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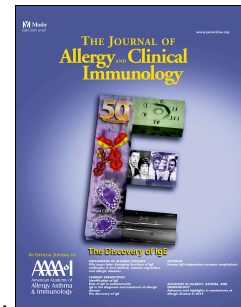
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**Role of C/EBP homologous protein (CHOP) and Endoplasmic Reticulum in
Asthma Exacerbation by Regulating the IL-4/STAT6/Tfec/IL-4R α Positive
Feedback Loop in M2 Macrophages**

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ABSTRACT

Background: C/EBP homologous protein (CHOP), a marker of endoplasmic reticulum (ER) stress, exhibits aberrant expression patterns during the development of asthma. However, its exact role in asthma pathogenesis is not fully understood.

Objectives: We aimed to determine the function and mechanism of CHOP in the pathogenesis of allergic asthma in patients and animals.

Methods: Studies were conducted in asthmatic patients and CHOP^{-/-} mice to dissect the role of CHOP and ER stress in asthma pathogenesis. An ovalbumin (OVA)-induced allergic airway inflammation model was employed to address the impact of CHOP deficiency on the development of asthma. Next, the effect of CHOP deficiency on macrophage polarization and related signaling pathways were investigated to demonstrate the underlying mechanisms.

Results: Asthmatic patients and mice after OVA induction exhibited aberrant CHOP expression along with ER stress. Specifically, CHOP was noted to be specifically overexpressed in macrophages, and mice deficient in CHOP were protected from OVA-induced allergic airway inflammation as manifested by attenuated airway inflammation, remodeling, and hyper-responsiveness. CHOP was found to exacerbate allergic airway inflammation by enhancing M2 programming in macrophages. Mechanistic studies characterized an interleukin-4/signal transducer and activator of transcription 6/transcription factor EC/IL-4R α (IL-4/STAT6/Tfec/IL-4R α) positive feedback regulatory loop, in which IL-4 induces CHOP expression, which then promotes STAT6 signaling to transcribe Tfec expression, and Tfec transcribes IL-4R α expression to promote M2 programming in macrophages.

Conclusions: CHOP and ER stress are implicated in asthma pathogenesis, which involves the regulation of M2 programming in macrophages.

Key Messages:

1. Mice deficient in CHOP were protected from OVA-induced allergic airway

inflammation as manifested by attenuated airway inflammation, remodeling, and hyper-responsiveness.

2. Loss of CHOP attenuated the IL-4/STAT6/Tfec/IL-4R α positive feedback loop essential for M2 programming in macrophages.
3. The number of sputum CHOP⁺CD206⁺ macrophages is positively correlated with asthma severity.

Capsule Summary: CHOP exacerbates allergic asthma by regulating the IL-4/STAT6/Tfec/IL-4R α positive feedback loop essential for M2 programming in macrophages, which are positively correlated with disease severity in asthmatic patients.

Keywords: CHOP, Asthma, Macrophage, Endoplasmic Reticulum Stress

Running title: CHOP in OVA-induced Allergic Airway Inflammation

Abbreviations: CHOP = C/EBP homologous protein; ER stress = endoplasmic reticulum stress; OVA = ovalbumin; AHR = airway hyper-responsiveness; LPS = lipopolysaccharide; TGF- β = transforming growth factor β ; Tfec = transcription factor EC; IL-4R α = IL-4 receptor alpha; ChIP = chromatin immunoprecipitation; BALF = bronchoalveolar lavage fluid; ATF4 = activating transcriptional factor 4; eIF2 α = eukaryotic initiation factor 2 α ; FIZZ1 = found in inflammatory zone-1; STAT6 = signal transducer and activator of transcription 6; FEV1 = forced expiratory volume in one second; FVC = forced vital capacity; PEF = peak expiratory flow.

INTRODUCTION

Allergic asthma is a chronic pulmonary disorder characterized by reversible episodes of airway obstruction, pronounced airway hyper-responsiveness (AHR), peribronchial inflammation, and airway remodeling¹. Macrophages are the most abundant inflammatory cells in the airways, where they produce copious amounts of cytokines such as interleukin (IL)-1 β , IL-13, and IL-17 to exacerbate asthma pathological processes²⁻⁴. Macrophages can be activated by interferon gamma (IFN- γ) or lipopolysaccharide (LPS) to manifest a classically activated phenotype (M1)⁵, or by IL-4 and IL-13 to display an alternatively activated phenotype (M2)⁶. Importantly, airways in allergic asthmatic patients are predominantly infiltrated by M2 macrophages during the course of disease development and progression⁷, and studies in animals revealed that M2 macrophages may represent a novel target for the prevention and treatment of asthma^{8; 9}. Indeed, M2 macrophages produce many factors such as IL-13, C-C motif chemokine ligand 17 (CCL17), CCL22, eotaxin, Ym1 and found in inflammatory zone 1 (FIZZ) contributing to airway inflammatory and remodeling responses during the course of asthma development^{3; 10-12}.

CHOP (also known as Gadd153 or CHOP10) is a marker of endoplasmic reticulum (ER) stress that is activated by eIF2 α phosphorylation and ATF-4 upregulation¹³. Recently, aberrant CHOP expression along with ER stress has been reported in asthmatic patients and animal models of asthma¹⁴⁻¹⁶; however, the significance of CHOP in the pathogenesis of asthma remains unclear. There is feasible evidence that ER stress might modulate the activation of M2 macrophages¹⁷, and we also noted that macrophages derived from induced sputum samples of asthma patients manifest a significant CHOP upregulation. We thus hypothesized that CHOP mediates the generation of M2 macrophages to facilitate the pathological changes associated with asthma development. To address this hypothesis, an OVA-induced allergic airway inflammation model was employed to assess the impact of CHOP deficiency on disease development. Remarkably, the loss of CHOP significantly attenuated

OVA-induced inflammation along with a marked reduction of M2 macrophage infiltration in the lungs. Mechanistic studies have revealed that CHOP regulates signal transducer and activator of transcription 6 (STAT6) phosphorylation, thereby enhancing the expression of mouse transcription factor EC (Tfec), which then transcribes IL-4 receptor alpha (IL-4R α) expression to promote M2 programming in macrophages. Together, our data provide novel insights into the role of ER stress in the pathogenesis of asthma.

MATERIALS AND METHODS

More details for materials and methods are included in the online supplement.

Animals

CHOP knockout (CHOP^{-/-}) mice were purchased from the Jackson's Laboratory (Bar Harbor, ME, USA). Wild-type (WT) mice were obtained from the Animal Experimental Center of Hubei Province (Wuhan, China). All animals were housed in a specific pathogen-free (SPF) animal facility at the Tongji Medical College under a 12 h light/dark photocycle with food and water available ad libitum. All experimental procedures were approved by the Animal Care and Use Committee of the Tongji Hospital of Huazhong University of Science and Technology.

Model for OVA-induced allergic airway inflammation in mice

Asthma was induced in WT and CHOP^{-/-} mice (8–10 weeks old) as previously described with minor modifications^{18; 19}. Briefly, the mice were sensitized using intraperitoneal (i.p.) injection of 100 µg OVA (grade V) emulsified in 1 mg of aluminum hydroxide gel (Thermo Scientific, Rockford, IL) in a total volume of 200 µl, on days 0, 7, and 14, respectively. Mice injected with an equal volume of saline served as controls. The sensitized mice were anesthetized with diethyl ether and intranasally challenged with 50 µl OVA (20 µg/µl) or saline for 7 consecutive days.

Macrophage depletion and macrophage adoptive transfer studies

Clodronate liposomes (40 µl) were administered for 2 consecutive days *via* intratracheal injection 7 days prior to OVA induction, and the severity of asthma was assessed following 7 consecutive days of OVA challenge. The adoptive transfer studies were conducted as previously described²⁰. Peritoneal macrophages derived from WT or CHOP^{-/-} mice were stimulated by IL-4 (10 ng/ml) or phosphate-buffered saline (PBS) for 12 h, and were then administered through intratracheal injection into

the lungs of clodronate liposome-treated WT and CHOP^{-/-} mice at a density of 1×10^6 cells/mouse (50 μ l) 1 day before OVA challenge as described above. The animals were then sacrificed for further analysis.

Statistical analysis

All data are presented as the mean $\pm$ SEM, and all *in vitro* studies were replicated a minimum of 3 times. Statistical analyses of the data were conducted using the GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA) and the standard one-way analysis of variance (ANOVA) or Dunnett's multiple comparison test when appropriate. For all statistical comparisons, $p < 0.05$ was considered statistically significant.

RESULTS

Asthmatic patients and mice exhibit aberrant CHOP expression and ER stress

We first examined the expression of CHOP in induced sputum samples obtained from asthmatic patients. CHOP was expressed at low levels in sputum derived from control subjects, whereas higher levels of CHOP were detected in induced sputum samples originated from asthmatic patients (Figure 1A). We also examined the expression of BIP, another marker of ER stress. Similar to CHOP, BIP was expressed at significantly higher levels in asthmatic patients compared with control subjects (Figure 1B).

To explore the function of CHOP in relation to allergic airway inflammation, we used a mouse model induced by OVA peptides. A two-fold increase in CHOP expression was observed in allergic mice compared with control mice (Figure 1C). Consistently, the levels of phosphorylated eIF2 α (p-eIF2 α) (Figure 1D), Atf4 (Figure 1E), and Bip (Figure 1F), as additional markers of ER stress, were also significantly increased in allergic mice compared with control mice. Collectively, these data support the notion that the pathogenesis of asthma is associated with ER stress.

