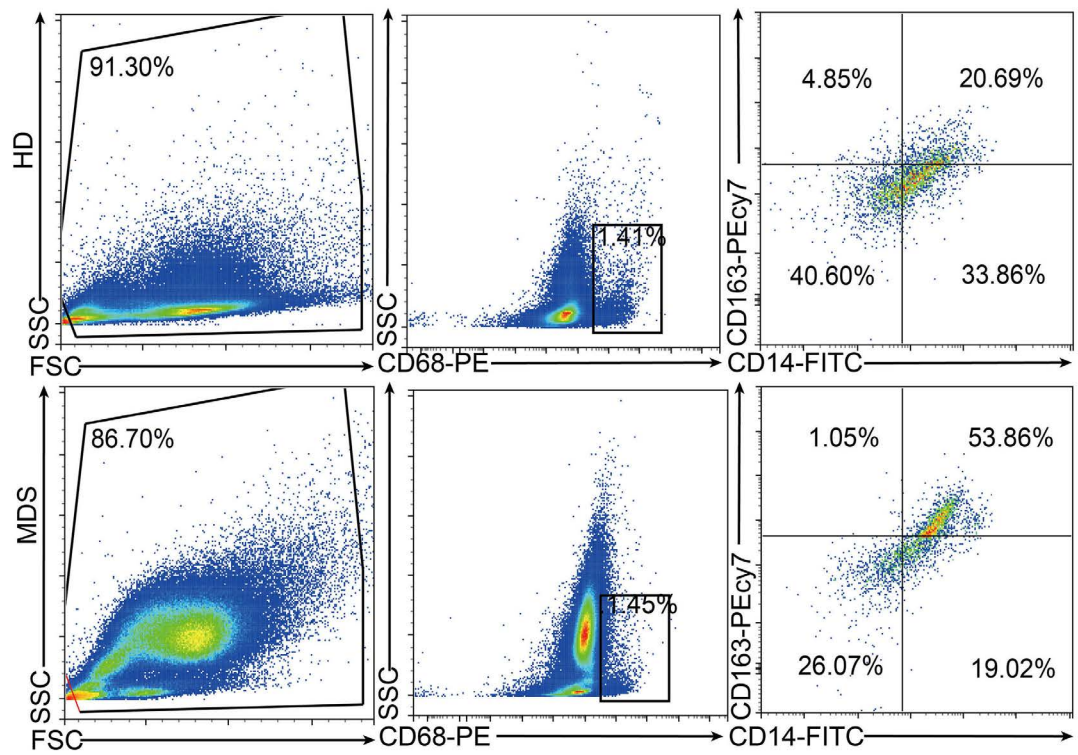

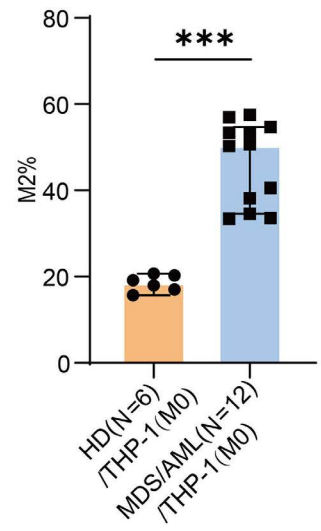

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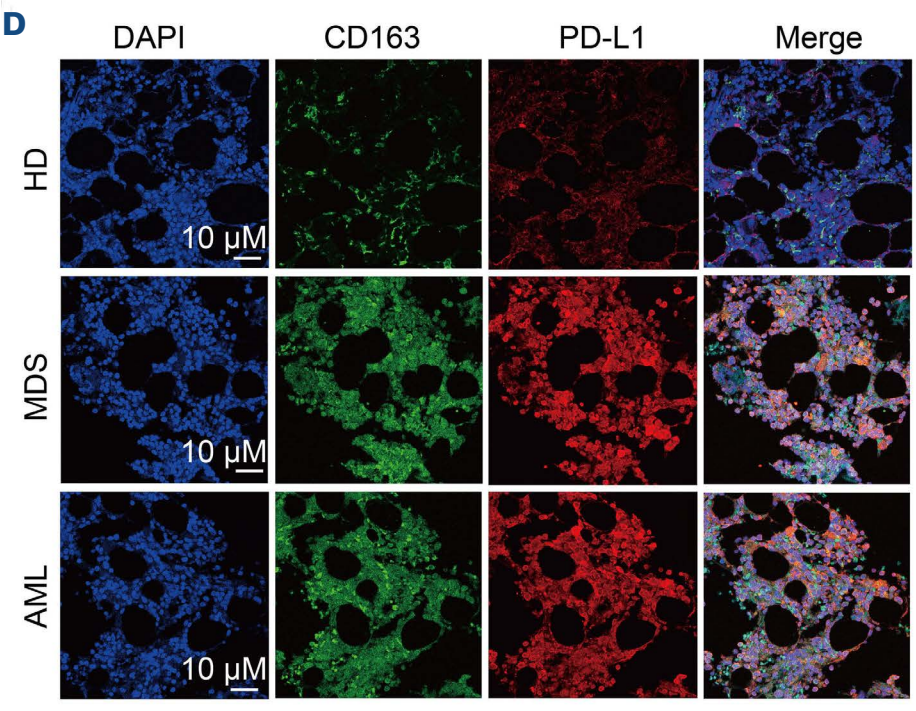

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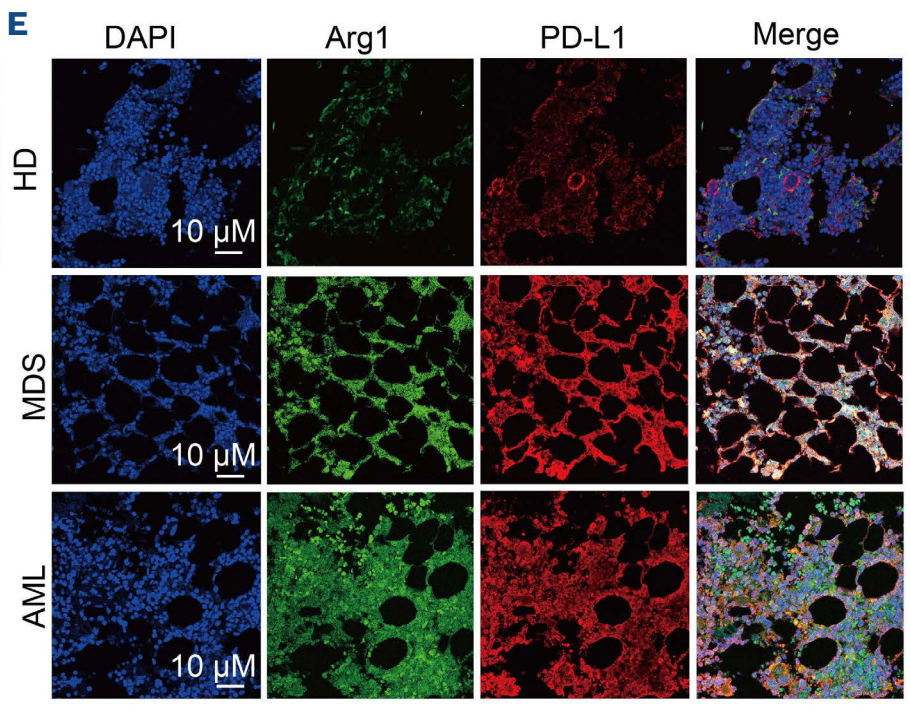

