

FOXM1 Promotes Allergen-Induced Goblet Cell Metaplasia and Pulmonary Inflammation

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Chronic airway disorders, including chronic obstructive pulmonary disease (COPD), cystic fibrosis, and asthma, are associated with persistent pulmonary inflammation and goblet cell metaplasia and contribute to significant morbidity and mortality worldwide. While the molecular pathogenesis of these disorders is actively studied, little is known regarding the transcriptional control of goblet cell differentiation and mucus hyperproduction. Herein, we demonstrated that pulmonary allergen sensitization induces expression of FOXM1 transcription factor in airway epithelial and inflammatory cells. Conditional deletion of the *Foxm1* gene from either airway epithelium or myeloid inflammatory cells decreased goblet cell metaplasia, reduced lung inflammation, and decreased airway resistance in response to house dust mite allergen (HDM). FOXM1 induced goblet cell metaplasia and *Muc5AC* expression through the transcriptional activation of *Spdef*. FOXM1 deletion reduced expression of *CCL11*, *CCL24*, and the chemokine receptors *CCR2* and *CX3CR1*, resulting in decreased recruitment of eosinophils and macrophages to the lung. Deletion of FOXM1 from dendritic cells impaired the uptake of HDM antigens and decreased cell surface expression of major histocompatibility complex II (MHC II) and costimulatory molecule *CD86*, decreasing production of Th2 cytokines by activated T cells. Finally, pharmacological inhibition of FOXM1 by ARF peptide prevented HDM-mediated pulmonary responses. FOXM1 regulates genes critical for allergen-induced lung inflammation and goblet cell metaplasia.

Asthma is one of the most common chronic respiratory diseases, affecting approximately 300 million individuals worldwide and representing a significant and growing cause of morbidity, mortality, and health care cost. Asthma is characterized by persistent pulmonary inflammation, goblet cell metaplasia, airway hyperresponsiveness, and lung remodeling (1). Eosinophilic inflammation and aberrant activation of T cells by pulmonary dendritic cell are important components in asthma pathogenesis. Cytokines/chemokines, including interleukin 4 (IL-4), IL-5, IL-13, IL-9, IL-17, IL-25, IL-33, and eotaxins, are produced by various resident and inflammatory cells in response to allergens that contribute to the pathogenesis of asthma (2–4). New therapeutic approaches are needed to improve patient care and reduce health care costs related to asthma.

Increased mucus production by goblet cells is a key feature in asthma and other chronic respiratory disorders, including chronic obstructive pulmonary disease (COPD), cystic fibrosis, and interstitial lung diseases (5, 6). In response to allergen stimulation, goblet cells differentiate from nonciliated epithelial cells in peripheral airways or basal cells in cartilaginous airways and trachea (7–9). Signaling pathways and transcriptional networks that control goblet cell differentiation have been increasingly studied. These include JAK/STAT-6, Notch, epidermal growth factor receptor (EGFR), mitogen-activated protein kinase (MAPK), and NF- κ B (8, 10–12). Notch signaling induces goblet cell metaplasia during lung development and lung injury (8). In response to allergen stimulation in mouse models, IL-4 receptor/STAT-6 signaling induces differentiation of goblet cells from Clara cells through upregulation of SPDEF and inhibition of TTF-1 and FOXA2 transcription factors (13). In the mouse, transgenic expression of SPDEF in Clara cells is sufficient to induce their differentiation into goblet cells, whereas *Spdef*^{-/-} mice lack airway goblet cells (13, 14). SPDEF induces expression of various goblet cell genes, including *Muc5ac*, *Muc16*, *Foxa3*, *Agr2*, and glycosyl-

transferase genes, that regulate mucus production associated with acquisition of the goblet cell phenotype (14). While SPDEF is both necessary and sufficient for differentiation of goblet cells, upstream regulators of SPDEF remain poorly understood.

