

ORIGINAL ARTICLE

WILEY

Asthma and Rhinitis

Role of autophagy in regulating interleukin-10 and the responses to corticosteroids and statins in asthma

Kittipong Maneechotesuwan¹  | Kanda Kasetsinsombat² | Adisak Wongkajornsilp² | Peter J Barnes³

¹Division of Respiratory Disease and Tuberculosis, Department of Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

²Department of Pharmacology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

³National Heart and Lung Institute, Imperial College, London, UK

Correspondence

Kittipong Maneechotesuwan, Division of Respiratory Disease and Tuberculosis, Department of Internal Medicine, Faculty of Medicine Siriraj Hospital, Mahidol University, 2 Prannok Road, Bangkok 10700, Thailand.
Email: kittipong.man@mahidol.ac.th

Funding Information

The funding of this study was provided by the Siriraj Grant for Research Development and Medical Education of the Faculty of Medicine Siriraj Hospital, Mahidol University.

Abstract

Background: Interleukin (IL)-10 is a key anti-inflammatory cytokine that may be reduced in asthma but is enhanced by corticosteroids, especially when combined with a statin, although the mechanisms of these effects are uncertain.

Objective: To study the role of autophagy in macrophages in promoting inflammation in asthma through reducing IL-10 secretion and how corticosteroids and statins may reverse this process.

Methods: We conducted a randomised double-blind placebo-controlled study in moderate to severe asthmatic patients (n = 44) to investigate the effect of an inhaled corticosteroid (budesonide 400 µg/day) and the combination of budesonide with an oral statin (simvastatin 10 mg/day) given for 8 weeks on autophagy protein expression in sputum cells by using immunocytochemistry and measurement of IL-10 release. In *in vitro* experiments, we studied cross-regulation between autophagy and IL-10 release by measuring the expression of autophagy proteins in M2-like macrophages and the effects of budesonide and simvastatin on these mechanisms.

Results: In asthmatic patients, inhaled budesonide inhibited airway macrophage autophagy (beclin-1, LC3) as well as autophagic flux (p62), which was enhanced by simvastatin and was correlated with increased sputum IL-10 and reduced IL-4 concentrations. In macrophages *in vitro*, budesonide and simvastatin inhibited rapamycin-induced autophagy as well as autophagic flux, with reduced expression of beclin-1 and LC3, but enhanced the accumulation of p62 and increased expression of IL-10, which itself further inhibited autophagy in macrophages. With siRNA-mediated silencing, LC3-deficient macrophages also showed a maximal induction of IL-10 transcription. Neutralisation of IL-10 with recombinant specific blocking antibody and silencing IL-10 transcription reversed the inhibitory effects of budesonide and simvastatin on macrophage autophagy.

Conclusion and clinical relevance: Inhibition by corticosteroids and a statin of macrophage autophagy enhances IL-10 production, resulting in the control of asthmatic inflammation.

Abbreviations: 3-MA, 3-methyladenine; AHR, airway hyperresponsiveness; ATG, autophagy; Bud, budesonide; eos, eosinophil; epi, airway epithelial cell; FEV, forced expiratory volume; GM-CSF, granulocyte-macrophage colony-stimulating factor; ICS, inhaled corticosteroids; IL, interleukin; LC3, microtubule-associated protein 1 light chain 3; lym, lymphocyte; mac, macrophage; Neu, neutrophil; rap, rapamycin; Sim, simvastatin.

KEYWORDS

autophagy, interleukin-10, macrophage

1 | INTRODUCTION

Interleukin (IL)-10 is an anti-inflammatory cytokine with a crucial and non-redundant role as a feedback regulator in limiting diverse inflammatory responses, including Th1 cell and Th2 cell responses to allergens and promoting resolution of the inflammatory response.¹ Although IL-10 is expressed by many cells of the innate and adaptive immune systems, including CD4⁺ Th1, Th2, Th17 cells, regulatory T cells, CD8⁺ T cells, B cells, dendritic cells, macrophages, mast cells, natural killer cells, eosinophils and neutrophils, M2 macrophages, regulatory T cells and myeloid dendritic cells are widely appreciated to be the dominant cellular sources.²⁻⁵ IL-10 expression in airway macrophages from steroid-naïve asthmatics is markedly reduced and dramatically enhanced by additive effects of corticosteroid and statin treatments,^{6,7} both of which can therefore be used as pharmacologic interventions to investigate IL-10 regulation in macrophages. Much is known about the anti-inflammatory mechanisms of IL-10 that are mediated through its engagement with the IL-10 receptor (IL-10R), resulting in the activation of signal transducer and activator of transcription-3 (STAT3). Via effects on macrophages, IL-10 suppresses airway eosinophilia in asthmatic patients through the induction of indoleamine-2,3-dioxygenase (IDO)⁷ and suppression of cytokines mediating eosinophil survival (IL-5 and GM-CSF), leading to eosinophil apoptosis.⁸ In contrast, little is known about the interaction between IL-10 induction and alteration in autophagy that may contribute to asthma pathogenesis.^{9,10}

Autophagy is critical cellular mechanism for the selective elimination of damaged potentially harmful cytosolic material by lysosomal degradation, thereby acting as a major cytoprotective system and as an adaptive process in response to different forms of cellular stress, including nutrient deprivation, growth factor depletion, infection and hypoxia.¹¹ However, excessive autophagy with increased autophagy markers occurs in asthma in association with enhancement of the type 2 (T2) immune response, eosinophilic airway

inflammation and airway remodelling.^{9,10,12-14} Macroautophagy is the best characterised form of autophagy and involves the formation of a double-membrane vesicles called autophagosomes that fuse with lysosomes, thus removing the damaged protein.¹¹ The initiation of the autophagic process requires beclin-1, a key initiator of autophagy by recruiting autophagy proteins involved in autophagosome formation.¹⁵ Autophagosome generation involves the ubiquitin-like molecule pro-light chain (LC)3 processed by autophagy-related cysteine proteinase-4 (ATG4) to active LC3 that mediates elongation of the phagophore and completion of autophagosome formation. However, the induction of autophagy is restricted under basal conditions by the kinase mammalian target of rapamycin (mTOR) by inhibiting the function of the unc-51-like kinase 1 (ULK1)/ULK2 complex, which plays an important role in autophagy initiation.^{16,17} Therefore, inhibition of mTOR with the inhibitor rapamycin promotes autophagosome biogenesis.¹⁸ In addition, the induction of autophagy accelerates the selective degradation of the stress-inducible cellular adapter protein p62 that serves as a signalling hub for mTOR activation on lysosomes and therefore p62 represents autophagic flux. Therefore, an impairment of autophagy results in the accumulation of p62 in the ubiquitin aggregate that activates mTOR with further suppression in autophagy (Figure 1).¹⁹⁻²¹

The regulation of autophagy by cytokines has been elucidated in the context of infection, particularly with *Mycobacterium tuberculosis*. Classical Th2 cytokines (IL-4 and IL-13) inhibit starvation- or IFN- γ -induced autophagosome formation, whereas IL-10 suppresses starvation-induced autophagy via the Akt pathway in mouse macrophage cell lines and rapamycin-induced autophagy via both the Akt and STAT3 pathways in macrophages/monocytic cells.²²⁻²⁴ As well as being regulated by cytokines, autophagy can itself directly modulate the transcription, processing and secretion of the pro-inflammatory cytokines IL-1 α , IL-1 β and IL-18 via the NLRP3 inflammasome.^{25,26} Although the contribution of autophagy to secretion of proinflammatory cytokines is well established, its role in the

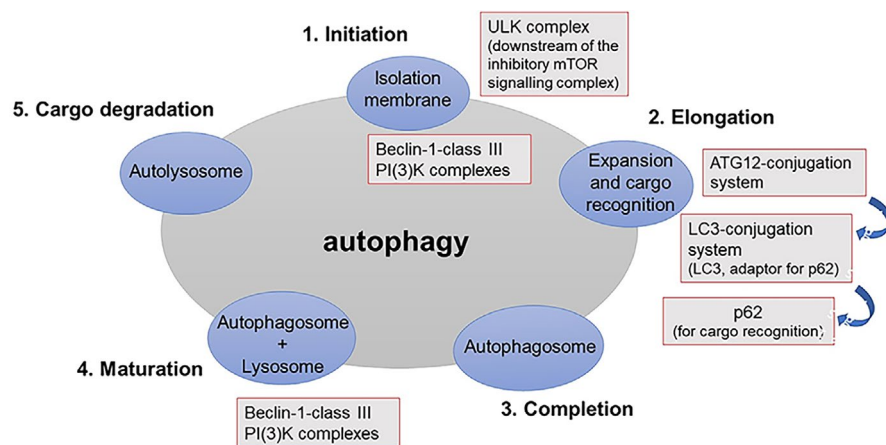


FIGURE 1 Schematic diagram illustrating the regulation of autophagy by autophagic mediators. ATG, autophagy; LC3, light chain 3; mTOR, mammalian target of rapamycin; PI3 K, phosphatidylinositol-3-OH kinase; ULK, Unc-55-like activating kinase.

regulation of IL-10 expression in asthmatic macrophages is uncertain. We hypothesised that autophagy may inhibit IL-10 expression in macrophages and thereby increase eosinophilic airway inflammation in asthma by reducing IL-10-induced eosinophil apoptosis and that corticosteroids and statins could restore this. We investigated the association between sputum macrophage autophagy and IL-10 production in asthmatic patients following inhaled corticosteroid (ICS) monotherapy or in combination with statin treatment. We also elucidated the negative cross-regulation between mTOR inhibition-induced autophagy and IL-10 expression in rapamycin-treated M2-like macrophages including the effects of corticosteroids and simvastatin *in vitro*.

2 | METHODS

2.1 | Study subjects

Eligible subjects, aged 18 to 75 years, had a documented history of asthma and had been treated with an ICS for at least 3 months before study enrolment. The diagnosis of asthma was based on the Global Initiative for Asthma (GINA) strategy.²⁷ Asthma diagnosis was based on typical symptoms with at least 200 mL and 12% improvement in FEV₁% predicted values or the presence of airway hyperresponsiveness (AHR).²⁸ This was subsequently confirmed by the presence of AHR with a provocative concentration of methacholine causing a 20% decrease in FEV₁ (PC₂₀) <4 mg/mL. Subjects were recruited with stable asthma requiring ICS at a dose of beclomethasone dipropionate ≥800 µg or equivalent daily by pressurised metered-dose inhaler or dry powder inhaler with or without a long-acting ICS/long-acting β₂-agonist (LABA) to maintain asthma control. Patients were clinically stable, with at least 4 weeks since an exacerbation. All subjects were non-smokers.