CHOP deficiency attenuates OVA-induced allergic airway inflammation in mice

Since asthmatic patients and mice were manifested by the altered expression of ER stress markers, we next sought to determine the impact of ER stress on the development of asthma by employing CHOP deficient mice. As expected, severe inflammatory responses were observed in WT mice following 7 days of repeated OVA challenges, as demonstrated by the infiltration of inflammatory cells into the peribronchiolar and perivascular connective tissues. However, CHOP^{-/-} mice exhibited markedly lower inflammatory scores, indicating that the loss of CHOP significantly attenuated OVA-induced inflammatory responses (1.74 ± 0.20 versus 2.96 ± 0.30 , $p < 0.01$; Figure 2A and D). Next, we examined mucus production using PAS staining. Severe goblet cell hyperplasia and mucus hypersecretion within the bronchi were

observed in OVA-challenged WT mice, and these defects were significantly less severe in OVA-challenged CHOP^{-/-} mice (2.32 ± 0.29 versus 1.31 ± 0.19 , $p < 0.05$; Figure 2B and E). Since the thickening of the airway wall is a characteristic feature of airway remodeling, we thus further assessed airway peribronchial collagen deposition using Masson's trichrome staining. Similarly, repeated exposure to OVA for 7 consecutive days increased peribronchial collagen deposition in WT mice compared with the saline-treated controls. However, the loss of CHOP dramatically attenuated OVA-induced peribronchial collagen deposition as well compared with WT mice (0.14 ± 0.02 versus 0.24 ± 0.02 , $p < 0.001$; Figure 2C and F).

Next, we compared the number and subtype of inflammatory cells in the bronchoalveolar lavage fluid (BALF) group. Consistent with the above-mentioned observations, the total number of inflammatory cells in the BALF derived from CHOP^{-/-} mice was significantly reduced compared with WT mice ($7.17 \pm 0.55 \times 10^5/\text{ml}$ versus $11.91 \pm 1.05 \times 10^5/\text{ml}$, $p < 0.01$; Figure 3A). Specifically, BALF derived from OVA-challenged CHOP^{-/-} mice contained significantly fewer eosinophils ($5.28 \pm 0.19 \times 10^5/\text{ml}$ versus $7.89 \pm 0.78 \times 10^5/\text{ml}$, $p < 0.05$; Figure 3B), lymphocytes ($0.90 \pm 0.09 \times 10^5/\text{ml}$ versus $2.17 \pm 0.38 \times 10^5/\text{ml}$, $p < 0.05$; Figure 3C), and macrophages ($0.83 \pm 0.04 \times 10^5/\text{ml}$ versus $1.47 \pm 0.17 \times 10^5/\text{ml}$, $p < 0.05$; Figure 3D) compared with OVA-challenged WT mice. However, the total number of neutrophils was not significantly different between OVA-challenged CHOP^{-/-} and WT mice ($0.48 \pm 0.10 \times 10^5/\text{ml}$ versus $0.48 \pm 0.09 \times 10^5/\text{ml}$, $p > 0.05$; Figure 3E). In addition, we compared the cytokine profiles of BALF samples obtained from OVA-challenged CHOP^{-/-} and WT mice by enzyme-linked immunosorbent assay (ELISA). BALF samples derived from CHOP^{-/-} mice were characterized by the significantly attenuated levels of IL-4, IL-5, IL-13, TGF- β 1, and CCL17 (Figure 3F-J) compared with WT mice. However, no significant differences in the levels of IFN- γ , IL-1 β , and IL-10 were observed (Figure 3K-M). Collectively, these results suggest that CHOP deficiency represses the production of Th2 cytokines by inflammatory cells.

To further evaluate the effect of CHOP deficiency on asthma pathogenesis, we examined AHR by assessing the resistance of the lung (R_n) in response to increasing concentrations of acetylcholine in mechanically ventilated mice. As expected, OVA-challenged WT mice developed AHR as demonstrated by the increase in R_n values induced by acetylcholine compared with the saline-treated control mice. Notably, OVA-challenged CHOP^{-/-} mice exhibited a marked reduction in R_n values in response to acetylcholine compared with OVA-challenged WT mice (25 mg/ml: 2.57 ± 0.25 cm H₂O s/ml versus 4.56 ± 0.33 cm H₂O s/ml, $p < 0.001$; 50 mg/ml: 3.83 ± 0.27 cm H₂O s/ml versus 7.69 ± 0.77 cm H₂O s/ml, $p < 0.001$; Figure 3N). Together, these data demonstrate that the loss of CHOP provides protection for mice against OVA-induced allergic airway inflammation.

CHOP deficiency impairs M2 macrophage induction

To dissect the mechanisms by which CHOP deficiency represses OVA-induced allergic inflammation and airway responsiveness, we determined the specific cell types associated with aberrant CHOP expression in BALF samples. CHOP was predominantly localized within the F4/80-positive macrophages in OVA-challenged WT mice compared with saline-treated controls (Figure 4A), and similar results were observed by analysis of lung sections (Supplementary Figure 1).

Macrophages are the most abundant type of inflammatory cells in the airway. Particularly, polarized M2 macrophages produce copious amount of cytokines in the airway to exacerbate asthma progression³. Based on these findings, we hypothesized that CHOP exacerbates OVA-induced allergic inflammatory responses and AHR by enhancing M2 programming in macrophages. Indeed, flow cytometry analysis revealed a significantly lower number of M2 macrophages in OVA-challenged CHOP^{-/-} mice as manifested by cluster of differentiation 206 (CD206) expression ($2.18 \pm 0.31\%$ versus $6.79 \pm 0.90\%$, $p < 0.05$; Figure 4B). Furthermore, western blot analysis of lung samples derived from OVA-challenged WT mice exhibited a six-fold increase in the levels of the M2 marker Arginase-1 compared with the saline-treated

control mice (Figure 4C). However, the expression of Arginase-1 was reduced by one-fold in CHOP^{-/-} mice following similar procedures for OVA challenges (Figure 4C). Similar results were observed by reverse transcription polymerase chain reaction (RT-PCR) analysis of four additional M2 markers, FIZZ, YM1 (Figure 4D and E), CD206, and CCL17 (Supplementary Figure 2A and B). Unlike its impact on M2 macrophages, CHOP deficiency did not seem to affect the induction of M1 macrophages, as we failed to detect perceptible differences in the number of CD11c⁺F4/80⁺ macrophages following OVA challenges (Supplementary Figure 2C). Together, these data suggest that the loss of CHOP protects mice from OVA-induced allergic airway inflammation by inhibiting the polarization of M2 macrophages in the airways.

M2 macrophages are required for CHOP deficiency-mediated protection against allergic airway inflammation

To investigate whether the protective effect observed in CHOP^{-/-} mice was dependent on the induction of M2 macrophages, we compared disease severity in OVA-challenged WT and CHOP^{-/-} mice after macrophage depletion. Macrophages in the lungs were depleted by intratracheally injecting mice with clodronate liposomes as described²⁰. Following depletion of macrophages by clodronate liposomes, both WT and CHOP^{-/-} mice were then challenged with OVA as described above. As expected, WT and CHOP^{-/-} mice displayed comparable disease severity as demonstrated by histological assays (Figure 5A). Consistently, the BALF samples derived from WT and CHOP^{-/-} mice exhibited comparable levels of inflammatory cells (Figure 5B). To further address this question, we examined the AHR following OVA challenges. Notably, WT and CHOP^{-/-} mice showed comparable Rn values in response to acetylcholine (Figure 5C).

Next, peritoneal macrophages from WT or CHOP^{-/-} mice were stimulated with IL-4 for induction of M2 macrophages, and equal numbers of IL-4-induced WT or CHOP^{-/-} macrophages were then adoptively transferred via intratracheal injection into

clodronate liposome-treated WT and CHOP^{-/-} mice 1 day prior to OVA challenges, respectively. The adoptive transferred WT and CHOP^{-/-} mice developed severe lung inflammation following OVA challenges. Remarkably, no detectable difference in disease severity was observed between WT and CHOP^{-/-} mice, as demonstrated by the comparable histological phenotype (Figure 5A) and similar number of inflammatory cells in the BALF between four groups of mice (Figure 5B). Consistently, comparable Rn values were observed between the adoptive transferred WT and CHOP^{-/-} mice (Figure 5C)

To confirm the above data, 1×10^6 of WT M0 macrophages were adoptively transferred into clodronate liposome-treated CHOP^{-/-} mice, and the same number of CHOP^{-/-} M0 macrophages was adoptively transferred into clodronate liposome-treated WT mice 1 day prior to OVA challenges. As expected, WT mice adoptively transferred with CHOP^{-/-} M0 macrophages developed less severe inflammatory responses following OVA challenges compared with CHOP^{-/-} mice adoptively transferred with WT M0 macrophages, as determined by histological analysis of lung sections, the number of inflammatory cells in the BALF, and Rn values (Supplementary Figure 3A-C). Collectively, our data support that CHOP deficiency-mediated protection for mice against OVA-induced allergic airway inflammation is dependent on the attenuation of M2 programming in macrophages.

CHOP promotes M2 programming by regulating an IL-4 positive feedback loop

To dissect the mechanisms by which CHOP promotes macrophage M2 programming, we first conducted studies in peritoneal macrophages to confirm that CHOP deficiency attenuates macrophage M2 programming following IL-4 stimulation. Western blot analysis revealed a significant reduction in the expression of Arginase-1 after 6 h of IL-4 stimulation in CHOP^{-/-} macrophages compared with WT macrophages (Figure 6A). Next, we examined the temporal expression pattern of CHOP in macrophages during the course of IL-4 stimulation. CHOP was lowly expressed in macrophages prior to IL-4 stimulation. However, a steady increase in

CHOP expression was observed upon IL-4 induction, and the highest expression levels were detected after 12 h of IL-4 stimulation (Figure 6A).

Levels of phosphorylated STAT6 (p-STAT6) have been reported to be critical to the optimal induction and maintenance of the M2 programming induced by IL-4 or IL-13 stimulation⁶. Therefore, we examined the effect of CHOP deficiency on STAT6 phosphorylation in IL-4-stimulated peritoneal macrophages. No significant difference in total STAT6 levels was observed between WT and CHOP^{-/-} macrophages, and p-STAT6 was undetectable in both types of macrophages prior to IL-4 stimulation. However, an increase in the p-STAT6 levels was detected after 0.5 h of IL-4 stimulation, and the highest levels were noted after 1 h of stimulation. After this time point, p-STAT6 underwent a steady decrease and became undetectable after 6 h of stimulation (Figure 6B). A similar trend was observed in CHOP^{-/-} macrophages, but the p-STAT6 levels were substantially reduced compared with WT macrophages, and were undetectable as early as 1 h after IL-4 stimulation (Figure 6B).

