Supplementary Information

Autophagy mediates epithelial cytoprotection in Eosinophilic Oesophagitis

Whelan et al.

Supplementary Materials and Methods

Patients and Oesophageal Biopsies

During diagnostic oesophagogastroduodenoscopy (EGD), additional pinch biopsies were obtained from the distal oesophagus of subjects (Supplementary Table S1). Patients who met clinical criteria of EoE[1] with histologic presence of ≥15 oesophageal mucosal eosinophils (eos) per high-powered field (hpf) were classified as "active EoE." EoE patients who demonstrated resolution of histologic inflammation and eosinophilia (<15 eos/hpf) upon follow up EGD were designated as "EoE Remission." "Normal" subjects include patients who reported symptoms warranting EGD, but demonstrated no endoscopic or histopathologic abnormalities in the upper gastrointestinal tract. 65 of 67 (97.0%) patients were treated with a proton pump inhibitor (PPI) for 4 to 6 weeks prior to endoscopy.

Transmission Electron Microscopy (TEM)

TEM images were assessed by two investigators (KAW and AKS), blind to clinical diagnosis. Early AVs were identified as double-membrane vesicles containing cellular material.[2] The presence of two membranes separated by an electron lucent cleft at minimum of one point in each AV was verified under high magnification (≥70,000X). Late AVs were identified were identified by a single membrane encasing electron dense material.[3] AV-positive (≥2 AVs/cell) cell percentage was determined for each sample by counting at least 25 keratinocytes within basal, parabasal and spinous cell layers. Each sample was scored as follows: Score 1, <10% AV-positive cells; Score 2, 10-20% AV-positive cells; Score 3, >20% AV-positive cells.

Histology and Immunohistochemistry

Sections were incubated with anti-cleaved LC3 polyclonal antibody (1:250; Abgent, San Diego, CA), anti-p63 monoclonal antibody (1:200; Santa Cruz Biotechnology, Dallas, TX) or anti-phospho-Histone H2A.X Ser139 (20E3) monoclonal antibody (1:250; Cell Signaling Technology, Danvers, MA) overnight at 4°C. Mouse IgGa2/κ was utilized as an isotype control for anti-p63 antibody (Supplementary Figure S1A). A synthetic peptide representing amino acids 89-120 of human cleaved LC3A (VSVSTPIADIYEQEKDEDGFLYMVYASQETFG; Biomatik, Cambridge, ON, Canada) was utilized at 10-fold molecular excess to validate cleaved LC3A antibody specificity (Supplementary Figure S1B). Phospho-Histone H2Ax (Ser139) blocking peptide #1260 (Cell Signaling Technology) was used according to the manufacturer's instructions (Supplementary Figure S1C).

A pathologist (AKS) blind to molecular and clinical data, scored cleaved LC3 stained specimens based on intensity and distribution as represented in Supplementary Figure S12B.[4 5] Score 1, marginal to mild stain affecting nuclei of basal and parabasal layers; Score 2, moderate to intense nuclear staining and occasional cytosolic stain in most layers, including superficial layers in which nuclear staining was either absent or less intense than in basal and parabasal layers; Score 3, very intense nuclear stain in all layers together with obvious cytosolic stain with or without puncta. Cytosolic LC3 puncta (Supplementary Figure S12A) were noted in approximately 20-30% of slides scored as 3, consistent with other systems of LC3 scoring.[4 6] Eosinophil counts for murine experiments were scored by a pathologist (KC) for using 3-4 oesophageal cross-sections per animal. Results are presented as average of maximum eosinophil count per mm^2 for each cohort. Quantification of p63 staining is presented as numerical averages of 3 non-overlapping high-power fields of view (400X) from 3 independent oesophagi for each cohort. For human biopsies used for TEM-based AV scoring, basal cell (BC) scoring was performed as previously described.[7] For human biopsy specimens used for cleaved LC3 IHC analysis, corresponding H&E stained slides were evaluated by a pathologist (AKS) blind to molecular and clinical data using the following criteria: Score 0, single row of basal cells

and occasionally one parabasal row (normal,); Score 1, 1 row of basal and 1 row of parabasal ovoid or quasi-columnar cells (normal); Score 2, 2-4 rows of basal cells (mild basal cell hyperplasia); Score 3, 5 rows of basal cells (moderate BCH); Score 4, >5 rows of basal cells (severe basal cell hyperplasia).

Human Oesophageal Cell Culture, Treatment, RNA Interference and Live Cell Imaging

Subconfluent cells were treated with chloroquine (Sigma-Aldrich, St. Louis, MO) alone or in combination with TNF-α, IL-4, IL-5 or IL-13 (R&D Systems (Minneapolis, MN). Cells were pre-treated with the antioxidant *N*-acetyl-L-cysteine (Sigma-Aldrich) at 10 mM for 1 h as previously described.[8] Small interfering RNA (siRNA) sequences directed against Beclin-1 (Silencer Select s16537 and s16358; Life Technologies), ATG-7 (s20650; Life Technologies) or a non-silencing control sequence (Silencer Select Negative Control #1; Life Technologies) were transfected with at a final concentration of 10 nm using Lipofectamine RNAi Max reagent (Life Technologies) as described previously.[8] EPC2-hTERT cells expressing GFP-LC3 were imaged using a Leica DM IRB inverted microscope (Leica Microsystems, Buffalo Grove, IL). GFP-LC3 puncta were identified using the Find Maxima feature in ImageJ (National Institutes of Health). At least 25 cells were counted for each condition.

Immunoblot Analysis

Whole cell lysates were prepared and subjected to Western blotting as described previously.[9] In brief, 50 µg of denatured protein was fractionated on a NuPAGE Bis-Tris 4–12% gel (Life Technologies). Following electrotransfer, Immobilon-P membranes (EMD Millipore, Billerica, MA) were blocked with Dulbecco's Phosphate-Buffered Saline (DPBS) containing 0.1% Tween-20 and 5% milk, followed by overnight incubation with the following primary antibodies: rabbit anti-LC3B (1:1000, Cell Signaling Technology), rabbit anti-Beclin-1 antibody (1:1000, Cell Signaling Technology), mouse-anti-p62 (1:1000, Sigma-Aldrich) rabbit-anti-ATG-7 (1:1000 Sigma-Aldrich) and mouse anti-β-actin (1:10000, Sigma-Aldrich) at 4°C. Secondary antibodies (Sigma-Aldrich) were used at 1:10000. OxyBlot (anti-carbonyl antibody; EMD Millipore) was used according to the manufacturer's guidelines. Targeted proteins were visualized using a chemiluminescence detection system (Amersham ECL or ECL Prime; GE Healthcare Life Sciences; Buckinghamshire, UK) and exposed to Blue Lite Autorad film (ISC-BioExpress, Kaysville, UT).