The study was approved by the Institutional Review Board, Faculty of Medicine Siriraj Hospital, Bangkok, Thailand (si481/2010), and registered on ClinicalTrials.gov (NCT01266434), and all patients provided written informed consent.

2.2 | Study design

A prospective, double-blinded, randomised placebo-controlled study was undertaken. Subjects were randomised to receive oral matched placebo or simvastatin 10 mg (Zocor; MSD) once a day for 8 weeks (1:1) using a concealed computer-generated randomisation sequence with four-element block design and stratified on a clinical centre basis. Both groups were administered budesonide (400 µg once daily) via a dry powder inhaler (Giona easy inhaler, Orion Pharma). Screening began at least 2 weeks before treatment administration. The use of all ICSs and long-acting bronchodilators was discontinued for 2 weeks before randomisation. Oral asthma medications such as theophylline and leukotriene receptor antagonists were also prohibited. Patients who were unable to

tolerate withdrawal of therapy (which was closely monitored) were excluded.

2.3 | Lung function measurements and methacholine challenge

FEV₁, FVC and PC₂₀ were measured at the start and the end of treatment period using a dry wedge spirometer (Vitalograph, Buckingham, UK). Values are expressed as the per cent of predicted normal values. Baseline values were measured after 15 min of rest and were taken as the highest of three readings. The methacholine PC₂₀ was determined by the linear interpolation of the concentration-FEV₁ response curve. Lung function measurement and methacholine challenge were performed at the screening visit and the end of run-in and treatment periods.

2.4 | Patient sputum specimens and blood collection

Sputum samples were collected from all of the trial participants before and after the treatment period, and venous blood samples for *in vitro* experiments were taken at the start of randomisation. Sputum specimens were collected by induction with hypertonic saline generated by the ultrasonic nebuliser and processed as previously described.²⁹ The supernatants were stored at -70°C until further analysis. For immunocytochemistry, cytopins were fixed with 4% paraformaldehyde (BDH Ltd., Poole, UK) and stored at -20°C. Total cell counts were recorded with a hemocytometer, using Kimura stain. Cell viability was determined by trypan blue exclusion before cytopins were undertaken. The slides were stained with May-Grunwald-Giemsa stain, and differential cell counts were made by a blinded observer. Four hundred inflammatory cells were counted on two slides for each sample in a blinded manner. Differential cell counts are expressed as the percentages of total inflammatory cells. Samples with cell viability of greater than 70% and less than 30% squamous cell contamination were considered adequate for analysis.

2.5 | Isolation and cell culture of monocyte-derived macrophages

Peripheral blood mononuclear cells were isolated from 30 mL of venous blood of all trial participants prior to randomisation by Ficoll density gradient centrifugation (IsoPrep, Robbins Scientific, Sunnyvale, CA). The mononuclear cell fraction was allowed to adhere over the tissue culture container at a density of 4×10^6 cells/mL for 1 h at 37°C. The retrieved adherent cells were resuspended in RPMI 1640 supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin and recombinant human macrophage colony-stimulating factor (M-CSF; 50 ng/mL; BioLegend, San Diego, CA), and incubated at 37°C in 5% CO₂ for 6 days to

induce differentiation into uncommitted macrophages (M0). The medium was changed on the fourth day. On day 6, differentiated macrophages (M0) were further polarised into alternatively activated (M2) macrophages using IL-4 (500 IU/ml, Amoytop Biotech, Xiamen, China)/IL-13 (20 ng/ml, BioLegend, San Diego, CA) for 2 days as previously described.³⁰ On day 8, cells were washed and used for further experiments. Population frequencies of M2 macrophages were assessed with the expression of two markers CD11b and CD209 (Figure S1).

2.6 | *In vitro* experiments

For autophagy and apoptosis assays, M2-like macrophages were cultured in 2% fetal blood serum (FBS) for 24 h prior to further incubation with or without rapamycin (1 μ M) for 18 h. After 12-h exposure to rapamycin, macrophages were treated with the autophagy inhibitor 3-methyladenine (3-MA, 0.5 mM; Sigma-Aldrich, St. Louis, MO), recombinant IL-10 (100 ng/ml, Biolegend), anti-IL-10 neutralising antibody (10 ng/ml, Biolegend), budesonide (100 μ g/mL, Sigma-Aldrich), simvastatin (2 ng/ml, Sigma-Aldrich) and combination for 6 h.

2.7 | Autophagy analysis

Quantification of autophagy was carried out using the Cyto-ID Green autophagy detection kit (Enzo Life Sciences, Farmingdale, NY) according to the manufacturer's instructions. Conditional M2-like macrophages were stained with Cyto-ID Green Detection Reagent, a dye that selectively labels accumulated autophagic vacuoles, at 37°C for 30 min and then analysed with CytoFLEX flow cytometry (Beckman Coulter, Indianapolis, IN). Values were normalised by protein concentration. Autophagic flux was demonstrated by the degree of p62 expression that means the accumulation of p62 in macrophages reflecting the interruption of autophagic flux.

2.8 | Small interfering RNA (siRNA)-mediated knockdown

For knockdown of transcription of targeted proteins, scrambled siRNA (control), Beclin-1, LC3, p62 and IL-10-specific siRNA (10 nM) (Qiagen, Germantown, MD) was transfected into M2-like macrophages for 20 h using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Beclin-1 was targeted by an siRNA with sequence: sense, 5'-GGU CUA AGA CGU CCA ACA ATT-3'; antisense, 5'-UUU CAC UAA CGU AUA CAG GTA-3'. LC3 was targeted by an siRNA with sequence: sense, 5'-CCU GUA UAC GUU AGU GAA ATT-3'; antisense, 5'-UUG UUG GAC GUC UUA GAC CCT-3'. p62 was targeted by an siRNA with sequence: sense, 5'-GGG UGC

AAG AAG CCA UUU ATT-3'; antisense, 5'-5'-UAA AUG GCU UCU UGC ACC CTA-3'. IL-10 was targeted by an siRNA with sequence: sense, 5'-TGA GTT TGA CAT CTT CAT CAA-3'; antisense, 5'-CAG CTG GAC AAC TTG TTG TTA-3'.

2.9 | Quantitative RT-PCR

Total RNA was isolated from M2-like macrophages using the Illustra RNAspin Mini RNA Isolation Kit (GE Healthcare Life Sciences, UK) and reverse transcribed to produce cDNA with the ImProm-II Reverse Transcription System (Promega, Madison, WI) according to the manufacturer's instructions. Quantitative RT-PCRs were performed using the KAPA SYBR® FAST qPCR kit Master mix ABI Prism (Kapasystems, Massachusetts, United States) and LightCycler® 480 Real-Time PCR system (Roche). Primers were purchased from First BASE Laboratory (Singapore): IL-10 sense: CTG GGT TGC CAA GCC TTG T, antisense: AGT TCA CAT GCG CCT TGA TG; Beclin-1 sense: CCG AGG GAT GGA AGG GTC TA, antisense: GGT GAC ACG GTC CAG GAT CT; LC3 sense: GGT GAT CAT CGA GCG CTA CAA, antisense: TGA CCA ACT CGC TCA TGT TGA; p62 sense: CGC CGT TGT GAC CAC TTT T, antisense: CCC GTA CAG AGA CCT GCA ATT C; GAPDH sense: GAA ATC CCA TCA CCA TCT TCC, antisense: AAA TGA GCC CCA GCC TTC TC. The 7300 System SDS software version 1.4.0.25 was used to analyse relative quantity of the target cDNA according to the $\Delta\Delta$ Ct method.

2.10 | Flow cytometry

Cells were stained with primary monoclonal antibodies against IL-10 PE (eBioscience, San Diego, CA; 1:20), LC3 Alexa Fluor 488 (Abcam, Cambridge, MA), Beclin-1 PC5 (Abcam), p62 Alexa Fluor 647 (Abcam), caspase 3 PE-Cy7 (Biotyl), CD11b and CD209 (BioLegend) as previously described.⁷ The cells were analysed by flow cytometry using a CytoFLEX (Beckman Coulter, Indianapolis, IN), and data were analysed using FlowJo software version 10.5.3. Gating strategy for exclusion of debris and doublets, which was the same for all stimulation conditions, was based on FSC height and area (Figure S2A). The histograms of beclin-1, LC3, IL-10 and p62 (Figure S2B-C) in the indicated treatment conditions were shown in the dark grey outline overlaid with the control (white outline) and the rapamycin treatment (light grey outline).

2.11 | Western blot analysis

Whole cell lysates were prepared in NP-40 lysis buffer (0.5% Nonidet P-40, 20 mM Tris-HCl [pH 7.5], 150 mM NaCl) in the presence of complete protease cocktail inhibitor. Lysates were centrifuged at 4°C for 10 min at 12,000 rpm in an Eppendorf microcentrifuge to remove cellular debris. Western blot analysis was performed as

previously described,³¹ using anti-beclin-1, anti-LC3, anti-p62 and anti-IL-10 (Cell Signaling Technology, MA, US). Immunoreactive proteins were detected using an enhanced chemiluminescence ECL kit (Amersham Biosciences).

2.12 | Cytokine analysis

IL-10, IL-4 and IL-13 concentrations in sputum supernatants were determined by enzyme-linked immunosorbent assay (ELISA) using a Colorimetric method (Abcam) according to the manufacturer's instructions. The sensitivity for IL-10, IL-4 and IL-13 detection was less than 5, 0.31 and 0.15 pg/ml, respectively.

2.13 | Statistical analysis

Statistical analysis was performed using PASW statistics 18 (SPSS Inc., Chicago, IL, USA). Data are shown as means $\pm$ SD otherwise indicated. The within-patient treatment and between-treatment differences calculated and then analysed by paired t test for parametric and Wilcoxon signed rank test and Mann-Whitney U test for non-parametric data. Relationship between changes in sputum cytokine levels and autophagic protein expression was determined using Spearman's rank correlation test and Pearson correlation coefficients as appropriate. Statistical analysis for multiple comparisons was performed using one-way ANOVA and post hoc with Bonferroni correction for equal variances assumed. All statistical tests were two-sided, and significance was accepted at the level of 95% and $p < 0.05$; * $p < .05$, ** $p < .01$ and *** $p < .001$.