There is evidence that Tfec, a transcription factor, is a downstream target of STAT6 signaling²¹. The above results prompted us to examine the impact of CHOP deficiency on Tfec expression following IL-4 stimulation in peritoneal macrophages. Remarkably, IL-4 induced a 32-fold increase in Tfec expression in WT peritoneal macrophages; however, IL-4-induced Tfec expression in CHOP^{-/-} macrophages was reduced by 3.2-fold compared with WT macrophages (Figure 6C), indicating that CHOP promotes STAT6 phosphorylation to enhance Tfec transcription.

The next key question is how CHOP-induced Tfec promotes M2 programming. To address this question, we examined all signaling molecules relevant to the IL-4 network.

IL-4 was found to induce an eight-fold increase in IL-4R α expression, a gene encoding the high-affinity IL-4 receptor²², in WT macrophages, while the loss of CHOP attenuated IL-4-induced IL-4R α expression by three-fold (Figure 6D). These

results prompted us to hypothesize that Tfec may transcribe the expression of IL-4R α . *In silico* analysis of the IL-4R α promoter identified 11 potential Tfec binding sites in the IL-4R α gene (Supplementary Figure 4). Chromatin immunoprecipitation (ChIP) was next conducted, and the resulting products were used to amplify the IL-4R α promoter regions flanking the above-indicated 11 potential Tfec binding sites. Indeed, Tfec manifested binding activity to the IL-4R α promoter in a region of -571 bp upstream of the transcriptional start site (transcriptional start site as +1, Figure 6E). IL-4R α promoter reporter assays were next employed to confirm these findings. A mutated IL-4R α promoter reporter (MU) was constructed, in which the Tfec binding motif (CAGTTG) was mutated to ACGTGT as described (Figure 6F, upper panel). Consistently, disruption of the Tfec binding site resulted in a 1.7-fold reduction in IL-4-induced luciferase reporter activities (Figure 6F). Collectively, our data support that CHOP promotes macrophage M2 programming by regulating an IL-4 positive feedback loop, in which IL-4 induces CHOP expression, which then promotes STAT6 signaling to transcribe Tfec expression, and Tfec next transcribes IL-4R α expression to exacerbate M2 programming in macrophages.

CHOP shRNA protects mice against OVA-induced allergic airway inflammation

To demonstrate the therapeutic potential of CHOP in OVA-induced allergic airway inflammation, we constructed lentiviruses expressing a CHOP shRNA. RT-PCR analysis of lung tissues after intratracheal transduction of 8×10^6 lentiviruses revealed a 66% reduction of CHOP expression in shRNA-transduced mice compared with mock shRNA-transduced mice (Figure 7A). Next, the mice were subjected to repeated OVA challenges, and lentiviruses were intratracheally delivered into the lungs as described above on day 2 of the OVA challenge. Remarkably, CHOP shRNA-transduced mice manifested reduced severity of inflammatory responses, attenuated mucus production and improved airway remodeling compared with mock shRNA-transduced mice (Figure 7B). In line with these observations, the total number of inflammatory cells in the BALF was significantly reduced ($7.58 \pm 0.73 \times 10^5/\text{ml}$ versus $12.11 \pm 1.33 \times 10^5/\text{ml}$,

$p < 0.05$; Figure 7C). Specifically, the CHOP shRNA-transduced mice displayed significantly lower amounts of eosinophils ($3.57 \pm 0.36 \times 10^5/\text{ml}$ versus $6.72 \pm 0.79 \times 10^5/\text{ml}$, $p < 0.05$) and lymphocytes ($2.14 \pm 0.24 \times 10^5/\text{ml}$ versus $3.67 \pm 0.17 \times 10^5/\text{ml}$, $p < 0.001$) (Figure 7C). Consistently, CHOP shRNA-transduced mice exhibited a marked reduction in Rn values compared with control mice in response to acetylcholine (50 mg/ml: 3.17 ± 0.46 cm H₂O s/ml versus 5.44 ± 0.42 cm H₂O s/ml, $p < 0.001$; Figure 7D). To confirm that the above-mentioned therapeutic effect of CHOP shRNA was dependent on the suppression of M2 macrophage production, we conducted RT-PCR to analyze the expression of M2 markers FIZZ1 and YM1 in the lung tissues. Indeed, the expression of FIZZ1 and YM1 was reduced by two-fold and one-fold, respectively. Together, our data indicate that knockdown of CHOP protects mice against OVA-induced allergic airway inflammation by suppressing the production of M2 macrophages.

Sputum CHOP⁺CD206⁺ macrophages positively correlate with asthma severity

To confirm the above-described notion in asthmatic patients, we first examined CHOP-expressing macrophages in sputum samples followed by correlation analysis of lung function relevant to asthma severity. It was noted that cells with aberrant CHOP expression were CD14⁺ macrophages as manifested by the analysis of CHOP⁺ and CD14⁺ (a marker for human macrophages) cells (Figure 8A). Co-immunostaining of CHOP and CD206 further confirmed that those CHOP-overexpressing macrophages were characterized by an M2 phenotype (Figure 8B). Next, we assessed the correlation between the number of sputum CHOP⁺CD206⁺ macrophages and disease severity. The asthma severity was estimated by the ratio between forced expiratory volume in one second (FEV1) and forced vital capacity (FVC) and the value of FEV1. As expected, the number of sputum CHOP⁺CD206⁺ macrophages was positively correlated with asthma severity, as manifested by the detection of a negative correlation with FEV1/FVC ratios ($R^2 = 0.4061$, $p < 0.01$; Figure 8C) and FEV1 values ($R^2 = 0.4382$, $p < 0.01$; Figure 8D).

DISCUSSION

In the present study, we demonstrated that asthmatic patients and mice with OVA-induced allergic airway inflammation exhibit aberrant CHOP expression along with ER stress. Interestingly, CHOP was overexpressed in macrophages in both asthmatic patients and mice with OVA-induced allergic airway inflammation. Accordingly, CHOP deficiency provided protection for mice against airway inflammation, remodeling, and AHR after repeated OVA challenges. Specifically, the loss of CHOP protected mice from OVA-induced asthma by inhibiting the polarization of M2 macrophages in the airways. Mechanistic studies have revealed that CHOP promotes macrophage M2 programming by regulating an IL-4 positive feedback loop, in which IL-4 induces CHOP expression, which then promotes STAT6 signaling to transcribe *Tfec* expression, and *Tfec* next transcribes *IL-4R α* expression to promote macrophage M2 programming (Figure 8E). These results not only provide novel insights into the understanding of the pathogenesis underlying asthma, but also demonstrate evidence that targeting of CHOP attenuates the IL-4/STAT6/*Tfec*/*IL-4R α* positive feedback loop to inhibit macrophage M2 programming, which may have great potential for the treatment of asthma in clinical settings.

Emerging evidence indicates that patients with asthma are associated with the presence of ER stress as manifested by the detection of altered BIP and CHOP expression in lung samples derived from asthmatic patients¹⁴⁻¹⁶. Studies in animals further revealed that suppression of ER stress could attenuate airway inflammation and bronchial hyper-responsiveness in a mouse model of allergic airways disease^{15; 16; 23}. Consistent with these findings, analysis of induced sputum samples originated from our asthmatic subjects also noted significantly higher levels of CHOP and BIP expression, and studies in lung homogenates derived from OVA-challenged asthmatic mice further demonstrated significant overexpressions for CHOP, p-eIF2 α , Atf4, and Bip. Collectively, these data support that CHOP and its related ER stress are

implicated in the pathogenesis of asthma, but the exact mechanisms are yet to be elucidated.

To dissect the mechanisms by which CHOP contributes to asthma pathogenesis, we first examined CHOP expression in inflammatory cells of sputum samples derived from asthmatic patients and in BALF samples and lung tissues originated from OVA-challenged mice. In general, CHOP was hardly detectable in macrophages derived from normal subjects and control mice. Remarkably, significantly higher levels of CHOP were detected in sputum macrophages originated from asthma patients compared with those from healthy controls. Similarly, CHOP was noted to be overexpressed in macrophages derived from OVA-induced asthmatic mice. Co-immunostaining of CHOP and CD206 confirmed that CHOP-overexpressing cells were M2 macrophages, and the number of CHOP⁺CD206⁺ macrophages was associated with decreased lung function in asthmatic patients. These results support the notion that altered CHOP expression and ER stress might trigger asthma pathogenesis by promoting the generation of M2 macrophages.

Macrophages are the most abundant inflammatory cells in the airways, and the activation of macrophages is vital to asthma pathogenesis. Indeed, there is evidence that both M1 and M2 macrophages are important for the development of asthma. For example, for severe forms of asthma in aged adults, macrophages are shown to become an M1 phenotype, which produces a great amount of pro-inflammatory mediators, including IL-1 β , to exacerbate lung injury and to accelerate airway remodeling²⁴. However, for the allergic form of asthma, M2 macrophages are found to be the key source of IL-4, IL-13, and CCL17, and suppression of the functions for M2 macrophages alleviates airway inflammation in OVA-induced asthmatic animals⁸.⁹. In the present study, no obvious difference in terms of IL-1 β production and the percent of CD11c⁺F4/80⁺ macrophages was noted between WT and CHOP^{-/-} mice following repeated OVA challenges, which in fact, is consistent with our previous report that CHOP deficiency does not affect macrophage M1 programming following

LPS stimulation²⁰. In sharp contrast, the number of M2 macrophages was significantly lower in CHOP^{-/-} mice compared with WT mice following OVA challenges as demonstrated by the significantly attenuated expression of M2 markers Arginase-1, Ym1, Fizz1, CD206, and CCL17. In line with these results, mice deficient in CHOP were protected from OVA-induced allergic airway inflammation.