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Figure 1. Alteration of tumor-associated macrophage associated immunity in the myelodysplastic syndromes and acute myeloid leukemia tumor microenvironment. (A) Uniform Manifold Approximation and Projection (UMAP) of bone marrow (BM) cells from 16 acute myeloid leukemia (AML) samples and 4 healthy donors (HD), revealing 10 major clusters. Each dot represents a single cell, colored by cluster. (B) Macrophage (M2) functional scores from single-cell RNA sequencing (scRNA-seq) data in the BM of HD and AML samples, assessed using gene set variation analysis (GSVA). The Wilcoxon rank-sum test was used for statistical comparison. (C) Fluorescence-activated cell sorting (FACS) analysis of M2 tumor-associated macrophages (TAM; $CD14^+$ $CD68^+$ $CD163^+$ cells) in HD (N=6) and patients with myelodysplastic syndromes (MDS; N=6) or AML (N=6). (D, E) Confocal microscopic analysis of CD163, Arginase 1 (Arg1), and programmed death-ligand 1 (PD-L1) expression in the BM of HD and patients with MDS (N=6) or AML (N=6). Data shown are mean ± Standard Error of Mean from three independent experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

functional characteristics, we applied Gene Set Variation Analysis (GSVA) using a predefined macrophage function-related marker list.[23] The results showed a higher M2 macrophage functional score in AML samples compared to healthy controls, indicating that macrophages in AML samples tend to exhibit M2-associated functions (Figure 1B, *Online Supplementary Figure S1C*). Uniform Manifold Approximation and Projection (UMAP) visualization also showed the expression of several common M2-related genes in the macrophage population, with high expression of CD163, MRC1, and CD36 (*Online Supplementary Figure S1B*). Additionally, we observed a decrease in the proportion of $CD8^+$ T cells in AML samples, suggesting a potential immunosuppressive microenvironment (*Online Supplementary Figure S1D*).

We next analyzed BM monocytes from MDS/AML patients and found a significantly higher proportion of M2-like TAM ($CD14^+CD68^+CD163^+$ cells) compared to HD (Figure 1C). Interestingly, we also found an increased level of M2 marker CD163 and Arginase 1 (termed Arg1), but a decreased level of M1 marker CD80, accompanied by enhanced expression of PD-L1, a major immune checkpoint ligand, in the BM of MDS/AML patients (Figure 1D, E, *Online Supplementary Figure S1E*). PD-L1 was co-localized with CD163 in BM of MDS/AML patients as well (Figure 1D).

Substantial evidence has revealed pro-tumoral TAM can either directly inhibit CD8 cytotoxic T lymphocyte (CTL) responses or indirectly regulate immunosuppression by reshaping the immune microenvironment.[24] Consistently, our data indicated that AML patients exhibit a significant increase in the proportion of M2 macrophages but a decrease in $CD8^+$ T cells compared to HD (*Online Supplementary Figure S1F, G*). Collectively, these data suggest that PD-L1 may mediate immunosuppression through the interaction between TAM and $CD8^+$ T cells. This crosstalk may be responsible for the alterations in immunity within the MDS/AML TME.

Bone marrow stroma cells regulated M2 polarization via secretion of TGFβ1

To mimic the MDS/AML BM microenvironment, we established a co-culture model of interactions between macrophages, BM stromal cells, and T cells (*Online Supplementary Figure S2A*). When educated by BM stroma cells of MDS/AML patients, characterized by $CD44^+CD90^+CD105^+CD146^+CD45^-$ (*Online Supplementary Figure S2B*), THP-1 (treated with 100 ng/mL PMA for 48 hr, termed as "M0" macrophages) were polarized to M2 subtypes, shown as decreased expression of TNF-α and enhanced expression of CD163 (Figure 2A). The expression of PD-L1 was also increased in M2-like macrophages (Figure 2A). In tumor eradication, $CD8^+$ T cells play a key role by secreting cytokines like IFN-γ and tumor necrosis factors (TNF), boosting their cytotoxicity and recruiting/activating other immune cells to amplify the anti-tumor response.[25] When THP-1 ("M0") were educated by BM stroma from MDS/AML patients, and then co-cultured with activated $CD8^+$ T cells, the production of TNF-α and IFN-γ by $CD8^+$ T cells were decreased (Figure 2B), suggesting an immunosuppressive effect in this co-culture system, potentially mediated by PD-L1.

Using quantitative proteomic analysis, we identified 150 differentially secreted proteins in BM stroma from MDS/AML patients compared to HD, including 29 up-regulated and 121 down-regulated proteins (Figure 2C, *Online Supplementary Figure S2C*). Notably, the expression of latency-associated peptide (LAP)-TGFβ1 (termed "L-TGFβ1"), the precursor of mature TGFβ1 (termed "m-TGFβ1"), was significantly increased in stroma from MDS/AML patients (*Online Supplementary Figure S2D*). The up-regulated m-TGFβ secreted by clinical stroma cells was further confirmed (Figure 2D). m-TGFβ1 regulates the polarization of TAM to M2 by activating both SMAD and non-SMAD (such as p-ERK) signaling pathways upon binding to the TGFβ receptor complex.[26] When THP-1 ("M0") were treated with rTGFβ1, PD-L1 and M2 marker CD163 were increased in macrophages, and p-Smad3 and p-ERK signaling pathways were activated (*Online Supplementary Figure S2E-J*), suggesting rTGFβ1 can promote M2 polarization via Smad and ERK signaling pathways. Co-culture with leukemic BM stroma can also stimulate THP-1 ("M0") into M2 subtypes and increase PD-L1 level (*Online Supplementary Figure S2H*). Furthermore, TNF-α and IFN-γ production was down-regulated in $CD8^+$ T cells when co-cultured with rTGFβ1-treated macrophages, but up-regulated when PD-L1 was neutralized by antibodies (*Online Supplementary Figures S2I, S3A*).

Subsequently, we investigated whether TGFβ1-induced TAM polarization would simultaneously affect PD-L1 expression and ultimately impact the efficacy of T-cell-mediated tumor cell killing *in vivo* (*Online Supplementary Figure S3B*). In BM macrophage-depleted mice (*Online Supplementary Figure S3C*), the number of GFP-labeled murine myeloid

leukemia cells (M-1-GFP) in peripheral blood were increased when co-transplanted with rTGFβ1-treated RAW264.7 (Figure 2E). Conversely, in the cohort treated with rTGFβ1, subsequent administration of the a-mPD-L1 antibody significantly reduced the population of M-1-GFP cells (Figure 2E). Simultaneously, TNF-α and IFN-γ production in plasma, as well as the number of $CD8^+$ T cells in BM was down-regulated in mice co-transplantation with rTGFβ1-treated RAW264.7, but up-regulated in mice that received the a-mPD-L1 antibody treatment (Figure 2F, *Online Supplementary Figure S3D*). These data suggest that BM stroma may contribute to M2 polarization of TAM and enhance its PD-L1 expression via secreting TGFβ, thereby influencing the proliferation of leukemic cells.

