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Autocrine Transforming Growth Factor-β Signaling Promotes Cell Motility and Chemokine Secretion in an Angiomyolipoma-Derived Cell Model of Lymphangioleiomyomatosis

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Lymphangioleiomyomatosis (LAM) is a rare systemic disease that affects young women and is classified as a low-grade metastasizing neoplasm. It is characterized by uncontrolled proliferation of LAM cells within the lung parenchyma, which results from loss-of-function mutations in tuberous sclerosis complex 2 (*TSC2*) or 1 (*TSC1*) and activation of the mechanistic target of rapamycin complex 1. Abnormal cell growth leads to cyst formation and lung damage. Rapamycin-based therapy is the only approved treatment. Although it stabilizes the lung function in most patients, it has several limitations. Therefore, new therapeutic strategies are needed. This study examined the role of transforming growth factor-β (TGF-β), a pleiotropic cytokine with well-established protumorigenic activity, in LAM cell biology. Using a TSC2-deficient angiomyolipoma-derived cell line indicated that $TSC2^{-/-}$ cells exhibited a higher expression of *TGFβ1* and *TGFβ3* than cells with restored TSC2 expression. Additionally, $TSC2^{-/-}$ cells expressed glycoprotein-A repetitions predominant and integrin β8, which promote TGF-β activation. Inhibition of TGF-β signaling in $TSC2^{-/-}$ cells reduced their migration in a wound healing assay, impaired transmigration through a three-dimensional matrix, and decreased the expression of monocyte chemoattractant protein-1. These findings provide new insights into the regulation of processes contributing to LAM progression and point to TGF-β as one of the potential targets for LAM treatment. *(Am J Pathol 2025, 195: 1394–1410; https://doi.org/10.1016/j.ajpath.2025.04.019)*

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Lymphangioleiomyomatosis (LAM) is a rare progressive lung disease affecting predominantly women of reproductive age.[1–5] Its pulmonary form can be associated with extrapulmonary LAM manifested as renal angiomyolipoma (AML) or lymphatic abnormalities.[6,7] The clinical manifestations of LAM include signs of pulmonary dysfunction, such as dyspnea and recurrent pneumothorax.[6] Histologically, pulmonary LAM is characterized by the presence of thin-walled cysts and nodules within lung parenchyma.[8] LAM arises from the infiltration of the lungs by smooth muscle-like cells (LAM cells), which originate from extrapulmonary tissue and spread through lymphatic and blood vessels.[9,10] The origin of LAM cells remains a subject of ongoing debate, with various theories suggesting potential sources, such as the uterus (uterine leiomyomas),[11,12] lung mesenchyme,[13,14] or lymphatic endothelium.[15] LAM cells are characterized by uncontrolled proliferation, a consequence of biallelic loss-of-function mutations in the tuberous sclerosis complex 2 (*TSC2*) or tuberous sclerosis complex 1 (*TSC1*).[2] LAM cells deprived of functional TSC1/TSC2 complexes display elevated levels of GTP-bound Ras homolog enriched in the brain (Rheb-GTP), which, in turn, sustains the activation of the mechanistic target of rapamycin (mTOR), a serine/threonine protein kinase, a component of mTOR complex 1 (mTORC1), which orchestrates crucial cellular processes, including protein biosynthesis, cell growth, and proliferation.[16] Although increased proliferation is the most profound feature of LAM cells, the lack of functional TSC1/TSC2 complex and dysregulated mTORC1 signaling pathway impacts the expression and activity of numerous proteins. Up-regulated expression of matrix metalloproteinases, especially matrix metalloproteinases 2, 7, and 9,[17,18] increased secretion of chemokines,[3] and overexpression of immune checkpoint proteins, such as programmed death ligand 1 and B7-H3 (CD276)[19,20] enables LAM cells to exploit cancer-specific mechanisms of surrounding tissue destruction, invasion, metastasis, and immune evasion. Consequently, LAM has been classified as a low-grade, metastasizing neoplasm.[21] So far, despite the enormous advancement in understanding the molecular mechanisms that govern LAM progression, a rapamycin-based therapy remains the sole US Food and Drug Administration–approved LAM treatment.[22] Rapamycin inhibits mTOR within mTORC1, displaying the potential to impede mTORC1-driven LAM progression and stabilize lung function.[22,23] Although a significant fraction of patients with LAM have a favorable response to rapamycin-based therapy, this treatment has several limitations.[24,25] Moreover, because of its cytostatic nature, rapamycin-based treatment must continue throughout life. However, this regimen may impose a risk of acquired drug resistance, which could significantly reduce its therapeutic efficacy.[26] This highlights the need for the identification of novel therapeutic approaches, either applied as monotherapy or in combination with rapamycin.[27]

Transforming growth factor-β (TGF-β) is a pleiotropic cytokine that plays a vital role in sustaining homeostasis through the regulation of a wide range of processes, including differentiation, proliferation, migration, and apoptosis.[28] Three TGF-β isoforms (namely, TGF-β1, TGF-β2, and TGF-β3) have been identified, with TGF-β1 displaying ubiquitous expression.[29] TGF-β is secreted from a cell in inactive form, complexed with latency-associated peptide (LAP) and latent TGF-β binding protein[30] or transmembrane glycoprotein-A repetitions predominant (GARP).[31] Active TGF-β may be released from its latent complexes through the proteolytic cleavage or due to the conformational changes imposed on the complex after binding of integrins to the Arg-Gly-Asp (RGD) integrin binding motif within LAP.[31–33] TGF-β signals through a heterotetrameric complex that consists of two types of TGF-β receptors displaying serine/threonine kinase activity: TGF-β type I receptor [alias activin-like kinase 5 (ALK5)] and TGF-β type II receptor.[34] TGF-β triggers the activation of the canonical Smad-dependent pathway and/or the noncanonical pathways, like mitogen-activated protein kinase, Rho family GTPase, and phosphatidylinositol 3-kinase/Akt.[28,35] The outcome of TGF-β signaling is highly context-dependent and may vary between the cell types and their developmental stages and may be determined by the type and activation status of the surrounding microenvironment.[36,37] The dual role of TGF-β is particularly manifested during tumorigenesis.[38] At the early stages of tumor development, TGF-β acts as a potent tumor suppressor, demonstrating robust anti-proliferative and pro-apoptotic activity.[39,40] However, during the advanced stages of tumor development, tumor cells acquire the ability to escape from TGF-β–mediated suppression, thereby converting TGF-β into a potent tumor-promoting agent.[41]

Elevated TGF-β1 levels have been observed in LAM lung nodules.[42] Given its protumorigenic role, it was hypothesized that TGF-β may contribute to LAM progression by directly stimulating LAM cells. In this study, a TSC2-null cell line, derived from the angiomyolipoma of a patient with LAM, exhibited increased expression of *TGFβ1* and *TGFβ3* compared with that in cells with restored TSC2 expression. Moreover, TSC2-null cells expressed GARP and integrin β8 involved in the membrane presentation and activation of TGF-β. Interestingly, even though TSC2-null cells exhibit sensitivity to TGF-β, they apparently reprogrammed the TGF-β signaling pathway to evade its anti-proliferative effects. Instead, TGF-β facilitated their migration and up-regulated the expression of monocyte chemoattractant protein-1 (MCP-1). Presented data point to TGF-β as one of the factors that may directly impact LAM cells and promote LAM progression.

Materials and Methods

Cell Lines and Cell Culture

TSC2-null 621-101 cell line, further referred to as TSC2^{-}, established from a renal angiomyolipoma of patient with

LAM[43] and its derivatives (621-103 cell line re-expressing TSC2 and 621-102 cell line transfected with a corresponding empty vector)[44] were obtained from Professor Elisabeth Henske (Brigham and Women's Hospital and Harvard Medical School, Boston, MA). Mink lung epithelial TGF-β reporter cell line, tMLEC, expressing luciferase under the control of plasminogen activator inhibitor 1 (PAI-1) promoter,[45] was provided by Professor Sophie Lucas (de Duve Institute, Brussels, Belgium). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Thermo Fisher Scientific, Waltham, MA) with 4.5 g/L glucose supplemented with 10% fetal bovine serum (FBS; Gibco), later referred to as a growth medium. The cells were cultured under standard conditions (37°C, 5% CO_2, and 95% humidity). *Mycoplasma* 16S rDNA PCR was conducted to check for *Mycoplasma* contamination.

Generation of TSC2^{-}-Derived Cell Line with Restored TSC2 Expression

TSC2 coding sequence was obtained from HEK-293 cells. Total RNA was isolated from the cells using thiocyanate-phenol-chloroform extraction method,[46] and 1 μg of RNA was reverse transcribed using M-MLV reverse transcriptase (Promega, Madison, WI) following manufacturer's recommendations using oligo(dT)$_{15}$ primer (Genomed SA, Warsaw, Poland). The coding sequence of TSC2 was PCR amplified using Q5 High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA) with the following primers, 5′-TGGCCTCTGAGGCCTCACCATGGCCAAACCAACAAGC-3′ (forward) and 5′-TGGCCTGACAGGCCTCACACAAACTCGGTGAAGTC-3′ (reverse). The primers were designed to contain SfiI-restriction sites GGCCNNNNGGCC partially complementary to the pSB vector sequences, ensuring proper orientation of the inserted sequence as well as providing Kozak sequence. PCR products were resolved in 1% agarose gel, and the band corresponding to TSC2 cDNA was cut out. DNA was purified using Gel/PCR ME MiniKit (Syngen Biotech, Warsaw Poland), cloned into pJET1.2/blunt using CloneJET PCR Cloning Kit (Thermo Fisher Scientific) and sequenced using sequencing pJET1.2 primers and five additional *TSC2*-specific primers (Table 1). The sequence was identified as a TSC2 transcript isoform 5 (NIH Nucleotide database, *https://www.ncbi.nlm.nih.gov/nucleotide*; record NM_001114382.3) using NIH Nucleotide BLAST (*https://blast.ncbi.nlm.nih.gov/Blast.cgi*).