3 | RESULTS

3.1 | Study population, baseline characteristics

Of 84 screened patients with moderate to severe persistent asthma, 52 fulfilled the entry requirements and were randomised into either the simvastatin tablet group ($n = 28$) or the placebo group ($n = 24$) (Figure 2). Forty-four patients completed the treatment phase and 8 patients discontinued the study before completion: 6 in the simvastatin arm and 2 in the placebo arm (7 developed acute exacerbation and one had poor compliance). The clinical characteristics of the patients are shown in Table 1. The inclusion of female patients with moderate to severe asthma was significantly high in proportion to male individuals.

There was an improvement in prebronchodilator (BD) FEV₁ but not PC₂₀ after budesonide treatment (Table 2). In contrast, none of the isolated and additive effects of combined budesonide-simvastatin on pre-BD FEV₁ and PC₂₀ was statistically significant compared with budesonide treatment alone (Table 2).

3.2 | Effects of ICS and simvastatin on airway inflammation

Budesonide treatment was associated with a mean reduction in the number of sputum eosinophils (from 3.37% at baseline to 0.75% after treatment, $p = .01$ for % eosinophils and $p = .002$ for eosinophil absolute numbers) (Table 2). Eosinophils were more markedly reduced after 8 weeks of treatment with combined-simvastatin treatment (from 4.0% at baseline to 0.5% after treatment, $p < .001$ for

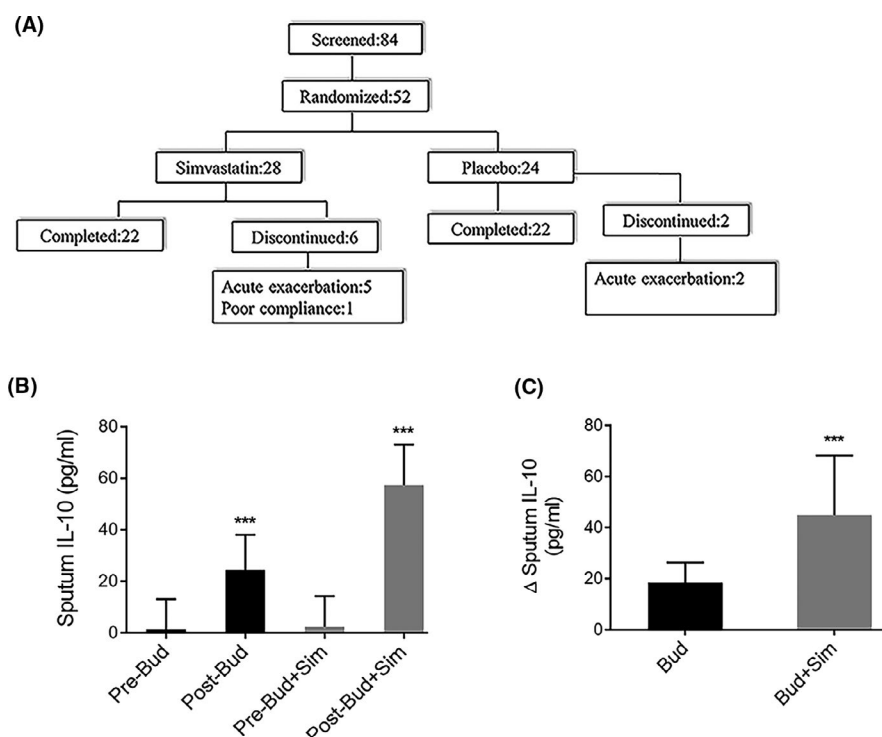


FIGURE 2 Enrolment of patients and completion of the study (A) and sputum IL-10 levels following treatment with inhaled corticosteroid (ICS) and ICS in combination with a statin. After treatment with budesonide (Bud) and Bud in combination with simvastatin (Sim) there were significantly increased IL-10 concentrations (B). The magnitude of therapeutic response was higher in the combination of Bud/Sim than Bud treatment (C). Results are expressed as medians (25th-75th percentiles), *** $p < .001$ compared with pretreatment and between treatments.

Variable	Bud	Bud +Sim	P-value
Subjects (n)	22	22	
Age (y) [§]	50.5 (42.0, 59.2)	51.5 (43.0, 60.5)	.67 ^a
Male [†]	10 (45.4)	2 (9.09)	<.01 ^b
Pre- β 2 FEV ₁ [§]	1.7 (1.1, 2.1)	1.5 (1.2, 1.8)	.35 ^a
Pre- β 2 FEV ₁ % predicted [§]	69.9 (45.5, 88.8)	70.6 (60.9, 86.3)	.46 ^a
PC ₂₀ [§]	1.8 (0.6, 3.1)	0.8 (0.5, 2.7)	.51 ^a
Beclomethasone equivalent ICS dose [§]	640 (475,1000)	800 (500, 1000)	.62 ^b
Treatment with ICS/LABA combination [†]	17 (77.3)	14 (63.6)	.22 ^b
Treatment with combined ICS/LABA with other controllers [†]	5 (22.7)	4 (18.1)	

TABLE 1 Demographic data and clinical characteristics of study subjects.

Note: Data are presented as numbers (percentages)[†] or median (25–75 percentiles)[§]

P-values are determined by Mann-Whitney U test^a and χ^2 test^b

Abbreviations: Bud, budesonide; CV, coefficient of variation; FEV₁, forced expiratory volume in 1 s; IQR, interquartile range; PC₂₀, concentration of methacholine that reduces the FEV₁ by 20%;SD, standard deviation; Sim, simvastatin.

% eosinophils and $p < .001$ for absolute eosinophil numbers). There was a significantly greater reduction in sputum eosinophils after combined budesonide-simvastatin than after budesonide alone (5.1% reduction versus 2.7% reduction, $p = .028$ for % eosinophils and $p = .043$ for absolute eosinophil numbers).

3.3 | Increased IL-10 production after treatment

Sputum IL-10 concentrations were significantly increased in ICS-treated asthmatic patients (median [IQR], 24.5 [15.8–38.0] pg/ml) compared with the baseline (1.5 [0.4–13.1] pg/ml), and further enhanced by the addition of simvastatin to ICS (increased by 46.6%). In contrast, significantly decreased concentrations of airway IL-4 were detected after ICS and ICS/simvastatin treatments (Table 2) (Figure 2).

3.4 | Effects of treatments on autophagy markers in asthmatic sputum

Previous studies have demonstrated sex dimorphism in constitutive autophagic protein expression, in particular LC3 and p62 in animal models,³² but although there were significant differences in the proportion of female asthmatic patients in ICS and ICS/simvastatin groups, baseline levels of beclin-1, LC3 and p62 expression were not significantly different between the treatment groups ($p = .87$, $p = .08$ and $p = .93$, respectively). The percentage of sputum macrophages and eosinophils with beclin-1 and LC3 expression in asthmatic patients were significantly reduced following ICS treatment (Table 3). In contrast, there was a dramatically increased p62 expression in macrophages and eosinophils after ICS treatment ($p < .001$ for both) compared with the baseline (Table 3). The addition of simvastatin further enhanced ICS effects on beclin-1 and p62 expression in

eosinophils ($p = .01$ and $p = .0027$, respectively) and on LC3 and p62 expression in macrophages ($p = .0018$ and $p = .036$, respectively) in association with the augmented reduction in sputum eosinophil numbers.

In sputum macrophages, the magnitude of upregulated IL-10 levels correlated negatively with the degree of changes in Beclin-1 ($p = .0019$, and $p = .0011$, respectively), while positively with p62 expression ($p = .024$ and $p = .0014$, respectively) after ICS and ICS/simvastatin treatment (Table 4). In contrast, the significant correlation with changes in LC3 was detectable after ICS but not ICS/simvastatin treatment ($p = .0007$ and $p = .05$, respectively). However, a significant correlation of changes in macrophage LC3 and p62 with IL-4 (Table 4) was detected, which was consistent with a significant suppression of IL-4 levels by ICS ($p = .0008$) and ICS/simvastatin ($p = .0011$) (Table 2).

3.5 | Cross-regulation between autophagy and IL-10 production in M2-like macrophages

We investigated whether IL-10 could regulate macrophage autophagy. Exogenous administration of IL-10 to M2-like macrophages in *ex vivo* cultures strongly suppressed rapamycin-induced autophagy, autophagic flux, beclin-1 and LC3 expression compared with rapamycin-treated macrophages in the absence of IL-10 ($p < .001$ for all) and control macrophages ($p < .001$ for autophagy and autophagic flux, and $p = 1.0$ for beclin-1 and LC3), and this effect on macrophage autophagy and autophagic flux was not significantly different compared with the autophagy inhibitor 3-MA ($p = 1.0$ and $p = .69$, respectively) (Figure 3A–D) (Supplementary Table 1). These effects of IL-10 on macrophage autophagy, autophagic flux, and beclin-1 and LC3 expression could be reversed, albeit partially, by IL-10 neutralisation with a specific recombinant monoclonal blocking antibody ($p < .001$ for all) (Figure 3A–D). Similarly,

TABLE 2 Induced sputum cytology and biochemical profiles.