Bisecting GlcNAc level of bone marrow stroma affected tumor-associated macrophage polarization

We previously reported that decreased levels of bisecting GlcNAc remodeled the BM niche by modulating melanoma cell adhesion molecule (MCAM) on stromal cells, and thereby affected proliferation of MDS/AML clonal cells.[20] Here, we found that increased CD163 level and decreased CD80 level, accompanied with enhanced PD-L1 expression which was co-localized with CD163 in the BM of *LepR-cre*; $MGAT3^{fl/fl}$ mice (Figure 3A, B). In contrast, when normal BM (NBM) stroma were treated with forskolin (FSK), an adenylyl cyclase activator that enhances bisecting GlcNAc levels (*Online Supplementary Figure S4A*), then co-cultured with THP-1 ("M0"), we observed an elevation in the expression of M1 markers, including CD86, CD80 and TNF-α, but a downregulation in the expression of PD-L1 and M2 markers, including CD163, IL10 and Arg1 in macrophages (*Online Supplementary Figure S4B-E*). Furthermore, TNF-α and IFN-γ production of $CD8^+$ T cells displayed a dramatic increase when co-cultured with FSK-NBM stroma-educated macrophages (*Online Supplementary Figure S4F*).

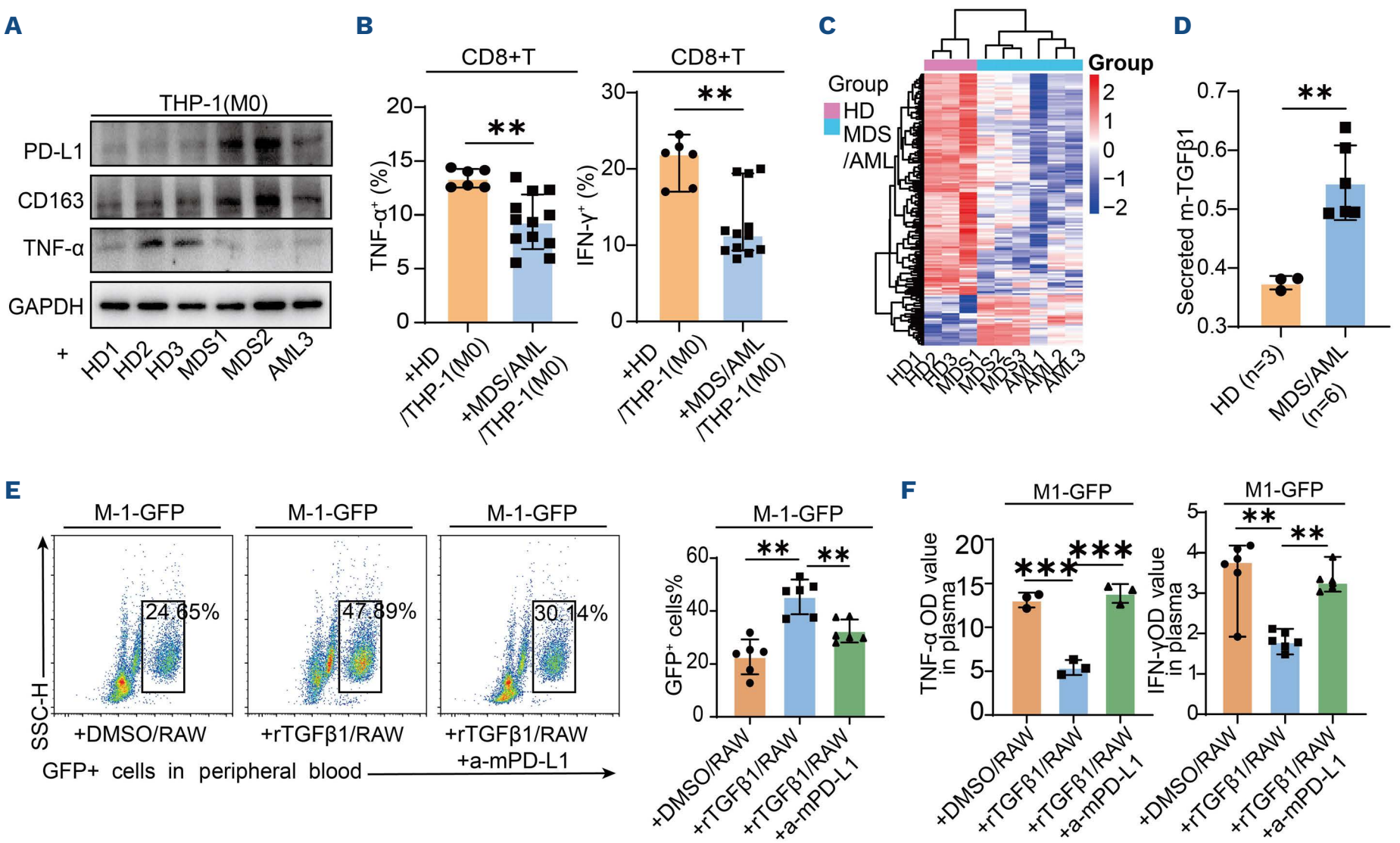

Figure 2. Mature transforming growth factor β1 secreted from bone marrow stroma cells of myelodysplastic syndromes and acute myeloid leukemia regulates tumor-associated macrophage polarization via Smad3 and ERK signaling. (A) Expression of Programmed death-ligand 1 (PD-L1), CD163, and tumor necrosis factor-α (TNF-α) in THP-1-derived undifferentiated macrophages (M0) after co-culture with bone marrow (BM) stroma cells from healthy donors (HD) or myelodysplastic syndromes (MDS) / acute myeloid leukemia (AML) patients. (B) $CD8^+$ T cells from HD were co-cultured with BM stroma-educated macrophages for 48 hours. TNF-α and interferon-γ (IFN-γ) production in $CD8^+$ T cells were determined by fluorescence-activated cell sorting (FACS). (C) Heatmap of differentially secreted proteins in BM stroma, with red indicating upregulation and blue indicating downregulation. (D) Secreted mature transforming growth factor β1 (m-TGFβ1) levels in the conditioned medium from BM stroma. (E) FACS analysis of M-1-GFP cells (GFP-labeled murine AML cell line) in peripheral blood after co-injection with RAW264.7 macrophages (RAW) pretreated with recombinant TGFβ1 (rTGFβ1) or vehicle (dimethyl sulfoxide, DMSO), followed by anti-mouse PD-L1 antibody (a-mPD-L1) treatment. (F) Relative levels of TNF-α and IFN-γ in mouse plasma. Data shown are mean ± Standard Error of Mean from three independent experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Similarly, when macrophages derived from primary normal monocytes (called "MDM") were co-cultured with FSK-NBM stroma, the expression of CD163 and PD-L1 was notably reduced, while CD80 expression increased (Figure 3C-E). Additionally, TNF-α and IFN-γ production by $CD8^+$ T cells was greatly enhanced when co-cultured with FSK-NBM stroma-educated MDM (Figure 3F).

To further validate this biological effect, we used two stromal cell lines, HS5 and HS27a, with different levels of bisecting GlcNAc. While their intrinsic differences may influence immune responses, they still provide supportive evidence for our findings. The expression of M1 markers was up-regulated, but the expression of PD-L1 and M2 markers was down-regulated in macrophages co-cultured with HS27a-M3 stroma cells, which expressed high bisecting GlcNAc level (*Online Supplementary Figure S4G-K*). The production of TNF-α and IFN-γ in $CD8^+$ T cells was also up-regulated (*Online Supplementary Figure S4L*). Conversely, when macrophages were co-cultured with HS5-shM3 cells, a MGAT3-knockdown variant (*Online Supplementary Figure S4M*), the opposite effects were observed (*Online Supplementary Figure S4N-R*). Upregulation of bisecting GlcNAc levels in stromal cells suppressed IL10 secretion in macrophages, whereas decreasing bisecting GlcNAc levels yielded the opposite results (*Online Supplementary Figure S5A*).

