TNF-α Suppresses Autophagic Flux inAcinar Cells in IgG4-Related Sialadenitis

X. Hong, S.N. Min, Y.Y. Zhang, Y.T. Lin, F. Wang, Y. Huang, G.Y. Yu,L.L. Wu, and H.Y. Yang

Appendix

Appendix methods

Enzyme-linked Immunosorbent Assay (ELISA)

Serum cytokine levels were measured by use of TNF alpha and IL-1 beta Human ELISA Kits (Invitrogen) following the manufacturer's instructions, with analytical sensitivity of 2.3 pg/mL and 0.3 pg/mL, respectively, and assay ranges of 7.8-500 pg/mL and 3.9-250 pg/mL, respectively.

Real-time Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from submandibular glands (SMGs) of the patients and controls using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. Reaction mixture (20 μL) containing 1 μg total RNA was reverse transcribed to cDNA using a PrimeScript RT Reagent Kit (Takara, Beijing, China) according to the manufacturer's instructions. RT-PCR was performed using SYBR Premix Ex Taq II kit (Takara, Beijing, China) and was monitored by the ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Life Technologies, Waltham, MA, USA). The primers used for TNF-α and GAPDH are listed in Appendix Table 3.

Western blotting

Total proteins of SMG-C6 cells and human SMGs were extracted using RIPA lysis buffer. For analysis of transcription factor EB (TFEB), cytoplasmic and nuclear proteins were extracted from SMG-C6 cells using NuclearCytosol-Mem Extraction kit (Applygen Technologies Inc., Beijing) as per manufacturer instructions. Protein concentrations were detected by a BCA Protein Assay Kit (Biosharp, Hefei, China). The extracted proteins were separated on 12% or 15% SDS-PAGE, transferred to polyvinylidene difluoride membranes, blocked with 5% nonfat milk, and probed with primary antibodies at 4°C overnight. The membranes were incubated with peroxidase-coupled secondary antibodies and visualized by enhanced chemiluminescence reagent (Millipore Sigma, Burlington, MA, USA). The band densities were quantified by use of ImageJ software.

Histological, immunohistochemical, and immunofluorescence staining

Hematoxylin and eosin (H&E) staining were performed on 4-μm-thick consecutive paraffin sections of SMG. For immunostaining, SMG sections or cells were blocked with 5% bovine serum albumin, then incubated with primary and HRP-conjugated secondary antibodies. Morphological changes were examined under a light microscope (BX53; Olympus, Japan). For immunofluorescence, the samples were incubated with a fluorescently labeled secondary antibody and 4,6-diamidino-2-phenylindole (DAPI) to label nuclei, and then fluorescence images were captured using a confocal microscope (LSM710; ZEISS, Germany).

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) assay

The 4-μm-thick paraffin sections of SMG were deparaffinized and rehydrated, then incubated with

Proteinase K for 30 min at 37°C. After addition of the TUNEL reaction mixture, sections were incubated for 60 min at 37°C according to the manufacturer's instructions (Roche, Mannheim, Germany), and observed under a fluorescence microscope (BX53; Olympus, Japan).

Transmission electron microcopy (TEM)

SMG tissues were fixed in 2% paraformaldehyde-1.25% glutaraldehyde for 24 h, and then fixed in 1% buffered osmium tetroxide, dehydrated with ethanol, and embedded in Eponate 12 resin (Ted Pella Inc., Redding, CA, USA). Ultrathin sections were cut on a Leica UC6rt ultramicrotome (Leica Microsystems, Bannockburn, USA) at 70-80 nm, placed on Formvar-coated grids, and counter-stained with aqueous uranyl acetate and lead citrate. Sections were examined by use of a transmission electron microscope (H-7500; Hitachi, Japan).

