Online Data Supplement

MTOR suppresses autophagy-mediated airway epithelial injury in allergic inflammation

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Supplemental material and methods

Reagents

Antibodies used were as follows: ACTB (Santa Cruz Biotechnology, sc-47778), p-S6 (Cell Signaling Technology, 4858), MTOR (Cell Signaling Technology, 2983), p-MTOR (Cell Signaling Technology, 5536), LC3B (Sigma-Aldrich, L7543), CC10 (Santa Cruz Biotechnology, sc-365992), TSC2 (Cell Signaling Technology, 3990), p-TSC2 (Cell Signaling Technology, 3611), p-TSC2 (Abcam, ab109403), IL25 neutralization antibody (R&D systems, MAB13992), Rat isotype control (R&D systems, MAB0061). RNAiso plus (9109), Reverse Transcription Reagents (DRR037A), and SYBR Green Master Mix (DRR041A) were purchased from Takara Biotechnology. Primers for *Tslp, Il13, Il25, Il33,* and *Muc5ac* were synthesized by Sangon Biotech, Shanghai. ELISA kits for mouse TSLP (MTLP00), IL13 (DY413), IL25 (DY1399), and mouse IL33 (M3300) were purchased from R&D systems. Doxycycline (D9891) and Baf A1 (B1793) were purchased from Sigma-Aldrich. Spautin1 (S7888) was purchased from Selleck. Recombinant Human IL33 (C091), human IL4 (CX03), and human TNF-α (C036) was purchased from Novoprotein, and recombinant Human IL13 (200-13) was a product from Peprotech.

Human samples

The diagnosis of bronchial asthma was based on the Global Initiative for Asthma (GINA) guidelines. The patients were relatively severe asthma who received a

bronchoscope to further confirm the clinical diagnosis. Exclusion criteria included systemic inflammation or other respiratory diseases. Healthy control subjects were participants with benign pulmonary nodules, who had normal lung function and no respiratory symptoms. We collected 3 sets of paraffin-embedded slides of human airway samples from Weifang Asthma Hospital (Weifang, China), State Key Lab of Respiratory Disease (Guangzhou, China), and the Second Affiliated Hospital of Zhejiang University (Hangzhou, China). We used the 1st set of samples (3 controls and 12 asthmatics, each 2 slides) for staining of p-S6 and LC3B. However, the number of asthmatic samples of LC3B staining was 11, because there were no epithelial cells in 1 slide for LC3B staining. And we used the 2nd set of samples (7 controls and 15 asthmatics) for staining of p-TSC2. We also collected another set of fresh airway biopsies (4 healthy controls and 4 asthmatics) for electronic microscopy analysis. In all the 3 sets of human samples, both age and sex distributions were similar for asthmatic patients and controls. The study was approved by the ethics committee of the three institutions. Written informed consent was obtained from all asthmatic or control subjects. The clinical information of each set of human subjects was shown in Table 1-2 and S3.

Mice

mtor$^{flox/flox}$ mice (C57BL/6; The Jackson Laboratory) were crossed with CC10-rtTA/(tetO)$_7$-cre transgenic mice (C57BL/6) to generate CC10-rtTA/(tetO)$_7$-cre-*mtor*$^{flox/flox}$ (*mtor*$^{\Delta/\Delta}$) mice. Transgene-negative littermates were used as the control (*mtor*$^{+/+}$) animals in the experiments. To induce expression of Cre,

6-wk-old *mtor*$^{\Delta/\Delta}$ mice and age- and sex-matched *mtor*$^{+/+}$ mice were fed with doxycycline in drinking water (2 mg/ml) for 20 days in advance of establishing the model of asthma, and the mice were maintained on doxycycline at all the time until they were killed. *lc3b*$^{-/-}$ mice were from Jackson laboratory. C57BL/6 mice were used as WT mice, which were purchased from Shanghai SLAC laboratory animal Co. Ltd. To delete both of the *mtor* and *lc3b* alleles in airway epithelial cells, CC10-rtTA/(tetO)7-cre-*mtor*$^{flox/flox}$- *lc3b*$^{-/-}$ (*mtor*$^{\Delta/\Delta}$*lc3b*$^{-/-}$) mice were generated by crossing the *mtor*$^{\Delta/\Delta}$ mice with *lc3b*$^{-/-}$ mice. CD45.1 mice were kindly provided by Dr. Lie Wang, Zhejiang University. All mice used in this study were 6-8-wk-old male and were housed under a constant 12-hour light/12-hour dark cycle in a specific pathogen-free facility and all experimental protocols were approved by the Ethical Committee for Animal Studies at Zhejiang University. During the process of animal studies no adverse events occurred.