To address the notion that the ability of CHOP deficiency to protect mice against OVA-induced allergic airway disease is dependent on the attenuation of M2 programming in macrophages, we conducted macrophage adoptive transfer studies. Clodronate liposomes were employed to deplete endogenous macrophages, and IL-4 polarized WT peritoneal M2 macrophages were then adoptively transferred into clodronate liposome-treated WT and CHOP^{-/-} mice as described, followed by OVA challenges as described. As expected, CHOP^{-/-} mice developed comparable severity of allergic airway inflammation to WT mice after adoptive transfer of WT M2 macrophages. Next, WT M0 macrophages were adoptively transferred into clodronate liposomes treated CHOP^{-/-} mice, or CHOP^{-/-} M0 macrophages were adoptively transferred into clodronate liposome-treated WT mice, followed by repeated OVA challenges as described. WT mice adoptively transferred with CHOP^{-/-} M0 macrophages displayed less severe airway inflammation than CHOP^{-/-} mice transferred with WT M0 macrophages. Together, these data support that the protection resulting from CHOP deficiency is dependent on its effect on the suppression of macrophage M2 programming.

The next critical issue is to dissect the pathways relevant to CHOP-regulated M2 programming in macrophages. Previous studies have suggested feasible evidence that Th2 cytokines (e.g., IL-4 or IL-13) could enhance the expression of Tfec following STAT6 phosphorylation, thereby inducing the expression of a subset of IL4-responsive genes²¹, while IL-4R α has a high binding affinity for IL-4 and has been noted to participate in a positive feedback loop in IL-6-induced macrophage M2 programming in adipose tissues²⁵. These discoveries prompted us to hypothesize that

CHOP may regulate a positive feedback regulatory loop to promote M2 polarization. To address this hypothesis, we first assessed the effect of CHOP deficiency on IL-4-induced STAT6 phosphorylation in macrophages. Indeed, CHOP deficiency markedly inhibited IL-4-induced STAT6 phosphorylation, and consistently, the expression of STAT6 downstream transcription factor, Tfec, was significantly reduced following IL-4 stimulation. It was further noted that CHOP deficiency also attenuated IL-4-induced IL-4 α expression, suggesting that IL-4 α could be a downstream target of Tfec transcription factor. Indeed, ChIP assays demonstrated that Tfec selectively bound to the IL-4 α promoter at position of -571 bp upstream of the transcriptional start site. Promoter reporter assays further confirmed that Tfec transcribed the expression of IL-4 α following IL-4 stimulation in macrophages. Taken together, we demonstrated experimental evidence indicating the existence of an IL-4/STAT6/Tfec/IL-4 α positive feedback regulatory loop in macrophages, while CHOP is likely to regulate this positive feedback regulatory loop to promote M2 programming in macrophages. In support of this notion, the number of CHOP⁺CD206⁺ M2 macrophages in induced sputum has been noted to be correlated with the severity of asthma.

Previous studies have provided suggestive evidence that CHOP could increase the secretion of inflammatory cytokines in a murine model of neutrophilic asthma²⁶, and promote epithelial apoptosis in HDM-induced allergic airway disease²⁷. In the current report, our studies in CHOP^{-/-} mice demonstrated additional evidence that CHOP deficiency protects mice against OVA-induced allergic airway inflammation, further suggesting a great potential for CHOP in the prevention and treatment of allergic airway diseases, such as asthma. Indeed, our studies using lentiviruses expressing a CHOP shRNA confirmed that knockdown of CHOP potentially alleviated the severity of airway inflammatory responses along with attenuated mucus production and improved airway remodeling in mice after repeated OVA challenges. Nevertheless, additional follow-up studies would be necessary to fully establish the therapeutic potential of CHOP in the prevention and treatment of airway inflammatory diseases in

clinical settings.

In summary, we have demonstrated that altered CHOP expression along with ER stress is a characteristic feature of asthmatic patients. Mice deficient in CHOP were found to be protected from OVA-induced airway inflammation and AHR. The most exciting discovery in the current report is the identification of the IL-4/STAT6/Tfec/IL-4R α positive feedback regulatory loop, which exacerbates asthma pathogenesis. CHOP has been found to regulate this feedback loop to enhance M2 programming in macrophages. Together, our data indicate that targeting CHOP and ER stress may represent a promising therapeutic approach for the prevention and treatment of asthma in clinical settings.

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Conflict of interest disclosure

The authors declare no competing financial interests.

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FIGURE LEGENDS:

Figure 1. Analysis of ER stress markers in asthmatic patients and OVA-challenged mice. **A:** CHOP expression in induced sputum samples from asthmatic patients (AP, N=6) and healthy subjects (HS, N=6). **B:** BIP expression in induced sputum from AP and HS. **C-F:** Expression of CHOP (**C**), p-eIF2 α (**D**), Atf4 (**E**) and Bip (**F**) in the lungs of mice after OVA challenge. The data were collected from 6 mice in each study group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 2. Comparison of the severity of lung inflammation between CHOP^{-/-} and WT mice after OVA challenge. **A:** H&E staining. **B:** PAS staining. **C:** Masson's trichrome staining. **D-F:** Bar graphs represent the quantitative mean score of inflammation (**D**), mucus production (**E**) and fibrosis ratio (**F**). Images were captured using a microscope at a magnification of 200 $\times$. The data were collected from 4-6 mice in each study group. *, $p < 0.05$; **, $p < 0.01$.

Figure 3. Comparison of inflammatory cells and cytokines in BALF samples between CHOP^{-/-} and WT mice after OVA challenge. **A:** Total number of inflammatory cells in BALF samples. **B-E:** Analysis of eosinophils (**B**), lymphocytes (**C**), macrophages (**D**) and neutrophils (**E**) in BALF samples. **F-M:** Inflammatory mediators in BALF samples. Six mice were included for each study group. **N:** The response to acetylcholine (N=6-7 for each group). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 4. CHOP is overexpressed in infiltrating macrophages in the lungs of OVA-challenged mice. **A:** Co-immunostaining of CHOP and F4/80 in BALF cells. **B:** Flow cytometry analysis of CD206 in macrophages following OVA challenges. **C:** Arginase-1 expression in the lung. **D-E:** FIZZ1 (**D**) and YM1 (**E**) expression in the lungs. The data were collected from studies of 4-6 mice in each study group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 5. CHOP deficiency protects mice from developing asthma by attenuating M2 programming in macrophages. **A:** Representative results of pathological staining (200×). **B:** Number of inflammatory cells in BALF samples obtained from each group of mice. **C:** The response to acetylcholine. Five mice were examined in each study group. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 6. CHOP regulates the IL-4/STAT6/Tfec/IL-4R α positive feedback regulatory loop to exacerbate M2 program. **A & B:** Temporal expression of Arginase-1, CHOP, STAT6, and p-STAT6 in macrophages after IL-4 stimulation. **C & D:** Expression of Tfec (**C**) and IL-4R α (**D**). **E:** ChIP analysis of the IL-4R α promoter. **F:** Results for IL-4R α promoter luciferase reporter assays. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Figure 7. CHOP shRNA attenuates OVA-induced allergic airway inflammation and M2 macrophage production. **A:** Expression of CHOP in the lung. **B:** Representative results of pathological staining (200×). **C:** Inflammatory cells in BALF samples. **D:** The response to acetylcholine. **E-F:** Analysis of FIZZ1 (**E**) and YM1 (**F**) expression in the lungs. Four to six mice were examined in each study group. *, $p < 0.05$; **, $p < 0.01$.

Figure 8. The number of M2 macrophages in induced sputum samples is highly correlated with asthma severity. **A:** Co-immunostaining of CHOP and CD14. **B:** Co-immunostaining of CHOP and CD206. **C & D:** Results for correlation analysis of CHOP⁺CD206⁺ macrophage counts and FEV1/FVC (**C**) and FEV1 (**D**) in patients with asthma ($n = 18$). AP: Asthmatic patients; HS: Healthy subjects. **, $p < 0.01$. **E:** The IL-4/STAT6/Tfec/IL-4R α positive feedback regulatory loop.