Flow Cytometry

Flow cytometry was performed using FACSCalibur, FACSCanto or LSR II cytometers (BD Biosciences, Franklin Lakes, NJ) and FlowJo software (Tree Star, Ashland, OR) for cells suspended in DPBS containing 1% bovine serum albumin (Sigma-Aldrich). AVs were determined with Cyto-ID® fluorescent dye (Enzo Life Sciences, Farmingdale, NY) by staining EPC2-hTERT and EPC425 primary cells 1:1000 in 1:1 keratinocyte serum free medium (KSFM):DPBS at 37°C for 30 minutes. ROS were determined with 2', 7'-dichlorodihydrofluorescein diacetate (DCF) dye (Life Technologies) as described previously.[8 10] Whole murine esophagi were dissected longitudinally then the epithelial layer was separated from subepithelium using fine-tipped forceps. Epithelial sheets were incubated two times in 0.25% trypsin for 5 minutes at 37°C. Liberated keratinocytes were then forced through a 40 µm strainer and trypsin activity was quenched using soybean trypsin inhibitor. Remaining tissue was minced and mashed through a 40 µm strainer using the end of a 1 ml syringe. Human biopsies were forced through a 40 µm strainer to obtain a single cell suspension. Single cell suspensions from human and murine specimens were stained with Cyto-ID at 1:100 in 1:1 KSFM:DPBS at 37°C for 30 min. 4',6-diamidino-2-phenylindole (DAPI) was used to determine viability. For autophagy flux assays, single cells suspensions from biopsies were incubated at 37°C in KSFM in the presence or absence of Chloroquine (50 µg/ml) for 3 hours prior to

staining. Unstained cells from each specimen were utilized to establish both the background fluorescence and the Cyto-ID-positive cut off for that specimen. The percent of Cyto-ID-positive cells was determined in the live cell fraction (DAPI-). The mean fluorescence in live cells was determined for each specimen and is presented after subtraction of background fluorescence.

Murine Ex Vivo Oesophageal 3D Organoid Culture

Using 24-well plates, 5,000 cells were seeded per well in 50µl Matrigel. After solidification, 500 µl of DMEM/F12 supplemented with 1X Glutamax, 1X HEPES, 1X N2 Supplement, 1X B27 Supplement, 0.1 mM *N*-acetyl-L-cysteine (Sigma-Aldrich), 50 ng/ml mouse recombinant EGF (R&D Systems), 2.0% Noggin/R-Spondin-conditioned media and 10 µM Y27632 (Tocris Biosciences, Bristol, UK) were added and replenished every other day. Organoids were grown for 4 days in growth media before addition of TNF-α and/or CQ for 7 days (Supplementary Figure S4). Organoids were recovered by digesting Matrigel™ (BD Biosciences, San Jose, CA) with Dispase I (BD Biosciences, San Jose, CA; 1U/ml) and fixed overnight in 4.0% paraformaldehyde. Specimens were embedded in 2.0% Bacto-Agar: 2.5% gelatin prior to paraffin embedding.

Murine EoE Models

In the MC903-OVA model, oesophagitis was induced in wild type (WT) BALB/c mice (Jackson Laboratory, Bar Harbor, ME) by epicutaneous sensitization with MC903 (Tocris Bioscience, Bristol, UK) and ovalbumin (OVA; Sigma-Aldrich) followed by oral administration of OVA for 18 days as previously described.[11] L2-IL5 mice underwent cutaneous sensitization and intra-gastric challenge with 4-ethoxymethylene-2-phenyl-2-oxazolin-one (oxazolone) to induce oesophagitis.[12] Krt5-rtTA tetO-IL13 mice were given Doxycycline (DOX; 2 mg/ml plus 50 mg/ml sucrose) in drinking water for 7 days to activate IL-13 expression in esophageal epithelia.[13] Krt5-rtTA mice treated with DOX served as controls. Data represent two independent experiments with similar results.

Supplementary References

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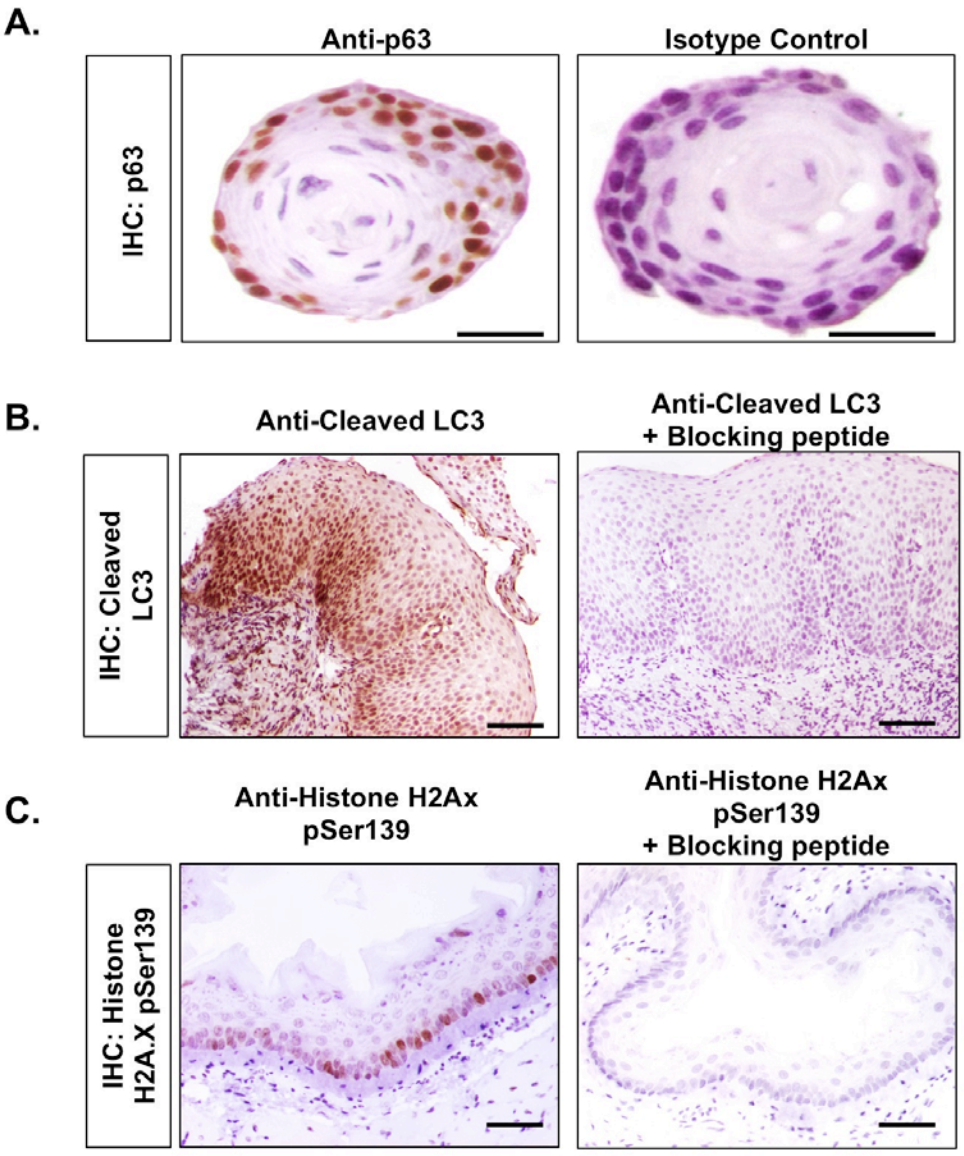
Supplementary Table S1. Patient Demographics