Transmission electron microscopy

For transmission electron microscopy (TEM) examination, the freshly isolated bronchus samples from mouse and fiberoptic bronchial biopsy specimens of asthmatic patients and healthy control were fixed in 2.5% glutaraldehyde in PBS overnight and embedded in paraffin. These samples were photographed using a TECNA1 10 transmission electron microscope (FEI, Hillsboro, Oregon, USA). For quantification, the area of the cell cytoplasm of ciliated cells was measured using Image-Pro Plus 6.0 (Media Cybernetics, Silver Spring, MD, USA), and all autophagic vacuoles (AVs) in human ciliated cells were counted. Data were represented as AVs

per 100 μm^2. While in mouse ciliated cells, Autophagosomes (APs) and autolysosomes (ALs) were counted. [1-3] The data were represented as APs and ALs per 100 μm^2, respectively.

Cell culture

HBE cells were purchased from American Type Culture Collection (ATCC@ PCS-300-100™) and were cultured in RPMI 1640 with 10% FBS. Each well of six-well plates was placed with 5×10^5 cells for 18h and treated with IL13 (10 ng/ml) or IL33 (100 ng/ml).

GFP-LC3 plasmid transfection

The GFP-LC3B plasmid transfection was performed with the PolyJet in vitro DNA Transfection reagent following the manufacturer's protocol. Briefly, cells were seeded to each well of six-well plates for 24 h before transfection. The infection medium was replaced with fresh growth medium after being incubated with HBE cells for 8 h. Confocal images were viewed using a Zeiss LSM confocal laser scanning microscope (Carl Zeiss, Göttingen, Germany).

Allergic animal models

Mice for HDM model were exposed to HDM (Greer Laboratories, 10.52 EU/mg endotoxin) via intratracheal injection of HDM (100 μg) in 50 μl saline (control mice injected pure saline) on days 0, 7 and 14 and were sacrificed on day 17 as previously described.[4] Mice for OVA model were sensitized on day 0 and day 14 by

intraperitoneal injection of 80 μg OVA in 0.1 ml saline and equal volume of aluminum hydroxide. On days 24, 25, and 26, mice were nebulized with 1.5% OVA in saline for 45 min. Control mice were sensitized and challenged with saline instead of OVA. Mice were analyzed in 24h after the last challenge.[5]

Measurement of airway hyperresponsiveness

we examined the airway hyperresponsiveness (AHR) by invasive plethysmography 48 h after the final HDM challenge using the Buxco FinePoint (Buxco Electronics, Troy, NY) as described previously.[6]

Bone marrow transplantation

Bone marrow transplantation (BMT) was performed by transplanting total BMCs (5×10^6) from 6-week-old mice (donor) into lethally irradiated (X-ray radiation at the dose of 8 Gy) 6-week-old mice (recipient) through tail vein. Allergic models were established by three weeks after BMT. Transplantation efficiency was evaluated by detecting the ratio of reconstituted $CD45.1^+$ or $CD45.2^+$ / total $CD45^+$ cells in bone marrow when mice were sacrificed.

In vivo spautin-1 treatment

Spautin-1 was dissolved in DMSO and was injected intraperitoneally (20 mg/kg) on days 12, 13, and 14 before the last HDM challenge. The controls were received the same volume of DMSO.

In vivo IL25 neutralization antibody treatment

IL25 neutralization antibody (2 μg) was dissolved in 50 μl PBS and was injected intratracheally on day 13, 14, 15, and 16. The controls were received the same volume of normal Rat isotype control or Goat isotype control, respectively.

BALF collection and analysis

72 h after the last exposure to HDM or 24 h after the last OVA challenge, mice were sacrificed to obtain BALF by injecting 0.4 ml PBS into the left lungs and withdrawing to collect the cells for three times. After counting the number of BALF total cells, the residual BALF was centrifuged at 6000 rpm for 10 min at 4℃. The supernatant was stored at -80℃ for the detection of LDH and cell precipitation suspended in PBS was spun onto glass slides. Cells were stained with Wright-Giemsa stain (Baso, BA4017) for differential counts by counting total of 200 cells.

Histological analyses

After treatment with HDM or OVA, the left lungs of experimental mice were fixed in formalin for 24 h, embedded in paraffin, and then stained with H&E or PAS according to standard procedures. The degree of peribronchial and perivascular inflammation was assessed on a subjective scale of 0–3 as previously described.[7] PAS-stained goblet cells in airway epithelium were measured using a numerical scoring system (0 =＜5% goblet cells; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 =＞75%＝, as described previously.[8] All quantification of histology was measured by observers blinded to group/treatment.

RNA isolation and quantitative real-time PCR analysis

Total RNA was extracted from lung homogenates by RNAiso plus. RNA was reverse-transcribed with Reverse Transcription Reagents and cDNA was used for quantitative real-time PCR with SYBR Green Master Mix on a StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA) to determine the levels of mouse *Il13*, *Il33*, *Il25*, *Tslp*, and *Muc5ac.* All procedures were according to the manufacturer's protocols. Data were normalized to *Actb* expression. The primers are listed in Table S1.