Appendix Table 1. Information of antibodies

Antibody	Manufacturer	Catalog No.	Clone No.	Application	Dilution
TNF-α	Abcam	ab1793	52B83	IHC	1:100
β-actin	Abcam	ab115777	SP124	WB	1:2000
GAPDH	Abcam	ab181602	EPR16891	WB	1:2000
Histone H3	Abcam	ab176842	EPR16987	WB	1:100000
Beclin-1	Abcam	ab62557	polyclonal	WB	1:1000
Phospho-extracellular signal-regulated kinase 1/2 (p-ERK1/2)	Abcam	ab32538	E337	IF	1:100
IgG4	Zhongshan Laboratories	ZA-0576	EP13	IHC	working solution
Microtubule-associated protein 1 light chain 3 (LC3)	Sigma-Aldrich	L7543	polyclonal	WB IF	1:1000 1:100
SQSTM1/p62	Proteintech Group	18420-1-AP	polyclonal	WB IF	1:1000 1:100
Transcription factor EB (TFEB)	Proteintech Group	13372-1-AP	polyclonal	WB IF	1:1000 1:100
Cathepsin D	Proteintech Group	21327-1-AP	polyclonal	WB	1:1000
Lysosome-associated membrane protein 2 (LAMP2)	Santa Cruz	sc-18822	H4B4	IF	1:100
Aquaporin 5 (AQP5)	Santa Cruz	sc-514022	D-7	IF, IHC WB	1:100 1:1000
Erk1/2	Cell Signal Technology	4695	137F5	WB	1:1000
p-ERK1/2	Cell Signal Technology	4370	D13.14.4E	WB	1:1000
Autophagy-related 5 (Atg5)	Cell Signal Technology	12994	D5F5U	WB	1:1000

IHC, immunohistochemical stain; *WB*, western blot; *IF*, immunofluorescence staining

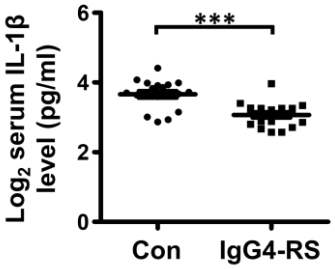
Appendix Table 2. Baseline information of IgG4-RS patients

Age (years)	51.8 ± 2.8
Male: female ratio	1.75:1 (21M: 12F)
Organ involvements, *n* (%)	
Submandibular gland	33 (100.0)
Parotid gland	12 (36.4)
Sublingual gland	12 (36.4)
Lacrimal gland	20 (60,6)
Pancreas	1 (3.0)
Biliary system	1 (3.0)
Retroperitoneal tissue	1 (3.0)
Serum IgG4 level, (mg/L)	8894 ± 1606

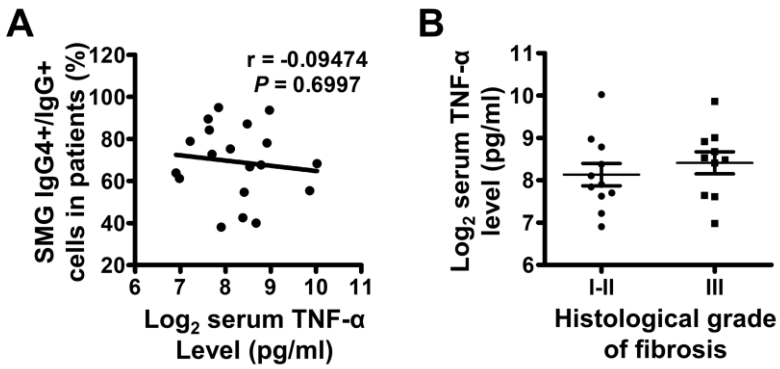
IgG4-RS IgG4-related sialadenitis; all data are presented as mean ± SEM

Appendix Table 3. Primers for human TNF-α and GAPDH mRNAs

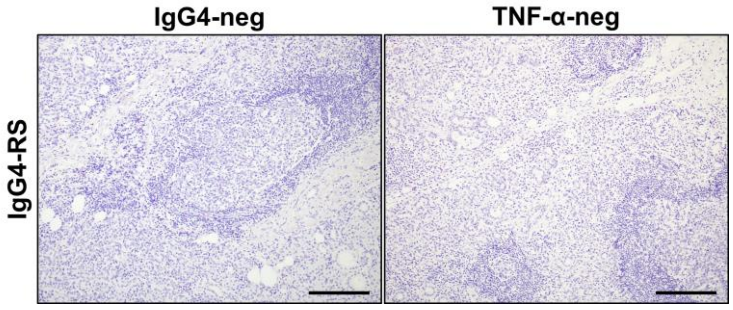
Gene	Forward primer (5′→3′)	Reverse primer (5′→3′)
TNF-α	CTGGGCAGGTCTACTTTGGG	CTGGAGGCCCCAGTTTGAAT
GAPDH	ACATCATCCCTGCCTCTACTG	CCTGCTTCACCACCTTCTTG