FOXM1 (previously known as HFH-11B, Trident, Win, or MPP2) is a member of the Forkhead box (FOX) family of transcription factors that share homology in the Winged Helix/Forkhead DNA binding domain (15, 16). Targeted deletion of the *Foxm1* gene in mice (*Foxm1*^{-/-}) is embryonic lethal due to multiple abnormalities in the development of the lung, liver, heart, and blood vessels (17–20). FOXM1 inactivation in cycling cells caused delays in DNA replication and mitosis, and altered expression of cell cycle regulatory genes (20–23). Consistent with an important role of FOXM1 in cell cycle progression, FOXM1 induced proliferation of tumor cells during development of lung, liver, brain, and prostate cancers (21, 24–27). Likewise, FOXM1 accelerated cellular proliferation during acute lung and liver injuries (28, 29).

While FOXM1 is an important regulator of cellular proliferation, a number of cell cycle-independent functions of FOXM1 were recently described (30). Depending on cell/tissue specificity, FOXM1 induces surfactant production by type II alveolar epithelial cells (31), increases differentiation of type I epithelial cells (32), regulates expression of tight junction proteins in endothelial cells

Received 12 July 2012. Returned for modification 27 August 2012.

Accepted 2 November 2012.

Published ahead of print 12 November 2012.

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doi:10.1128/MCB.00934-12

(33, 34), and increases macrophage recruitment after liver injury (35). Deletion of *Foxm1* from embryonic lung epithelium causes respiratory failure after birth (31), whereas deletion of *Foxm1* from alveolar type II cells of adult mice impairs alveolar barrier repair after acute lung injury (32). Although these studies demonstrated that FOXM1 is a critical transcriptional regulator of alveolar epithelial cells, the role of FOXM1 in the airway epithelium remains unknown. Herein, we demonstrate that house dust mite allergen (HDM) increases FOXM1 expression in airway epithelium and inflammatory cells. We used genetic and pharmacological approaches to inhibit FOXM1 and identify molecular mechanisms by which FOXM1 influences pulmonary allergic responses.

MATERIALS AND METHODS

Mouse strains. Generation of a *Foxm1*^{lox/lox} (*Foxm1*^{fl/fl}) mouse line, which contains LoxP sequences flanking DNA binding and transcriptional activation domains of the *Foxm1* gene (exons 4 to 7), was previously described (19). The *Foxm1*^{fl/fl} mice were bred with *CCSP-rtTA*^{tg/tg}/*Otet-Cre*^{tg/tg} mice (line 2) (14) to generate *CCSP-rtTA*^{tg/tg}/*Otet-Cre*^{tg/tg}/*Foxm1*^{fl/fl} mice (*CCSP-Foxm1*^{fl/fl}). *Foxm1* deletion from Clara cells was achieved by doxycycline (Dox; 625 mg/kg; Harlan Teklad, Madison, WI), which was administered to mice in their food (36). Controls included *CCSP-Foxm1*^{fl/fl} mice without Dox, Dox-treated *Foxm1*^{fl/fl} littermates, and mice expressing Cre alone (Dox-treated *CCSP-rtTA*^{tg/tg}/*Otet-Cre*^{tg/tg}/*Foxm1*^{wt/wt} mice). *LysM-Cre*^{tg/tg} *Foxm1*^{fl/fl} mice were generated and described previously (35). Transgenic mice were maintained in a C57BL/6 genetic background. *LysM-Cre* deletes *Foxm1* in cells of myeloid lineage (37) as well as in a subset of alveolar type II cells (38).

Allergen stimulation and treatment with ARF peptide. Animal studies were approved by the Animal Care and Use Committee, and human studies were approved by the institutional review board (IRB) of Cincinnati Children's Hospital Research Foundation. Ovalbumin (OVA) was given intraperitoneally (i.p.) on days 0, 7, and 14 (100 µg of OVA mixed with 1 mg of aluminum hydroxide) followed by two intranasal treatments of OVA (50 µg) or saline on days 24 and 27 as described previously (39, 40). HDM extract (50 µg diluted in saline; Greer Laboratories) was given intratracheally (i.t.) on days 0 and 14. Twenty-four hours after the last HDM or OVA challenge, lungs were harvested and used for bronchoalveolar lavage fluid (BALF) collection, paraffin embedding, and preparation of RNA. The following enzyme-linked immunosorbent assay (ELISA) kits were used to measure mouse cytokines and chemokines in BALF: IL-4, IL-5, IL-13, IL-12p70, and CCL2 (all from eBioscience), and eotaxin (Abcam). Airway resistance was assessed on tracheostomized 8-week-old mice using a computerized FlexiVent system (SCIREQ, Montreal, Canada) as described previously (41). Methacholine was delivered using an Aeroneb nebulizer (SCIREQ).