Immunohistochemistry (IHC) staining

Lung tissues and fiberoptic bronchial biopsy specimens of asthmatic patients and healthy controls were fixed in 4% paraformaldehyde and embedded in paraffin. Then lung sections were immunostained with antibodies against p-S6, LC3B, and p-TSC2 using the standard methods.[9] These samples imaged using an Olympus BX53 inverted microscope (Olympus, Melville, NY). Image quantitative analysis was performed by observers blinded to group/treatment. To evaluate the alteration of the expression of p-S6, LC3B, and p-TSC2, results were provided as the percentage of positive cells in total epithelial cells.

Immunofluorescence staining

Lung sections were stained together with anti-p-S6 and anti-CC10 according to the manufacturer's protocol. Fluorescent images were captured with a Zeiss LSM laser scanning confocal microscope.

Western blot assay

Lung tissue homogenates were prepared with RIPA lysis buffer (Beyotime, P0013B) in the presence of protease-inhibitor cocktail (Roche Diagnostics GmbH, 04-693-116-001). Lysates were loaded to SDS–PAGE and immunoblotted with relevant antibodies using standard methods.

ELISA

Lung tissue homogenates were prepared by adding 300 μl RIPA to every 10 mg lung tissues. The concentration of TSLP, IL25, IL33, and IL13 in lung homogenates was determined with ELISA kits following the manufacturer's protocol.

Supplemental references

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Supplementary figures

Figure S1. Semi-quantification. (A) Semi-quantification of figure 3D. (B) Semi-quantification of figure 3H. Error bars, median ± IQR.

Figure S2. Inflammatory cytokines IL4 and TNF-α are not able to inactivate MTOR and induce autophagy in HBE cells. (A and B) Immunoblotting analysis of MTOR-autophagy-related molecules in HBE cells treated with IL4 (A) or TNF-α (B) at 100 ng/ml for indicated time points.

Figure S3. Activation of TSC2 in the airway epithelium of asthmatic patients or allergic mice, or in HBE cells treated with IL13. (A and B) Representative images (A) and semi-quantification (B) of the expression of immunohistochemistry staining of

p-TSC2 in bronchial specimens from asthmatic patients. Scale bar: 50 μm. Data are representative of 7 healthy controls and 15 asthmatic patients. (C and D) Representative images (C) and semi-quantification (D) of the expression of immunohistochemistry staining of p-TSC2 in mouse lung sections 72 h after HDM challenge. Scale bar: 50 μm. Data are representative of 6 mice per group. (E-G) Western blot analyses of the levels of TSC2 and p-TSC2 in IL13-treated HBE cells (E), and the semi-quantification results (F and G). Data are representative of 3-4 independent experiments. Error bars, mean ± SEM (B and D) or median ± IQR (F).

Figure S4. The generation of airway epithelium-specific *mtor*$^{\triangle/\triangle}$ mice. (A) Schematic map of the generation of *mtor*$^{\triangle/\triangle}$ mice. (B) Genotyping of *mtor*$^{\triangle/\triangle}$ mice by PCR using genomic DNA from mouse tails. (C) Representative immunofluorescence images of p-S6 (red), nuclei (DAPI, blue), airway epithelial cells (with clara cell antigen 10, CC10, green) in *mtor*-deficient airway epithelium. Data are representative of 3 independent experiments.

Figure S5. Expression of certain cytokines and airway responsive in HDM-exposed *mtor*$^{\triangle/\triangle}$or *lc3b*$^{-/-}$ mice. (A-C) Relative mRNA expression of *Il13* (A), *Il33* (B), or *Muc5ac* (C) in *mtor*$^{\triangle/\triangle}$ mouse lungs. (D-H) Relative mRNA expression of *Il13* (D) *Il33* (E), *Muc5ac* (F), *Eotaxin 1* (G), or *Il5* (H) in *lc3b*$^{-/-}$ mouse lungs. Data are representative of 4-12 mice and were replicated in 3 independent experiments. Error bars, mean ± SEM (A to D, J) or median ± IQR (E to I). *P < 0.05.

Figure S6. Spautin-1 attenuates HDM-induced airway inflammation.

HDM-induced allergic airway inflammation was described in Supplemental Methods. On days 12, 13, and 14, mice were intraperitoneally injected with spautin-1 (20 mg/kg) before the last HDM challenge. (A) The numbers of total cells and eosinophils in BALF. (B to E) Relative mRNA expression of *Il13* (B), *Il25* (C), *Il33* (D), and *Muc5ac* (E) in mouse lungs exposed to HDM. (F) AHR in response to methacholine in Spautin-1 treated mice was measured 48 h after the last exposure to HDM. Data are representative of 5-7 mice for each group and were replicated in 3 independent experiments. Error bars, mean ± SEM.