For the generation of the Sleeping Beauty transposon vector, a SfiI-flanked *TSC2* sequence was subcloned from pJET1.2 into SfiI-linearized pSBbi-GP-PuroR[47] [a gift from Eric Kowarz (Institute of Pharmaceutical Biology, Goethe University, Frankfurt, Germany); Addgene (Watertown, MA) plasmid number 60511]. The obtained vector was named pSBbi-GP-PuroR-TSC2.

TSC2^{-} cells (8×10^4 per well of 12-well plate in 1 mL of culture medium) were cotransfected with pSBbi-GP-PuroR-TSC2 or empty pSBbi-GP-PuroR (975 ng) and pCMV(CAT)T7-SB100 coding for transposase (25 ng) [a gift from Zsuzsanna Izsvak (Max Delbrück Center for Molecular Medicine, Berlin, Germany); Addgene plasmid number 34879][48] using Lipofectamine LTX (Thermo Fisher Scientific), following a manufacturer's protocol. The next day, the cells were transferred to 6-well plates, and stably transfected cells were selected by puromycin (10 μg/mL).

Table 1 Sequences of pJET1.2 Sequencing Primers and TSC2-Specific Primers

Primer	Sequence
pJET1.2 reverse sequencing primer	5′-AAGAACATCGATTTTCCATGGCAG-3′
pJET1.2 forward sequencing primer	5′-CGACTCACTATAGGGAGAGCGGC-3′
TSC2_pJET_seq_1	5′-TACAACATGTGCCACCTCATG-3′
TSC2_pJET_seq_2	5′-TTCAGACCAAGCTGTACACC-3′
TSC2_pJET_seq_3	5′-CCCATCACGTCATAGCCATG-3′
TSC2_pJET_seq_4	5′-GCTGATGAGCCTGGAGAAC-3′
TSC2_pJET_seq_5	5′-TGTTCCTGCAGCTCTACCATTC-3′

TSC, tuberous sclerosis complex.

Analysis of Gene Expression by Quantitative RT-PCR

Total cellular RNA was isolated from the TSC2^{-} and TSC2^{-}-derived cells, and the samples of 1 μg were reverse transcribed as described in *Generation of TSC2^{-}-Derived Cell Line with Restored TSC2 Expression*. The levels of particular cDNAs were analyzed by real-time quantitative PCR on an Eco Real-Time PCR System (Illumina, San Diego, CA) using Start-Warm HS-PCR Mix (A&A Biotechnology, Gdansk, Poland) and specific primers (Table 2; TGF-β type II receptor–specific primers from Primer Bank, identifier 133908633c1[49]). The annealing temperature for all primers was 60°C. The evaluated efficiency of all primer pairs exceeded 90%. The relative changes in transcript levels were assessed using the delta-delta cycle threshold ($\Delta\Delta C_T$) method with *TBP* as a housekeeping gene. The analyzed sublines did not show any differences in *TBP* expression levels.

Assessing the Impact of TSC2 and mTORC1 Signaling Pathway on the Levels of Selected Genes

TSC2^{-} and TSC2^{-}-derived cell lines were plated on 6-well plates (2×10^5 cells/well) and cultured overnight in the growth medium. Next, the growth medium was replaced with DMEM containing FBS reduced to 2% concentration, further referred to as DMEM + 2% FBS. The cells were cultured in DMEM + 2% FBS for 48 hours in the presence or absence of rapamycin (20 nmol/L; Millipore, Sigma-Aldrich, Inc., St. Louis, MO) or its solvent, dimethyl sulfoxide, and subjected to quantitative RT-PCR analysis, or harvested from a plate using trypsin/EDTA, or, in the case of analyzing membrane-displayed GARP–LAP–TGF-β1 complexes with 3 mmol/L EDTA/phosphate-buffered saline (PBS), and then subjected to flow cytometry analysis.

Table 2 qPCR Primer Sequences

cDNA	Primers
GARP	F: 5′-GCTGCACAACACCAAGACAA-3′
	R: 5′-GCTGATCTCATTGGTGCTCA-3′
Integrin β8	F: 5′-CACCCCGAAAGGATTCATAA-3′
	R: 5′-CCAGCAGCAATCTTTTAGCC-3′
MCP-1	F: 5′-TCTGTGCCTGCTGCTCATAG-3′
	R: 5′-CAGATCTCCTTGGCCACAAT-3′
TGF-β1	F: 5′-CACGTGGAGCTGTACCAGAA-3′
	R: 5′-CACGTGGAGCTGTACCAGAA-3′
TGF-β2	F: 5′-CTGTCCCTGCTGCACTTTTGTA-3′
	R: 5′-TGTGGAGGTGCCATCAATACCT-3′
TGF-β3	F: 5′-AGATCCTTCGGCCAGATGAG-3′
	R: 5′-TCTCCATTGGGCTGAAAGGT-3′
PAI-1	F: 5′-CTCTCTCTGCCCTCACCAAC-3′
	R: 5′-GTGGAGAGGCTCTTGGTCTG-3′
TβR1	F: 5′-ACAACGTCAGGTTCTGGCTC-3′
	R: 5′-TTCTTCTCCCCGCCACTTTC-3′
TβR2[49]	F: 5′-GTAGCTCTGATGAGTGCAATGAC-3′
	R: 5′-CAGATATGGCAACTCCCAGTG-3′
TBP	F: 5′-TAGAAGGCCTTGTGCTCACC-3′
	R: 5′-GAGCCATTACGTCGTCTTCC-3′

F, forward; GARP, glycoprotein-A repetitions predominant; MCP-1, monocyte chemoattractant protein-1; PAI-1, plasminogen activator inhibitor 1; qPCR, real-time quantitative PCR; R, reverse; TBP, TATA-box binding protein; TβR1, TGF-β type I receptor; TβR2, TGF-β type II receptor.

Assessing the Impact of TGF-β on the Expression of Selected Cytokines

TSC2^{-} cells were seeded on 6-well plates (2×10^5 cells/well) and cultured overnight. Then, the growth medium was replaced with DMEM + 2% FBS and the following inhibitors of TGF-β signaling were added to some wells: SB431542 (10 μmol/L), an inhibitor of ALK4, ALK5, and ALK7, later referred to as ALK4/5/7 inhibitor[50]; and LY364947 (10 μmol/L), an inhibitor of ALK5, later referred to as ALK5 inhibitor (Sigma-Aldrich). An appropriate volume of dimethyl sulfoxide (a solvent for inhibitors) was added to control wells. In parallel experiments, TSC2^{-} cells were incubated for 24 hours in DMEM + 2% FBS in the presence or absence of exogenous TGF-β1, TGF-β2, or TGF-β3 (2 ng/mL; PeproTech, Cranbury, NJ). The levels of MCP-1 were measured in culture media using enzyme-linked immunosorbent assay (Human CCL2/MCP-1 Duo-Set ELISA; R&D Systems, Minneapolis, MN), according to a manufacturer's protocol. The levels of MCP-1 transcript were analyzed by quantitative RT-PCR.

Co-Culture of TSC2-Null Cells with TGF-β Reporter Cell Line (tMLEC)

TSC2^{-} cells seeded on 96-well plates (1×10^4/well or 0.5×10^4/well) were cultured overnight. Next, they were washed with PBS, and the growth medium was replaced either with DMEM + 2% FBS or DMEM + 2% FBS containing SB431542 (ALK4/5/7 inhibitor; 10 μmol/L), or the monoclonal antibody (clone MHG8; 10 μg/mL) that blocks the release of active TGF-β1 from GARP—LAP—TGF-β1 complexes,[51] later referred to as α-GARP. An appropriate volume of dimethyl sulfoxide or mouse IgG2a antibodies (10 μg/mL) was added to the corresponding control wells. Next, 1×10^4 tMLEC cells were added to each well and co-cultured with TSC2^{-} cells for 24 hours. Monocultured tMLEC cells served as a control for each condition. The activity of luciferase was assessed by a Luciferase assay system (Promega), according to a manufacturer's protocol. The activity of luciferase in each co-culture condition was normalized to luciferase activity in its respective control monocultured tMLEC cells.

Immunohistochemistry

The LAM tissue study was approved by the Ethics Committee of Bellvitge Institute for Biomedical Research and the University Hospital of Bellvitge (reference PR104/20). The samples corresponded to 10 lung biopsies of women with a clinical LAM diagnosis based on outlined guidelines.[52,53] The immunohistochemical assays were performed on serial formalin-fixed, paraffin-embedded tissue sections (4 μm thick) using an EnVision kit (Dako, Agilent, Santa Clara, CA). Following antigen retrieval using EDTA (pH 8.0) for anti-CD68 (Abcam, Cambridge, UK; ab955), anti—TGF-β2 (Invitrogen, Thermo Fisher Scientific, Waltham, MA; MA5-37505), anti—TGF-β3 (Invitrogen; PA5-78197), and citrate buffer (pH 6.0) for anti—MCP-1 (R&D Systems; MAB679) and anti—TGF-β1 (R&D Systems; AB-246-NA), sections were blocked with 3% hydrogen peroxide and a serum-based blocking solution. Primary antibodies were applied overnight at 4°C at the following dilutions: anti-CD68 (1:200), anti—chemokine (C-C motif) ligand 2 (1:25), anti—TGF-β1 (1:200), anti—TGF-β2 (1:50), and anti—TGF-β3 (1:500).