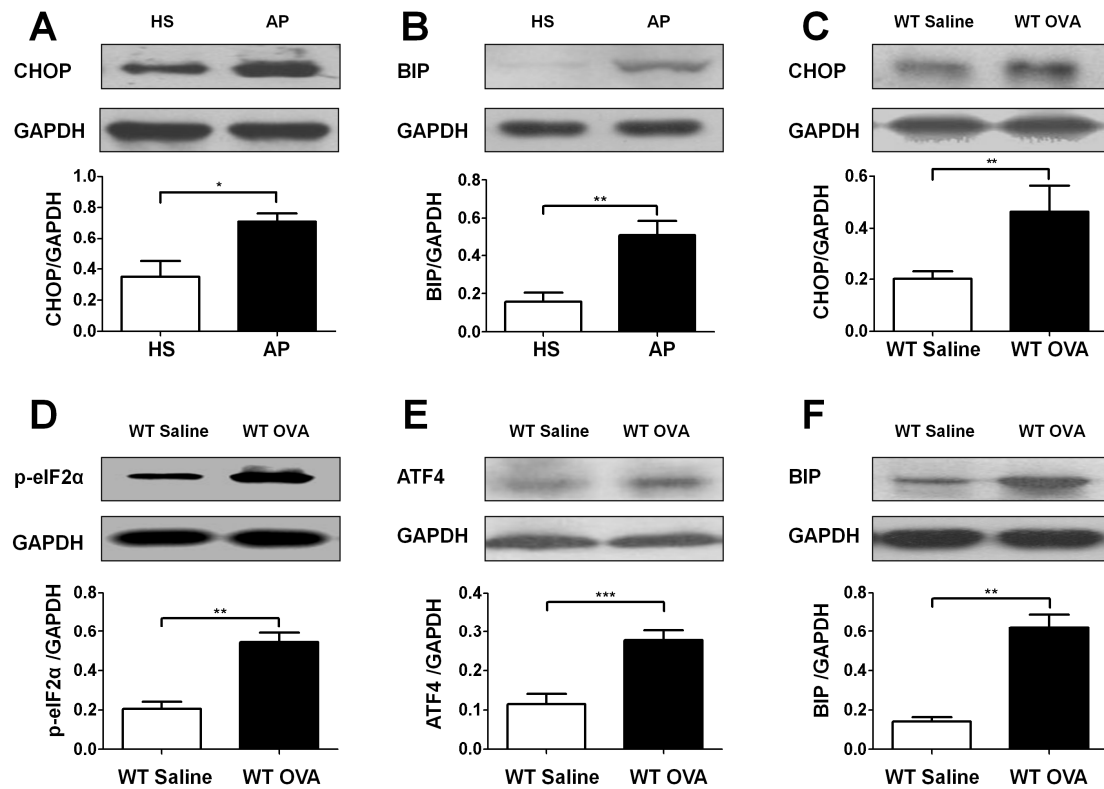


Figure 1

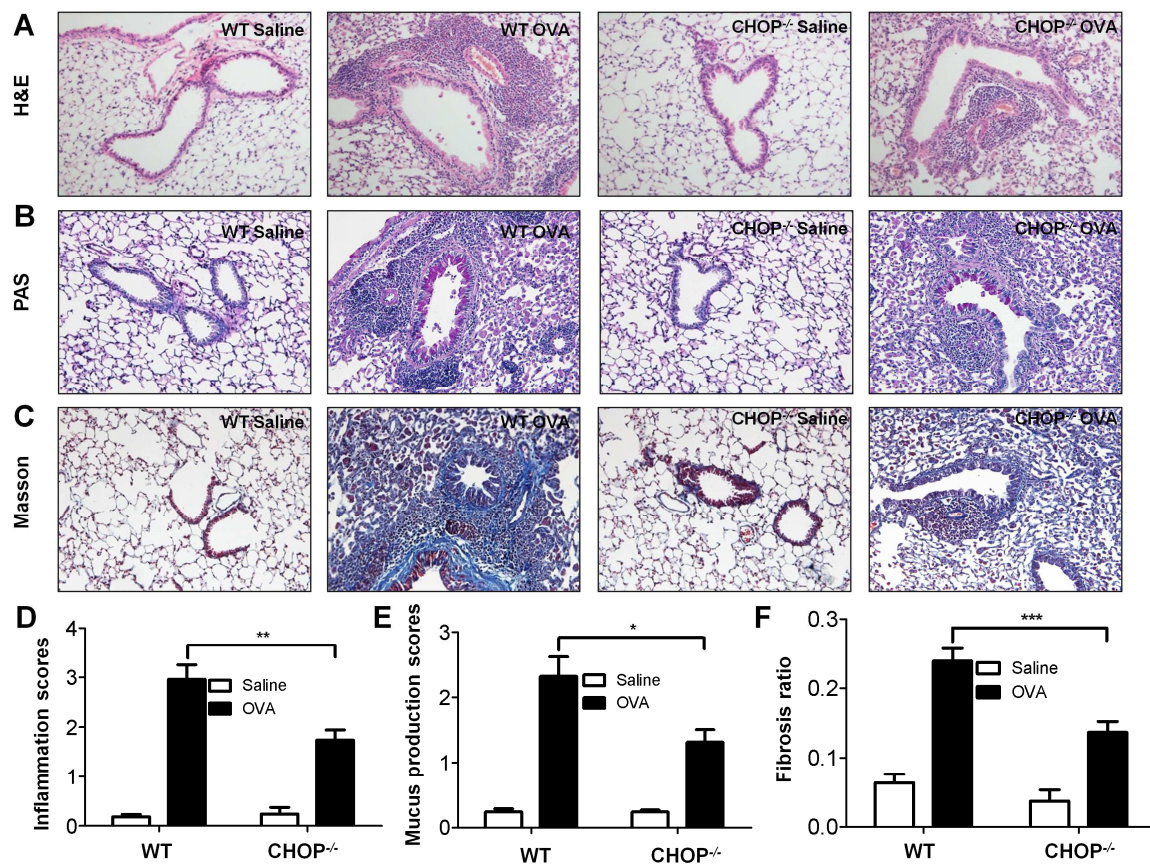


Figure 2

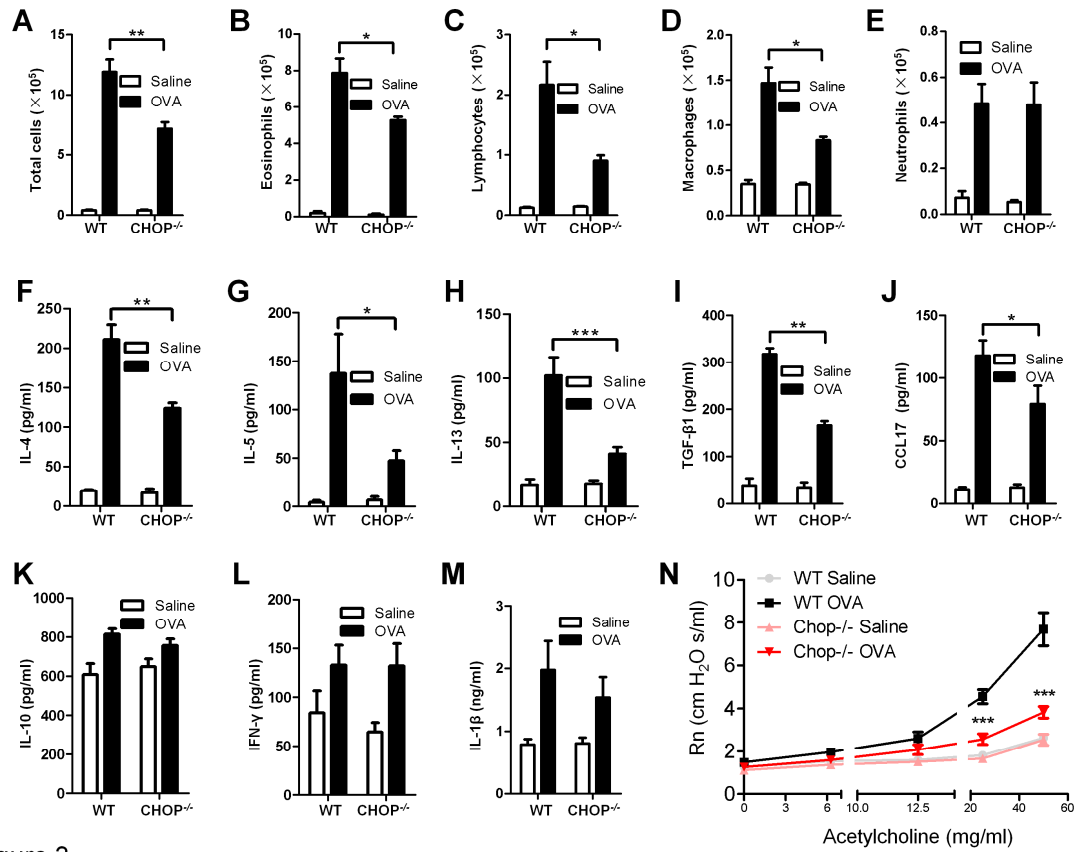


Figure 3

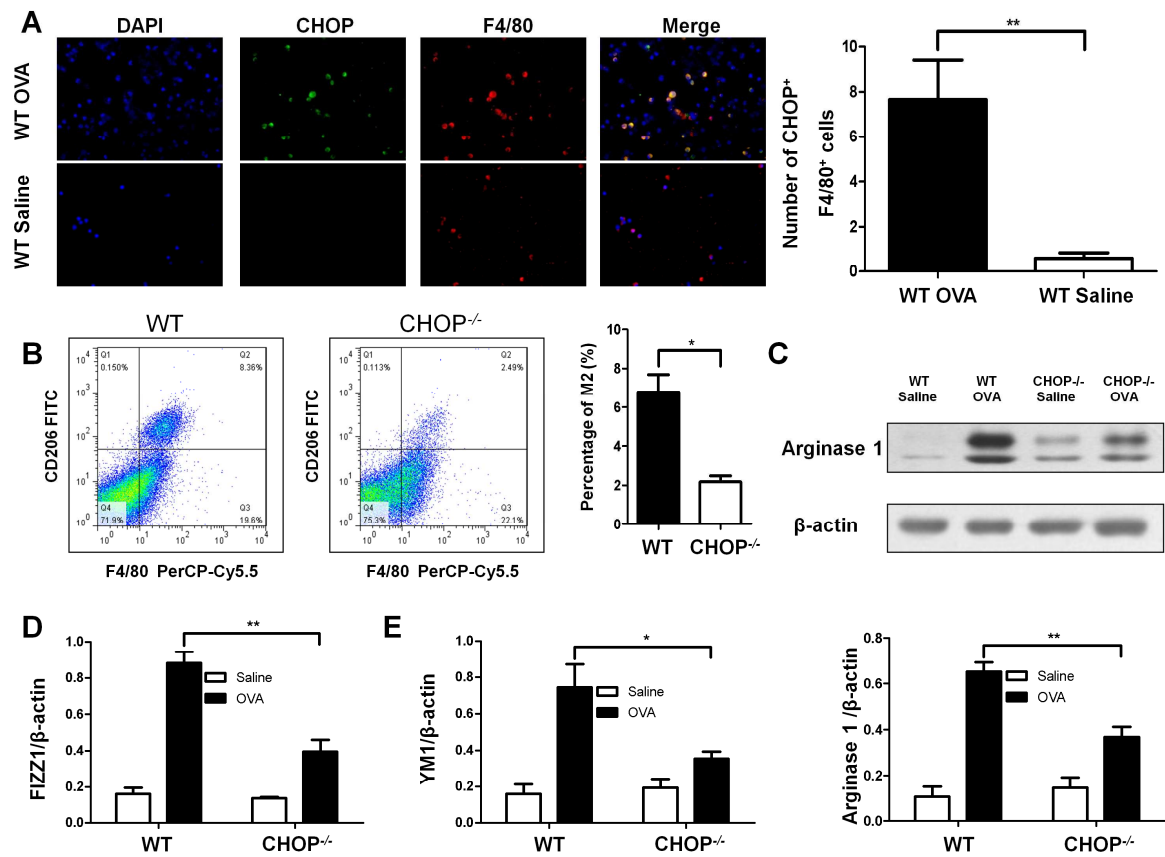


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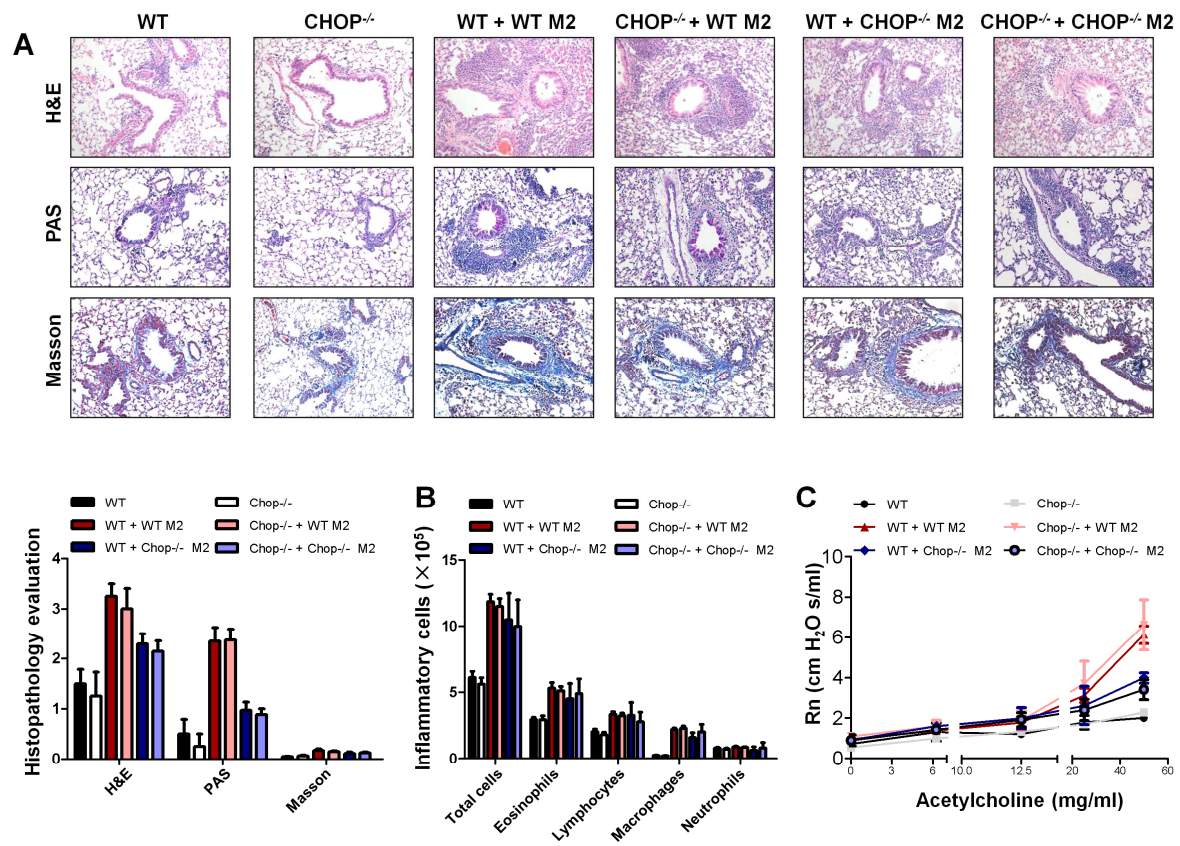