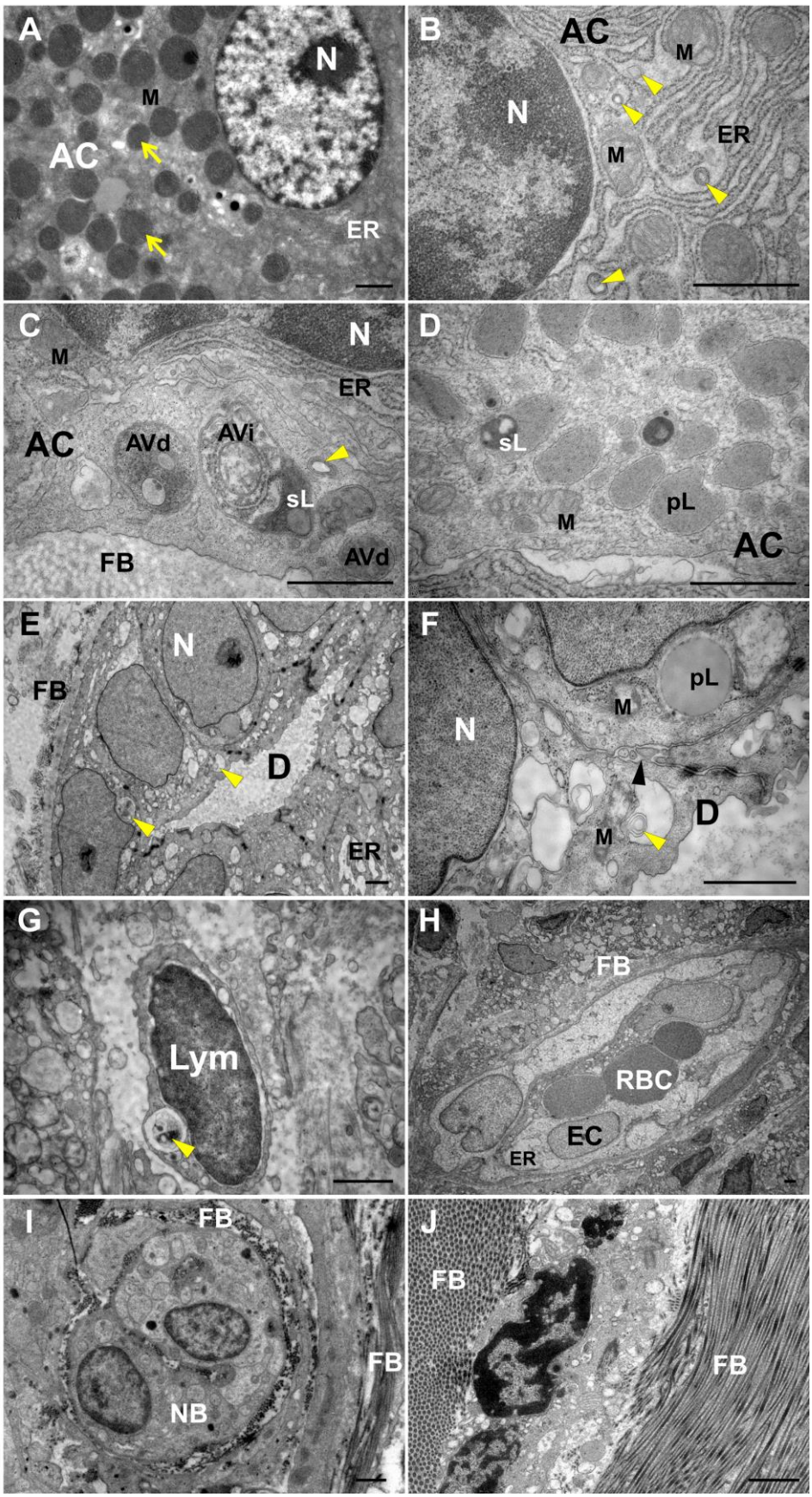
Appendix Figure 1. Serum levels of interleukin (IL)-1β. Serum levels of IL-1β in healthy controls (Con) and IgG4-related sialadenitis (IgG4-RS) patients was measured by enzyme-linked immunosorbent assay (n = 19). All data are presented as mean ± SEM. ****P* < 0.001.