Secondary peroxidase-conjugated antibodies (Envision+ system-HRP; Dako) were used. Sections were hematoxylin counterstained and examined with a Nikon Eclipse 80i microscope (Nikon Instruments, Melville, NY). Images were captured under a bright field at ×4 to ×40 magnification using a Nikon Digital Sight color video camera linked to a computer system. The positive control tissue was defined on the basis of evidence from the Human Protein Atlas[54] and included normal colon and skin tissue.

Flow Cytometry Analysis

The cells were detached from the plate with Trypsin/EDTA or as in the case of analysis of GARP—LAP—TGF-β1 complexes with 3 mmol/L EDTA/PBS, washed twice with PBS, and incubated with the LIVE/DEAD fixable dead cell stain (Thermo Fisher Scientific), according to a manufacturer's protocol to exclude dead cells from the analysis. Next, the cells were incubated for 20 minutes at 4°C with the following primary antibodies or their respective isotype controls diluted in

PBS supplemented with 5% FBS: phosphatidylethanolamine-conjugated anti-human GARP/mouse IgG2b (1 μg/mL; clone 7B11; BioLegend, San Diego, CA), unconjugated anti-human integrin β8/mouse IgG2b (2 μg/mL; clone 416922; R&D Systems), and unconjugated anti-human GARP−LAP−TGF-β1/mouse IgG2a (10 μg/mL; clone MHG8[51]). The cells incubated with unconjugated primary antibodies were next stained for 20 minutes at 4°C with the phosphatidylethanolamine-conjugated goat anti-mouse IgG (Thermo Fisher Scientific) diluted 1:1000 in PBS supplemented with 5% FBS. The cells were analyzed using LSR Fortessa flow cytometer and FlowJo software version 10.7.1 (BD Bioscience, Franklin Lakes, NJ).

Wound Healing Assay

$TSC2^-$ cells were seeded on 24-well plates (1.3×10^5 cells/well) and cultured overnight. The medium was replaced for DMEM + 2% FBS, and the cells were treated with inhibitors of TGF-β signaling, as described in *Assessing the Impact of TGF-β on the Expression of Selected Cytokines*. After 24 hours, cell monolayers were manually wounded with a white pipette tip, detached cells were washed out with PBS, and fresh DMEM + 2% FBS enriched with relevant inhibitors was added to appropriate wells. For each wound, images of three randomly selected regions were captured right after wounding (day 0) and after 18 hours (day 1) using Leica DM IL LED fluorescent microscope (Leica Microsystems, Wetzlar, Germany). The wound area at both time points was analyzed using ImageJ software version 1.54p (NIH, Bethesda, MD; *http://imagej.org*). To assess the rate of cell migration, the percentage of wound closure was calculated according to the following formula and normalized to the corresponding control: wound closure (%) = [(area day 0 − area day 1)/(area day 0)] × 100%.

Three-Dimensional Collagen Type I/Matrigel Transmigration Assay

$TSC2^-$ cells seeded on 6-well plates (1.2×10^5/well) were cultured overnight. The growth medium was replaced with DMEM + 2% FBS, and the cells were treated with inhibitors of TGF-β signaling, as described in *Assessing the Impact of TGF-β on the Expression of Selected Cytokines*. After 24 hours, the cells were collected from the plate, counted, and 1.2×10^5 cells from each experimental group were embedded in a matrix composed of 10% Matrigel and 2% collagen type I and placed in 12-mm inserts with an 8-μm pore membrane (Corning, Sigma-Aldrich). DMEM + 2% FBS medium enriched with respective inhibitors or controls was added to the lower and upper chambers. After 18 hours, gels were removed from the inserts, and non-transmigrated cells were removed from the upper chamber with a cotton swab. The membranes were fixed in 2% paraformaldehyde in PBS and stained with DAPI (1 μg/mL; Thermo Fisher Scientific). Images of five random areas of the insert were captured using a Leica DM IL LED fluorescent microscope. The cells were counted using ImageJ software version 1.54p, and the average number of transmigrated cells was normalized to the respective control within each experiment.

Statistical Analysis

GraphPad Prism 10.4.1 (GraphPad Software Inc., San Diego, CA) was used for the statistical analysis and generation of graphs. Two groups were compared using an unpaired two-tailed *t*-test. More than two groups were analyzed with one- or two-way analysis of variance with the Bonferroni test for multiple comparison correction. Real-time quantitative PCR data were analyzed using unpaired two-tailed *t*-test with Welch correction or repeated-measures one-way analysis of variance or repeated-measures two-way analysis of variance, with Geisser-Greenhouse correction and the Bonferroni test for multiple comparison correction. Data are presented as means ± SD; $P < 0.05$ was regarded as statistically significant.

Results

AML-Derived $TSC2^-$ Cells Display Up-Regulated Expression of *TGFβ1* and *TGFβ3* and Retain Responsiveness to TGF-β

LAM lesions enriched in proliferating diseased cells exhibit significant deposition of TGF-β1 in the extracellular matrix. In contrast, in normal lung tissue, TGF-β1 primarily localizes to the bronchial epithelium and vascular endothelium.[42] Data from a single-cell transcriptomic analysis of the lung, uterus, and kidney of patients with LAM deposited in the LAM Cell Atlas (LCA) database[55] indicate that LAM cells also express two other isoforms of *TGFβ*, *TGFβ2* and *TGFβ3*, both of which are known to play roles in tumor progression.[56,57]

To gain a better insight into the regulation of TGF-β expression in LAM cells, the role of TSC2 in the regulation of *TGFβ1*, *TGFβ2*, and *TGFβ3* expression was assessed. To this end, 621-101 cell line, the TSC2-null cell line derived from AML of a patient with LAM (further referred to as $TSC2^-$) was used along with the sublines transfected with either empty- (621-102 cells) or TSC2-coding vector (621-103 cells), further referred to as EV-1 and $TSC2^+$-1, respectively. Given that the clonal sublines may not fully represent the heterogeneity of the parental cell population,[58] a second parallel pair of 621-101−derived sublines, EV-2 and $TSC2^+$-2, was generated. To preserve the heterogeneity of the parental cell line, the Sleeping Beauty transposon system was used, enabling efficient stable integration of transgenes into the genome.[59]

Growth factors present in serum may hamper the activity of TSC2. Thus, to ensure the full manifestation of the activity of restored TSC2, $TSC2^+$ cells should be cultured in serum-reduced conditions. Because the complete removal of FBS reduces the expression of certain genes of interest

(Supplemental Figure S1), all experimental procedures were conducted in DMEM containing FBS reduced to 2% concentration, further referred to as DMEM + 2% FBS. Cells with restored expression of TSC2 cultured in the presence of 2% FBS displayed decreased activity of the mTORC1 pathway (assessed by the decreased level of phosphorylated S6 ribosomal protein) and lower proliferation rate compared with that in $TSC2^{-}$ parental cell line (Supplemental Figure S2). To compare the expression levels of *TGFβ1*, *TGFβ2*, and *TGFβ3* in $TSC2^{-}$, $TSC2^{+}$-1, and $TSC2^{+}$-2 cells, quantitative RT-PCR analysis was conducted, and a significant decrease in *TGFβ1* and *TGFβ3* transcript levels following the restoration of TSC2 expression was observed (Figure 1A). The negative correlation between *TGFβ2* and TSC2 expression was not obvious. Although there was a significant decrease in *TGFβ2* levels in $TSC2^{+}$-1 cells, its expression was elevated in the $TSC2^{+}$-2 cell line compared with the corresponding control lines, EV-1 and EV-2. The possible source of this discrepancy is proposed in the *Discussion* section.

Given that lack of TSC2 may affect the expression of certain genes in an mTOR-independent manner,[60] the role of mTORC1 signaling pathway in the up-regulation of *TGFβ1* and *TGFβ3* in $TSC2^{-}$ cells was examined next. $TSC2^{-}$ cells were treated with rapamycin, an inhibitor of mTORC1 (Figure 1B). Although the levels of *TGFβ1* were elevated in $TSC2^{-}$ cells compared with both cell lines with restored TSC2 expression, it remained unaffected by rapamycin treatment, implying that its up-regulation is not driven by mTORC1 signaling pathway. In contrast, inhibition of mTORC1 led to a significant decrease in the *TGFβ3* levels, suggesting mTORC1-mediated up-regulation of its expression in LAM cells.

The expression of TGF-β isoforms was analyzed in lung tissue biopsies from women with LAM. TGF-β1 was detected in diseased areas of all 10 patient samples (Figure 1C and Supplemental Figure S3). TGF-β1 staining in diseased nodules was quantified and compared with the negative control (tissue samples processed without primary antibody). Clear differences in staining relative to the negative controls were observed. The distribution of TGF-β1 positivity in LAM diseased areas appeared variable, potentially reflecting heterogeneity among the lesions in terms of cellular composition, histologic features, and disease state (Supplemental Figure S3). In addition to TGF-β1, TGF-β3 showed prominent expression within LAM lung nodules, whereas TGF-β2 was not detected (Figure 1C). Normal skin and colon tissue sections were used as a positive control for the expression of these factors (Supplemental Figure S4).