For pharmacological inhibition of FOXM1, we synthesized the (D-Arg)₉-ARF(26–44) peptide containing a fluorescent tetramethylrhodamine (TMR) tag and nine N-terminal D-Arg residues to enhance the cellular uptake (21, 42). Eight-week-old BALB/c mice were subjected to i.t. administration of HDM on days 0 and 14. ARF peptide or control mutant peptide (21, 42) was administered i.t. on days 13 and 15 (1 mg/kg of body weight, diluted in saline). Forty-eight hours after the last peptide treatment, mice were sacrificed.

Immunohistochemical staining. Lungs were inflated, fixed in 4% paraformaldehyde, and embedded in paraffin blocks. Five-µm sections were stained with hematoxylin and eosin (H&E) or Alcian blue or used for immunohistochemistry as described previously (26, 31, 43). The following antibodies were used for immunostaining: FOXM1 (1:1,000, K-19, sc500; Santa Cruz Biotechnology), Cre recombinase (1:15,000, 69050-3; Novagen), Clara cell-secreted protein (CCSP; 1:2,000, WRAB-CCSP; Seven Hill Bioreagents), Ki-67 (1:500, clone Tec-3; Dako), PH3 (1:500, sc8656; Santa Cruz Biotechnology), FOXA2 (1:4,000, WRAB-FoxA2;

Seven Hills Bioreagents); FOXA3 (1:200, sc5361; Santa Cruz Biotechnology), β-tubulin (1:100, MU178-UC, BioGenex), SPDEF (1:2,000; generated in the lab of J. A. Whitsett [14]), MUC5AC (1:100, 45M1, ab3649; Abcam), alpha-smooth muscle actin (αSMA, 1:10,000, clone A5228; Sigma), and proSP-C (1:2,000) (31). Antibody-antigen complexes were detected using biotinylated secondary antibody followed by avidin-biotin-horseradish peroxidase complex (ABC), and 3,3'-diaminobenzidine (DAB) substrate (all from Vector Lab). Sections were counterstained with nuclear fast red (Vector Laboratories, Burlingame, CA). For colocalization experiments, secondary antibodies conjugated with Alexa Fluor 488 or Alexa Fluor 594 (Invitrogen/Molecular Probes) were used as previously described (43, 44). Slides were counterstained with DAPI (4',6'-diamidino-2-phenylindole; Vector Lab). Fluorescent images were obtained using a Zeiss Axioplan2 microscope equipped with an Axiocam MRm digital camera and Axiovision 4.3 software (Carl Zeiss Microimaging, Thornwood, NY).

Flow cytometry. Inflammatory cells were prepared from lung tissue of HDM-treated *LysM-Cre Foxm1*^{fl/fl} and control *Foxm1*^{fl/fl} mice as described previously (45). Briefly, lungs were perfused with phosphate-buffered saline (PBS), removed, gently minced with scissors, and then placed in RPMI 1640 medium containing Liberase (37.5 µg/ml; Roche Diagnostics) and DNase I (0.5 mg/ml, Sigma) for 40 min at 37°C in a CO₂ incubator. After digestion, lung tissue was forced through a 40-µm cell strainer. Red blood cells were lysed with ammonium-chloride-potassium (ACK) lysis buffer (Invitrogen).