These findings clearly demonstrate that upregulation of bisecting GlcNAc levels in the stroma hinder the polarization of TAM toward the M2 phenotype and concurrently reduce their PD-L1 expression.

Bisecting GlcNAc modification of L-TGFβ1 affect M2 polarization

In BM stroma from MDS/AML patients, bisecting GlcNAc expression is down-regulated, while L-TGFβ1 expression is elevated (Figure 4A), indicating that L-TGFβ1 expres-

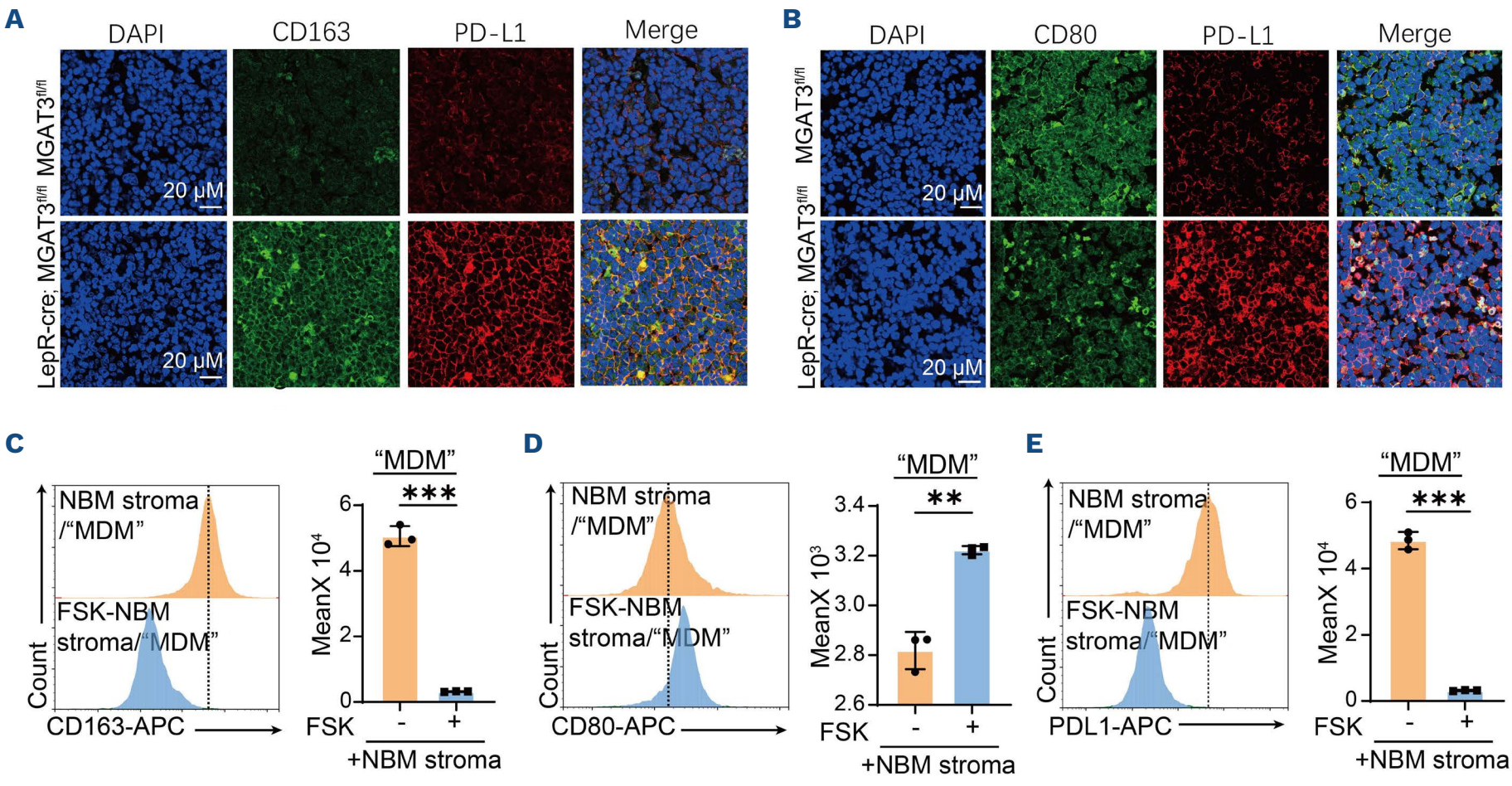

Figure 3. Bisecting N-acetylglucosamine level of stroma cells affects polarization of tumor-associated macrophages. (A and B) Confocal microscopic analysis of CD163, CD80 and Programmed death-ligand 1 (PD-L1) expression in bone marrow (BM) of $MGAT3^{fl/fl}$ and *LepR-cre*; $MGAT3^{fl/fl}$ mice. (C-E) Normal BM stroma (NBM stroma) cells were treated with 10 μM forskolin (FSK) for 12 hours (hr), and then macrophages from primary monocytes (termed as monocyte-derived macrophages, MDM) were co-cultured with FSK-treated (FSK-NBM stroma) or untreated NBM stroma for 48 hr. The expression of CD163, CD80 and PD-L1 in MDM were analyzed by FACS. (F) MDM were educated by NBM stroma or FSK-NBM stroma for 48 hours and subsequently co-cultured with activated $CD8^+$ T cells. Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production in $CD8^+$ T cells was determined by FACS. Data shown are mean ± Standard Error of Mean from three independent experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

sion may be affected by bisecting GlcNAc levels in stroma. Consistently overexpression or silencing of MGAT3 in both human BM stroma (HS27a, HS5) and murine normal BM stroma (mNBM stroma), characterized by $CD105^{+}CD90^{+}CD11b^{-}CD45^{-}$ (*Online Supplementary Figure S5B*), led to downregulation and upregulation of L-TGFβ1 and m-TGFβ1 expression, respectively (Figure 4B, *Online Supplementary Figure S5C, D*). Similarly, FSK treatment reduced both L-TGFβ1 and m-TGFβ1 expression in these stromal cells (*Online Supplementary Figure S5C, D*). Furthermore, m-TGFβ1 secretion was inversely proportional to bisecting GlcNAc levels, decreasing as GlcNAc levels rose and increasing when they were reduced (*Online Supplementary Figure S5E, F*). L-TGFβ1 expression in HS27a-M3 was blocked by lysosomal inhibitor chloroquine, but unaffected by proteasome inhibitor MG132 (Figure 4C). Co-localization of L-TGFβ1 and lysosome marker Lamp2, further confirmed that bisecting GlcNAc modification affects L-TGFβ1 stability and causes L-TGFβ1 degradation via a lysosomal pathway (Figure 4D, *Online Supplementary Figure S5G*).