	Bud (n = 22)			Bud +Sim (n = 22)					
			P			P	Δ Post-pre	Δ Post-pre	P
Variables	Pre	Post	value	Pre	Post	value	Bud	Bud +Sim	value
Lung function									
Pre-β2 FEV1	1.7 (1.1, 2.1)	1.8 (1.5, 2.2)	.004	1.5 (1.2, 1.8)	1.4 (1.3, 1.8)	0.41	0.1 (−0.1, 0.5)	0.03 (−0.1, 0.1)	.1
PC ₂₀	1.8 (0.6, 3.1)	2.1 (0.9, 3.3)	0.4	0.9 (0.5, 2.7)	1.9 (0.5, 3.6)	0.22	0.24 (−0.9, 1.1)	0.5 (−0.9, 1.1)	.99
Differential cell count and proportion									
Total cell count (×10 ⁶)	0.9 (0.7,1.3)	0.7 (0.6, 1.0)	.009	0.81 (0.65, 1.1)	0.7 (0.6, 1.1)	0.96	−0.2 (−0.6, −0.1)	−0.1 (−0.3, −0.5)	0.08
Eos (×10 ⁶)	0.03 (0.0, 0.1)	0.01 (0.0, 0.02)	.002	0.03 (0.02, 0.1)	0.003 (0.0, 0.01)	< .001	−0.02 (−0.1, 0.0)	−0.03 (−0.1, −0.02)	.043
Eos (%)	3.4 (0.8, 5.6)	0.8 (0.5, 2.1)	.01	4.0 (2.7, 7.5)	0.5 (0.0, 1.0)	< .001	−1.5 (−5.0, −0.1)	−3.5 (−7.0, −2.4)	.028
Neu (×10 ⁶)	0.4 (0.3, 0.7)	0.3 (0.2, 0.4)	.06	0.36 (0.3, 0.5)	0.3 (0.2, 0.7)	.44	−0.1 (−0.2, 0.1)	−0.1 (−0.2, 0.3]	.61
Neu (%)	39.1 (31.9, 51.9)	44.9 (30.6, 49.3)	.23	43.9 (34.8, 56.2)	40.1 (28.9, 53.6)	.37	−4.5 (−8.4, 5.5)	−4.0 (−17.1, 7.7)	.87
Mac (×10 ⁶)	0.5 (0.3, 0.7)	0.4 (0.3, 0.6)	.13	0.4 (0.3, 0.5)	0.43 (0.4, 0.6)	.33	−0.03 (−0.3, 0.0)	−0.7 (−1.5, 0.06)	.06
Mac (%)	52.0 (40.8, 58.4)	53.5 (47.1, 68.6)	.01	49.0 (35.0, 55.9)	57.6 (45.7, 70.6)	.04	7.0 (−2.5, 13.7)	9.3 (−4.0, 21.2)	.67
Lym (×10 ⁶)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.62	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.34
Lym (%)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	1.0	0.0 (0.0, 0.1)	0.0 (0.0, 0.0)	.56	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.34
Epi (×10 ⁶)	0.0 (0.01,0.04)	0.0 (0.0, 0.01)	.023	0.0 (0.0, 0.02)	0.0 (0.0, 0.01)	0.08	−0.01 (−0.02, 0.0)	−0.002 (−0.02, 0.0)	.54
Epi (%)	1.8 (0.5, 3.3)	0.8 (0.0, 1.8)	.031	0.75 (0.4, 1.8)	0.5 (0.0, 2.0)	.23	−1.1 (−2.3, 0.6)	−0.3 (−1.0, 0.5)	.0002
Sputum cytokines									
IL−4 (pg/ml)	5.2 (3.1, 5.9)	1.8 (0.5, 2.7)	.0008	5.0 (2.8, 6.2)	2.3 (0.7, 4.0)	.0011	−3.0 (−4.6, −0.5)	−1.7 (−4.1, −0.2)	.58
IL−13 (pg/ml)	103.5 (90.7,175.6)	119.7 (97.5,171.8)	.309	111.0 (97.8,190.7)	62.5 (43.3,90.3)	<.001	5.6 (−9.3, 44.6)	−54.8 (−133.4, −22.3)	<.001
IL−10 (pg/ml)	1.5 (0.4, 13.1)	24.5 (15.8, 38.0)	<.001	2.4 (1.0, 14.2)	57.4 (33.0, 73.1)	<.001	18.5 (12.3, 26.4)	45.0 (29.5, 68.2)	<.001

Note: Data are presented as median (25–75 percentiles)

Bold font indicates results of statistical analyses where $p < .05$

corticosteroids, simvastatin and combination treatment exerted their suppressive effects on autophagy and autophagic flux in rapamycin-treated macrophages ($p < .001$ for all) (Figure 3A-B) but not in rapamycin-untreated macrophages (Figure S3A) (Supplementary Table 2) Corticosteroids, simvastatin and both treatments in combination also markedly downregulated rapamycin-enhanced Beclin-1 ($p < .001$ for all) and LC3 expression ($p < .001$ for all) (Figure 4A-B) (Supplementary Table 3) and inhibited rapamycin-driven autophagy ($p < .001$ for all, respectively) and rapamycin-accelerated autophagic flux ($p < .001$ for all) in parallel in macrophages (Figure 3A-B). We also demonstrated the greater effects of combination therapy on

autophagic flux, Beclin-1 and LC3 compared to simvastatin ($p < .001$, $p = .006$ and $p = .003$, respectively) and budesonide treatment alone ($p = .003$, $p = .002$ and $p = .012$, respectively) and the additive effects of both treatments on macrophage autophagy compared with either treatment alone ($p < .001$ for both) (Figure 3A-B and Figure 4A-B). In addition, despite significant pharmacological suppression in rapamycin-induced autophagy in macrophages, there was no significant impact on cell survival (Figure S3B) (Supplementary Table 4). These inhibitory effects on macrophage autophagy and autophagic flux appeared to be IL-10-dependent as downregulation of IL-10 expression by siRNA, albeit only 86% of IL-10 knockdown (Figure S4A-B),

TABLE 3 The expression of autophagic proteins in induced sputum in response to ICS and simvastatin treatment.

	Bud (n = 22)			Bud +Sim (n = 22)					
			P			P			
Cell subsets	Pre	Post	value	Pre	Post	value	Δ Post-pre Bud	Δ Post-pre Bud +Sim	P value
Beclin-1									
Total cells (%)	51.2 (34.4, 64.5)	29.9 (24.3, 51.8)	.0053	50.4 (38.8, 54.1)	28.2 (23.3, 39.1)	.0017	-15.7 (-31.5, 2.4)	-16.5 (-29.2, -6.5)	.82
Neu (%)	1.6 (0.9, 3.0)	1.3 (0.9, 2.2)	.52	1.3 (0.9, 2.4)	1.5 (0.7, 2.8)	.7	-0.7 (-1.7, 1.2)	-0.13 (-1.6, 1.8)	.98
Eos (%)	1.9 (0.5, 3.9)	0.5 (0.0, 1.3)	<.001	3.2 (1.7, 4.5)	0.6 (0.0, 1.3)	<.001	-1.2 (-1.9, -0.3)	-2.5 (-3.6, -1.4)	.01
Mac (%)	43.0 (28.5, 56.4)	26.3 (17.8, 44.4)	.01	44.1 (35.3, 47.9)	24.9 (19.4, 31.8)	<.001	-10.6 (-27.6, -0.6)	-14.3 (-24.9, -7.8)	.3
Lym (%)	0.0 (0.0, 0.1)	0.0 (0.0, 0.5)	.1	0.0 (0.0 to 0.0)	0.0 (0.0, 0.0)	.43	0.0 (0.0, 0.5)	0.0 (0.0, 0.0)	.97
Epi (%)	1.9 (0.5, 3.9)	1.0 (0.4, 1.8)	.031	1.7 (1.2, 2.8)	0.36 (0.0, 2.0)	.015	-0.6 (-1.9, 0.1)	-1.2 (-2.2, 0.3)	.66
LC3									
Total cells (%)	47.2 (30.2, 54.9)	25.6 (11.8, 45.2)	<.001	51.4 (45.0, 55.8)	20.5 (15.1, 24.5)	<.001	-16.5 (-27.7, -5.5)	-27.6 (-34.0, -22.2)	.0006
Neu (%)	0.0 (0.0, 1.2)	0.2 (0.0, 1.6)	.27	0.5 (0.0, 1.6)	0.6 (0.0, 2.0)	.72	0.02 (-0.1, 1.5)	0.23 (-0.9, 0.8)	.76
Eos (%)	2.3 (1.2, 3.8)	0.2 (0.0, 0.6)	<.001	2.4 (1.4, 3.4)	0.0 (0.0, 0.6)	<.001	-2.1 (-2.9, -0.6)	-2.4 (-3.2, -1.4)	.48
Mac (%)	42.2 (27.1, 47.9)	21.4 (11.0, 41.7)	<.001	44.9 (39.2, 49.3)	17.3 (12.2, 22.5)	<.001	-14.5 (-24.5, -4.8)	-26.4 (-32.3, -19.9)	.0018
Lym (%)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	>.999	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.5	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.89
Epi (%)	1.2 (0.0, 1.9)	0.6 (0.0, 2.0)	>.999	0.7 (0.7, 2.0)	0.5 (0.2, 1.5)	.07	0.002 (-1.3, 1.1)	-0.7 (-1.5, 0.06)	.22
p62									
Total cells (%)	21.3 (11.3, 27.8)	45.2 (30.3, 53.3)	<.001	22.1 (16.6, 24.5)	52.7 (45.4, 57.2)	<.001	20.9 (10.3, 31.4)	28.8 (22.2, 36.3)	.019
Neu (%)	0.0 (0.0, 2.4)	1.5 (0.0, 2.0)	.52	0.7 (0.0, 2.2)	0.7 (0.0, 2.2)	.91	0.9 (-0.96, 1.5)	-0.2 (-0.7, 0.8)	.21
Eos (%)	0.0 (0.0, 0.2)	1.0 (0.5, 2.2)	<.001	0.0 [0.0 to 0.5]	2.4 (1.4, 3.6)	<.001	0.9 (0.4, 1.8)	2.3 (1.4, 3.5)	.0027
Mac (%)	18.9 (10.7, 23.8)	41.1 (27.4, 47.6)	<.001	18.5 (12.2, 22.5)	45.4 (39.2, 50.2)	<.001	18.6 (6.7, 28.9)	26.3 (19.9, 32.4)	.036
Lym (%)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.43	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.5	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)	.64
Epi (%)	0.7 (0.0, 1.2)	1.2 (0.0, 2.2)	.12	0.9 (0.3, 1.5)	1.5 (0.7, 2.0)	.042	0.0 (-0.7, 2.0)	0.6 (-0.1, 1.7)	.59

Note: Data are presented as median (25–75 percentiles). The percentage of difference in autophagic protein expression in cell subsets was calculated by subtraction of the post- and pre-treatment values.

Bold font indicates results of statistical analyses where $p < .05$

resulted in a reversal of anti-autophagic effects of the combination therapy in rapamycin-treated M2-like macrophages ($p < .001$ for both) (Figure 3A-B).