Tumor cells may escape anti-proliferative and pro-apoptotic signals triggered by TGF-β by down-regulating their responsiveness to this cytokine.[61,62] To evaluate whether this applies to LAM cells, the expression levels of TGF-β receptors in $TSC2^{-}$ cells were assessed. Both *TGFBR1* (*ALK5*) and *TGFBR2* were expressed in $TSC2^{-}$ cells at levels comparable to TSC2-restored sublines (Figure 1D), and their expression levels were not changed by rapamycin (Figure 1E). Next, the responsiveness of $TSC2^{-}$ cells to TGF-β stimulation was analyzed. To this end, $TSC2^{-}$ cells were treated with TGF-β1, TGF-β2, or TGF-β3, and the expression of *PAI-1*, a TGF-β–upregulable gene, was measured (Figure 1F). All three TGF-β isoforms significantly stimulated *PAI-1* expression in $TSC2^{-}$ cells.

Altogether, these results suggest that $TSC2^{-}$ cells retain responsiveness to TGF-β–mediated signaling and demonstrate up-regulated expression of *TGFβ1* and *TGFβ3*, with the latter being expressed in a rapamycin-sensitive manner.

$TSC2^{-}$ Cells Express GARP and Integrin β8 Involved in TGF-β Activation at the Cell Membrane

The localization of TGF-β activation within the extracellular space is determined by the type of protein associated with the secreted latent LAP–TGF-β complex.[32] When secreted in a complex with latent TGF-β binding protein, latent TGF-β is deposited within the extracellular matrix.[63] Certain cancer cells, along with regulatory T cells and endothelial cells, secrete latent TGF-β in a complex with the transmembrane protein GARP. Consequently, latent TGF-β is displayed at the cell membrane.[64–66] Such localization may facilitate the activation of TGF-β in close proximity to the cell membrane, leading to a relatively high local concentration of activated TGF-β.[51,67] According to the LCA database, LAM cells express GARP mRNA, and its expression levels are up-regulated compared with those in control lung mesenchymal cells.[55] In line with the LCA data, LAM patient-derived $TSC2^{-}$ cells expressed GARP both at the mRNA and protein levels (Figure 2, A and B). To verify whether GARP expression was controlled by the active TSC2, the level of GARP expression in two sublines with the restored expression of TSC2 was assessed. Unexpectedly, both control, empty vector-transfected sublines, EV-1 and EV-2, displayed significantly decreased expression of GARP compared with the parental $TSC2^{-}$ cells. Although the restoration of TSC2 expression resulted in the complete inhibition of GARP expression in $TSC2^{+}$-1 cells, it did not further down-regulate GARP expression in $TSC2^{+}$-2 cells compared with the corresponding control lines (Figure 2, A and B). The potential mechanism that underlines this discrepancy is discussed in the *Discussion* section.

On the basis of these results, it was not possible to conclude whether TSC2 affected GARP expression.

GARP levels were significantly down-regulated in rapamycin-treated $TSC2^{-}$ cells, which suggested mTORC1-dependent regulation of its expression (Figure 2, C and D). In GARP-expressing cells, LAP–TGF-β preferentially interacted with GARP and was expressed at the cell membrane as a GARP-bound complex.[31] To verify the

cell surface expression of GARP-bound LAP—TGF-β in TSC2$^-$ cells, MHG-8 monoclonal antibody that specifically recognizes GARP—LAP—TGF-β1 complexes was used.[51,68] Approximately 10% of TSC2$^-$ cells displayed GARP-bound LAP—TGF-β1 at the cell surface (Figure 2E). The release of active TGF-β from the GARP-bound complexes is mediated via interaction between the LAP RGD motif and integrin β6 or β8.[31,33,64] The

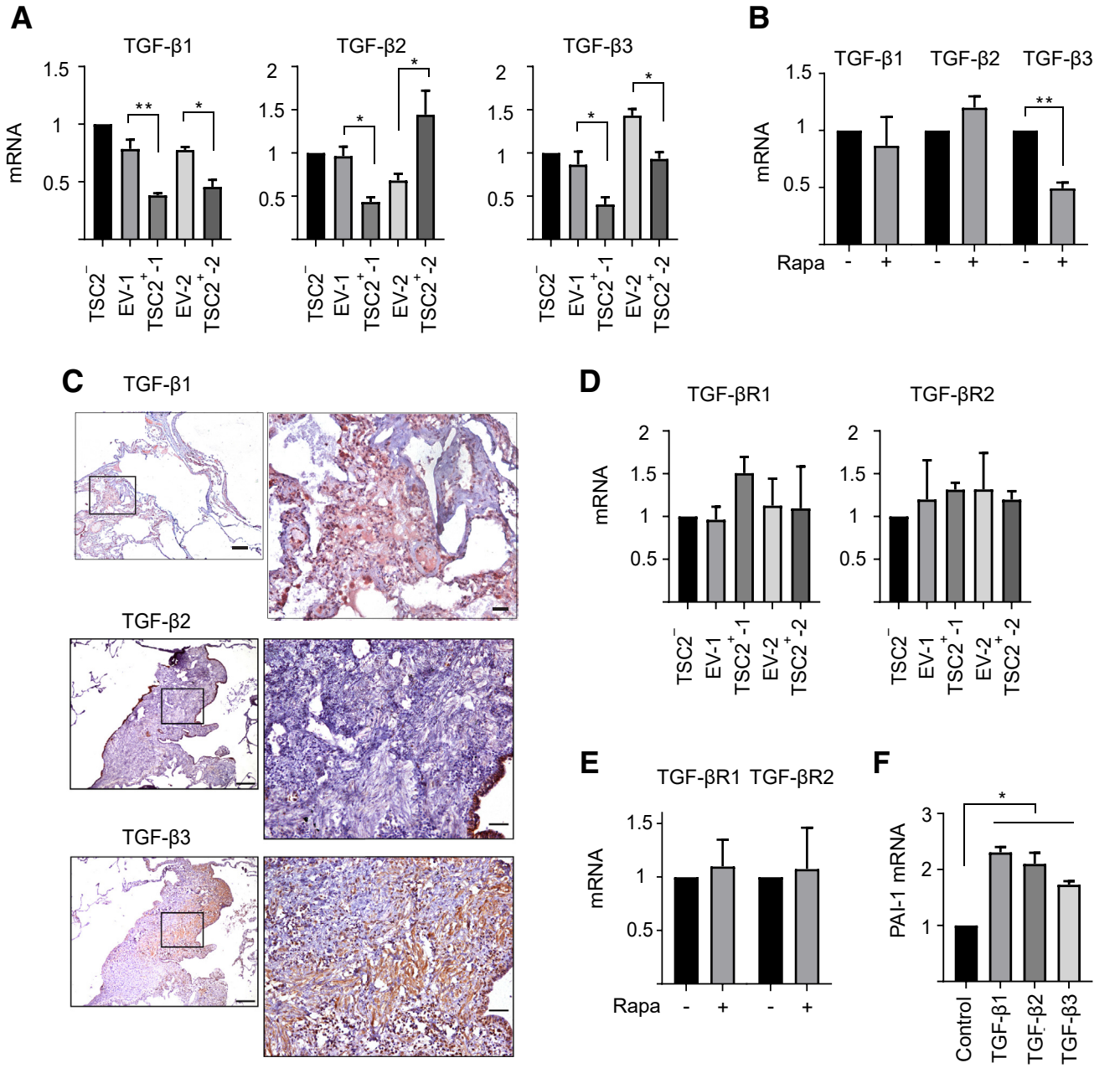

Figure 1 Tuberous sclerosis complex 2 (TSC2)$^-$ cells overexpress *TGFβ1* and *TGFβ3* and retain responsiveness to TGF-β. **A** and **D:** The levels of *TGFβ1*, *TGFβ2*, and *TGFβ3* transcripts (**A**) and *TGFBR1* and *TGFBR2* transcripts (**D**) in TSC2$^-$ cells, in two TSC2$^-$-derived sublines with restored TSC2 expression, TSC2$^+$-1 and TSC2$^+$-2, and in corresponding control cell lines EV-1 and EV-2, determined by quantitative RT-PCR (RT-qPCR) after 48 hours culture of the cells in fetal bovine serum (FBS)—reduced medium [Dulbecco's modified Eagle's medium (DMEM) + 2% FBS]. **B** and **E:** The levels of *TGFβ1*, *TGFβ2*, and *TGFβ3* (**B**) and *TGFBR1* and *TGFBR2* transcripts (**E**) in TSC2$^-$ cells cultured for 48 hours in DMEM + 2% FBS in the absence or presence of rapamycin (Rapa; 20 nmol/L) determined by RT-qPCR. **A**, **B**, **D**, and **E:** The expression levels of each analyzed gene in untreated TSC2$^-$ were set as 1. **C:** Representative immunohistochemistry images showing the expression of TGF-β1, TGF-β2, and TGF-β3 in a lymphangioleiomyomatosis (LAM) lung nodule. The **boxed areas** in the **left panels** are magnified in the **right panels.** All 10 analyzed LAM cases exhibited TGF-β1 positivity. TGF-β2 shows no positivity except for the normal epithelial layer. TGF-β3 shows frequent positivity in the diseased cell area, in addition to the normal epithelial layer. **F:** The levels of *PAI-1* transcript in TSC2$^-$ cells stimulated for 24 hours with different isoforms of TGF-β (2 ng/mL) were determined by RT-qPCR. The expression level of *PAI-1* in untreated (control) cells was set as 1. Data show means ± SD (**A**, **B**, and **D**—**F**). $n = 3$ independent experiments performed in duplicates (**A**, **B**, and **D**—**F**). $^*P < 0.05$, $^{**}P < 0.01$ using one-way analysis of variance. Scale bars: 250 μm (**C**, **left panels**); 50 μm (**C**, **right panels**).