Appendix Figure 2. (**A**) Correlation of serum tumor necrosis factor-α (TNF-α) level in IgG4-related sialadenitis (IgG4-RS) patients with $IgG4^+/IgG^+$ ratio in submandibular gland (SMG) was analyzed by Spearman test (n = 19). (**B**) Serum TNF-α level of IgG4-RS patients classified according to fibrosis grade (n = 11–10).

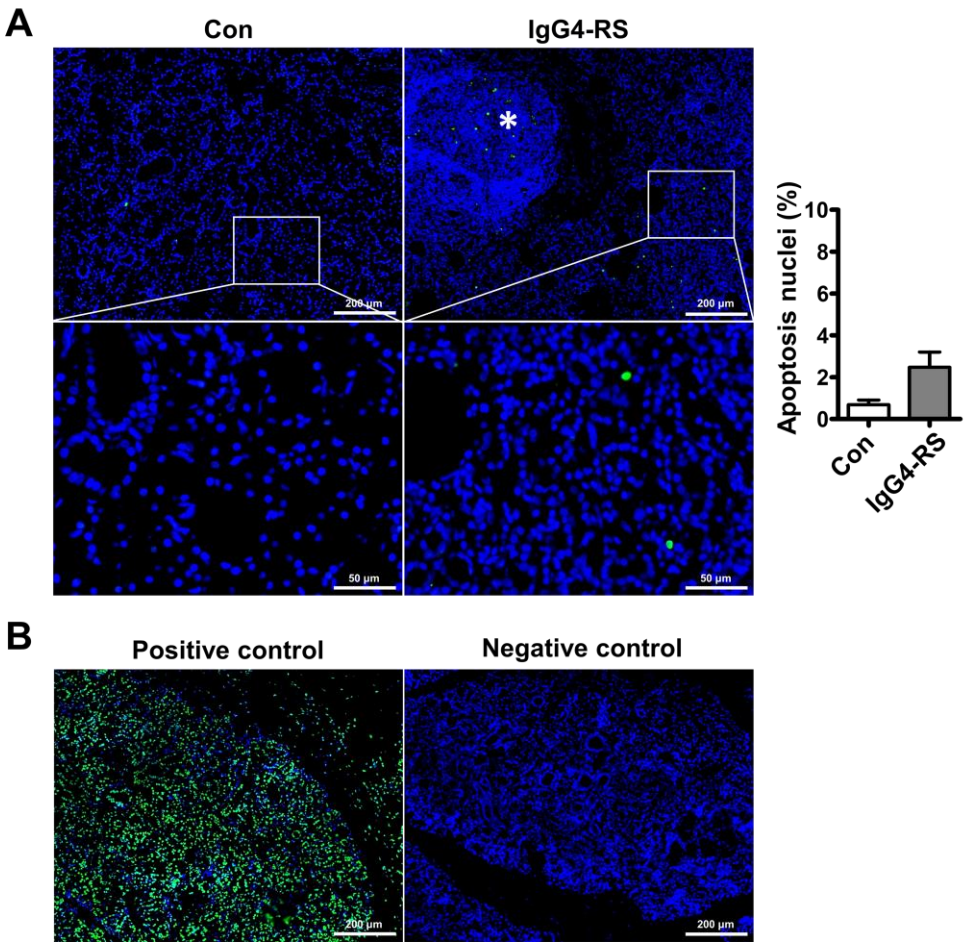


Appendix Figure 3. Negative controls of IgG4 (**A**) and tumor necrosis factor-α (TNF-α) (**B**) for immunohistochemical staining in IgG4-related sialadenitis (IgG4-RS) patients. 5% albumin from bovine serum (BSA) was used instead of primary antibodies. Bars: 200 μm.