There was reciprocal interaction between autophagy and IL-10 expression in M2-like macrophages. Rapamycin-treated macrophages had low expression of IL-10 transcripts ($p = .014$), with the lower number of IL-10-expressing macrophages than control

($p < .001$) (Figure 5A-B). In contrast, the suppression of rapamycin-induced macrophage autophagy with 3-MA dramatically enhanced IL-10 expression at both mRNA ($p < .001$ for both) and protein levels compared with rapamycin-treated and control macrophages ($p < .001$ for both). Similarly, rapamycin-treated macrophages under various culture conditions in the presence of corticosteroids, simvastatin, and both in combination exhibited

greater expression of IL-10 transcripts ($p < .001$ for all) and IL-10 protein compared with those without these treatments ($p < .001$ for all), with greater effects of combination treatment on IL-10 transcripts and protein expression than simvastatin and budesonide treatment ($p < .001$ for all). The expression of IL-10 in M2 macrophages was strongly correlated with the degree of autophagy inhibition in macrophages from all culture conditions ($r = -.89, p < .001$) (Figure 5C).

TABLE 4 Induced sputum cytology and biochemical profiles.

	Bud Δ IL-10 (pg/ml)	Bud +Sim Δ IL-10 (pg/ml)	Bud Δ IL-4 (pg/ml)	Bud +Sim Δ IL-4 (pg/ml)
Sputum macrophages (n = 22)				
Δ Beclin-1%	-0.62 ^{§**}	-0.64 ^{**}	0.15	0.029
Δ LC3%	-0.66 ^{§**}	-0.4	0.83 ^{§***}	0.78 ^{**}
Δ p62%	0.47 ^{§*}	0.63 ^{**}	-0.54 ^{**}	-0.43 [*]

Note: Pearson and Spearman[§] r values reported.

* $p < .05$

** $p < .01$

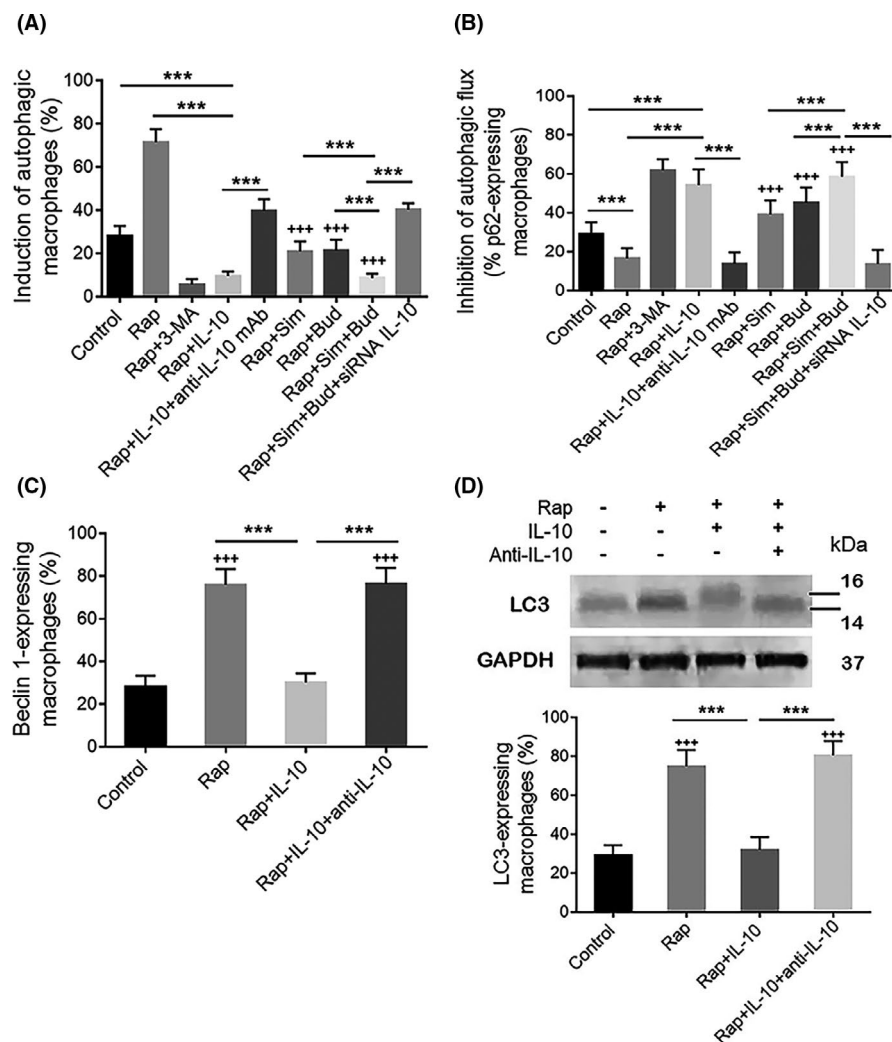
*** $p < .001$

We next sought to determine the degree of IL-10 mRNA expression after transcriptional silencing of specific autophagic proteins (Beclin-1, LC3, p62) with siRNA directed against these proteins in rapamycin-induced autophagic macrophages, with comparable silencing (59.8%, 61.4% and 63.7%, respectively) (Figure S4A-B). We found that IL-10 transcription in siRNA LC3-treated macrophages was increased to the greatest extent, followed by silencing with p62 and Beclin-1, despite the absence of statistical significance for the former treatment when compared with control ($p < .001$, $p = .083$ and $p < .001$) and scrambled control siRNA-treated macrophages ($p < .001$, $p < .001$ and $p = .003$, respectively) (Figure 5D) (Supplementary Table 5). This suggests that IL-10 expression in macrophages was more dependent on LC3 than on other autophagy proteins.

4 | DISCUSSION

The present study has demonstrated for the first time that the production of macrophage-derived IL-10, a key anti-inflammatory cytokine in asthma, is negatively regulated by autophagy, and this provides a potential novel therapeutic target for the new asthma

FIGURE 3 (A) Induction of macrophage autophagy by rapamycin (Rap) was markedly inhibited by 3-MA, IL-10, budesonide (Bud), simvastatin (Sim) and both in combination, all of which were significantly reversed by anti-IL-10 neutralizing antibody and siRNA IL-10 (n = 7). (B) Autophagic flux was significantly induced by rapamycin as reflected by p62 accumulation in macrophage and markedly suppressed by 3-MA, IL-10, Bud, Sim and both in combination, whereas IL-10 neutralisation and siRNA IL-10 could reverse the suppressive effects of IL-10 and combination treatment, respectively (n = 9). IL-10 significantly inhibited Rap-enhanced Beclin-1 (C) and LC3 expression in M2-like macrophages (D), whereas IL-10 neutralisation could reverse these effects (n = 6). Similarly, combination treatment with Bud/Sim provided greater suppression in macrophage autophagy and autophagic flux than either single treatment. Results are expressed as the means (STDEV). Multiple comparisons were performed with Bonferroni correction. * $p < .05$, ** $p < .01$ *** $p < .001$ compared with indicated conditional macrophages and *** $p < .001$ with Rap-treated macrophages.



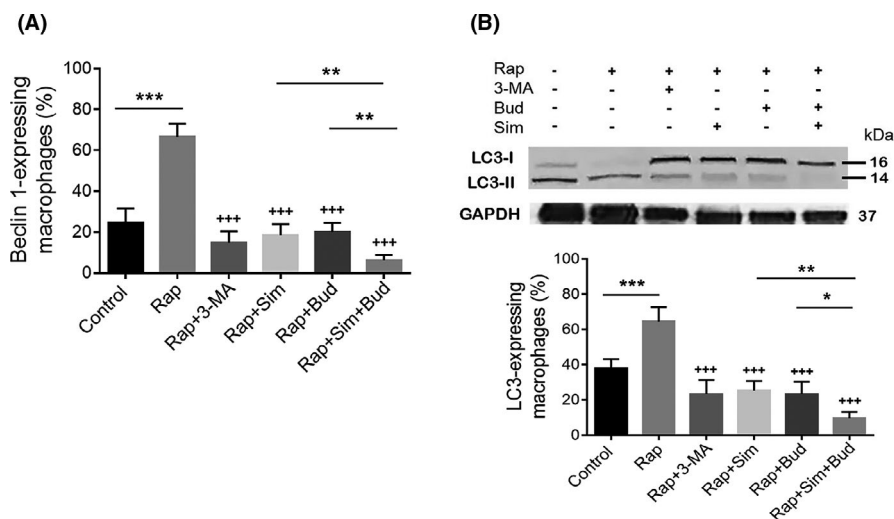


FIGURE 4 Rapamycin (Rap) significantly induced Beclin-1 (A) and LC3 expression (B) in M2-like macrophages whereas 3-MA, simvastatin (Sim), budesonide (Bud) and both in combination markedly suppressed rapamycin effects ($n = 6$). In addition, combination treatment with Bud/Sim inhibited rapamycin effects on Beclin-1 and LC3 expression to a significantly greater extent than either single treatment. Results are expressed as the means (STDEV). Multiple comparisons were performed with Bonferroni correction. * $p < .05$, ** $p < .01$ *** $p < .001$ compared with indicated conditional macrophages and +++ $p < .001$ with Rap-treated macrophages.

treatment development. We investigated the role of autophagy in worsening eosinophilic airway inflammation in asthma by accelerating inhibition of macrophage-derived IL-10 release, resulting in delayed resolution of inflammation. This was remarkably reversed by corticosteroid/statin treatment through the marked suppression of macrophage beclin-1, LC3 and autophagic flux, leading to enhanced IL-10 secretion, which further suppressed macrophage autophagy. We also determined the impact of depleting different autophagy molecules on IL-10 regulation in M2-like macrophages *in vitro* and demonstrated that silencing of macrophage LC3 expression had a greater effect in inducing IL-10 transcription than knockdown of Beclin-1 and p62.