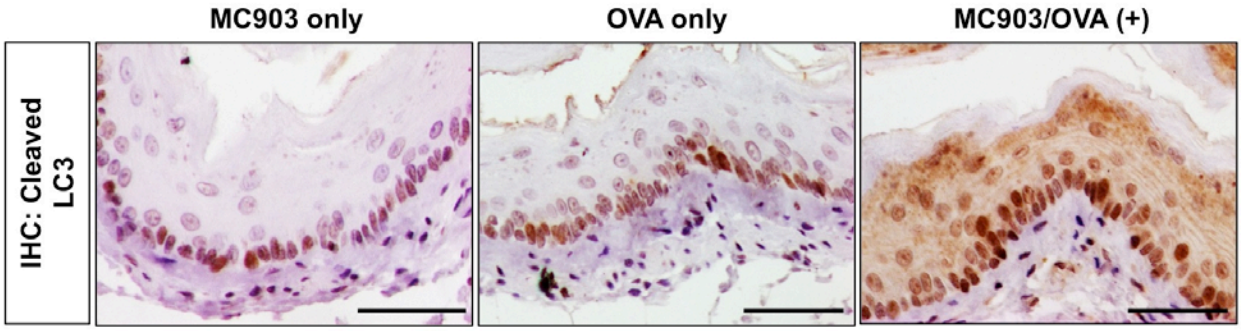
ID	Age (Y, M)	Gender	Diagnosis	Eos/HPF	BC Score	AV Score (TEM)
1	16,8	Female	Normal	0	ND	1
2	14	Female	Normal	0	0	1
3	8	Female	Normal	0	0	1
4	13	Female	Normal	0	0	1
5	17	Female	Normal	0	0	1
6	11	Male	Normal	0	0	1
7	12	Male	Normal	0	0	2
8	6,10	Female	Active EoE	15	ND	3
9	13	Female	Active EoE	20	1	2
10	14	Male	Active EoE	10	1	1
11	14	Male	Active EoE	70	3	3
12	6	Male	Active EoE	32	2	3
13	9	Male	Active EoE	29	2	3
14	12	Male	Active EoE	30	1	1
15	3	Male	Active EoE	20	1	1
16	9	Male	Active EoE	65	0	2
17	12,9	Male	EoE Remission	5	ND	1
18	10,8	Male	EoE Remission	10	ND	2
19	4	Male	EoE Remission	0	1	1
20	8	Male	EoE Remission	0	0	1
21	7	Male	EoE Remission	1	0	1
22	6	Male	EoE Remission	0	0	1
23	11	Male	EoE Remission	7	0	2
24	4	Male	EoE Remission	1	0	1
25	4	Male	EoE Remission	1	1	1
26	3	Male	EoE Remission	0	2	1
27	9	Male	EoE Remission	6	3	1
ID	**Age (Y, M)**	**Gender**	**Diagnosis**	**Eos/HPF**	**BC Score**	**Cleaved LC3 Score (IHC)**
28	15,4	Female	Normal	0	1	2
29	13,1	Female	Normal	0	1	1
30	17,.2	Male	Normal	0	1	1
31	11,3	Male	Normal	0	2	2
32	14,5	Male	Normal	0	1	1
33	3,6	Male	Normal	0	0	2
34	4,4	Male	Normal	0	0	1
35	9,11	Male	Normal	0	0	2
36	2,9	Male	Normal	0	1	1
37	5,5	Male	Normal	0	0	1
38	13,11	Female	Active EoE	100	4	3

39	14,4	Male	Active EoE	40	1	1
40	15,0	Male	Active EoE	30	4	3
41	12,0	Male	Active EoE	28	2	1
42	10,4	Male	Active EoE	18	4	3
43	6,6	Male	Active EoE	100	4	3
44	17,3	Male	Active EoE	15	3	2
45	7,9	Male	Active EoE	30	4	2
46	7,9	Male	Active EoE	25	3	2
47	15,4	Male	Active EoE	20	3	2
48	9,0	Male	Active EoE	50	4	2
49	13,8	Male	Active EoE	30	4	3
50	11,5	Male	Active EoE	50	2	3
51	12,4	Male	Active EoE	50	3	2
52	14,7	Male	EoE Remission	0	1	2
53	4,9	Male	EoE Remission	0	1	1
54	12,3	Male	EoE Remission	3	2	1
55	17,7	Male	EoE Remission	0	2	2
56	17,8	Male	EoE Remission	0	1	1
57	9,8	Male	EoE Remission	0	1	1
58	12,11	Male	EoE Remission	0	0	1
59	8,6	Male	EoE Remission	10	2	2
ID	**Age (Y, M)**	**Gender**	**Diagnosis**	**Eos/HPF**	**BC Score**	**Cyto-ID+ (%)**
60	14,11	Male	Normal	0	ND	5.3
61	6,9	Female	Normal	0	ND	5.6
62	10,7	Male	Active EoE	22	ND	4.7
63	10,3	Male	Active EoE	25	ND	27.8
64	10,0	Female	Active EoE	60	ND	12.9
65	5,9	Female	Active EoE	20	ND	32.4
66	13,0	Male	EoE Remission	2	ND	6.5
67	9,6	Male	EoE Remission	0	ND	2.8