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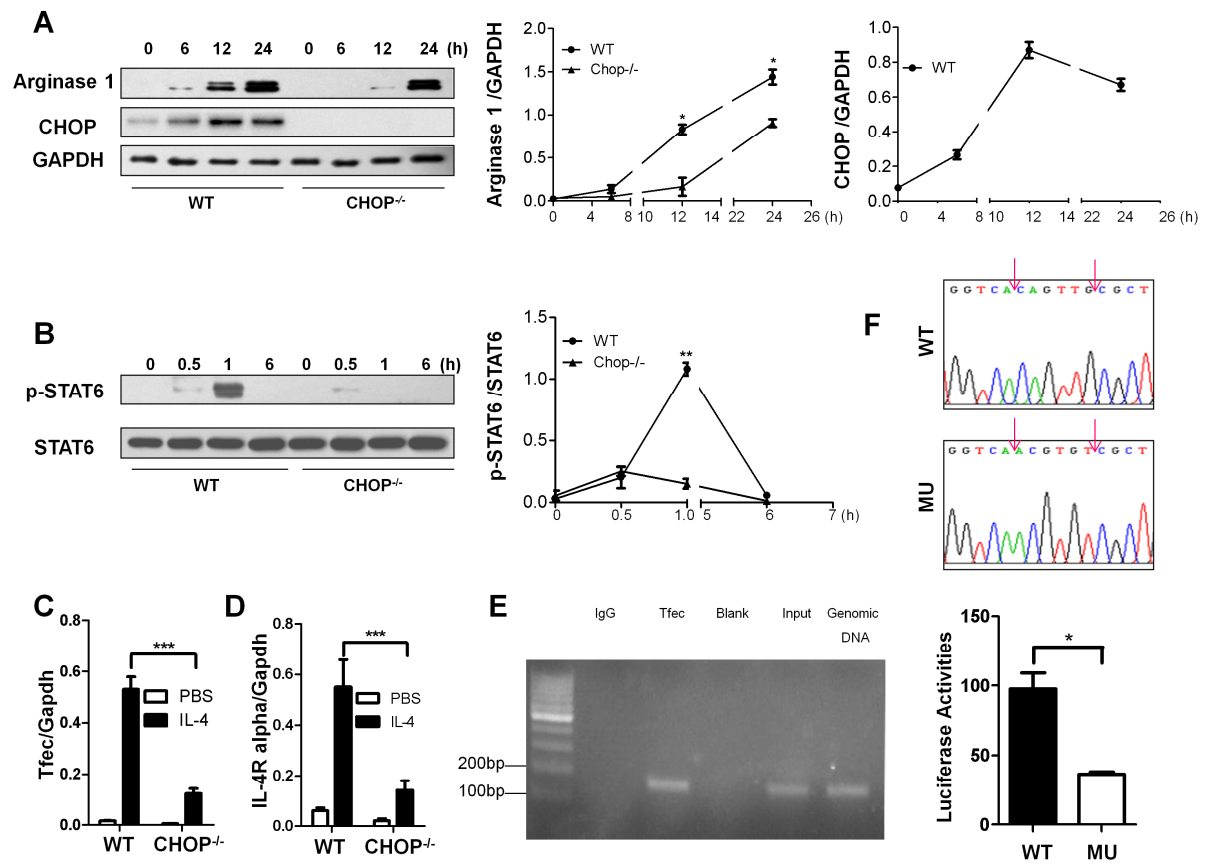


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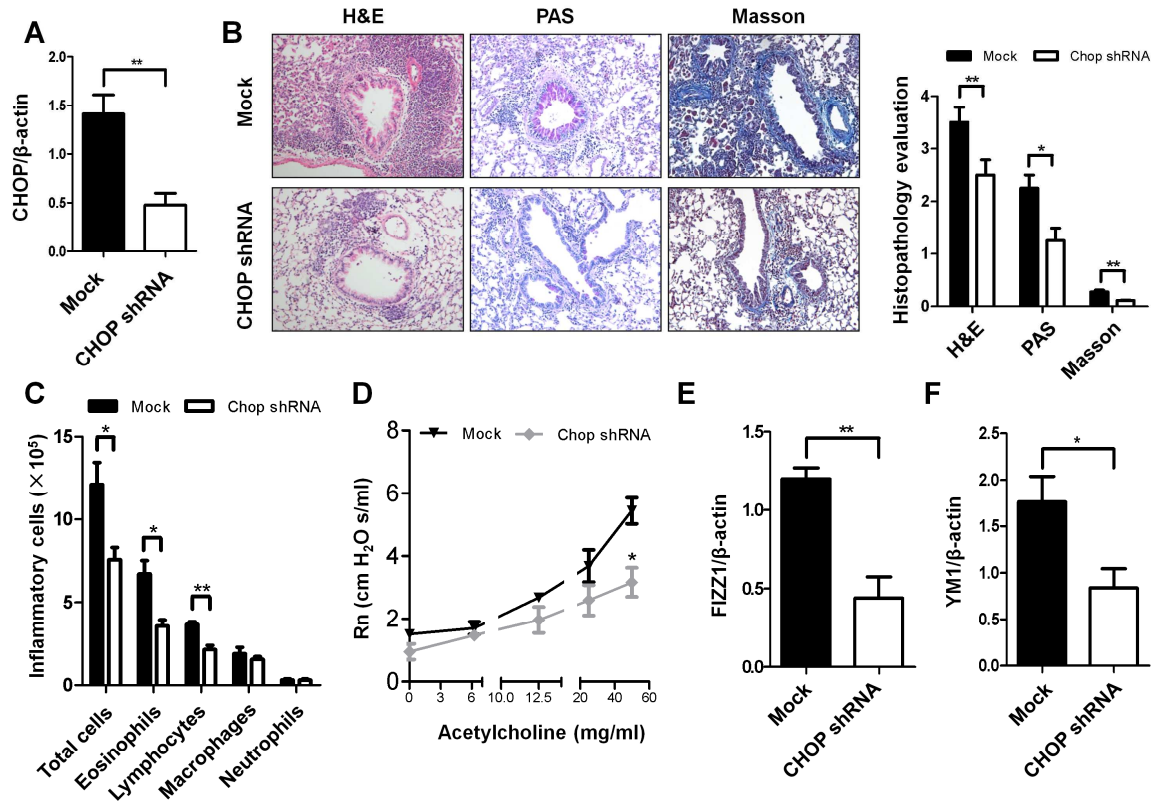
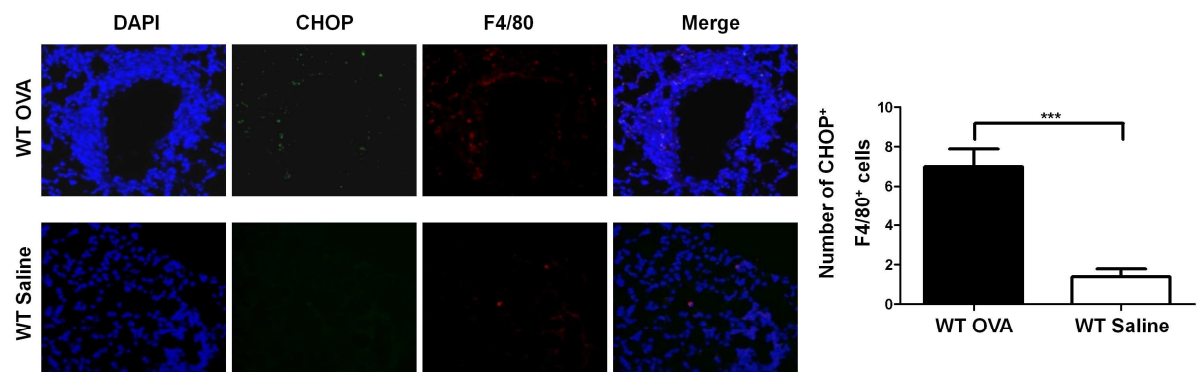
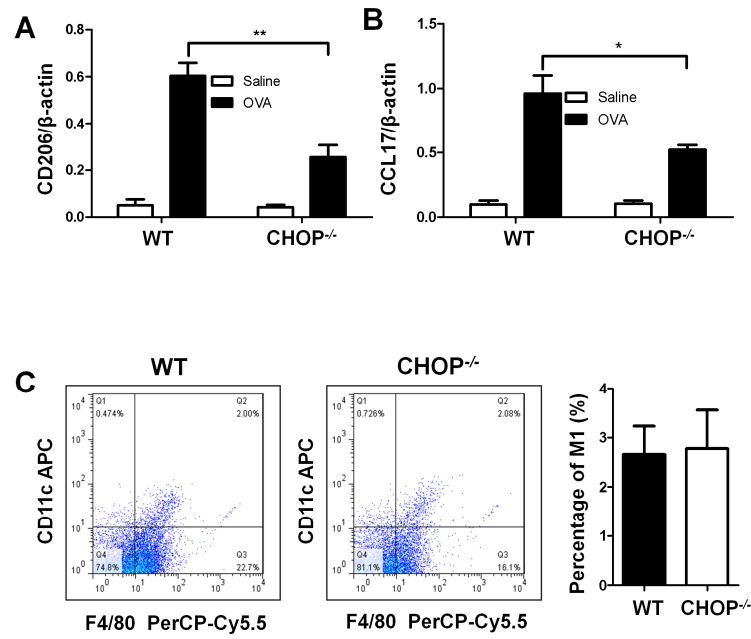