Inflammatory cells were stained with fluorescently labeled antibodies against CD11b, CD45, Ly-6C, Ly-6G, F4/80, SiglecF, Gr-1, CD11c, CD317, major histocompatibility complex II (MHC II), and CD86 as previously described (35, 46). Staining was performed at 4°C following incubation with Fc-Block (anti-mouse CD16/32, clone 93; eBiosciences, San Diego, CA) for 30 min. Following antibodies were used: anti-F4/80 (clone BM8; eBiosciences), anti-CD11b (clone M1/70; eBiosciences), anti-Ly-6C (clone HK1.4; BioLegend, San Diego, CA), anti-Ly-6G (clone 1A8; BioLegend), anti-CD86 (clone PO3; BioLegend), anti-CD317 (clone eBio927, eBiosciences), anti-MHC II (clone M5/114.15.2; eBiosciences), anti-CD11c (clone N418; eBiosciences), anti-SiglecF (clone E50-2440; BD Pharmingen), anti-Gr1 (RB6-8C5; eBiosciences), and anti-CD45 (clone 30-F11; BD Pharmingen). Dead cells were excluded using 7-aminoactinomycin (7-AAD) stain (eBiosciences). Stained cells were separated using cell sorting (five-laser FACSARIA II; BD Biosciences). The following specific cell subsets were identified by using the indicated surface marker phenotypes: eosinophils, CD45⁺ CD11b⁺ CD11c⁻ SiglecF⁺; neutrophils, CD45⁺ CD11b⁺ Ly6C⁺ Ly6G⁺; macrophages, CD45⁺ CD11b⁺ Ly6C⁻ Ly6G⁻ F4 80⁺; monocytes, CD45⁺ CD11b⁺ Ly6C^{hi} Ly6G⁻ F4 80⁺; myeloid dendritic cells (mDC), CD45⁺ CD11b⁺ CD11c⁺ Gr1⁻ CD317⁻ SiglecF⁻; and plasmacytoid dendritic cells (pDC), CD45⁺ CD11b⁻ CD11c⁺ Gr1⁺ CD317⁺. Purified cells were used for RNA preparation and cell culture.

Coculture of mDCs and T cells. mDC were isolated from lung tissue of HDM-treated mice, restimulated *in vitro* with 15 µg/ml of HDM labeled with IRD700 (Licor), and then cocultured with CD4⁺ T cells purified from spleens of HDM-treated wild-type (WT) mice (5 T cells/1 mDC). After 5 days in culture, cells were used for fluorescence-activated cell sorting (FACS) analysis to examine IRD700, MHC II, and CD86 in dendritic cells. ELISA was used to measure IL-4, IL-5, and IL-13 in culture media.

Quantitative real-time RT-PCR (qRT-PCR). RNA was prepared from whole lung tissue, FACS-sorted inflammatory cells, and epithelial cells isolated by laser capture microdissection. The Veritas microdissecting system (Molecular Devices, Sunnyvale, CA) was used for the laser capture microdissection of frozen lung sections as described previously (44). A StepOnePlus real-time PCR system (Applied Biosystems, Foster City, CA) was used as described previously (31, 47). Samples were amplified with TaqMan gene expression master mix (Applied Biosystems) combined with inventoried TaqMan gene expression assays for the gene of

TABLE 1 TaqMan primers for qRT-PCRs

Mouse gene in TaqMan expression assay	Catalog no.
<i>foxm1</i>	Mm01184444_g1
<i>beta-actin</i>	Mm00607939_s1
<i>ccl2</i>	Mm99999056_m1
<i>ccl3</i>	Mm00441259_g1
<i>ccl4</i>	Mm00443111_m1
<i>ccl5</i>	Mm01302427_m1
<i>ccl11</i>	Mm00441238_m1
<i>ccl17</i>	Mm01244826_g1
<i>ccl24</i>	Mm00444701_m1
<i>ccr2</i>	Mm00438270_m1
<i>ccr3</i>	Mm00515543_s1
<i>cx3cl1</i>	Mm00436454_m1
<i>cx3cr1</i>	Mm02620111_s1
<i>foxa2</i>	Mm00839704_mH
<i>foxa3</i>	Mm00484714_m1
<i>gm-csf</i>	Mm01290062_m1
<i>gm-csfra</i>	Mm00438331_g1
<i>il-1a</i>	Mm99999060_m1
<i>il-1b</i>	Mm01336189_m1
<i>il-4</i>	Mm00445260_m1
<i>il-5</i>	Mm00439646_m1
<i>il-6</i>	Mm01210733_m1
<i>il-10</i>	Mm99999062_m1
<i>il-12p35</i>	Mm00434165_m1
<i>il-13</i>	Mm00434205_g1
<i>il-33</i>	Mm00505403_m1
<i>lta4s</i>	Mm00521864_m1
<i>mcl-1</i>	Mm00725832_s1
<i>muc5ac</i>	Mm01276725_g1
<i>ptgs2</i>	Mm00478374_m1
<i>sox-2</i>	Mm00488369_s1
<i>Spdef</i>	Mm00600221_m1
<i>Integrin αM</i>	Mm01271259_g1
<i>tnfa</i>	Mm00443258_m1

interest (Table 1). Reactions were analyzed in triplicate. Expression levels were normalized to β -actin mRNA.