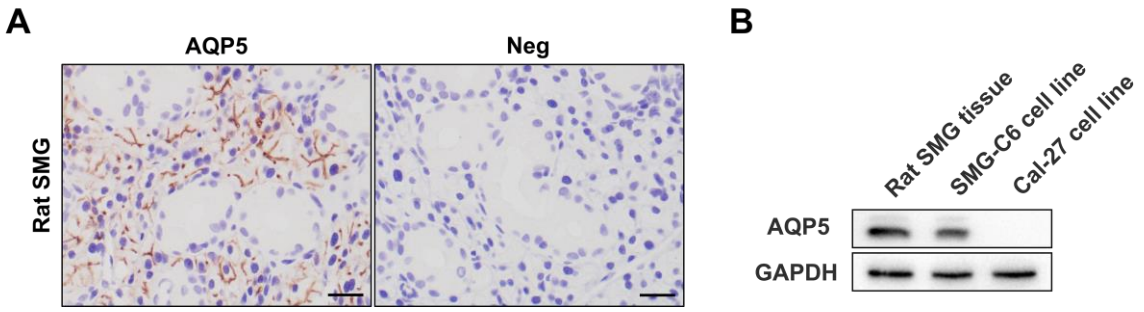


Appendix Figure 4. Transmission electron microscopy (TEM) of submandibular gland (SMG)

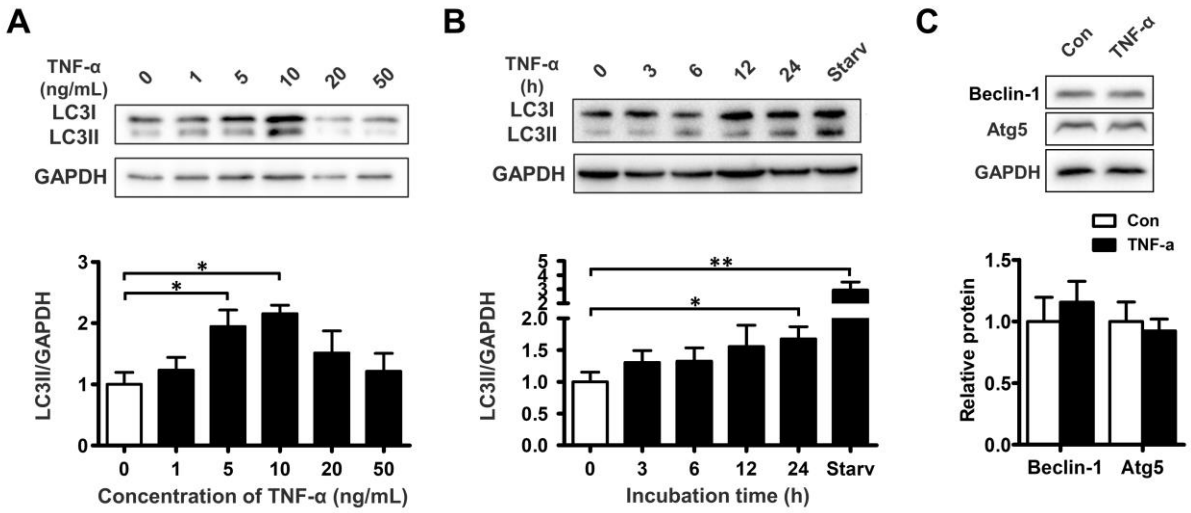
specimens from controls and IgG4-related sialadenitis (IgG4-RS) patients. (**A–F**) Ultrastructural images of acinar cells from controls (**A**) and IgG4-RS patients (**B–D**), and ducts of the lesions (**E, F**). Acini and ducts were surrounded by fibrous bundles and intercellular spaces were widened in certain places, mitochondria were swelling together with a clear decrease in number, the endoplasmic reticulum was expanded, and autophagic vacuoles were commonly detected (yellow triangles). The accumulation of large autophagic vacuoles (**C**) and lysosomes (**D**) were primarily observed in acinar cells. (**G**) Autophagic vacuole in a lymphocyte of IgG4-RS patient. (**H**) Blood vessels of IgG4-RS patient contained swollen endothelial cells harboring expanded endoplasmic reticulum. (**I**) Medullated nerve bundle of IgG4-RS patient with fibrous bundles surrounding and within it. (**J**) Fibroblastic cells of IgG4-RS patient surrounded by dense fibrous bundles. Bars = 1 μm; AC, acinus; N, nucleus, M, mitochondria; ER, endoplasmic reticulum; FB, fibrous bundles; AVi, early autophagic vacuole; AVd, late autophagic vacuole; pL, primary lysosome; sL, secondary lysosome; D, duct; Lym, lymphocyte; EC, endothelial cell; RBC, red blood cell; NB, nerve bundle; yellow triangles, autophagic vacuoles; black triangles, intercellular spaces; yellow arrows, secretory granules.