Figure 7

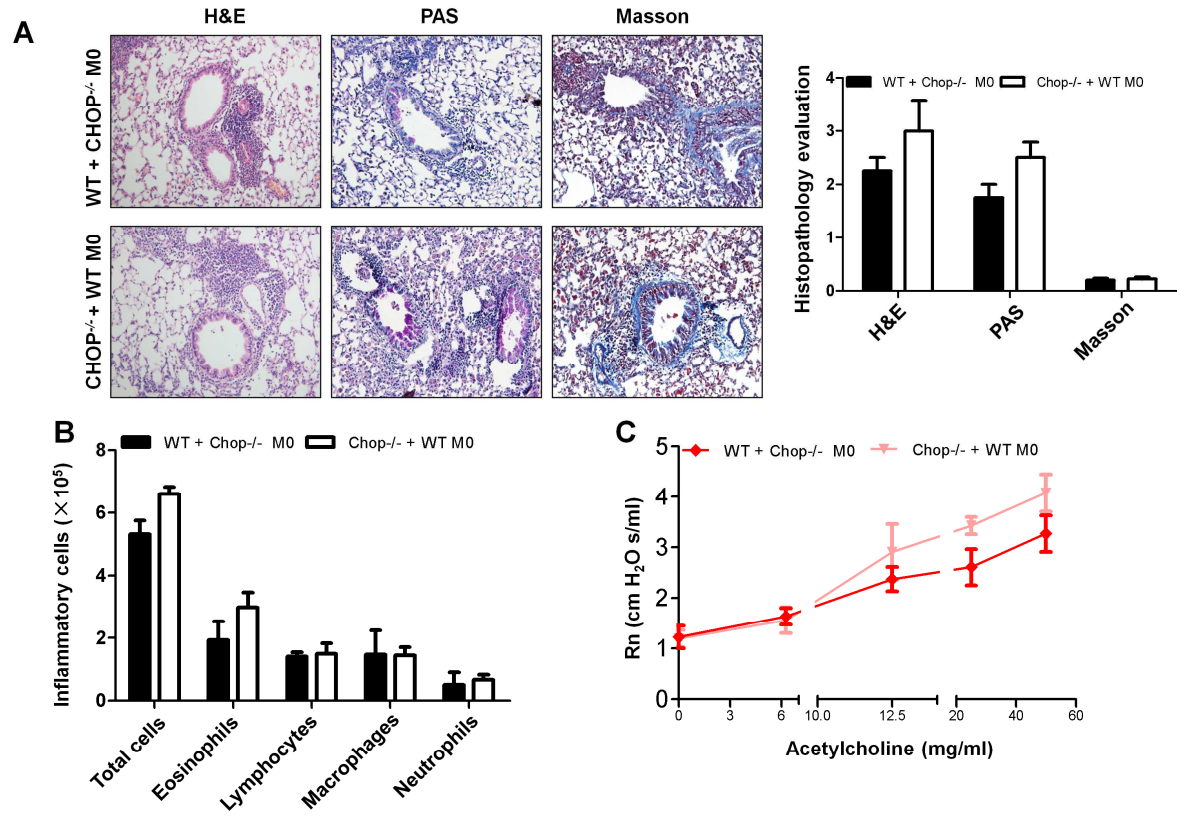




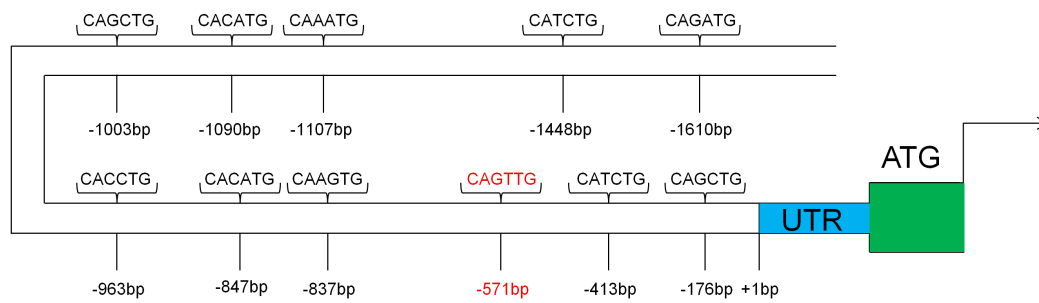
Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3

In Silico analysis of the IL-4R α promoter for Tfec binding sites

Supplementary Figure 4

MATERIALS AND METHODS

Human samples

Induced sputum samples were obtained from healthy volunteers (n = 15) and asthmatic patients (n = 18) enrolled in Tongji Hospital. All of the study subjects provided informed consent. The diagnosis of asthma was based on the asthma guidelines provided by the American Thoracic Society. None of patients in the asthma group had undergone inhaled corticosteroid (ICS) therapy prior to blood tests and lung function tests¹. All studies in human subjects were conducted in accordance with the NIH guidelines and were approved by the Institutional Review Board (IRB) of Tongji Hospital. The clinical characteristics of all study subjects are provided in Table

Animals

CHOP knockout (CHOP^{-/-}) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Wild-type (WT) mice were obtained from the Animal Experimental Center of Hubei province (Wuhan, China). All animals were housed in a specific pathogen-free (SPF) animal facility at the Tongji Medical College under a 12 h light/dark photocycle with diet and water available ad libitum. All experimental procedures were approved by the Animal Care and Use Committee of the Tongji Hospital of Huazhong University of Science and Technology.

Reagents and antibodies

Murine recombinant IL-4 was obtained from PeproTech (Rocky Hill, NJ, USA). Antibodies against ATF4 and Arginase-1 were purchased from Abcam (Cambridge, MA, USA), and antibodies against p-eIF2 α and STAT6 were obtained from Cell Signalling (Danvers, MA, USA). Antibodies against CHOP, BIP, GAPDH and p-STAT6 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and Tfec antibody was obtained from Proteintech (Wuhan, China). Murine enzyme-linked immunosorbent assay (ELISA) kits for IL-4, IL-5, IL-13, TGF- β 1, IFN- γ , IL-1 β , IL-10, and CCL17 were purchased from BD Biosciences (San Jose, CA, USA). All other reagents were purchased from Sigma (St. Louis, MO, USA), unless otherwise stated.

Genotyping

The genotypes of the mice used in this study were confirmed by PCR. The primers used to amplify the WT CHOP allele were WT-F 5'-CAG ATC CTC ATA CCA GGC TTC C-3' and WT-R 5'-CTC CAC CCT CTG CCA ATG TAG-3', while primer MU-F 5'-GCC AGG GTT TTC CCA GTC AC-3' along with the above WT-R primer were employed to amplify the CHOP *null* allele.

OVA-induced allergic airway inflammation model in mice

Asthma was induced in WT and CHOP^{-/-} mice (8–10 weeks old) as previously described with minor modifications^{2; 3}. Briefly, the mice were sensitized using intraperitonea (i.p.) injection of 100 µg OVA (grade V) emulsified in 1 mg of aluminum hydroxide gel (Thermo Scientific, Rockford, IL) in a total volume of 200 µl, on days 0, 7 and 14. Mice injected with an equal volume of saline were served as controls. The sensitized mice were anesthetized with diethyl ether and intranasally challenged with 50 µl OVA (20 µg/µl) or saline for 7 consecutive days. CHOP shRNA lentiviral vectors (AAG AGA ACG AGC GGC TCA A, 8×10^6 IFU) were injected into the anesthetized animals via intratracheal 1 days after the first challenge with OVA. A mock virus (negative control shRNA) were used as controls. The mice were sacrificed after 7 consecutive days of OVA challenges.