Cotransfection studies. U2OS cells were transfected with either cytomegalovirus (CMV)-FoxM1b or control empty CMV plasmids, as well as with a luciferase (LUC) reporter driven by the 2.8-kb mouse *Spdef* promoter (13, 14). The 2.8-kb mouse *Spdef* promoter was subcloned into the pGL3-LUC vector. CMV-*Renilla* was used as an internal control to normalize transfection efficiency. A dual LUC assay (Promega) was performed as described previously (31).

Chromatin immunoprecipitation (ChIP) assays. ChIP assays were performed using *in situ* cross-linked human bronchial epithelial Beas-2B cells as described previously (31, 48, 49). Rabbit anti-Foxm1 antibodies (Abs) (C-20; Santa Cruz) and control rabbit IgG (Vector Lab) were used for ChIP. Promoter DNA was amplified using sense and antisense primers shown in Table 2.

Statistical analysis. *Post hoc* analysis of variance (ANOVA) was used for multiple-group comparison. For comparison between two groups, Student's *t* test was used to determine statistical significance. *P* values of ≤ 0.05 were considered significant. Values for all measurements were expressed as means \pm standard deviations (SD).

RESULTS

Pulmonary FOXM1 expression is increased after allergen stimulation. Two mouse models of allergic airway diseases were used to assess the expression of FOXM1 after allergen stimulation, in-

TABLE 2 Primers for ChIP assays

Primer ^a	Sequence
hSPDEF (S)	CCTGCAAGGGTAAATCAGGAGCCT
hSPDEF (AS)	GCAGTGTGGACACGGCAGAGTGCA
hCCL24 (S)	GACCTCTCATTGTCTGTTACACAC
hCCL24 (AS)	CTGAGCCTCAGTTACTTCAGCTG
hCCL11 (S)	TTGGTCTACTCTACTTCATTGCTGA
hCCL11 (AS)	AACCTCAGTGGCTCAGTGGAA

^a S, sense; AS, antisense.

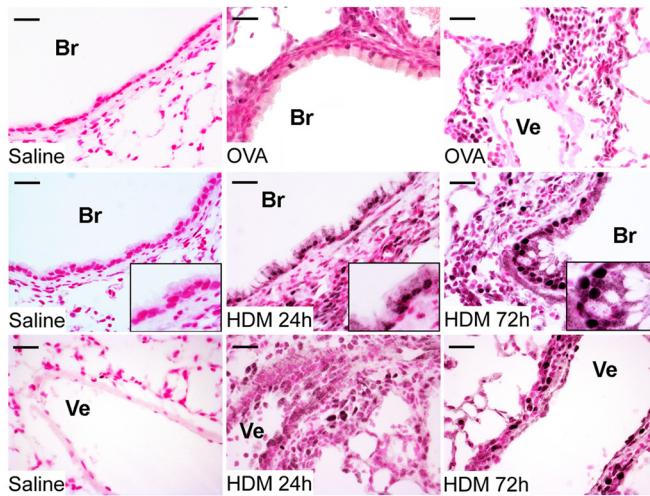
cluding treatment with OVA and house dust mite extract (HDM). Although FOXM1 was not detected in lungs of mice treated with saline, FOXM1 was induced in subsets of bronchiolar epithelial cells and inflammatory cells in each of the mouse models of experimental asthma (Fig. 1A). Increases in FOXM1 staining and Foxm1 mRNA were observed 24 to 72 h after final HDM treatment (Fig. 1A and B). Consistent with data from mouse lung tissue, FOXM1 staining was increased in bronchiolar epithelium and inflammatory cells of patients with severe asthma and chronic obstructive pulmonary disease (COPD) (Fig. 1D). In contrast, FOXM1-expressing cells were rarely seen in normal human lungs (Fig. 1D). Thus, allergen stimulation induced FOXM1 expression in mouse and human lungs.