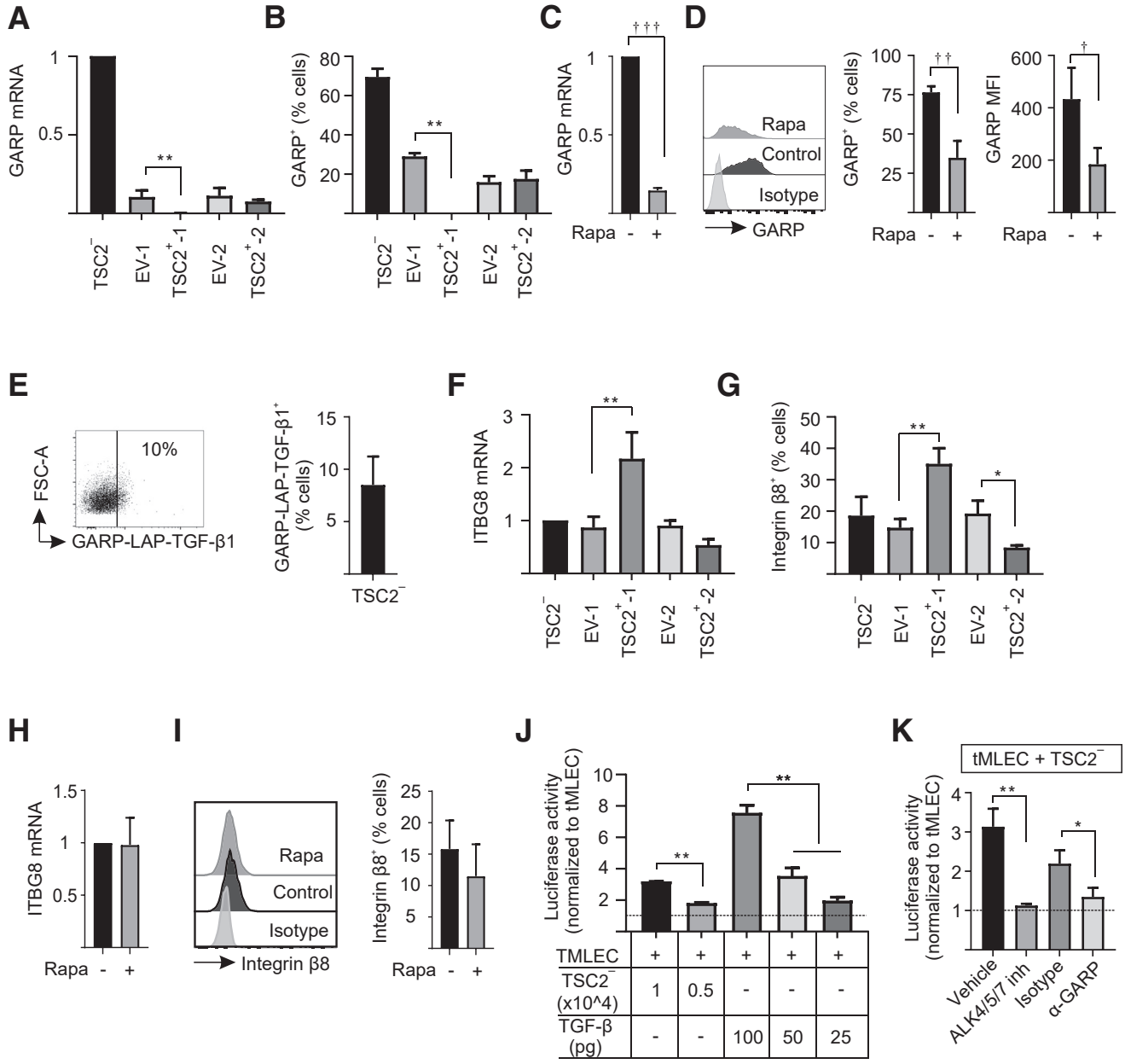

Figure 2 Tuberous sclerosis complex 2 (TSC2)$^{-}$ cells express glycoprotein-A repetitions predominant (GARP) and integrin β8, and activate TGF-β. **A** and **B:** GARP expression in TSC2^{-} cells and TSC2^{-}-derived sublines, cultured for 48 hours in Dulbecco's modified Eagle's medium (DMEM) + 2% fetal bovine serum (FBS). **A:** *GARP* transcript levels determined by quantitative RT-PCR (RT-qPCR); *GARP* levels in TSC2^{-} cells were set as 1. **B:** Percentage of GARP-expressing cells assessed by flow cytometry. **C** and **D:** The effect of rapamycin (Rapa; 20 nmol/L) on GARP expression in TSC2^{-} cells cultured for 48 hours in DMEM + 2% FBS. **C:** *GARP* transcript levels determined by RT-qPCR. **D:** Surface GARP expression analyzed by flow cytometry. **Left panel:** Representative histogram of GARP expression. **Middle panel:** Frequency of GARP-expressing cells. **Right panel:** Median intensity [mean fluorescent intensity (MFI)] of GARP expression. **E:** Expression of GARP—latency-associated peptide (LAP)—TGF-β1 complexes assessed by flow cytometry. **Left panel:** Representative dot plot. **Right panel:** Frequency of GARP—LAP—TGF-β1—positive cells. **F** and **G:** Expression of integrin β8 in TSC2^{-} and TSC2^{-}-derived cells cultured for 48 hours in DMEM + 2% FBS. **F:** Transcript levels assessed by RT-qPCR; *ITBG8* mRNA levels in TSC2^{-} were set as 1. **G:** Protein surface levels presented as a percentage of integrin β8—positive cells determined by flow cytometry. **H** and **I:** The effect of rapamycin (20 nmol/L) on integrin β8 level in TSC2^{-} cells cultured for 48 hours in DMEM + 2% FBS. **H:** *ITBG8* transcript levels determined by RT-qPCR. **I:** Surface integrin β8 expression analyzed by flow cytometry. **Left panel:** Representative histogram of integrin β8 expression. **Right panel:** Frequency of integrin β8—expressing cells. **J** and **K:** Evaluation of activity of TGF-β released from TSC2^{-} cells. Reporter tMLEC cells were co-cultured for 24 hours with 1×10^4 or 0.5×10^4 TSC2^{-} cells or monocultured in the presence of 100, 50, or 25 pg of recombinant TGF-β1 (**J**) or co-cultured with 1×10^4 TSC2^{-} cells in the presence of antibody blocking GARP—LAP—TGF-β1 complexes (α-GARP; 10 μg/mL) or its isotype control (isotype; 10 μg/mL), or ALK 4/5/7 inhibitor (ALK4/5/7 inh; 10 μmol/L; **K**). Luciferase activity was assessed at the end of the co-culture and for each culture condition was normalized to luciferase activity detected in its respective control monocultured tMLEC cells. Luciferase activity in monocultured tMLEC cells was set as 1 and is represented by a **dotted line**. Data show means ± SD (**A**—**K**). $n = 3$ independent experiments performed in duplicates (**A**—**K**). $^{*}P < 0.05$, $^{**}P < 0.01$ using one-way analysis of variance; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$, $^{\dagger\dagger\dagger}P < 0.001$ using *t*-test. FSC, forward scatter.

expression of integrin β6 could not be detected, but the expression of integrin β8 was verified in the TSC2$^-$ cell line and two pairs of its sublines, those with restored TSC2 expression and corresponding control lines (Figure 2, F and G). The LCA database points to integrin β8 expression in LAM cells.[55] In accordance with these data, TSC2$^-$ cells expressed integrin β8 in the cell membrane. The effect of TSC2 restoration on integrin β8 expression varied among TSC2-reexpressing sublines. Compared with control cells, an increase in integrin β8 levels was observed in TSC2$^+$-1 cells, whereas there was a decrease in integrin β8 levels in TSC2$^+$-2 cells. A possible interpretation of this discrepancy is addressed in the *Discussion* section. Unlike GARP, the expression of integrin β8 was not significantly altered by rapamycin treatment, suggesting that its expression is not regulated by mTORC1 (Figure 2, H and I). However, the possible mTORC1-independent role of TSC2 in the regulation of integrin β8 remains to be elucidated.