Although ICS potentiate airway epithelial autophagy to maintain epithelial integrity and to promote antiviral activity as shown in several studies,^{33,34} their effects on autophagy in inflammatory cells, such as macrophages, have not been reported. The effect of statins on macrophage autophagy in asthma has also not been reported, although statins have been shown to induce autophagy-mediated retardation of macrophage-driven atherosclerotic plaque progression and to reduce *Mycobacterium tuberculosis* burden.³⁵⁻³⁷ However, statins are able to both induce and inhibit excessive autophagy resulting in different cellular outcomes, depending on the cell type, disease condition and drug concentration.^{38,39} We have demonstrated that anti-autophagy effects of ICS and statins in asthma with the additive enhancement, which was exhibited in sputum macrophages. In animal studies, inducing effects of statins on autophagy are mediated through mTOR-dependent and independent mechanisms.^{40,41} In contrast, our data demonstrated that corticosteroids, simvastatin and their combination significantly inhibit rapamycin-induced macrophage autophagy, while lacking any autophagic effects and IL-10 upregulation on macrophages not treated with rapamycin (Figure S3C) (Supplementary Table 2). MTOR activation is regulated by the

phosphoinositide-3-kinase (PI3 K) and Av-akt murine thymoma viral oncogene homolog (Akt)/protein kinase B (PKB) pathway resulting in suppression of autophagy. We postulated that the potential mechanisms underlying the anti-autophagic effects of corticosteroids may be associated with activation of the PI3 K-Akt/mTOR signaling pathway, and may be enhanced by simvastatin, as demonstrated in previous studies.⁴²⁻⁴⁴ In addition, corticosteroids may mediate their anti-autophagic effects by suppressing TANK-binding kinase 1 (TBK1), which coordinates assembly and function of the autophagic machinery by phosphorylating the autophagic adaptor p62, and therefore reduce the maturation of autophagosomes.⁴⁵ Changes in autophagy activity may be associated with alteration in transcription of IL-10 that is primarily regulated by transcription factors Sp1 and Sp3 as autophagy induction could downregulate Sp1 protein expression through inhibition of mTOR cascade.⁴⁶⁻⁴⁸ This raises the possibility that corticosteroid- and statin-mediated suppression in macrophage autophagy lead to upregulation of Sp1 expression with increased transcriptional activity to bind to the IL-10 transcriptional motif. However, further investigations to elucidate these mechanisms are needed.

Although insights have been obtained into the role of autophagy in pro-inflammatory cytokines,^{25,26,49,50} it is less clear whether macrophage autophagy has been implicated in transcriptional and translational control of IL-10 expression. The present study has uncovered a previously unappreciated role for macrophage autophagy in dampening IL-10 secretion. Rapamycin inhibited the expression of IL-10 at a protein level in M2-like macrophages. Conversely, treatment with the autophagy inhibitor 3-MA, budesonide, simvastatin and both in combination enhanced the production of IL-10 in M2-like rapamycin-treated macrophages. We confirmed the involvement of macrophage autophagy in regulating IL-10 at transcription level by transcriptional inhibition of specific autophagic

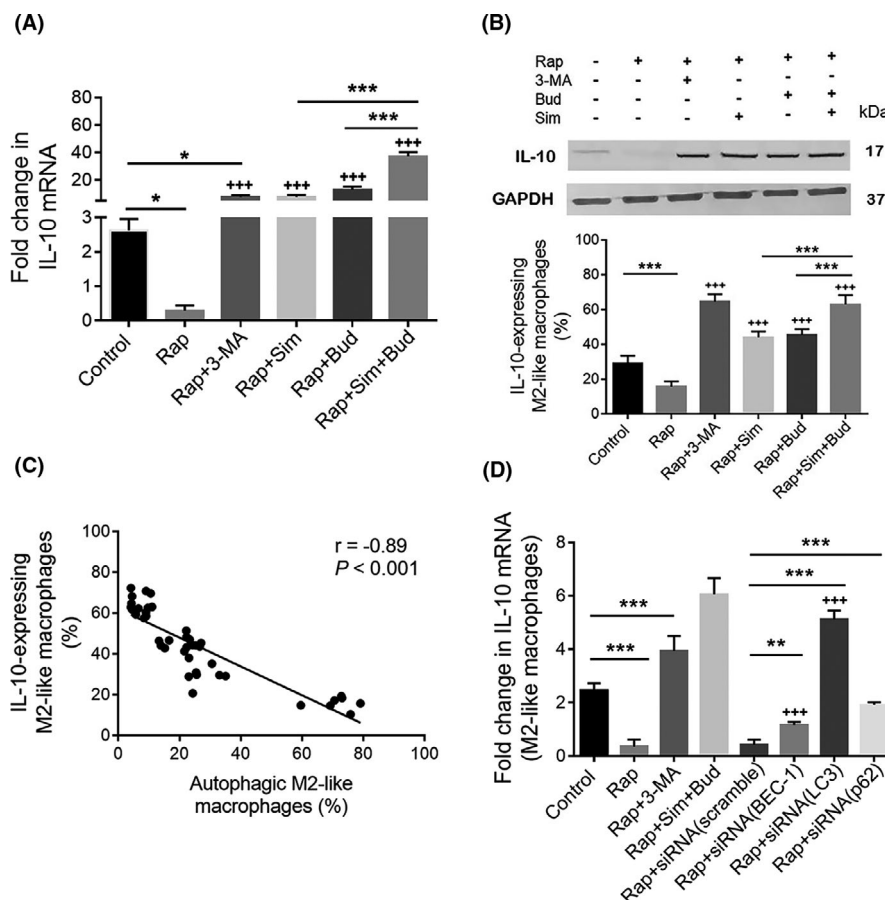


FIGURE 5 Rapamycin significantly induced downregulation of IL-10 transcription (A) ($n = 12$) and protein expression measured by flow cytometry (B) ($n = 7$), whereas 3-MA, simvastatin (Sim), budesonide (Bud) and both in combination could inhibit these inhibitory effects of rapamycin on IL-10 in M2-like macrophages. In addition, the degree of IL-10-expression was inversely correlated with the extent of macrophage autophagy as analysed by Spearman's rank correlation test ($n = 42$) (C). (D) Small interference RNA (siRNA)-mediated knockdown of LC3 transcription induced by far the highest upregulation of IL-10 transcripts in Rap-treated M2-like macrophages compared with Beclin-1 (BEC-1) and p62 silencing ($n = 8$). This observation did not result from the different efficacy of individual siRNAs (shown no discrepancy in Figure S4A). Results are expressed as the means (STDEV). Multiple comparisons were performed with Bonferroni correction. * $p < .05$, ** $p < .01$ and *** $p < .001$ compared with the indicated conditional macrophages and **** $p < .001$ with Rap-treated macrophages.

proteins (Beclin-1 and LC3) and autophagic flux-related proteins (p62) using specific siRNA knockdown of these proteins in macrophages. Silencing of LC3 was the most effective intervention in augmenting IL-10 mRNA expression compared to the other autophagy proteins. Our results are supported by previous studies suggesting that inhibition of mTOR-mediated autophagy is associated with IL-10 induction, despite different cell types and unclear mechanisms underlying this regulation.⁵¹ In macrophages, IL-10 expression can be induced by TLR-dependent signals including extracellular signal-regulated kinase (ERK) and p38 MAPK, both of which are regulated by autophagy-related proteins.⁵² In addition, microRNA-27a can enhance IL-10 transcription through ERK activation and regulates molecular features of autophagy, although whether the autophagic machinery *per se* regulates the cellular fate of microRNA-27a in macrophages remains unknown.⁵³ Therefore, autophagy possibly suppresses IL-10 production by modulation of kinase activity in favour of p38 MAPK, although this requires further elucidation.

In asthma, IL-13 activates autophagy to enhance the formation of airway goblet cells leading to further pathobiology,⁵⁴ while IL-4 induces autophagy in B cells both *in vitro* and *in vivo* to extend B-cell survival and enhance antigen presentation, resulting in worsening of asthma,⁵⁵ suggesting that Th2 cytokine-driven autophagy is detrimental in asthma. In the present study, ICS and ICS/statin significantly suppressed IL-4 secretion, which directly correlated with the downregulation of LC3 expression in macrophages, suggesting the reciprocal interaction of Th2 cytokines with autophagy. Although IL-10 broadly inhibits starvation-, rapamycin-, LPS-induced autophagy via Akt and STAT3 pathways, its anti-autophagic effects in asthma require further investigation. Given that IL-4, IL-13 and IL-10 are largely secreted by monocytes/macrophages and lymphocyte subsets, T cell-mediated and auto-crine regulation of autophagy by these cytokines in macrophages may be a mechanism driving the inflammatory response in asthma. Corticosteroid/statin suppressed autophagy and enhanced IL-10 secretion, which is important for the control of asthmatic airway

inflammation, but can further inhibit macrophage autophagy through autoregulation via IL-10 secretion from macrophages, with the induction of cross-talk between the IL10-IL10R-STAT3 and IL10-Akt-mTOR pathways.⁵⁶

In summary, the present study demonstrates that there is the negative regulation between macrophage autophagy and IL-10 secretion in asthma and that this can be overcome by corticosteroids, especially if combined with a statin. Further studies should explore the ways of reducing of macrophage autophagy activity to optimise IL-10 levels for optimal suppression of eosinophilic inflammation in asthma without compromising other physiological functions of autophagy.

ACKNOWLEDGEMENTS

We would like to thank Faculty of Medicine Siriraj Hospital for providing research funding (R015433008). We thank Jirawat Assawapoom for project coordination and technical assistance and Suthipol Udompunturak for help with statistical analysis advice.

CONFLICT OF INTERESTS

KM, KK and AW have no conflicts of interest to declare. PB has served on Scientific Advisory Boards of AstraZeneca, Boehringer-Ingelheim, Bepak, Chiesi, Daiichi-Sankyo, DeepBreeze, GlaxoSmithKline, Glenmark, Johnson & Johnson, Merck, Novartis, Nycomed/Takeda, Pfizer, Prosonix, RespiVert, Sun Pharmaceuticals, Teva and UCB and has received research funding from Aquinox Pharmaceuticals, AstraZeneca, Boehringer-Ingelheim, Chiesi, Daiichi-Sankyo, GSK, Novartis, Nycomed/Takeda, Pfizer, Prosonix, Sun Pharmaceuticals. He is also a co-founder of RespiVert (now part of Johnson & Johnson), which has discovered novel inhaled anti-inflammatory treatments for asthma and COPD.

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

AUTHOR CONTRIBUTIONS

KM is the guarantor of the paper and takes responsibility for the integrity of the work as a whole and contributed to the study conception and design, data acquisition, analysis and interpretation. KK contributed to experimental and clinical works. AW and PB contributed to the study design and the interpretation of results. KM drafted the initial manuscript. All authors discussed the results and reviewed the manuscript. All authors read and approved the final manuscript.