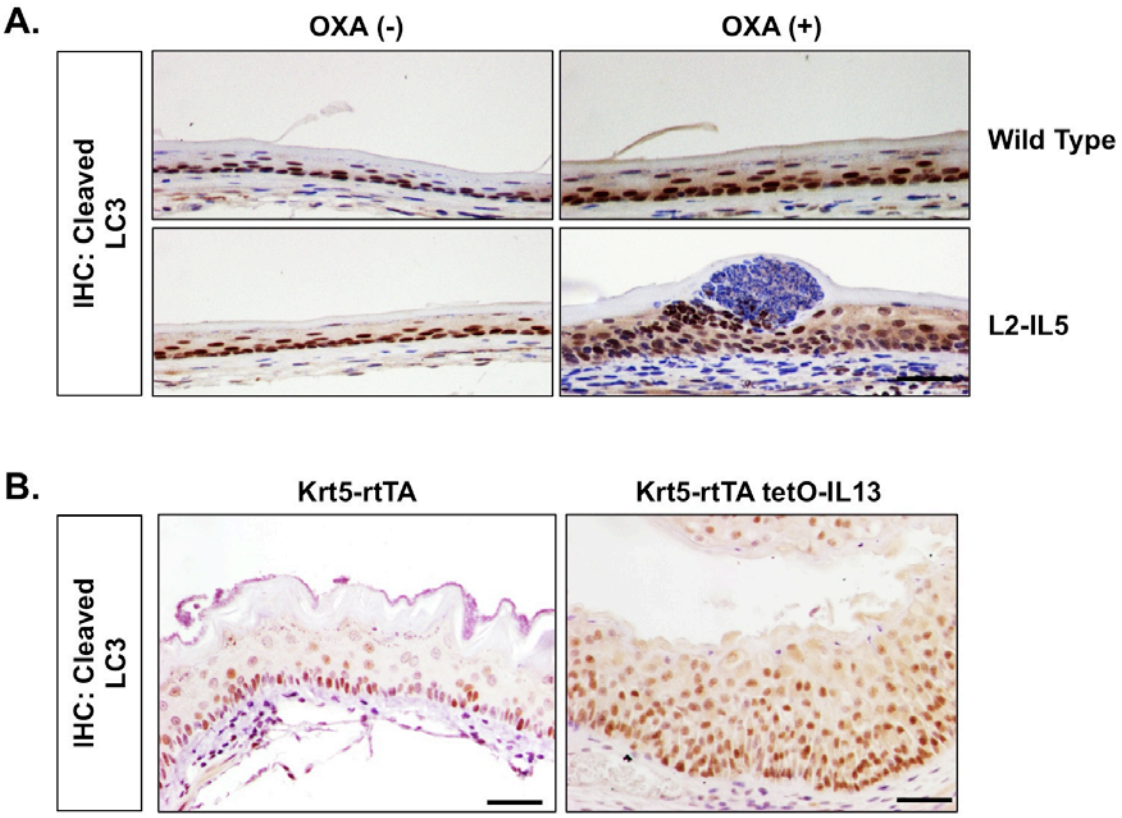
AV, Autophagic Vesicle
BC, Basal cell
Eos, Eosinophils
HPF, High power field
IHC, Immunohistochemistry
ND, Not determined
TEM, Transmission electron microscopy



Supplementary Figure S1. Verification of specificity of antibodies used for IHC.
(A) Oesophageal organoid sections incubated with anti-p63 primary antibody or isotype control (Mouse IgG2a/κ) were subjected to IHC staining for p63. (B) Human oesophageal tissue sections incubated with anti-cleaved LC3 primary antibody alone or in combination with blocking peptide were subjected to IHC staining for cleaved LC3. (C) Murine oesophageal tissue sections incubated with anti-Histone H2A.X pSer139 primary antibody alone or in combination with blocking peptide were subjected to IHC staining for Histone H2Ax pSer139. Scale bars, 25 μm in (A); 50 μm in (B, C).

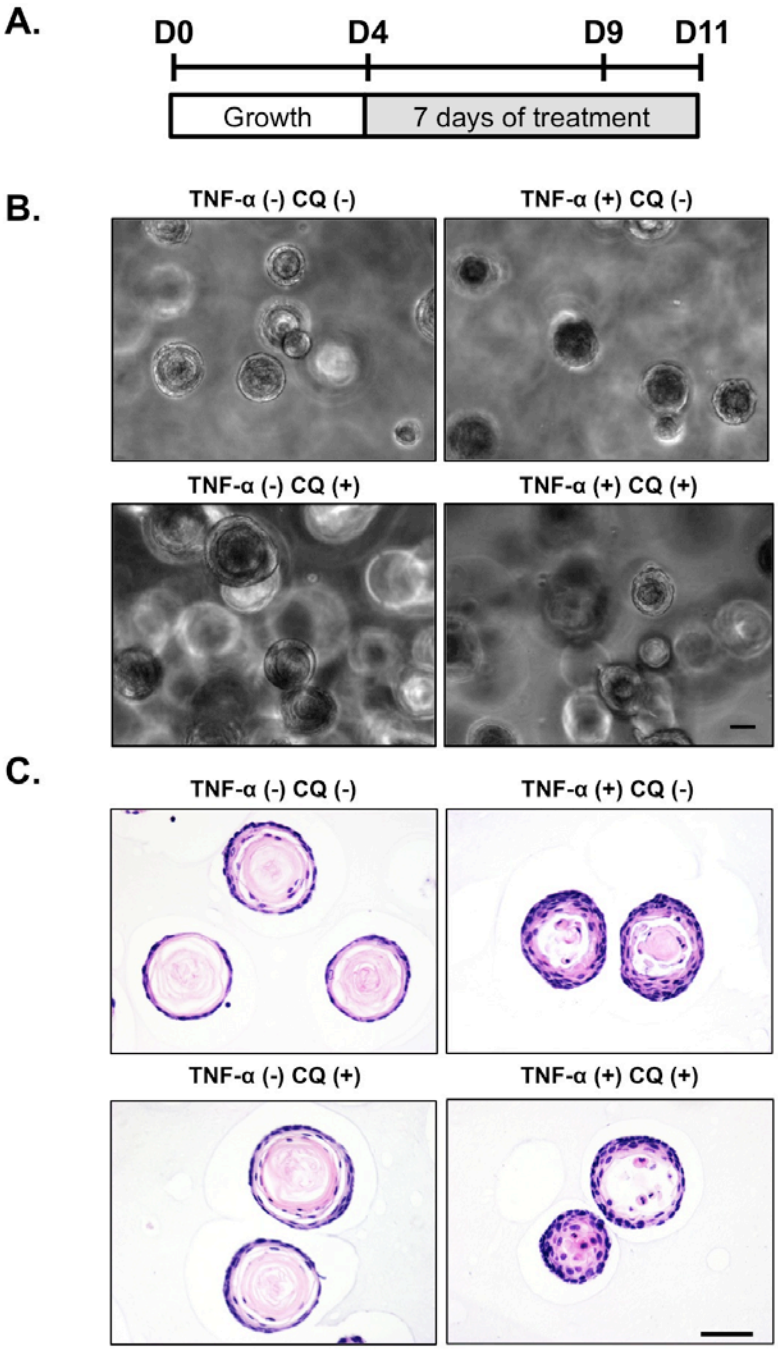


Supplementary Figure S2. Single agent controls for MC903/OVA murine EoE model.
BALB/c mice were epicutaneously treated with MC903 and/or OVA as indicated for 14 days. To induce EoE-like oesophageal inflammation, mice treated with MC903 and OVA in combination were orally challenged with OVA. All mice were sacrificed 18 days after initiation of sensitization. Oesophageal tissues were evaluated by IHC for cleaved LC3 (autophagy). Scale bars, 50 μm. (n=5 animals/treatment group)