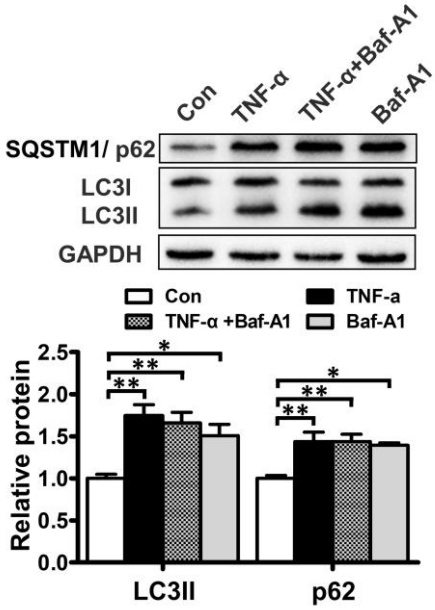
Appendix Figure 5. Detection of apoptosis in controls and IgG4-related sialadenitis (IgG4-RS) patients. (**A**) Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling (TUNEL) staining of submandibular glands (SMGs) from controls (Con) and IgG4-RS patients (n = 4). Apoptotic nuclei appear green, and cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI; blue). The higher magnification image of patient shows the region of residual acini. At least 10 random high-power fields from each sample were quantified. Asterisk, lymphoid follicle. (**B**) Positive control for TUNEL staining was incubated with DNase I (300 U/mL, at 37°C for 30 min) before adding TUNEL reaction mixture, and negative control was incubated with Label Solution instead of TUNEL reaction mixture. All data are presented as mean ± SEM.



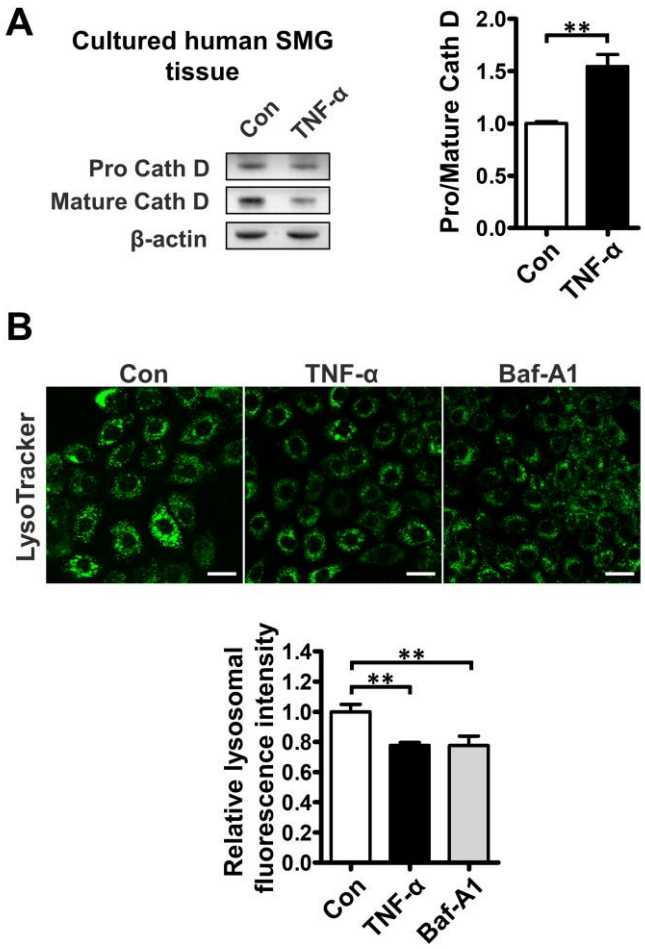
Appendix Figure 6. Identification of SMG-C6 cell line as rat acinar cell line. (A) Immunohistochemical staining of submandibular gland (SMG) from Sprague Dawley rat revealed expression of aquaporin 5 (AQP5) in acinar cells, but not in ductal cells. Bars: 20 μm. (B) The expression of AQP5 in SMG-C6 cells was detected by western blotting. Rat SMG tissue was used as positive control and Cal-27 cell line, a human tongue squamous cell carcinoma cell line, as negative control.