HS27a-M3 cells secreted lower levels of m-TGFβ1 (*Online Supplementary Figure S5E*). When THP-1 ("M0") were co-cultured with HS27a-M3 cells, the SMAD3 and ERK signaling pathways were suppressed, TAM polarized towards M1 but not M2 subtype, and PD-L1 expression was decreased. However, adding rTGFβ1 reversed this process (Figure 4E, F). In contrast, HS5-shM3 cells secreted higher levels of m-TGFβ1 (*Online Supplementary Figure S5E*). When THP-1 ("M0") were co-cultured with HS5-shM3 cells,

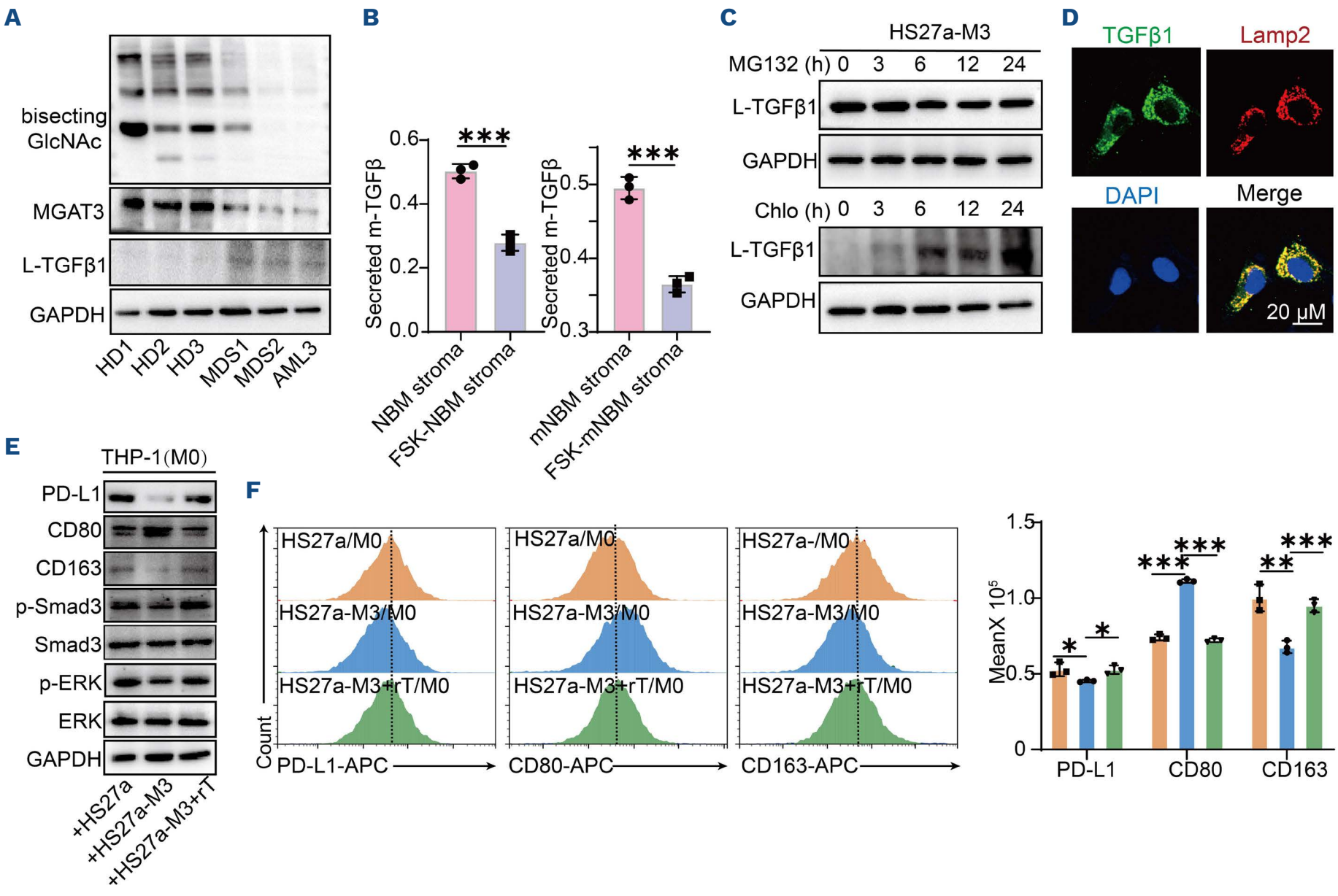

Figure 4. Identification and function of bisecting N-acetylglucosamine modified latency-associated peptide transforming growth factor β1 in bone marrow stroma. (A) Expression of N-acetylglucosaminyltransferase III (MGAT3), bisecting N-acetylglucosamine (GlcNAc), and latency-associated peptide transforming growth factor β1 (L-TGFβ1) in bone marrow (BM) stroma cells from healthy donors (HD) and patients with myelodysplastic syndromes or acute myeloid leukemia (MDS/AML). (B) Levels of secreted mature transforming growth factor β1 (m-TGFβ1) in normal bone marrow (NBM) stroma, forskolin-treated NBM stroma (FSK-NBM stroma), murine normal BM stroma (mNBM stroma), and FSK-mNBM stroma. (C) HS27a cells overexpressing MGAT3 (HS27a-MGAT3, referred to as HS27a-M3) were treated with chloroquine (Chlo) or MG132 for the indicated time, and L-TGFβ1 expression was evaluated. (D) Co-localization of L-TGFβ1 and lysosomal-associated membrane protein 2 (Lamp2) in lysosomes of HS27a-M3 cells. (E, F) THP-1-derived undifferentiated macrophages (M0) were co-cultured with HS27a, HS27a-M3, or HS27a-M3 in the presence of recombinant transforming growth factor β1 (rTGFβ1). Expression of PD-L1, CD80, and CD163 as well as activation of ERK and Smad3 signaling pathways were analyzed by western blotting (E), and cell surface marker expression was analyzed by fluorescence-activated cell sorting (FACS) (F). Data shown are mean ± Standard Error of Mean from three independent experiments. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