ORCID

Kittipong Maneechotesuwan  <https://orcid.org/0000-0001-5940-7436>

REFERENCES

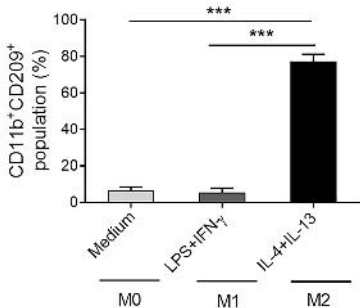
- Saraiva M, O'Garra A. The regulation of IL-10 production by immune cells. *Nat Rev Immunol*. 2010;10(3):170-181.
- Moore KW, de Waal MR, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol*. 2001;19:683-765.
- O'Garra A, Vieira P. T(H)1 cells control themselves by producing interleukin-10. *Nat Rev Immunol*. 2007;7(6):425-428.
- Roncarolo MG, Gregori S, Battaglia M, Bacchetta R, Fleischhauer K, Levings MK. Interleukin-10-secreting type 1 regulatory T cells in rodents and humans. *Immunol Rev*. 2006;212:28-50.
- Maynard CL, Weaver CT. Diversity in the contribution of interleukin-10 to T-cell-mediated immune regulation. *Immunol Rev*. 2008;226:219-233.
- Maneechotesuwan K, Supawita S, Kasetsinsombat K, Wongkajornsilp A, Barnes PJ. Sputum indoleamine-2, 3-dioxygenase activity is increased in asthmatic airways by using inhaled corticosteroids. *J Allergy Clin Immunol*. 2008;121(1):43-50.
- Maneechotesuwan K, Kasetsinsombat K, Wamanuttajinda V, Wongkajornsilp A, Barnes PJ. Statins enhance the effects of corticosteroids on the balance between regulatory T cells and Th17 cells. *Clin Exp Allergy*. 2013;43(2):212-222.
- Ogawa Y, Duru EA, Ameredes BT. Role of IL-10 in the resolution of airway inflammation. *Curr Mol Med*. 2008;8(5):437-445.
- Liu JN, Suh DH, Trinh HK, Chwae YJ, Park HS, Shin YS. The role of autophagy in allergic inflammation: a new target for severe asthma. *Exp Mol Med*. 2016;48(7):e243.
- Ban G-Y, Pham DL, Trinh THK, et al. Autophagy mechanisms in sputum and peripheral blood cells of patients with severe asthma: a new therapeutic target. *Clin Exp Allergy*. 2016;46(1):48-59.
- Mizushima N, Levine B. Autophagy in Human Diseases. *N Engl J Med*. 2020;383(16):1564-1576.
- Pham DL, Ban G-Y, Kim S-H, et al. Neutrophil autophagy and extracellular DNA traps contribute to airway inflammation in severe asthma. *Clin Exp Allergy*. 2017;47(1):57-70.
- McAlinden KD, Deshpande DA, Ghavami S, et al. Autophagy Activation in Asthma Airways Remodeling. *Am J Respir Cell Mol Biol*. 2019;60(5):541-553.
- Poon A, Eidelman D, Laprise C, Hamid Q. ATG5, autophagy and lung function in asthma. *Autophagy*. 2012;8(4):694-695.
- Liang XH, Jackson S, Seaman M, et al. Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature*. 1999;402(6762):672-676.
- Jung CH, Jun CB, Ro S-H, et al. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. *Mol Biol Cell*. 2009;20(7):1992-2003.
- Nazio F, Strappazzon F, Antonioli M, et al. mTOR inhibits autophagy by controlling ULK1 ubiquitylation, self-association and function through AMBRA1 and TRAF6. *Nat Cell Biol*. 2013;15(4):406-416.
- Galluzzi L, Baehrecke EH, Ballabio A, et al. Molecular definitions of autophagy and related processes. *EMBO J*. 2017;36(13):1811-1836.
- Rogov V, Dotsch V, Johansen T, Kirkin V. Interactions between autophagy receptors and ubiquitin-like proteins form the molecular basis for selective autophagy. *Mol Cell*. 2014;53(2):167-178.
- Komatsu M, Ichimura Y. Physiological significance of selective degradation of p62 by autophagy. *FEBS Lett*. 2010;584(7):1374-1378.
- Knaevelsrud H, Simonsen A. Fighting disease by selective autophagy of aggregate-prone proteins. *FEBS Lett*. 2010;584(12):2635-2645.
- Harris J, De Haro SA, Master SS, et al. T helper 2 cytokines inhibit autophagic control of intracellular Mycobacterium tuberculosis. *Immunity*. 2007;27(3):505-517.
- Park H-J, Lee SJ, Kim S-H, et al. IL-10 inhibits the starvation induced autophagy in macrophages via class I phosphatidylinositol 3-kinase (PI3K) pathway. *Mol Immunol*. 2011;48(4):720-727.
- Van Grol J, Subauste C, Andrade RM, Fujinaga K, Nelson J, Subauste CS. HIV-1 inhibits autophagy in bystander macrophage/monocytic cells through Src-Akt and STAT3. *PLoS One*. 2010;5(7):e11733.
- Harris J, Hartman M, Roche C, et al. Autophagy controls IL-1 β secretion by targeting pro-IL-1 β for degradation. *J Biol Chem*. 2011;286(11):9587-9597.

26. Saitoh T, Fujita N, Jang MH, et al. Loss of the autophagy protein Atg16L1 enhances endotoxin-induced IL-1 β production. *Nature*. 2008;456(7219):264-268.
27. Bateman ED, Hurd SS, Barnes PJ, et al. Global strategy for asthma management and prevention: GINA executive summary. *Eur Respir J*. 2008;31(1):143-178.
28. Coumou H, Westerhof GA, de Nijs SB, Zwinderman AH, Bel EH. Predictors of accelerated decline in lung function in adult-onset asthma. *Eur Respir J*. 2018;51(2).
29. Maneechotesuwan K, Essilfie-Quaye S, Kharitonov SA, Adcock IM, Barnes PJ. Loss of control of asthma following inhaled corticosteroid withdrawal is associated with increased sputum interleukin-8 and neutrophils. *Chest*. 2007;132(1):98-105.
30. Tarique AA, Logan J, Thomas E, Holt PG, Sly PD, Fantino E. Phenotypic, functional, and plasticity features of classical and alternatively activated human macrophages. *Am J Respir Cell Mol Biol*. 2015;53(5):676-688.
31. Ito K, Jazrawi E, Cosio B, Barnes PJ, Adcock IM. p65-activated histone acetyltransferase activity is repressed by glucocorticoids: mifepristone fails to recruit HDAC2 to the p65-HAT complex. *J Biol Chem*. 2001;276(32):30208-30215.
32. Olivan S, Calvo AC, Manzano R, Zaragoza P, Osta R. Sex differences in constitutive autophagy. *Biomed Res Int*. 2014;2014:652817.
33. Kim S-R, Song J-H, Ahn J-H, et al. Antiviral and anti-inflammatory activity of budesonide against human rhinovirus infection mediated via autophagy activation. *Antiviral Res*. 2018;151:87-96.
34. Trybus E, Krol G, Obarzanowski T, et al. In vivo and in vitro studies on multidirectional mechanism of anti-allergic activity of budesonide. *J Physiol Pharmacol*. 2017;68(6):907-919.
35. Huang B, Jin M, Yan H, et al. Simvastatin enhances oxidized low density lipoprotein induced macrophage autophagy and attenuates lipid aggregation. *Mol Med Rep*. 2015;11(2):1093-1098.
36. Parihar SP, Guler R, Khutlang R, et al. Statin therapy reduces the mycobacterium tuberculosis burden in human macrophages and in mice by enhancing autophagy and phagosome maturation. *J Infect Dis*. 2014;209(5):754-763.
37. Hu HJ, Zhou SH, Liu QM. Blockade of mTOR pathway inhibition in the neointimal hyperplasia and promoting macrophage autophagy - Effect of statin-eluting stents to reduce in-stent restenosis. *Int J Cardiol*. 2015;187:31-32.
38. Su F, Shi M, Zhang J, et al. Simvastatin Protects Heart from Pressure Overload Injury by Inhibiting Excessive Autophagy. *Int J Med Sci*. 2018;15(13):1508-1516.
39. Zhao W, Fu H, Chang F, et al. Effects of various doses of atorvastatin on vascular endothelial cell apoptosis and autophagy in vitro. *Mol Med Rep*. 2019;19(3):1919-1925.
40. Ashrafizadeh M, Ahmadi Z, Farkhondeh T, Samarghandian S. Modulatory effects of statins on the autophagy: A therapeutic perspective. *J Cell Physiol*. 2020;235(4):3157-3168.
41. Asakura K, Izumi Y, Yamamoto M, et al. The cytostatic effects of lovastatin on ACC-MESO-1 cells. *J Surg Res*. 2011;170(2):e197-209.
42. Chartoumpekis D, Ziros PG, Psyrogiannis A, Kyriazopoulou V, Papavassiliou AG, Habeos IG. Simvastatin lowers reactive oxygen species level by Nrf2 activation via PI3K/Akt pathway. *Biochem Biophys Res Commun*. 2010;396(2):463-466.
43. Wu H, Lu D, Jiang H, et al. Simvastatin-mediated upregulation of VEGF and BDNF, activation of the PI3K/Akt pathway, and increase of neurogenesis are associated with therapeutic improvement after traumatic brain injury. *J Neurotrauma*. 2008;25(2):130-139.
44. Jang HJ, Hong EM, Kim M, et al. Simvastatin induces heme oxygenase-1 via NF-E2-related factor 2 (Nrf2) activation through ERK and PI3K/Akt pathway in colon cancer. *Oncotarget*. 2016;7(29):46219-46229.
45. Wang J, Wang R, Wang H, et al. Glucocorticoids Suppress Antimicrobial Autophagy and Nitric Oxide Production and Facilitate Mycobacterial Survival in Macrophages. *Sci Rep*. 2017;7(1):982.
46. Tone M, Powell MJ, Tone Y, Thompson SA, Waldmann H. IL-10 gene expression is controlled by the transcription factors Sp1 and Sp3. *J Immunol*. 2000;165(1):286-291.
47. Brightbill HD, Plevy SE, Modlin RL, Smale ST. A prominent role for Sp1 during lipopolysaccharide-mediated induction of the IL-10 promoter in macrophages. *J Immunol*. 2000;164(4):1940-1951.
48. Kim J, Lee HW, Rhee DK, Paton JC, Pyo S. Pneumolysin-induced autophagy contributes to inhibition of osteoblast differentiation through downregulation of Sp1 in human osteosarcoma cells. *Biochim Biophys Acta Gen Subj*. 2017;1861(11 Pt A):2663-2673.
49. Liu T. Regulation of Inflammasome by Autophagy. *Adv Exp Med Biol*. 2019;1209:109-123.
50. Nakahira K, Haspel JA, Rathinam VA, et al. Autophagy proteins regulate innate immune responses by inhibiting the release of mitochondrial DNA mediated by the NALP3 inflammasome. *Nat Immunol*. 2011;12(3):222-230.
51. Deng H, Zheng M, Hu Z, Zeng X, Kuang N, Fu Y. Effects of daphnetin on the autophagy signaling pathway of fibroblast-like synovocytes in rats with collagen-induced arthritis (CIA) induced by TNF- α . *Cytokine*. 2019;127:154952.
52. Martinez-Lopez N, Singh R. ATGs: Scaffolds for MAPK/ERK signaling. *Autophagy*. 2014;10(3):535-537.
53. Saha B, Bruneau JC, Kodys K, Szabo G. Alcohol-induced miR-27a regulates differentiation and M2 macrophage polarization of normal human monocytes. *J Immunol*. 2015;194(7):3079-3087.
54. Dickinson JD, Alevy Y, Malvin NP, et al. IL13 activates autophagy to regulate secretion in airway epithelial cells. *Autophagy*. 2016;12(2):397-409.
55. Xia F, Deng C, Jiang Y, et al. IL4 (interleukin 4) induces autophagy in B cells leading to exacerbated asthma. *Autophagy*. 2018;14(3):450-464.
56. Shi J, Wang H, Guan H, et al. IL10 inhibits starvation-induced autophagy in hypertrophic scar fibroblasts via cross talk between the IL10-IL10R-STAT3 and IL10-AKT-mTOR pathways. *Cell Death Dis*. 2016;7:e2133.