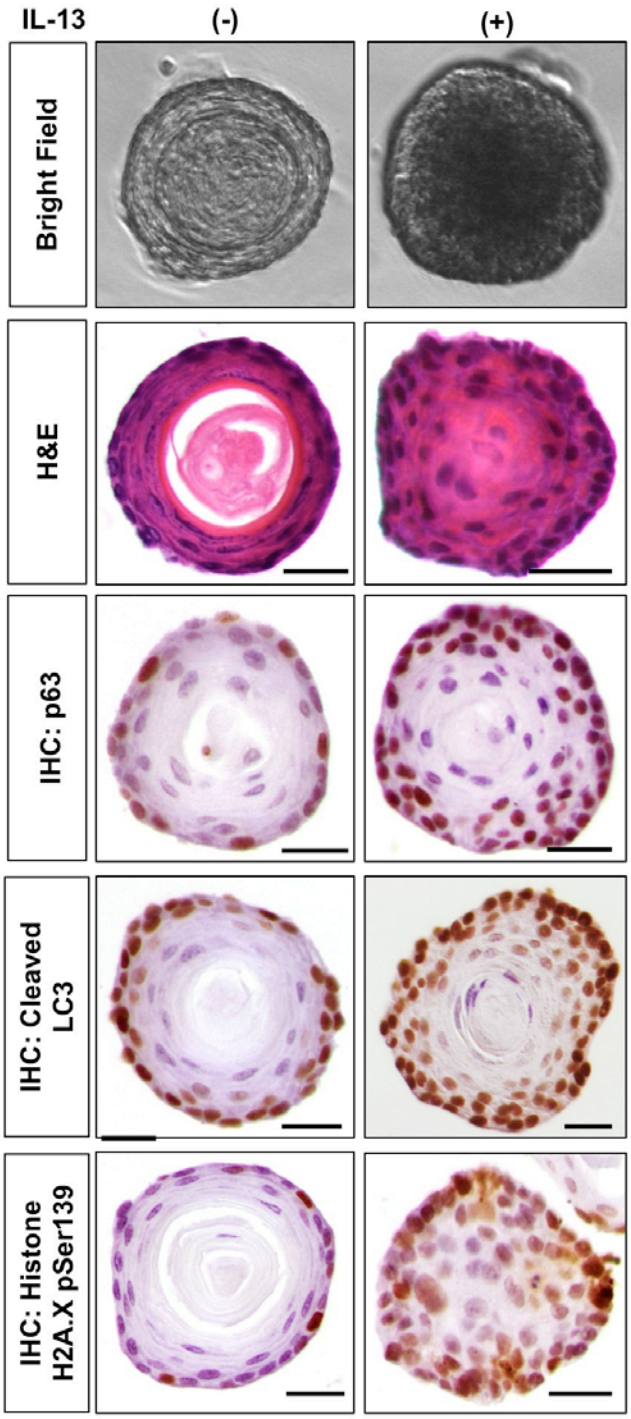


Supplementary Figure S3. Evidence of autophagy in esophageal epithelia of L2-IL5/OXA and Krt5-rtTA tetO-IL13/DOX mice.

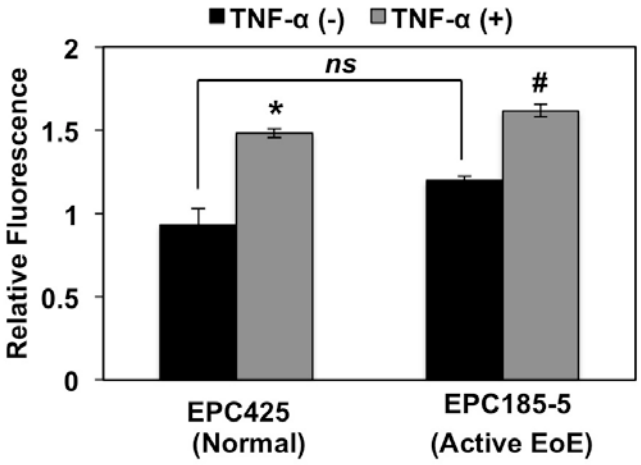
Oesophageal tissues isolated from mice of indicated genotype/treatment groups were stained via IHC for cleaved LC3. (A) Wild type (n=5) and L2-IL5 (n=5) mice were subjected to OXA sensitization/topical challenge for 12 days with vehicle-treated wild type (n=5) and L2-IL5 (n=5) mice as controls. (B) Krt5-rtTA (n=3) and Krt5-rtTA tetO-IL13 (n=5) mice were treated with DOX in drinking water for 7 days. Mice were sacrificed 14 days after DOX treatment. Scale bars, 100 μm in (A); 50 μm in (B).



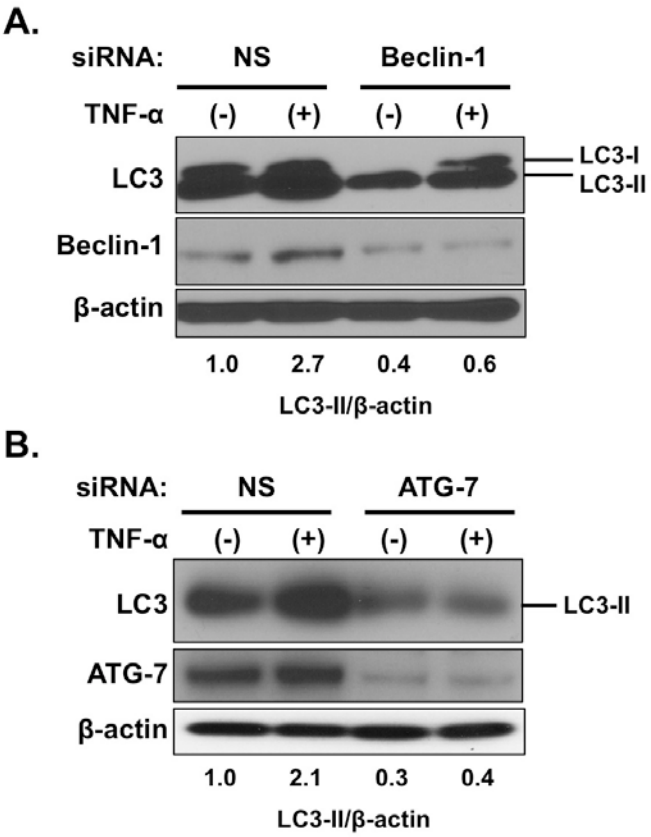
Supplementary Figure S4. Generation of *ex vivo* oesophageal 3D organoids.
Freshly isolated murine oesophageal keratinocytes were seeded as a single cell suspension at day 0. After 4 days of organoid growth, medium was supplemented with or without 40 ng/ml TNF-α or 1 μg/ml CQ alone or in combination. At day 9, TNF-α and/or CQ was replenished. Organoids were fixed at Day 11. (A) Schematic of experimental timeline. (B) Bright field images of organoids taken at day 11, prior to fixation. Scale bar, 50 μm. (C) H&E staining of organoid structures. Scale bar, 100 μm.