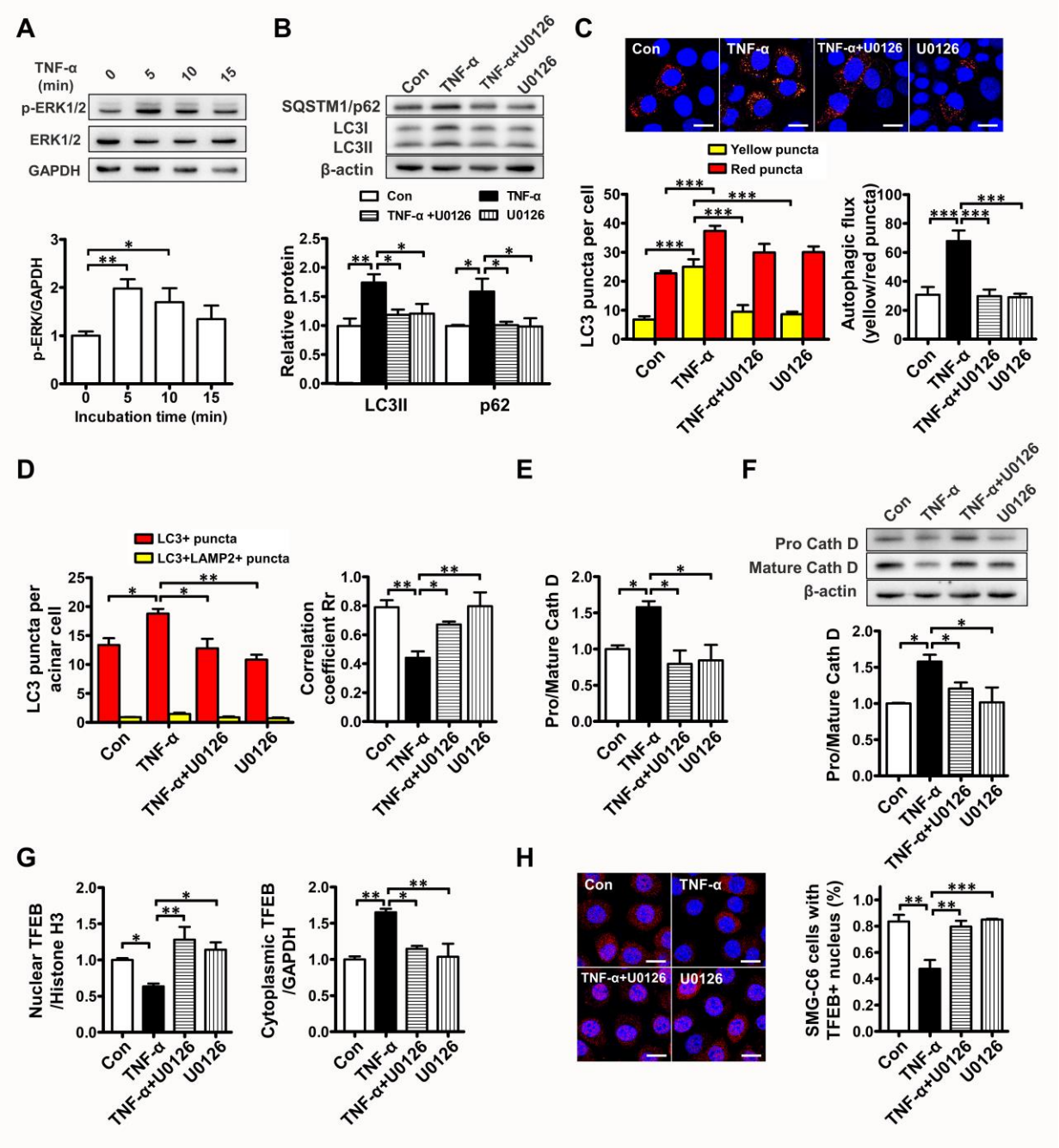
Appendix Figure 7. Effect of tumor necrosis factor-α (TNF-α) on expression of autophagic-related proteins in SMG-C6 cells. (**A**) SMG-C6 cells were incubated with TNF-α at different concentrations for 24 h. (**B**) SMG-C6 cells were incubated with 10 ng/mL TNF-α for different periods. The medium without fetal bovine serum (Starv; 24 h) was used as a positive control. (**C**) SMG-C6 cells were incubated with 10 ng/mL TNF-α for 24 h, and Beclin-1 and autophagy-related 5 (Atg5) proteins were measured by western blotting. Data expressed as mean ± SEM of 4 independent experiments. **P* < 0.05 and ***P* < 0.01.