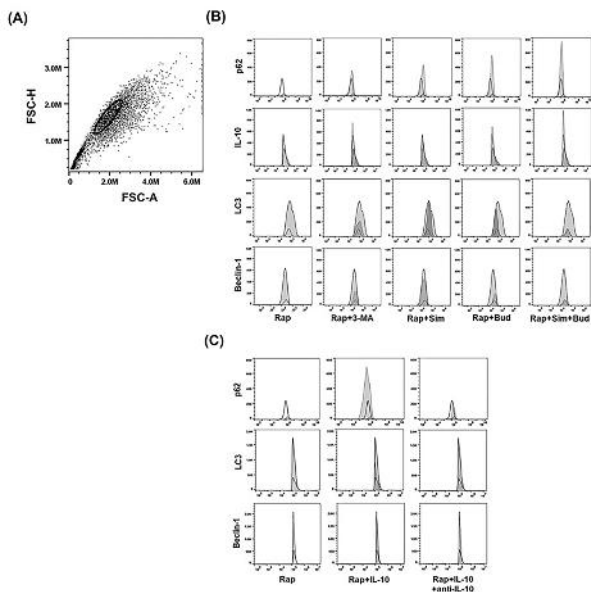
SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

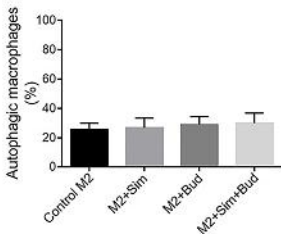
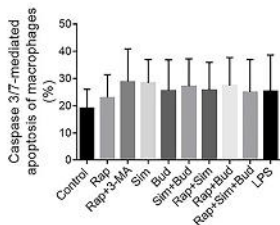
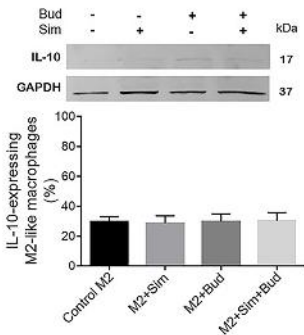
How to cite this article: Maneechotesuwan K, Kasetsinsombat K, Wongkajornsilp A, Barnes PJ. Role of autophagy in regulating interleukin-10 and the responses to corticosteroids and statins in asthma. *Clin Exp Allergy*. 2021;00:1-13. <https://doi.org/10.1111/cea.13825>



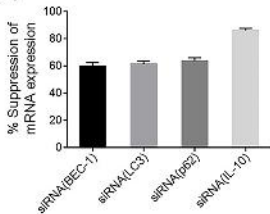
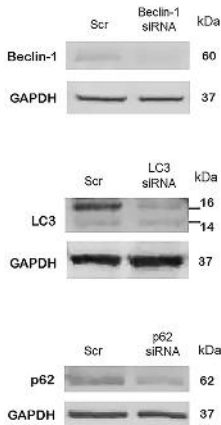
Supplementary Figure 1



Supplementary Figure 2

(A)**(B)****(C)**

Supplementary Figure 3

(A)**(B)****Supplementary Figure 4**

SUPPLEMENTARY TABLE 1 Autophagy modification by IL-10 and pharmacologic agents

Markers	Control	Rap	Rap + 3-MA	Rap + IL-10	Rap + IL10 + anti-IL10	Rap + Sim	Rap + Bud	Rap + Sim + Bud	Rap + Bud + Sim + siRNA IL-10
Autophagic M2 (%) N = 7	28.1 ± 4.7	71.4 ± 6.1	5.8 ± 2.5	9.5 ± 2.0	39.8 ± 5.3	21.1 ± 4.6	21.4 ± 5.0	8.7 ± 1.9	40.3 ± 3.0
P value (vs control) (vs Rap)		<.001	<.001 <.001	<.001 <.001	<.001 <.001	.089 <.001	.134 <.001	<.001 <.001	<.001 <.001
Autophagic flux (% p62 expressing M2) N = 9	29.1 ± 6.1	16.6 ± 5.3	61.9 ± 5.6	54.3 ± 8.1	13.9 ± 5.8	39.2 ± 7.0	45.2 ± 7.8	58.6 ± 7.6	13.8 ± 7.2
P value (vs control) (vs Rap)		.008	<.001 <.001	<.001 <.001	<.001 1.0	.079 <.001	<.001 <.001	<.001 <.001	<.001 1.0
Beclin-1 (%) N = 6	28.4 ± 5.0	76.0 ± 7.3	- -	30.1 ± 4.4	76.4 ± 7.5	-	-	-	-
P value (vs control) (vs Rap)	-	<.001	- -	1.0 <.001	<.001 1.0	-	-	-	-
LC3 (%) N = 6	29.2 ± 5.3	75.0 ± 8.3	- -	32.0 ± 6.6	80.7 ± 7.2	-	-	-	-
P value (vs control) (vs Rap)	-	<.001	- -	1.0 <.001	<.001 1.0	-	-	-	-

SUPPLEMENTARY TABLE 2 Pharmacological effects on autophagy and IL-10 expression in rapamycin-untreated macrophages

	Control	Sim	Bud	Sim+ Bud
Autophagic M2 (%) N=5	25.3 ± 4.7	26.2 ± 7.3	28.0 ± 5.5	29.0 ± 7.1
P value (vs control)		1.0	1.0	1.0
IL-10-expressing M2 (%) N=6	29.9 ± 3.2	28.7 ± 5.1	29.3 ± 5.7	30.4 ± 5.5
P value (vs control)		1.0	1.0	1.0

Note: Data are presented as mean (SD)

SUPPLEMENTARY TABLE 3 The effects of pharmacological treatment on autophagic markers and IL-10 expression

	Control	Rap	Rap + 3-MA	Rap+Sim	Rap+ Bud	Rap+ Sim+Bud
Beclin 1-expressing M2 (%)						
N=6	24.8 ± 6.8	66.6 ± 6.4	14.8 ± 5.7	18.7 ± 5.3	20.2 ± 4.3	6.3 ± 2.6
P value						
(vs control)		<.001	.046	.85	1.0	<.001
(vs Rap)			<.001	<.001	<.001	<.001
LC3-expressing M2 (%)						
N=6	38.2 ± 5.1	64.6 ± 8.2	23.4 ± 8.0	25.4 ± 5.3	23.4 ± 7.0	9.6 ± 3.7
P value						
(vs control)		<.001	.006	.026	.006	<.001
(vs Rap)			<.001	<.001	<.001	<.001
IL-10-expressing M2 (%)						
N=7	29.2 ± 4.3	15.8 ± 3.0	64.7 ± 4.2	44.1 ± 3.3	45.4 ± 3.3	63.0 ± 5.3
P value						
(vs control)		<.001	<.001	<.001	<.001	<.001
(vs Rap)			<.001	<.001	<.001	<.001
IL-10 mRNA (fold change)						
N = 12	2.7 ± 0.3	0.3 ± 0.2	7.8 ± 1.3	7.8 ± 1.3	12.9 ± 2.3	37.3 ± 3.0
P value						
(vs control)		.014	<.001	<.001	<.001	<.001
(vs Rap)			<.001	<.001	<.001	<.001

SUPPLEMENTARY TABLE 4 Pharmacological induction of macrophage apoptosis

[illegible]

SUPPLEMENTARY TABLE 5 The effects of siRNA-mediated transcriptional silencing of specific autophagic proteins on IL-10 transcription

	Control	Rap	Rap + 3-MA	Rap + Sim+Bud	Rap + scramble	Rap + SiRNA BEC-1	Rap + siRNALC3	Rap + siRNAp62
IL-10 mRNA (fold change) N = 8	2.5 ± 0.3	0.4 ± 0.3	4.0 ± 0.6	6.1 ± 0.6	0.4 ± 0.2	1.2 ± 0.1	5.1 ± 0.3	1.9 ± 0.1
P value (vs control)		<.001	<.001	<.001	<.001	<.001	<.001	0.083
(vs scramble siRNA)	<.001	1.0	<.001	<.001		.003	<.001	<.001