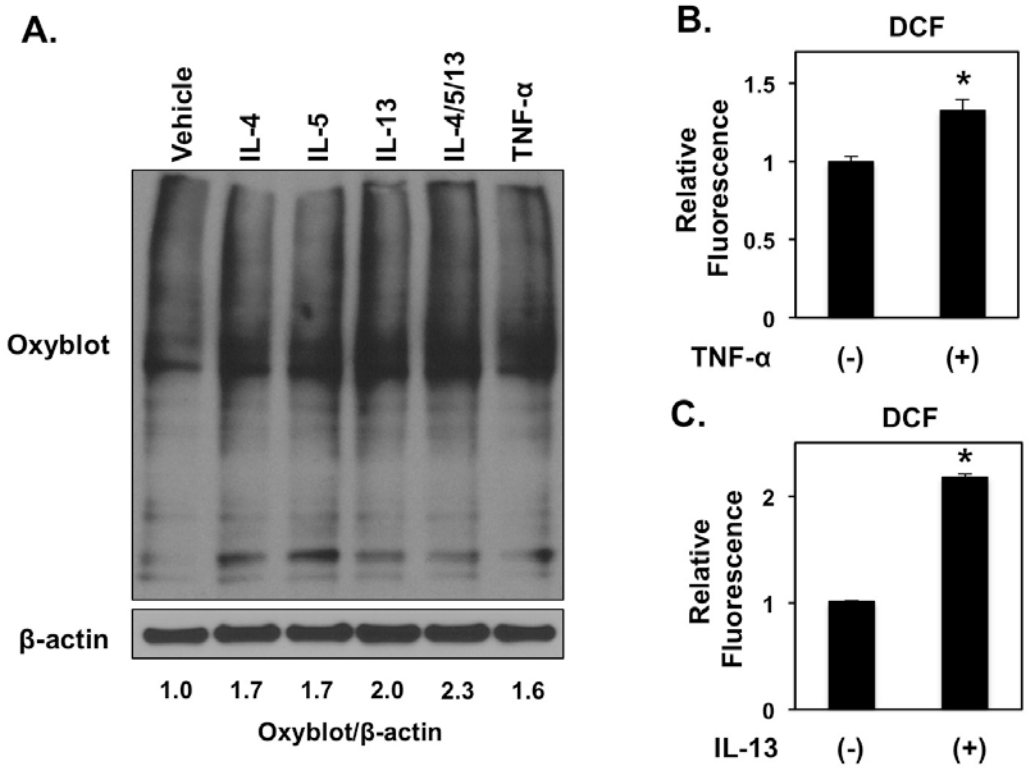
Supplementary Figure S5. IL-13 promotes basal cell proliferation, cleaved LC3 expression and oxidative stress in oesophageal organoids.
Murine oesophageal organoids were cultured in the presence or absence of 10 ng/ml IL-13 from days 4 through 11 and subjected to bright field imaging, H&E staining or IHC for p63 (basal cells), cleaved LC3 (autophagy) and Histone H2A.X pSer139 (oxidative stress). Scale bars, 25 μm.



Supplementary Figure S6. EoE-relevant cytokines promote AV accumulation in primary oesophageal keratinocytes.
Primary oesophageal keratinocytes derived from mucosa of a normal patient (EPC425) or an active EoE patient (EPC185-5) were stimulated with TNF-α (40 ng/ml) for 72 hours. Staining with Cyto-ID was performed to assess AV content by flow cytometry. All data is represented as relative to EPC-425 TNF-α (-). Histogram represents relative Cyto-ID level from one of at least three independent experiments*, $p<0.05$ vs. EPC425 TNF-α (-). *ns*, not significant; (n=3).

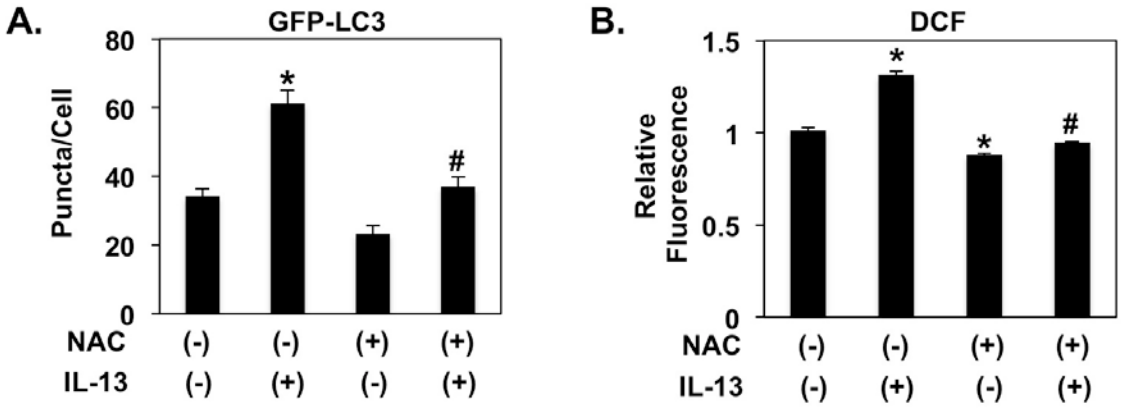


Supplementary Figure S7. Genetic inhibition of autophagy blunts TNF-α-mediated AV accumulation in oesophageal keratinocytes.
EPC2-hTERT cells were transfected with control non-silencing (NS) siRNA or siRNA directed against Beclin-1 in (A) or ATG-7 in (B). Cells were then stimulated with TNF-α (40 ng/ml) for 72 hours and immunoblot analysis determined levels of indicated proteins. Densitometry values were indicated below.