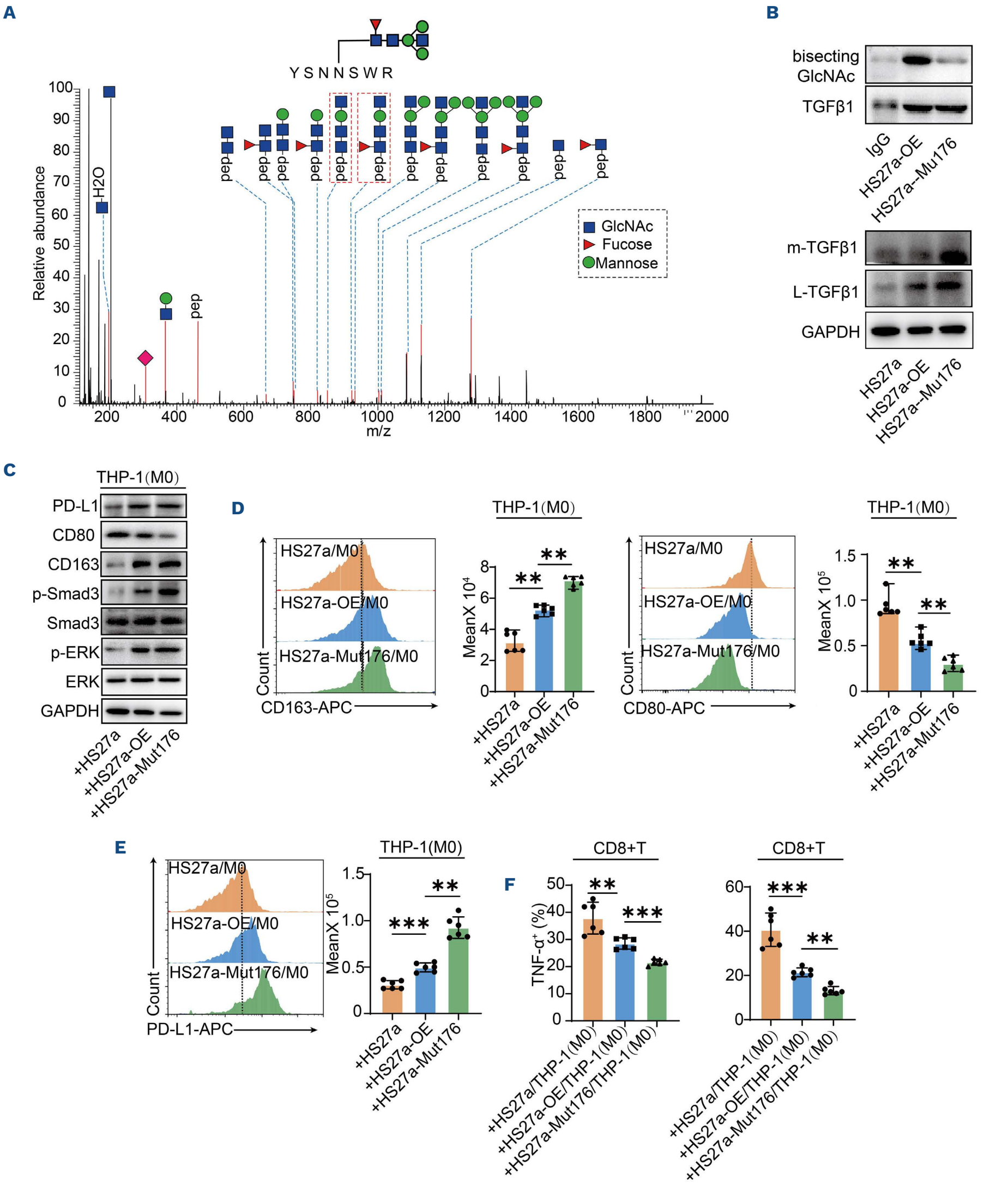

Figure 5. Effect of bisecting N-acetylglucosamine modification of latency-associated peptide transforming growth factor β1 on macrophage polarization. (A) Representative tandem mass spectrometry (MS/MS) spectra of the peptide carrying bisecting N-acetylglucosamine (GlcNAc) on latency-associated peptide transforming growth factor β1 (L-TGFβ1). (B) Levels of mature TGFβ1 (m-TGFβ1), L-TGFβ1, and bisecting GlcNAc in HS27a cells overexpressing either wild-type or N176D-mutated L-TGFβ1 (termed

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HS27a-OE and HS27a-Mu176, respectively), detected by immunoprecipitation and western blotting. (C-E) THP-1-derived undifferentiated macrophages (M0) were co-cultured with HS27a, HS27a-OE, or HS27a-Mu176 cells. The expression levels of PD-L1, CD163, and CD80 and the activation of ERK and Smad3 pathways were analyzed by western blotting (C), and surface expression of CD80, CD163, and PD-L1 was assessed by fluorescence-activated cell sorting (FACS) (D, E). (F) THP-1 (M0) macrophages were pre-educated by HS27a, HS27a-OE, or HS27a-Mu176 for 48 hours, then co-cultured with activated $CD8^+$ T cells. Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production in $CD8^+$ T cells was determined by FACS after 48 hours. Data shown are mean ± Standard Error of Mean from three independent experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

the SMAD3 and ERK signaling pathways were activated, leading to enhanced M2 polarization, and increased PD-L1 expression. The addition of a TGFβ1 receptor inhibitor (TRi) reversed this process (*Online Supplementary Figure S5H, I*).

Identification and function of bisecting GlcNAcylated L-TGFβ1 in bone marrow stroma

With a combination of MAX enrichment and LC-MS/MS, we identified a bisecting GlcNAc modified peptide YSNN#SWR of L-TGFβ1, characterized by the signature of pep+HexNAc3Hex1 at Asn 176 (Figure 5A). Notably, the expression of L-TGFβ1 and m-TGFβ1 was significantly up-regulated in the N176D (Asn replaced by Asp) mutant of L-TGFβ1, compared to its wild-type counterpart in stroma HS27a cells, designated as HS27a-Mu-176 and HS27a-OE, respectively (Figure 5B). The modification of L-TGFβ1 with bisecting GlcNAc was rarely observed in HS27a-Mu-176, indicating that Asn 176 serves as the primary site for this modification (Figure 5B) and the secretion of m-TGFβ1 was enhanced in HS27a-Mu-176 cells compared to HS27a-OE (*Online Supplementary Figure S6A*). When co-cultured with HS27a-Mu-176, THP-1 (“M0”) showed activation of SMAD3 and ERK signaling pathways, enhanced PD-L1 expression, and polarization towards M2 (Figure 5C-E). And the production of TNF-α and IFN-γ of $CD8^+$ T cells was significantly reduced when co-cultured with HS27a-Mu-176 educated THP-1 (“M0”), compared with that co-cultured with HS27a-OE educated THP-1 (“M0”) (Figure 5F).

To elucidate how bisecting GlcNAc modification affects L-TGFβ1 degradation, IP-MS identified that Rab7, a late endosome marker, was one key protein interacting with L-TGFβ1 (*Online Supplementary Figure S6B*). Rab7 exhibited a stronger binding affinity to wild-type L-TGFβ1 compared to mutant L-TGFβ1 (*Online Supplementary Figure S6C*). Moreover, knockdown of Rab7 expression enhanced the expression of L-TGFβ1 and the secretion of m-TGFβ1 (*Online Supplementary Figure S6D-F*). We also assessed the expression of autophagy-related proteins (*Online Supplementary Figure S6G*) to explore a potential autophagic mechanism, but found no significant changes, indicating that autophagy does not play a major role in L-TGFβ1 degradation under these conditions.

Effect of a reprogrammed tumor-associated myelodysplastic syndrome/acute myeloid leukemia microenvironment

Since bisecting GlcNAc levels in BM stromal cells can affect expression of L-TGFβ1 and the secretion of m-TGFβ1, we investigated whether this in turn could influence macrophage polarization. When BM stroma cells were treated with FSK to elevate the bisecting GlcNAc levels, the expression of L-TGFβ1 and m-TGFβ1 was significantly down-regulated (Figure 6A). This, in turn, inhibited THP-1 (“M0”) polarization towards M2-phenotype and diminished PD-L1 expression (Figure 6B, C). Furthermore, TNF-α and IFN-γ production by $CD8^+$ T cells displayed a remarkable increase when co-cultured with macrophages educated by FSK-treated BM stroma cells from MDS/AML patients (Figure 6D).

The above findings were further validated using transplantation experiments (*Online Supplementary Figure S6J*). Pre-treatment of RAW264.7 cells with conditioned medium from mNBM stroma-shM3 cells, which up-regulated m-TGFβ1 secretion and PD-L1 expression (*Online Supplementary Figure S6H, I*), led to an increase in M-1-GFP cells in peripheral blood when co-transplanted with these cells (Figure 6E). This increase was reduced by using a-mPD-L1 antibody (Figure 6E). In contrast, in mice co-transplanted with mNBM stroma-shM3 CM-treated RAW264.7 cells, TNF-α and IFN-γ levels in plasma, as well as the number of $CD8^+$ T cells in the BM, were reduced. But TNF-α and IFN-γ levels, and $CD8^+$ T-cell number were increased in mice that received a-mPD-L1 antibody treatment (Figure 6F, *Online Supplementary Figure S6K*).