Appendix Figure 8. Tumor necrosis factor-α (TNF-α) induces autophagic flux suppression in SMG-C6 cells. SMG-C6 cells were incubated with 10 ng/mL TNF-α for 24 h combined with 50 nM Baf-A1 for 4 h, and then LC3II and SQSTM1/p62 proteins were quantified by western blotting. Data expressed as mean ± SEM of 5 independent experiments. **P* < 0.05, ***P* < 0.01.



Appendix Figure 9. Tumor necrosis factor-α (TNF-α)-induced decrease in lysosome acidification in SMG-C6 cells and cultured human submandibular gland (SMG) tissues. (**A**) Cultured human SMG tissues were incubated with 10 ng/mL TNF-α for 4 h. Expression of cathepsin D (Cath D) was quantified by western blotting. (**B**) SMG-C6 cells were incubated with 10 ng/mL TNF-α for 24 h. Baf-A1 (50 nM, 4 h) was used as a positive control. LysoTracker DND-26 (100nM) was added for 5 min at 37℃ after TNF-α or Baf-A1 treatment, and the fluorescence intensity of the lysosome was analyzed. At least 10 random visual fields for each independent experiment were selected. All data are presented as mean ± SEM of 4 independent experiments. Bars: 20 μm. $^{**}P < 0.01$.



Appendix Figure 10. Extracellular signal-regulated kinase 1/2 (ERK1/2) activation is required for tumor necrosis factor-α (TNF-α)-induced autophagic flux suppression, lysosome impairment and redistribution of transcription factor EB (TFEB). **(A)** SMG-C6 cells were treated with 10 ng/mL TNF-α for 5, 10, and 15 min, and ERK1/2 phosphorylation was assessed by western blotting. (**B-H**) U0126 (10 μM) was administrated to SMG-C6 cells or cultured human submandibular gland (SMG) tissues 0.5 h before TNF-α incubation (10 ng/mL, 24 h for cells and 4 h for tissues). (**B**) LC3II and

SQSTM1/p62 protein levels of cultured human SMG tissues were quantified by western blotting. (**C**) SMG-C6 cells were transfected with Ad-mCherry-GFP-LC3B before treatment, and autophagic flux was analyzed based on the amount of red and yellow puncta in each cell, as described in Fig. 2. (**D**) Quantification of immunofluorescence labeling for LC3 and LAMP2 and 4′,6-diamidino-2-phenylindole (DAPI) staining in cultured human SMG tissues, as described in Fig. 3. (**E**) Quantification of Cathepsin D (Cath D) in SMG-C6 cells detected by western blotting. (**F**) Cathepsin D in cultured human SMG tissues was quantified by western blotting. (**G**) Quantification analysis of nuclear and cytoplasmic TFEB levels in SMG-C6 cells detected by western blotting. (**H**) Changes of TFEB distribution revealed by immunofluorescence staining in SMG-C6 cells. At least 15 random acini of SMGs, or 10 random visual fields of cells for each independent experiment, were selected for quantification analysis. Data expressed as mean ± SEM of 5 independent *in vitro* experiments or 4 independent *ex vivo* experiments. Bars: 20 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.