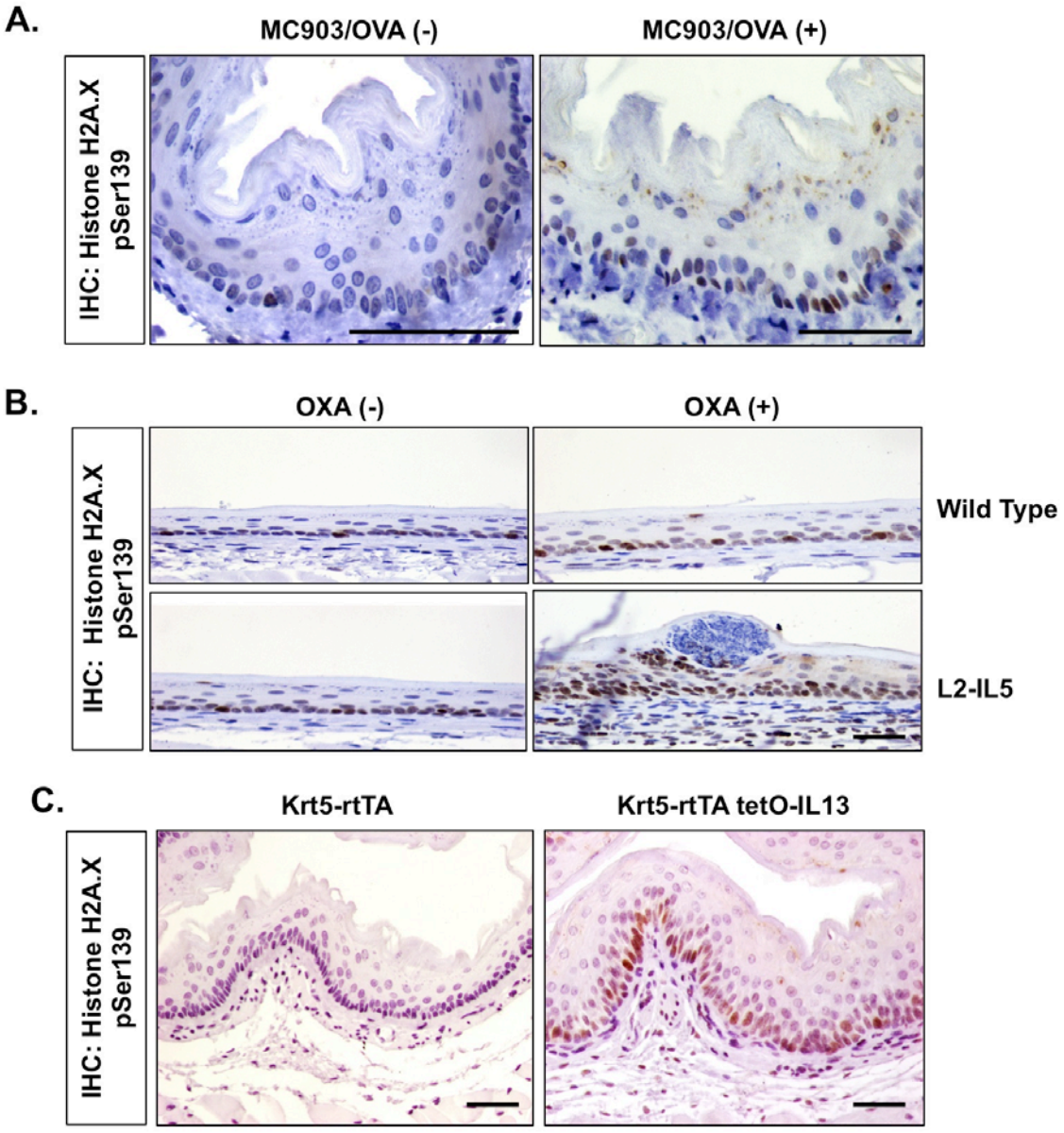


Supplementary Figure S8. EoE-relevant cytokines promote oxidative stress in oesophageal keratinocytes.

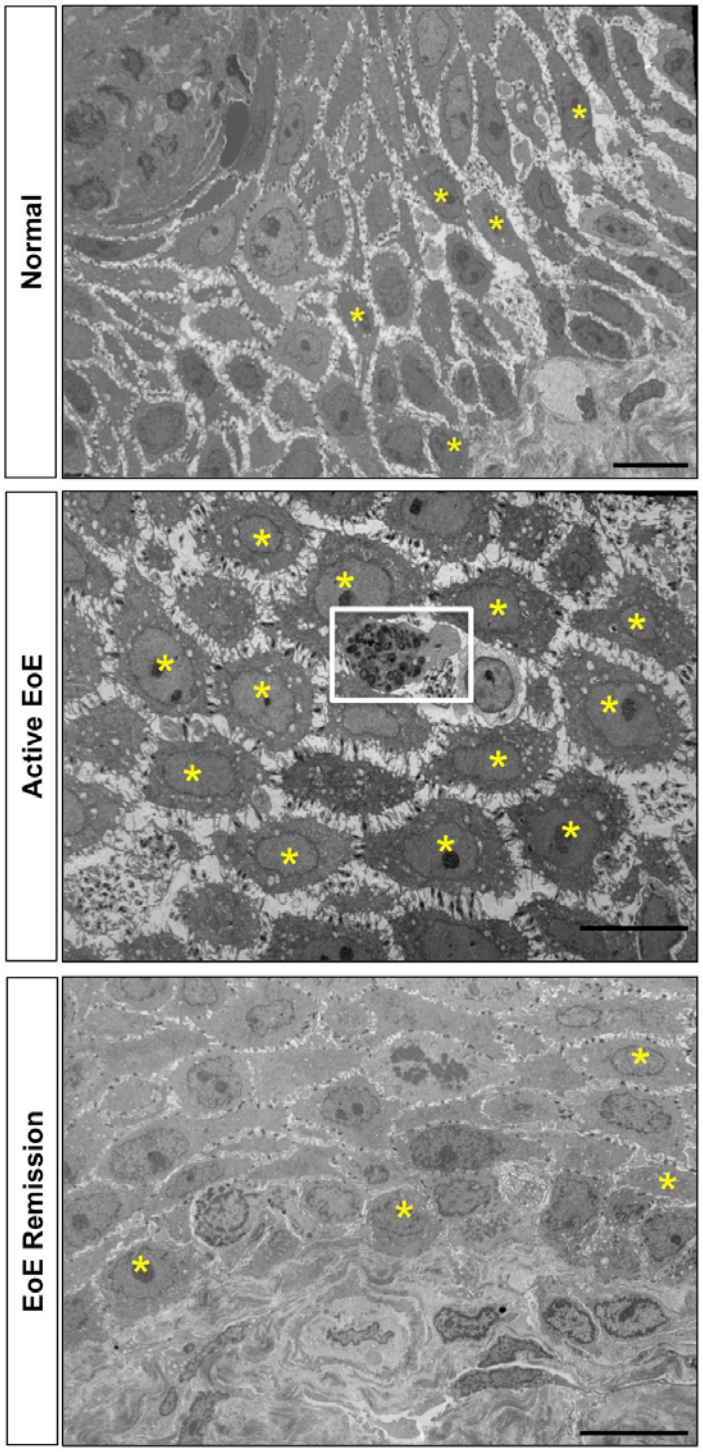
(A) EPC2-hTERT cells were stimulated with indicated cytokines for 24 hours. TNF-α was utilized at a final concentration of 40 ng/ml. IL-4, IL-5 and IL-13 were utilized at a final concentration of 10 ng/ml. Oxyblot analysis was performed to assess protein oxidation with β-Actin as a loading control. Relative expression of Oxyblot signal was determined by densitometry as indicated below. (B and C) EPC425 primary oesophageal keratinocytes were cultured for 72 hours in the presence or absence of 40 ng/ml TNF-α in (B) or 10 ng/ml IL-13 in (C). Cells were stained with DCF for flow cytometry to assess ROS. Histograms represent relative DCF level from one of at least three independent experiments. *, $p<0.05$ vs. TNF-α (-) or IL13 (-); (n=3).