Figure S7. Bone marrow reconstitution of WT or *Ic3b*$^{-/-}$ mice for allergic models. (A) Schematic image of the bone marrow transplantation (BMT). (B) Representative flow cytometry analysis of the expression of CD45.1 or CD45.2 in mouse bone marrow, and the efficiency of bone marrow transplantation (planted CD45.1^{+} or CD45.2^{+} / total CD45^{+} cells). Data were representative of 21 mice. (C) Amounts of eosinophils in BALF induced by HDM challenge. (D and E) Relative mRNA expression of *Il13* (D) and *Il25* (E) in mouse lungs exposed to HDM. Data are representative of 5-9 mice for each group and were replicated in 3 independent experiments. Error bars, median ± IQR.

Figure S8. Expression of IL33 and TSLP in *mtor*$^{\triangle/\triangle}$ and *Ic3b*$^{-/-}$ mice. (A) Representative images of immunohistochemistry staining of IL33 in mouse lung sections 24 h after HDM challenge. Scale bar: 20 μm. (B and D) The mRNA expression of *Tslp* in mouse lung tissues of *mtor*$^{\triangle/\triangle}$ (B) and *Ic3b*$^{-/-}$ (D) mice 72 h after

HDM challenge. (C and E) The protein expression of TSLP in BALF of *mtor*$^{\triangle/\triangle}$ (C) and *lc3b*$^{-/-}$ (E) mice 72 h after HDM challenge. Data are representative of 5-11 mice and were replicated in 3 independent experiments.

Figure S9. Summarization of the role of MTOR-LC3B axis in asthmatic epithelial injury. Allergen-initiated inflammatory mediators such as early initiated IL33 and later produced IL13 activate TSC2, which then suppresses MTOR and subsequently induces autophagy in airway epithelial cells. The induction of autophagy results in the production of certain pro-allergic cytokines such as IL25, thereby promoting the type 2 response and the overall airway inflammation in asthma

Table S1. Primers used for quantitative real-time PCR analysis.

Species	Genes	Primer sequence (5'-3')
Mouse	*Actb*	Forward AGAGGGAAATCGTGCGTGAC
		Reverse CAATAGTGATGACCTGGCCGT
Mouse	*Muc5ac*	Forward CTGTGACATTATCCCATAAGCCC
		Reverse AAGGGGTATAGCTGGCCTGA
Mouse	*Il13*	Forward CCTGGCTCTTGCTTGCCTT
		Reverse GGTCTTGTGTGATGTTGCTCA
Mouse	*Il33*	Forward ATTTCCCCGGCAAAGTTCAG
		Reverse AACGGAGTCTCATGCAGTAGA
Mouse	*Il25*	Forward TATGAGTTGGACAGGGACTTGA
		Reverse TGGTAAAGTGGGACGGAGTTG
Mouse	*Tslp*	Forward ACTGCAACTTCACGTCAATTACG

		Reverse TTGCTCGAACTTAGCCCCTTT
Human	*ACTB*	Forward CATGTACGTTGCTATCCAGGC
		Reverse CTCCTTAATGTCACGCACGAT
Human	*IL25*	Forward CCAGGTGGTTGCA-TTCTTGG
		Reverse TGGCTGTAGGTGTGGGTTCC

Table S2. Primers used for genotyping.

Genes	Primer sequence (5'-3')
Cc10	Forward AAA ATCTTGCCAGCTTTCCCC
	Reverse ACTGCCCATTGCCCAAACAC
Teto	Forward TGCCACGACCAAGTGACAGCAATG
	Reverse AGAGACGGA AATCCATCGCTCG
Mtor	Forward TTATGTTTGATAATTGCAGTTTTGGCTAGC AGT
	Reverse TTTAGGACTCCTTCTGTGACATAC ATTTCCT
Lc3b	Lc3b-1 GACACCTGTACACTCTGATGCACT
	Lc3b-2 CCTGCCGTCTGCTCTAAGCTG
	Lc3b-3 CCACTCCCACTGTCCTTTCCTAAT

Table S3. Characteristics of human subjects for p-TSC2 Expression.

	Healthy controls	Asthma	P-Value
Number	7	15	-
Age, yrs	52±3	49±2	0.547
Sex (M/F)	4/3	7/8	-

Bronchodilator reversibility test	negative	positive	-
Blood eosinophils (%)	1.77±0.62	7.23±1.72	0.048
FEV1, % predicted	86.38±7.38	65.74±5.22	0.034

Data are presented as Mean ± SEM. Differences between groups were assessed by t-test.