Discussion

Cancer progression is dictated not only by the intrinsic alterations acquired by cancer cells but also by its surrounding microenvironment. Being an essential part of the tumor microenvironment, TAM are often associated with poor prognosis due to their pro-tumorigenic functions. In this study, we observed a significantly higher proportion of M2-like TAM in MDS/AML patients compared to HD. TAM can polarize into various phenotypes including immunosuppressive subtypes, depending on external stimuli. For example, leukemia cells secrete growth factor independence 1 and Arg1, which can generate pro-tumor M2 macrophages.[14,27] Wang *et al*. discovered that reprogrammed host macrophages by stroma cells are required for stroma cell-mediated microenvironment restoration and leukemia inhibition.[19]

We found that the increase of M2 TAM was accompanied by elevated levels of PD-L1 expression. Targeting PD-1/PD-L1

has been approved for tumor treatment with durable clinical benefits. It was originally thought tumor cells were the main source of PD-L1-mediated suppression. However, recent studies have indicated that PD-L1 expression on dendritic cells (DC) or TAM play a more dominant role in determining efficacy of PD-1 immune checkpoint blockade therapy.[28-30] An increase in PD-L1 expression on TAM could inhibit T-cell activity, thereby promoting tumor metabolism and metastasis.[30,31] Based on immunohistochemistry and FACS assays, we found a negative correlation between M2 TAM and $CD8^+$ T cells in MDS/AML BM biopsy. Interestingly, our investigations revealed that BM stroma from MDS/AML patients prompted PD-L1 expression in co-cultured TAM and the educated TAM further inhibited the function of $CD8^+$ T cells.

By investigating these mechanisms, our data indicated BM stroma cells from MDS/AML patients can secrete elevated levels of m-TGFβ to promote macrophage polarization towards M2 subtype. Expression of L-TGFβ1, the precursor of m-TGFβ1, was significantly increased in stroma cells from MDS/AML patients. TGF-β1 has long been identified with its intensive involvement in early embryonic development and organogenesis, immune supervision, tissue repair, and adult homeostasis.[32] A recent study revealed that, upon activation by integrin αvβ8, TGF-β orchestrates both autocrine and paracrine signaling, enabling cells to regulate their own signaling pathways and fine-tune immune responses and developmental processes, thereby offering new therapeutic possibilities.[33] Once TGF-β1 binds to its low-affinity het-

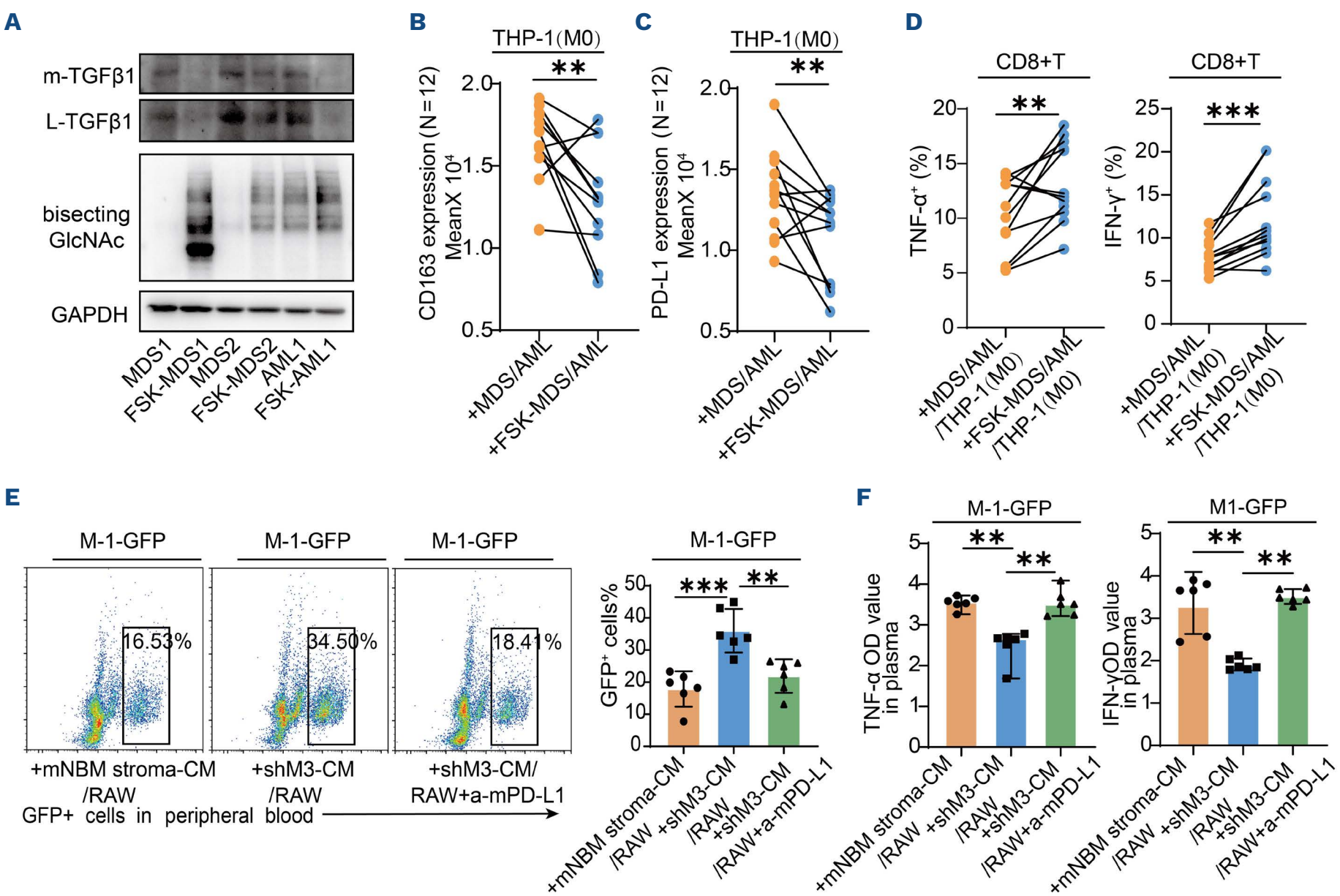

Figure 6. Effect of reprogrammed tumor-associated macrophages in the myelodysplastic syndromes and acute myeloid leukemia microenvironment. (A) Relative expression of bisecting N-acetylglucosamine (GlcNAc), mature transforming growth factor β1 (m-TGFβ1), and latency-associated peptide TGFβ1 (L-TGFβ1) in bone marrow (BM) stroma or forskolin-treated BM stroma from patients with myelodysplastic syndromes or acute myeloid leukemia (MDS/AML). (B, C) Relative expression of CD163 (B) and programmed death-ligand 1 (PD-L1) (C) in THP-1-derived undifferentiated macrophages (M0) co-cultured with untreated or forskolin-treated BM stroma from MDS/AML patients. (D) M0 macrophages were educated by BM stroma or forskolin-treated BM stroma for 48 hours (hr) and then co-cultured with activated $CD8^+$ T cells. Tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) production in $CD8^+$ T cells was determined by fluorescence-activated cell sorting (FACS) after 48 hr. (E) FACS analysis of M-1-GFP cells (a GFP-labeled murine acute myeloid leukemia cell line) in peripheral blood after co-injection with RAW264.7 macrophages (RAW) pretreated with conditioned medium (CM) derived from either wild-type murine normal bone marrow (mNBM) stromal cells or mNBM stroma with knockdown of N-acetylglucosaminyltransferase III (MGAT3, referred to as shM3), and subsequent treatment with or without anti-mouse PD-L1 antibody (a-mPD-L1). (F) Relative levels of TNF-α and IFN-γ in mice from the same groups. Data shown are mean ± Standard Error of Mean from three independent experiments. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