Because TSC2$^-$ cells express at the cell surface both LAP—TGF-β1 complexed with GARP and integrin β8 capable of releasing active TGF-β1, GARP-dependent activation of TGF-β in these cells was verified. A TGF-β reporter cell line, tMLEC, which expresses luciferase in response to TGF-β, was used.[45] The co-culture of tMLEC with TSC2$^-$ cells led to a significant, TSC2$^-$ cell number correlated increase in luciferase expression, as evidenced by increased luciferase activity, indicating TSC2$^-$ cell-dependent activation of TGF-β (Figure 2J). The activity of TGF-β secreted and activated by 1×10^4 cells was comparable to the activity displayed by 50 pg of recombinant TGF-β1 added to tMLEC culture for the same period. To evaluate the involvement of GARP in TGF-β activation, MHG-8 monoclonal antibody (later referred to as α-GARP antibody), which prevents the release of active TGF-β1 from GARP complexes, was used.[51,68] Therefore, the significant reduction of tMLEC luciferase activity in their co-culture with TSC2$^-$ cells in the presence of α-GARP antibody (Figure 2K and Supplemental Figure S5) suggested that active TGF-β1 derived from GARP—LAP—TGF-β1 is, at least partially, responsible for the activation of tMLEC cells. Whether TGF-β2 and/or TGF-β3 is also secreted in GARP-bound complexes and if GARP participates in their activation remains to be elucidated.

Taken together, TSC2$^-$ cells can tether LAP—TGF-β1 to the cell membrane via GARP and possess a capacity to activate TGF-β at least partially through its release from GARP-bound complexes.

Inhibition of TGF-β Compromises the Migratory Potential of TSC2$^-$ Cells

Certain tumor cells, including colon, pancreatic, or prostate cancer cells, while maintaining sensitivity to TGF-β, have developed mechanisms to evade its anti-proliferative and pro-apoptotic effects, instead using TGF-β—triggered signals to promote proliferation and migration.[61,62,69] Given the dysregulated proliferation and migration observed in LAM cells,[2,21] the potential role of TSC2$^-$ cell-derived TGF-β in regulating these processes was investigated. The autocrine TGF-β signaling in TSC2$^-$ cells was impeded by inhibiting TGF-β type I receptor activity. An ALK4/5/7 inhibitor (SB431542)[50] or a specific ALK5 inhibitor (LY364947) was used to suppress TGF-β type I receptor activity. The inhibition of TGF-β signaling did not markedly affect the proliferation of TSC2$^-$ cells (data not shown). To evaluate the role of TGF-β in the migration of TSC2$^-$ cells, the wound healing assay was used (Figure 3A). When compared with the control cells, the cells treated with ALK4/5/7 or with specific ALK5 inhibitor showed impaired wound closure, indicating that the migration rate of TSC2$^-$ cells was hampered when TGF-β signaling was blocked. To better mimic the complex interaction between migrating cells and extracellular matrix, the impact of TGF-β signaling inhibition on transmigration of TSC2$^-$ cells through collagen/Matrigel matrix was analyzed. Both ALK5 inhibitors significantly reduced the number of cells that transmigrated through a three-dimensional matrix (Figure 3B). To verify whether the lack of TSC2 determines the observed response to TGF-β inhibition, the impact of TGF-β signaling inhibitors on the migration of two sublines with the restored expression of TSC2 was assessed. Although LAM cells are characterized by enhanced migration, the restoration of TSC2 expression decreased the migration rate of TSC2$^+$-1 cells, but had no impact on TSC2$^+$-2 cells compared with the corresponding empty vector—transfected control lines (Figure 3C). Despite variations in the impact of TSC2 restoration on the migration rate, both TSC2 re-expressing cell lines exhibited impaired migration on treatment with ALK5 inhibitors, suggesting a TSC2-independent response to TGF-β (Figure 3C).

Taken together, these data indicate that TGF-β expressed by TSC2$^-$ cells stimulates their migration rate and may contribute to their invasiveness.

TGF-β Regulates MCP-1 Expression in TSC2$^-$ Cells

TSC2$^-$ cells overexpress MCP-1 in a TSC2-dependent manner, and LAM nodules have an increased level of MCP-1.[70,71] Given that TGF-β may regulate MCP-1 expression,[72,73] TGF-β—mediated regulation of MCP-1 expression in TSC2$^-$ cells was verified. First, the colocalization of these two cytokines within LAM nodules was analyzed. TGF-β1 and MCP-1 expression were evaluated in serial lung tissue sections from 10 patients with LAM. In all cases, both MCP-1 and TGF-β1 were detected in diseased areas (Figure 4A and Supplemental Figure S6A). Moreover, there was significant colocalization of MCP-1 and TGF-β1 within LAM lesions, as most diseased areas tested positive for both cytokines (Figure 4A). Given that in TSC2-null xenograft tumors, overexpression of MCP-1 has been

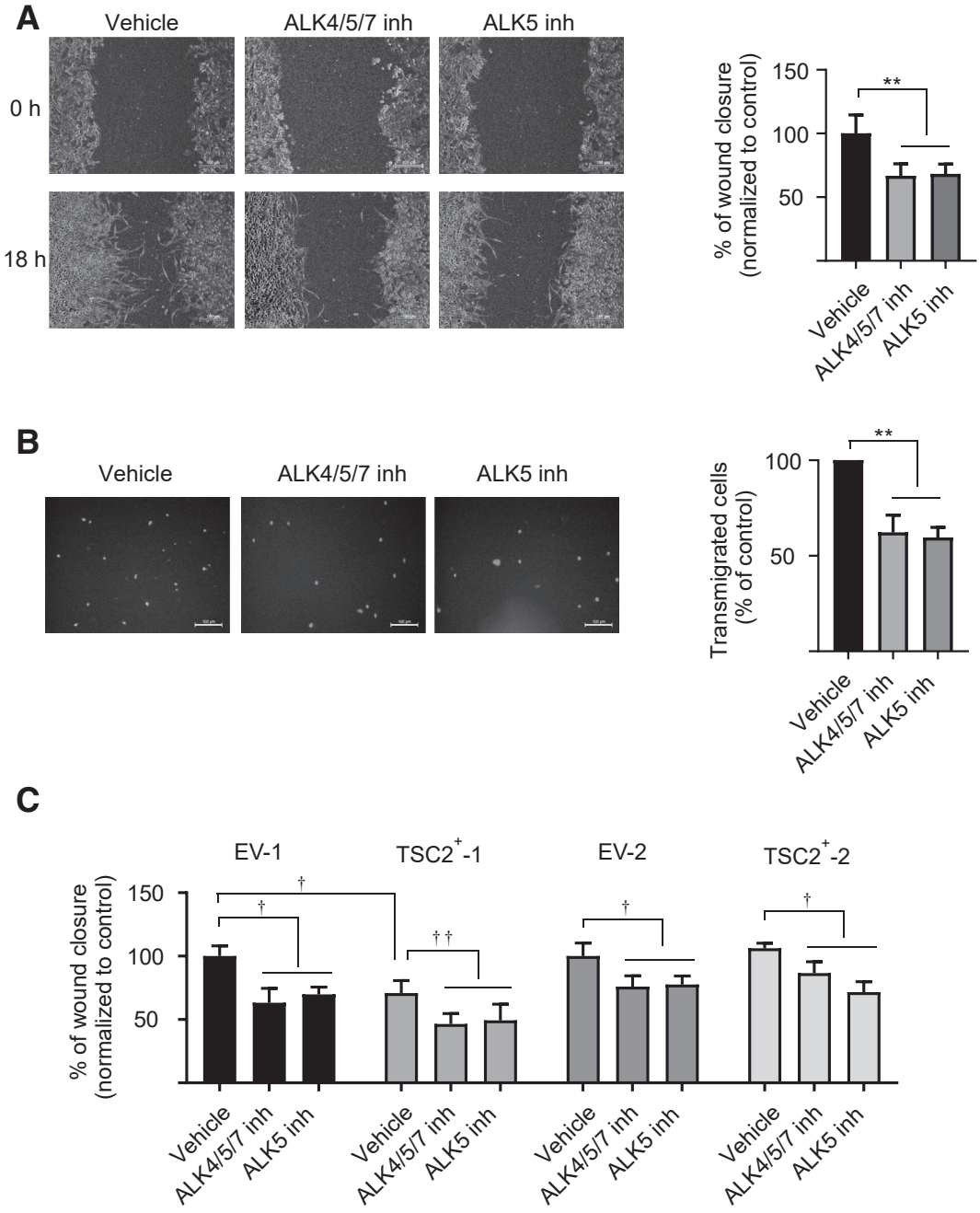

Figure 3 Inhibition of TGF-β signaling impairs migration of tuberous sclerosis complex 2 (TSC2)$^{-}$ cells. **A** and **B:** TSC2^{-} cells were pretreated for 24 hours with inhibitors of TGF-β signaling: ALK4/5/7 inhibitor (ALK4/5/7 inh) or ALK5 inhibitor (ALK5 inh; both 10 μmol/L), or dimethyl sulfoxide (vehicle). **A:** The wound healing assay. **Left:** Representative bright-field images of wound area captured 0 and 18 hours from scratching. **Right:** Quantification of a migration rate expressed as a percentage of wound closure 18 hours from scratching normalized to the corresponding control. **B:** Transmigration assay. **Left:** Representative bright-field images of transmigrated cells **Right:** Quantification of the cells transmigrated through three-dimensional collagen/Matrigel matrix expressed as a percentage of corresponding control. Inhibitors were present throughout both assays. **C:** TSC2^{-}-derived sublines were cultured with inhibitors of TGF-β signaling as in (**A**). The migration rate was determined in the wound healing assay and expressed as a percentage of wound closure 18 hours from scratching, normalized to the corresponding empty vector control. Data show means ± SD (**A**–**C**). $n = 3$ independent experiments performed in duplicates (**A**–**C**). $^{**}P < 0.01$ using one-way analysis of variance; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ using two-way analysis of variance. Scale bars = 100 μm (**A** and **B**, **left**).

correlated with increased infiltration of CD68^{+} tumor-associated macrophages at the tumor edges,[70] the presence of CD68^{+} cells was evaluated in lung tissue from 10 patients with LAM. The results demonstrated the presence of CD68^{+} cells dispersed throughout the lung parenchyma, with some areas showing prominent clusters of positive cells. In some cases, CD68^{+} cells were localized near lesion edges, and their infiltration was prominent only in larger, presumably more advanced, lesions (Figure 4A and Supplemental Figure S6B).