Analysis of airway responsiveness

Airway responsiveness to acetylcholine (Ach) was assessed 24 h after the last OVA challenge using a computer-controlled small animal ventilator system (SCIREQ Scientific Respiratory Equipment, Inc., Montréal, QC, Canada) as previously described⁴. Briefly, the mice were anesthetized with 10 mg/kg ketamine and 1 mg/kg xylazine, and the values for airway resistance (Rn, cm H₂O s/ml) in response to incremental doses of Ach (0.00, 6.25, 12.5, 25 and 50 mg/ml) administered by nebulization via airway were recorded.

Preparation of bronchoalveolar lavage fluid (BALF)

BALF was collected by cannulating the trachea and lavaging the lung with 0.6 ml of sterile PBS using previously described techniques⁵. Approximately 0.4 ml of BALF was recovered from each animal. BALF was centrifuged at 300 g for 5 min, and the supernatants were stored at -80°C prior to cytokine measurements. The total cell suspension was resuspended in 1 ml of PBS. Cells were counted using a haemocytometer. Differential cell counts were measured after Diff-Quick staining.

Histological analysis and immunostaining

The left lung was inflated with 4% neutral buffered paraformaldehyde using 25 cm of H₂O pressure for 1 min. The lungs were subsequently removed and placed in fresh 4% neutral buffered paraformaldehyde for 24 h at room temperature. After embedding the tissues in paraffin, they were sliced into 5-µm sections and subjected to haematoxylin and eosin (H&E), periodic acid-Schiff (PAS) and Masson staining using the established techniques⁶. For immunostaining, fresh frozen sections (8-µm) and smears of the induced sputum samples from asthma patients were prepared as previously described⁷. The slides were co-incubated with primary antibodies against F4/80 and CHOP, CHOP and CD206 or CHOP and CD14, followed by probing with Alexa 594-labeled or Alexa 488-labeled secondary antibodies (Invitrogen, Carlsbad, CA, USA). The results were assessed by two pathologists using a brightfield or fluorescent

microscope in a blinded fashion.

ELISA analysis of inflammatory cytokines

The levels for inflammatory cytokines IL-4, IL-5, IL-13, TGF- β 1, IFN- γ , IL-1 β , IL-10 and CCL17 in BALF samples were measured using commercial ELISA kits as previously described⁸.

Western blot analysis

Lung tissues and cultured peritoneal macrophages were homogenized in lysis buffer [300 mM NaCl, 50 mM Tris-HCl, and 1% Triton X-100 (pH 7.5)] using a BBX24 Bullet Blender homogenizer (Next Advance Inc., Averill Park, NY, USA) according to the manufacturer's protocol. Fifty micrograms of extracted proteins were separated using 12% SDS-PAGE, and the separated proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA, USA). The membranes were first probed with indicated primary antibodies, followed by incubating with an HRP-conjugated secondary antibody. The reactive bands were visualized using an ECL Plus Western Blot Kit (Pierce, Rockford, IL, USA) as previously reported⁹.

Flow cytometry analysis

The mononuclear cells in the lung tissue samples were obtained as reported previously¹⁰. The cells were then stained with anti-mouse F4/80-PerCP/Cy5.5 (1:100), CD11c-APC (1:100) and CD206-FITC (1:100). After washes, the cells were analysed by flow cytometry as previously described¹¹. Data analysis was performed using the FACS Express V3 software (De Novo Software) as instructed.

Quantitative RT-PCR analysis

Quantitative RT-PCR analysis was conducted using SYBR Premix Ex Taq (Takara) as previously described¹². Briefly, total RNAs were extracted from lung or macrophages using the RNAiso plus (TaKaRa, Japan) according to the supplier's instructions. Real-time RT-PCR was conducted to assess CD206, FIZZ1, YM1, CCL17, Tfec and IL-4R alpha expression using an ABI prism 7500 Sequence Detection System (Applied Biosystems, San Francisco, CA, USA). β -actin or Gapdh was used for normalization, and relative expressions for each target gene were calculated using the $2^{-\Delta\Delta C_t}$ approach as reported. The primers used to amplify each target gene are listed in Table 2.

Isolation and culture of peritoneal macrophages

Mouse peritoneal macrophages were prepared as previously described¹³. The isolated macrophages were cultured in RPMI 1640 for 30 min to remove nonadherent cells. The adherent cells were then cultured in RPMI 1640 supplemented with 10% FBS,

100 U/ml penicillin/streptomycin and recombinant IL-4 (10 ng/ml) for the indicated periods of time.

Macrophage depletion and macrophage adoptive transfer studies

Clodronate liposomes (40 μ l) were administered for two consecutive days *via* intratracheal injection 7 days prior to OVA induction, and the severity of asthma was assessed following 7 consecutive days of OVA challenges. The adoptive transfer studies were conducted as previously described¹⁴. Peritoneal macrophages derived from WT mice were stimulated by IL-4 (10 ng/ml) for 12 h and then administered through intratracheal injection into the lungs of clodronate liposome-treated WT and CHOP^{-/-} mice at a density of 1×10^6 cells/mouse (50 μ l) one day before challenge with OVA as described above. The animals were then sacrificed for further analysis.

Chromatin immunoprecipitation (ChIP) assay and IL-4R α promoter reporter assay

ChIP assays were conducted using a CHIP assay kit (Haimen, JS, China) as previously described¹⁵. The primers used in the ChIP assay are as follows: F1: 5' AGCCAACCCAATCCATAA3' and R1: 5'CCCTAGAAACCACGCAAA3'. A dual luciferase reporter system (Promega, Madison, WI, USA) was used for the IL-4R α promoter luciferase reporter assays, in which the Tfec binding site within the IL-4R α promoter was disrupted as previously reported¹⁶.

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156 **Statistical analysis**

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158 All data are presented as the mean $\pm$ SEM, and all *in vitro* studies were replicated a
159 minimum of 3 times. Statistical analyses of the data were conducted using the
160 GraphPad Prism 5 software (GraphPad Software Inc., San Diego, CA, USA) and the
161 standard one-way analysis of variance (ANOVA) or Dunnett's multiple comparison
162 *test* when appropriate. For all statistical comparisons, $p < 0.05$ was considered
163 statistically significant.

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FIGURE LEGENDS:

Supplementary Figure 1. CHOP is overexpressed in infiltrating macrophages in the lung sections derived from OVA-challenged mice. CHOP was stained in green, while F4/80 was stained in red. ***, $p < 0.001$.

Supplementary Figure 2. CHOP selectively modulates the M2 programme in macrophages. Analysis of CD206 (**A**) and CCL17 (**B**) expression in the lung. C: Flow cytometry analysis of CD11c expression in macrophages following OVA challenges. *, $p < 0.05$; **, $p < 0.01$.

Supplementary Figure 3. Adoptive transfer studies for M0 macrophages. WT M0 macrophages were adoptively transferred into clodronate liposome-treated CHOP^{-/-} mice, or CHOP^{-/-} M0 macrophages were adoptively transferred into WT mice, followed by repeated OVA challenges. A: Representative results of H&E, PAS and Masson staining. B: Inflammatory cells in BALF samples. C: The response to acetylcholine.

Supplementary Figure 4. *In Silico* analysis of the IL-4R α promoter for potential Tfec binding sites.

265 **Table 1. Characteristics of subjects for induced sputum samples.**

	induced sputum samples	
	Asthma (N=18)	Control (N=15)
Age, years	40.17 ± 3.95	40.06 ± 7.50
Sex		
Female	14 (77.88%)	14 (93.33%)
Male	4 (22.22%)	1 (6.67%)
FEV1 (L)	2.11 ± 0.25*	3.21±0.60
FVC (L)	3.42 ± 0.66	3.73±0.72
FEV1/FVC%	53.44 ± 3.62*	85.08±4.46
PEF (L)	3.96 ± 0.82*	7.56±2.26
Atopic [#]	12 (66.67%)	
BMI	22.26 ± 0.43	22.91 ± 1.06

266 FEV1: Forced expiratory volume in one second; FVC: Forced vital capacity; PEF:
 267 Peak expiratory flow. *, p < 0.05 asthma vs. control. #Atopic status was confirmed by
 268 allergic skin prick test responses.

269

270 **Table 2. Primers for RT-PCR**

CD206	5'-TTG GAC GGA TAG ATG GAG GG-3'
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	5'-CCA GGC AGT TGA GGA GGT TC-3'
<i>Fizz1</i>	5'-TCC CAG TGA ATA CTG ATG AGA-3'
	5'-CCA CTC TGG ATC TCC CAA GA-3'
<i>YM1</i>	5'-GGG CAT ACC TTT ATC CTG AG-3'
	5'-CCA CTG AAG TCA TCC ATG TC-3'
<i>Ccl17</i>	5'-TGG CTG CCC TGC TTC TG-3'
	5'-AAT GGC CCC CTT GAA GTA GTC-3'
<i>Tfec</i>	5'-GGA CAA CCA CAA CCT CAT TGA-3'
	5'-CCA GCG CAT ATC AGG ATC ATT-3'
<i>IL-4Rα</i>	5'-TGA CCT CAC AGG AAC CCA GGC-3'
	5'-GAA CAG GCA AAA CAA CGG GAT-3'
<i>β-actin</i>	5'-TGA CGT TGA CAT CCG TAA AGA CC-3'
	5'-CTC AGG AGG AGC AAT GAT CTT GA-3'
<i>Gapdh</i>	5'-CGA CTT CAA CAG CAA CTC CCA CTC TTC C-3'
	5'-TGG GTG GTC CAG GGT TTC TTA CTC CTT-3'