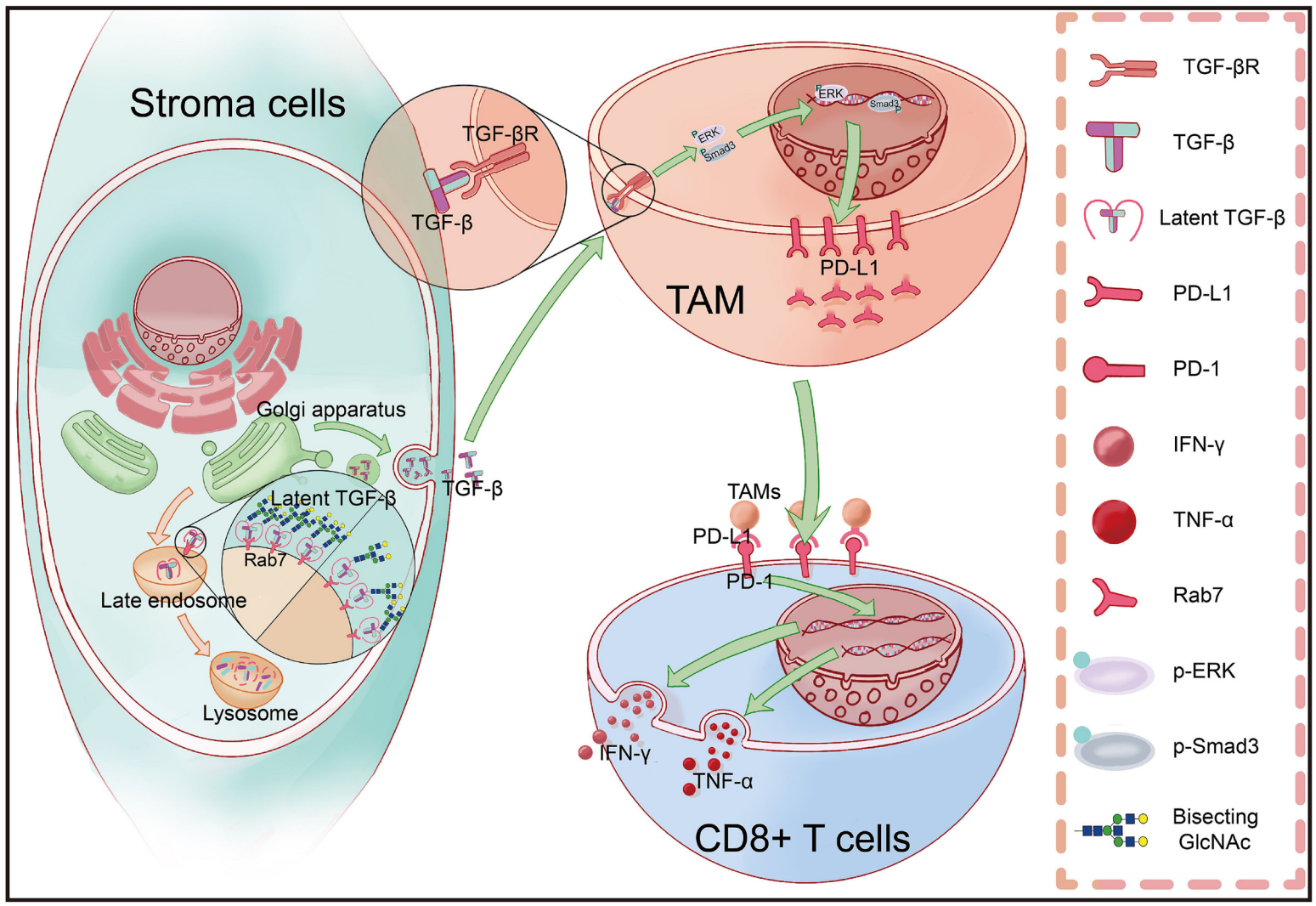

Figure 7. Diminished bisecting N-acetylglucosamine level in bone marrow stroma promotes mature transforming growth factor β1 secretion and induces PD-L1 upregulation in tumor-associated macrophages, facilitating immune evasion in myelodysplastic syndromes and acute myeloid leukemia (concept model).

eromeric receptor complex composed of TβRI and TβRII, it could activate the SMAD and ERK signaling pathways.[34] Our data suggest that stroma cell-derived TGFβ1 promoted TAM to M2 polarization by activating p-SMAD3 and p-ERK signaling in MDS/AML. Targeting TGF-β1 signal transduction by TRi can inhibit TAM to M2 polarization, decrease PD-L1 expression, and consequently enhance the function of CD8⁺ T cells.

In our previous study, we observed one specific glycan, bisecting GlcNAc, was down-regulated in BM stroma of MDS/AML patients, which promoted malignant clonal cell proliferation and survival while suppressing normal hematopoiesis.[20] Bisecting GlcNAcylation is a specific type of N-glycosylation that affects adhesion, migration, and other cellular functions by modifying adhesion molecules and receptors (notably E-cadherin, integrins, tetraspanins, and EGFR).[35-37] Here, we revealed that stromal cell with lower levels of bisecting GlcNAc secreted increased amounts of m-TGFβ1, and Asn 176 on L-TGFβ1 was the key bisecting GlcNAc-bearing site. Interestingly, the expression of both L-TGFβ1 and m-TGFβ1 was enhanced in HS27a-Mu-176 cells compared to HS27a-OE, inducing TAM polarization towards M2 phenotype and elevating PD-L1 expression, and consequently inhibiting the function of CD8⁺ T cells. Both rTGFβ1 and conditioned medium from mNBM stroma-shM3 cell-treated macrophages can significantly increase the proliferation of leukemic cells and impaired CD8⁺ T-cell function. Importantly, these effects were reversed by enhancing the bisecting GlcNAc level in stroma. This suggests that reduced bisecting GlcNAc levels in stroma boost m-TGFβ1 secretion, fostering an immunosuppressive environment that supported malignant cell proliferation while impairing CD8⁺ T-cell function.

In conclusion, we observed that down-regulated bisecting GlcNAc levels in the stromal cells of MDS/AML patients promoted the expression and secretion of TGFβ1, which drove TAM polarization towards the M2 phenotype and elevated PD-L1 expression. This, in turn, enhanced TAM-mediated inhibition of CD8⁺ T cells, facilitating immune escape and the progression of MDS/AML (Figure 7). Therefore, the strategic modulation of glycan level from leukemic stromal cells could represent novel therapeutic strategies for the management of hematopoietic malignancy.

Disclosures

No conflicts of interest to disclose.

Contributions

XL conceptualized the study, acquired funding and managed the project, supervised the research and validated

the findings, and reviewed and edited the manuscript. JF curated the data, conducted the formal analysis, contributed to visualization, carried out the investigation, designed the methodology, and wrote the original draft. JG designed the methodology and carried out the investigation. WW, YM, XR, CZ, XC, LL and ZT carried out the investigation. YW provided resources. FG supervised the research, validated the findings, and reviewed and edited the manuscript. All authors have reviewed and approved the final version of the manuscript for publication.

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Data-sharing statement

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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