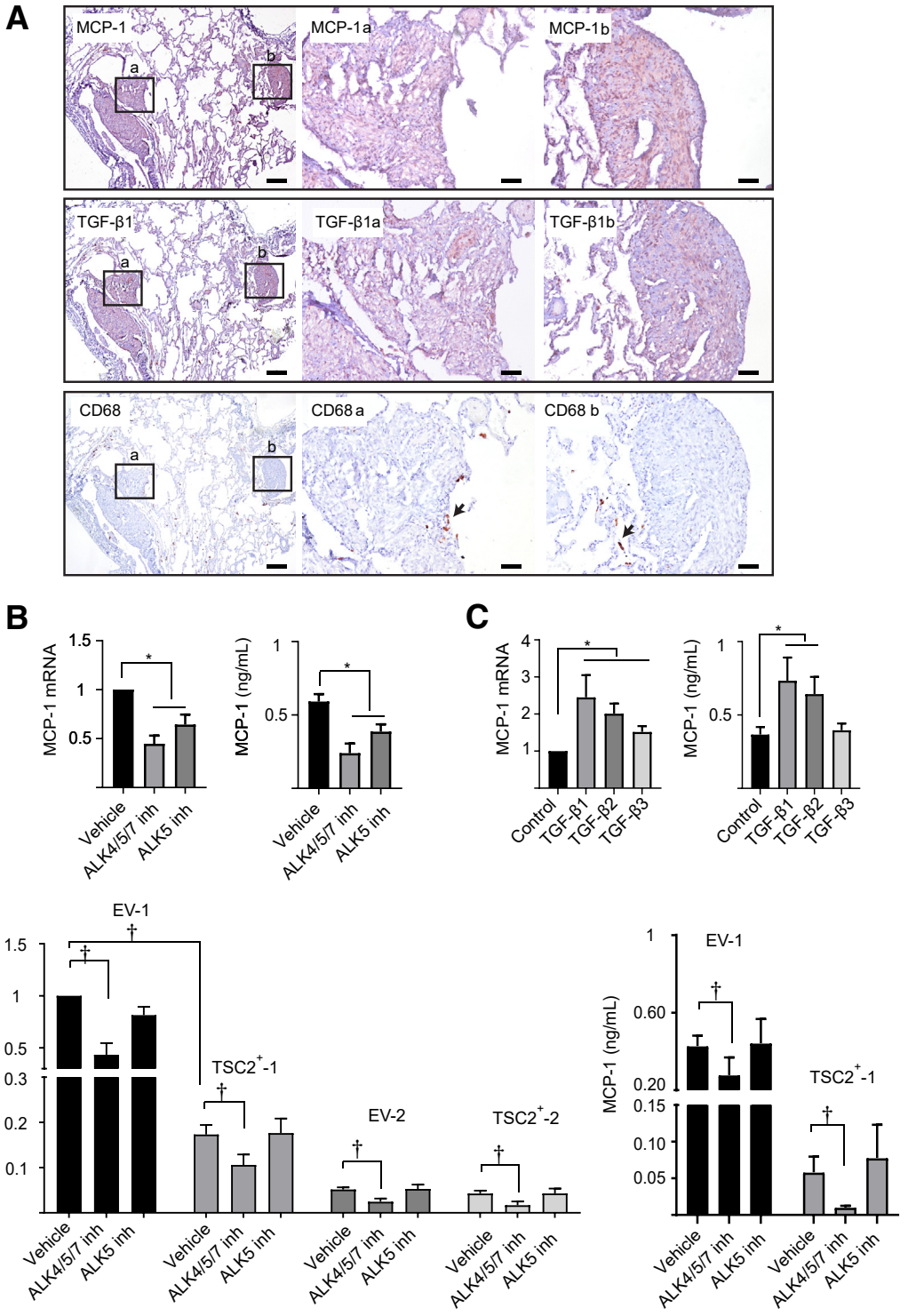

Figure 4 Analysis of TGF-β—mediated regulation of monocyte chemoattractant protein-1 (MCP-1) expression in tuberous sclerosis complex 2 (TSC2)$^{-}$ cells. **A:** Representative immunohistochemistry images showing the colocalization of MCP-1, TGF-β1, and CD68 in serial sections of lymphangioleiomyomatosis lung biopsies. **Left panels:** Low-magnification views of the tissue. **Right panels:** Higher magnification of the **boxed areas**, highlighting positively stained diseased regions. **Arrows** mark CD68^{+} cells infiltrating diseased areas. **B**—**D:** TSC2^{-} cells (**B** and **C**) or TSC2^{-}-derived sublines (**D**) were cultured for 24 hours with (**B** and **D**) inhibitors of TGF-β signaling: ALK4/5/7 inhibitor (ALK4/5/7 inh) or ALK5 inhibitor (ALK5 inh; both 10 μmol/L), or dimethyl sulfoxide (vehicle) or (**C**) different isoforms of TGF-β (2 ng/mL). **B**—**D:** The expression level of *MCP-1* was determined by quantitative RT-PCR (**left panels**) or enzyme-linked immunosorbent assay (**right panels**). **B**—**D:** *MCP-1* mRNA levels in control TSC2^{-} cells (**B** and **C**) and control EV-1 cells (**D**) were set as 1. Data show means ± SD (**B**—**D**). $n = 3$ independent experiments performed in duplicates (**B**—**D**). $^{*}P < 0.05$ with one-way analysis of variance; $^{\dagger}P < 0.05$ with two-way analysis of variance. Scale bars: 250 μm (**A**, **left panels**); 50 μm (**A**, **right panels**).

Given the increased level of MCP-1 observed in LAM lesions, the autocrine regulation of its expression by TGF-β in $TSC2^{-}$ cells was analyzed. The inhibition of TGF-β signaling by ALK 4/5/7 or ALK5 inhibitors led to a significant decrease in MCP-1 expression, both at mRNA and protein levels (Figure 4B). This suggested that in $TSC2^{-}$ cells MCP-1 expression was stimulated by TGF-β. To compare the impacts of the TGF-β isoforms on MCP-1 expression in $TSC2^{-}$ cells, cells were treated with TGF-β1, TGF-β2, or TGF-β3 (Figure 4C). Both TGF-β1 and TGF-β2 significantly up-regulated the levels of *MCP-1* mRNA and protein, with TGF-β1 demonstrating a more pronounced effect. On the contrary, the addition of TGF-β3 led to only a slight increase in *MCPI* mRNA levels, which did not result in elevated protein levels.

To determine whether the absence of TSC2 drives the observed response to TGF-β inhibition, the effect of TGF-β signaling was examined on inhibitors on MCP-1 expression in two sublines with restored TSC2 expression (Figure 4D). Although $TSC2^{-}$ cells overexpress MCP-1,[70,71] reduction in MCP-1 expression was observed in the present study only in one TSC2-reexpressing subline, $TSC2^{+}$-1, compared with the respective empty vector control. Interestingly, another control subline, EV-2, exhibited significantly lower *MCP-1* expression than EV-1, with the protein level below detection. Unlike $TSC2^{+}$-1 cells, $TSC2^{+}$-2 cells did not show a further reduction in *MCP-1* expression on TSC2 restoration. Inhibition of TGF-β signaling by ALK4/5/7 inhibitor decreased MCP-1 expression levels in both empty vector controls and $TSC2^{+}$-1 and $TSC2^{+}$-2 cells, suggesting TSC2-independent regulation. However, although ALK5 inhibitor decreased MCP-1 expression in parental $TSC2^{-}$ cells, it did not have the same effect in either the empty vector controls or TSC2-reexpressing sublines. Given these discrepancies, the findings obtained with these sublines should be interpreted with caution.

Taken together, these results show that the expression of MCP-1 by $TSC2^{-}$ cells is regulated by TGF-β. Using inhibitors that target the TGF-β signaling pathway may have the potential to decrease MCP-1 expression. However, the role of TSC2 in the TGF-β–regulated MCP-1 expression remains to be elucidated.

Discussion

TGF-β1, a factor with a well-documented role in the progression of established tumors, has been detected within LAM nodules.[42] Moreover, elevated levels of phosphorylated SMAD2, observed in both LAM and AML tissue, indicate that LAM-expressed TGF-β is active.[74] Interestingly, the expression of TGF-β isoforms and an elevated TGF-β signaling are also observed in uterine leiomyoma that spontaneously arise in Eker rats that carry loss-of-function mutation in *TSC2* gene, and inhibition of TGF-β reduces occurrence of uterine leiomyoma.[75] To get a better insight into the role of TGF-β in the biology of LAM cells, a TSC2-deficient cell line derived from AML of a patient with LAM was used, and the impact of TGF-β on cellular processes important for LAM progression was analyzed in the current study. Presented data suggest that $TSC2^{-}$ cells not only overexpressed and activated TGF-β but also retained responsiveness to its signaling. This autocrine TGF-β stimulation, along with other factors, may support the progression of LAM.