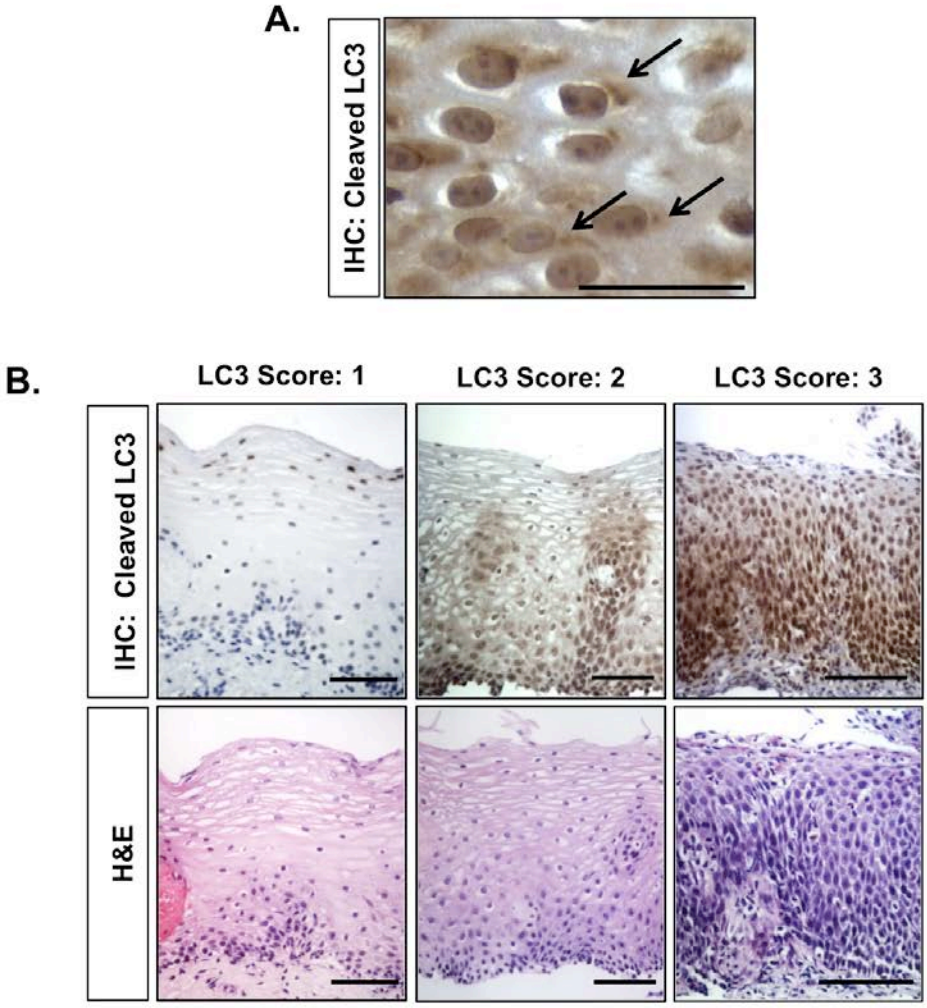
Supplementary Figure S9. IL-13-mediated induction of autophagy is ROS-dependent. EPC2-hTERT cells were pretreated with 10 mM NAC pretreatment for 1 hour then stimulated with 10 ng/ml IL-13 for 72 hours. (A) Live cell imaging was performed to determine the average GFP-LC3 puncta/cell. (B) Flow cytometry for DCF was used to evaluate ROS. Histograms represent data from one of at least three independent experiments. *, $p<0.05$ vs. IL-13 (-) NAC (-); #, $p<0.05$ vs. IL-13 (+) NAC (-); (n=3).



Supplementary Figure S10. Oxidative stress is induced in murine models of EoE.
Oesophageal tissues isolated from mice of indicated genotype/treatment groups were stained via IHC Histone H2A.X pSer139 to detect oxidative stress. (A) BALB/c mice were treated with MC903 and OVA to induce EoE-like esophageal inflammation for 18 days (n=3) with untreated mice as controls (n=4). (B) Wild type (n=5) and L2-IL5 (n=5) mice were subjected to OXA sensitization/ topical challenge for 12 days with vehicle-treated wild type (n=5) and L2-IL5 (n=5) mice as controls. (C) Krt5-rtTA (n=3) and Krt5-rtTA tetO-IL13 (n=5) mice treated with DOX in drinking water for 7 days then sacrificed 14 days after DOX treatment. Scale bars, 50 μm in (A and C); 100 μm in (B).

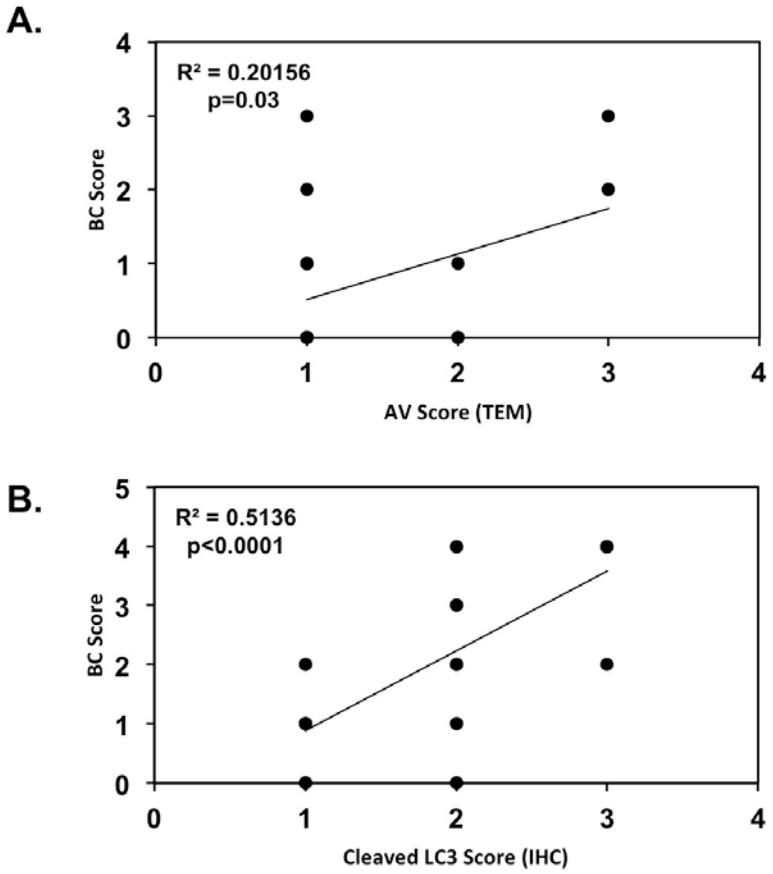


Supplementary Figure S11. TEM findings in human oesophageal biopsies.
Representative images for indicated patient groups. Asterisks denote AV-positive keratinocytes. White box denotes an eosinophil in active EoE specimen. Scale bars, 10 μm.



Supplementary Figure S12. Cleaved LC3 IHC scoring in human oesophageal biopsy specimens.

(A) Cytoplasmic cleaved LC3 puncta (arrows). (B) *Top*, Cleaved LC3 IHC staining in epithelial compartment was scored based on intensity and distribution as illustrated by representative patients scored as indicated. *Bottom*, Corresponding H&E staining. Scale bars, 100 μm.



Supplementary Figure S13. Correlation between basal cells (BC) and AV content in human oesophageal biopsy specimens.
Histogram representing relationship between BC score and AV content as determined by TEM in (A) and IHC for cleaved LC3 in (B).