The overexpression of *TGFβ1* in $TSC2^{-}$ cells remained unaffected by rapamycin, indicating that it is not directly influenced by the mTORC1 signaling pathway. Certain genes, which are typically overexpressed in LAM cells, are not down-regulated in response to rapamycin, suggesting that molecules upstream of mTORC1 are involved in their regulation.[60] TSC2, identified as a transcription factor, negatively regulates the expression of some genes.[76] Thus, the absence of TSC2 in $TSC2^{-}$ cells may lead to the uninhibited expression of its target genes, including *TGFβ1*. It is also possible that *TGFβ1* expression in $TSC2^{-}$ cells is directly influenced by GTP-bound Rheb, elevated in $TSC2^{-}$ cells because of the absence of active TSC2, as Rheb-GTP regulates the expression of certain genes in a noncanonical, mTORC1-independent manner.[77]

In addition to TGF-β1, TGF-β2 and TGF-β3 are also overexpressed in various tumor types.[56,57,78] Interestingly, elevated expression of TGF-β3 has been demonstrated in uterine leiomyoma, which is regarded as a potential source of LAM cells.[11] TGF-β3 stimulates the proliferation of leiomyoma cells and the expression of extracellular matrix proteins.[79] Presented findings reveal that in comparison to the TSC2-restored cell lines, the AML-derived $TSC2^{-}$ cell line mirrored leiomyoma cells in overexpressing *TGFβ3*. The overexpression of *TGFβ3* by $TSC2^{-}$ cells was dependent on mTORC1, as rapamycin significantly decreased its expression. The expression of *TGFβ3* was also observed within LAM lung nodules.

The regulation of *TGFβ2* expression in LAM cells remains unclear. First, although expression of *TGFβ2* was detected in $TSC2^{-}$ cells, it was not detected in LAM lung nodules. Second, the impact of TSC2 restoration on *TGFβ2* expression varied among TSC2 re-expressing sublines. A decreased level of *TGFβ2* transcript was observed in the clonal TSC2 re-expressing subline, $TSC2^{+}$-1. However, in the subline derived from a more heterogeneous population of cells, $TSC2^{+}$-2, TSC2 expression resulted in an increased level of *TGFβ2*. In contrast to $TSC2^{+}$-1 subline derived from a randomly selected clone, the heterogeneous $TSC2^{+}$-2 (obtained via transposon transfection) is free from clonal bias. The clone(s)-derived cell lines may have features, resulting from different gene expression patterns, that are far from the average characteristic of the initial population.[58,80] Thus, the results obtained with a heterogeneous cell population should be more reliable. However, in both cases, the restoration of TSC2 expression as well as antibiotic selection might promote the growth of cells with

certain features not necessarily directly related to the reversal of the prior deficiency.[81,82] Thus, all results obtained with TSC2 re-expressing cell lines should be treated with caution.

TGF-β is either secreted into the extracellular matrix as part of the LAP—TGF-β—latent TGF-β binding protein complex or presented on the cell membrane bound to the transmembrane protein GARP.[32,67] Blood and lymphatic endothelial cells, regulatory T cells, and platelets are among the cells known to express GARP.[65,66,83,84] Also, certain cancer cells exhibit GARP-bound TGF-β on their cell membranes. TGF-β released via GARP-integrin αvβ6 or integrin αvβ8 interaction may exert its protumorigenic effects.[66,85,86] The presented study added TSC2$^-$ cells to the list of GARP-expressing cells. TSC2$^-$ cells also express integrin β8, which suggests their capability to activate TGF-β, thereby potentially elevating the concentration of TGF-β in close proximity to the cell membrane. Tumor cells expressing integrin β8 can activate TGF-β bound to the membranes of neighboring cells, including immune cells.[87] Therefore, integrin β8-expressing TSC2$^-$ cells may function as activators of TGF-β release not only for themselves but also for a more heterogeneous population of cells. Knowledge regarding the regulation of GARP expression in tumor cells is scarce. In TSC2$^-$ cells, GARP expression was driven by the mTORC1 signaling pathway, as rapamycin treatment led to a significant reduction of its expression. The picture of GARP expression in the cell lines with restored TSC2 is complex. First, GARP expression was strongly reduced in control cells transfected with either empty vector. This raises the question of whether the cells exhibiting low levels of GARP were preferentially selected during the generation of control sublines. On restoration of TSC2, GARP expression was entirely abolished in clonal TSC$^+$-1 cells, which is in line with the LCA data[55] and with the effect of rapamycin, but only slightly further decreased in heterogeneous TSC$^+$-2. The strong effect of empty vectors prevents a reliable analysis of the impact of TSC2 restoration on GARP expression. In contrast to GARP, the level of integrin β8 was not impacted by rapamycin treatment, suggesting its mTORC1-independent expression. However, the direct regulation of integrin β8 expression by TSC2 remains unclear, as the impact of TSC2 restoration varied among TSC2 re-expressing sublines.

Certain tumor cells retain responsiveness to TGF-β—triggered signals but reconfigure TGF—β signaling pathways to disconnect apoptotic and anti-proliferative signals and preserve signals that support proliferation, survival, migration, and invasion.[61,62] During tumorigenesis, TGF-β stimulates epithelial-to-mesenchymal transition, which is associated with the enhanced migratory and invasive potential of the cells[88,89] and may involve the mTORC1 pathway.[90,91] Interestingly, LAM cells display some features of epithelial-to-mesenchymal transition that may be driven by dysregulated TSC2/mTORC1 pathway and account for their increased migration rate.[74,92] Presented data indicate that TGF-β may be considered as one of the factors that regulate migration of TSC2$^-$ cells.

Chemokines, including MCP-1, may additionally promote LAM disease progression.[3,93] Altered expression of MCP-1 occurs in LAM cells as well as in TSC2-deficient Eker rat embryonic fibroblasts and uterine leiomyoma-derived cells.[70,71] MCP-1 expressed by LAM cells exerts its activity in an autocrine manner by enhancing the motility of LAM cells and promoting their homing to the lungs.[93] Moreover, MCP-1 shapes the microenvironment of TSC2-null xenograft tumors by promoting CD68$^+$ tumor-associated macrophage infiltration, which correlates with increased vascular endothelial growth factor-D levels.[70] Consequently, the Syk-dependent signaling pathway that governs MCP-1 expression is a potential novel target for LAM therapy.[70] TGF-β regulates MCP-1 expression in a wide range of cells.[72,73] Presented data point to TGF-β as another factor that stimulated MCP-1 expression in LAM cells and suggest that TGF-β inhibition is another possible approach to decrease the level of MCP-1 and block its disease-promoting activity.

The presented study showed that TGF-β stimulated migration and MCP-1 expression in TSC2$^-$ cells in a TSC2-independent manner. This suggests that the observed response of TSC2$^-$ cells to TGF-β is a feature of the cells from which LAM directly originates and that aberrant mTORC1 signaling in LAM TSC2$^-$ cells may stimulate this response by up-regulating TGF-β expression. Another possibility is that the reexpression of TSC2 in TSC2$^-$ cells does not fully restore the functional properties of the cells that give rise to LAM, particularly in terms of the response to TGF-β—triggered signaling. Thus, whenever possible, the findings should be validated in cells of origin for LAM.

Because of its well-characterized protumorigenic activity, TGF-β is considered a promising target for anti-cancer therapies, and various strategies to inhibit its activity have been extensively tested in both preclinical and clinical models.[94,95] Presented data suggest that TGF-β may be added to the list of factors that support the progression of LAM and TGF-β inhibitors could be combined with other therapeutic strategies to enhance treatment efficacy. However, the current study has some limitations. First, given that the outcome of TGF-β—induced signaling is highly context-dependent, these results must be verified in the *in vivo* models. Second, although the AML-derived 621-101 cell line is widely used as a substitute for pulmonary LAM cells, it may not fully represent their known heterogeneity.[14,96] The process of isolation, immortalization, and propagation of this cell line may have led to the selection of cells that do not recapitulate the average characteristic of the parental cells. Therefore, the findings should be interpreted with caution and, whenever possible, validated using a more representative cell model of pulmonary LAM cells.

Declaration of Generative AI and AI-Assisted Technologies in the Writing Process

During the preparation of this work, the authors used ChatGPT (GPT-4; OpenAI, San Francisco, CA) to refine language and correct grammar. After using this tool, the authors reviewed and edited the content as needed and take full responsibility for the content of the publication.

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Author Contributions

A.M., R.M., J.B., and R.M.-K. designed the experiments; A.M., R.M., and R.M.-K. performed experiments and analyzed the data; A.B., I.G., L.R.A., F.M.G., and M.A.P. performed immunohistochemistry analysis; E.R. provided some of lymphangioleiomyomatosis lung biopsies; M.W. designed the pSBbi-GP-PuroR-TSC2 vector and generated tuberous sclerosis complex 2 (TSC2) re-expressing subline; J.K. and A.H. analyzed the data; S.L. provided valuable suggestions as well as anti-human glycoprotein-A repetitions predominant—latency-associated peptide—transforming growth factor-β1 antibodies and tMLEC cell line; and J.B. and R.M.-K. wrote the manuscript with input from all authors and supervised the project. R.M.-K. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Disclosure Statement

None declared.

Supplemental Data

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