To identify airway epithelial cells expressing FOXM1 in response to HDM challenge, colocalization experiments were performed. The majority of FOXM1-positive cells expressed the Clara cell-secreted protein (CCSP) (Fig. 1E), a marker of Clara cells. FOXM1 was also found in goblet cells expressing low levels of MUC5AC, whereas FOXM1 was absent from goblet cells expressing high levels of MUC5AC (Fig. 1E). FOXM1 was also found in subsets of Clara and goblet cells from patients with severe asthma (Fig. 1F). To identify inflammatory cells expressing FOXM1 after allergen stimulation, cells were isolated from lungs of HDM-treated mice and purified by flow cytometry-based cell sorting (Fig. 2). High levels of Foxm1 mRNA were found in neutrophils, monocytes and macrophages from HDM-treated lungs, whereas myeloid dendritic cells (mDCs) and lymphocytes expressed low levels of Foxm1 mRNA (Fig. 1C). Foxm1 mRNA was undetectable in eosinophils (Fig. 1C). FOXM1 protein and mRNA were absent from airway epithelial and myeloid cells of saline-treated mice (Fig. 1A and data not shown), indicating that HDM treatment was required to induce FOXM1 expression in the lung.

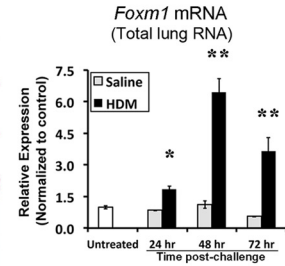
Conditional deletion of FOXM1 from myeloid cells. To determine the role of FOXM1 in myeloid cells after allergen exposure, *LysM-Cre Foxm1^{fl/fl}* mice were used. The *LysM-Cre Foxm1^{fl/fl}* mice contain LoxP-flanked *Foxm1*-floxed alleles (*Foxm1^{fl/fl}*) (19) and the *LysM-Cre* transgene (37), mediating a conditional deletion of exons 4 to 7, which encode DNA-binding and C-terminal transcriptional activation domains of the FOXM1 protein (19). *LysM-Cre* deletes *Foxm1* in cells of myeloid lineage, including macrophages, monocytes, DCs, and granulocytes (37), and in a subset of alveolar type II cells (38). In untreated *LysM-Cre Foxm1^{fl/fl}* mice, lung structure and cell counts in bone marrow and peripheral blood were unaltered (33, 35). To assess requirements for FOXM1 in the asthma model, *LysM-Cre Foxm1^{fl/fl}* and control *Foxm1^{fl/fl}* mice were subjected to the HDM sensitization/challenge protocol.

To address the efficiency of FOXM1 deletion, inflammatory cells were isolated from lungs of *LysM-Cre Foxm1^{fl/fl}* and control *Foxm1^{fl/fl}* mice 48 h after the last HDM challenge. A 70 to 90%

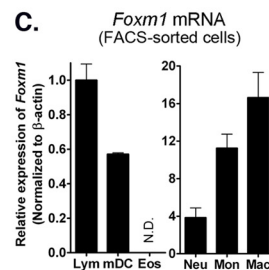
A. Foxm1 in Mouse lungs



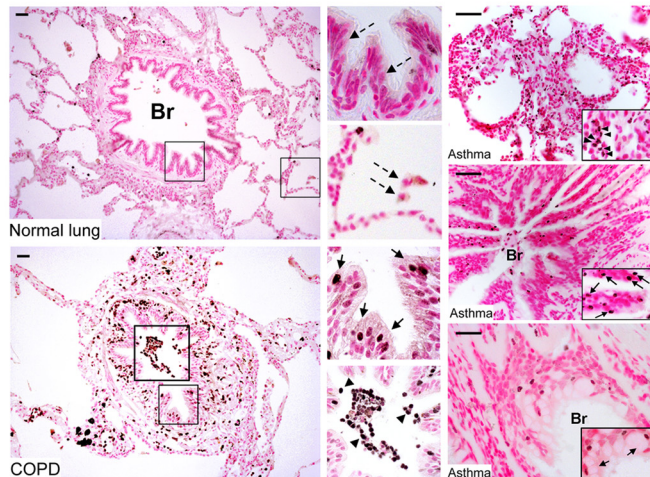
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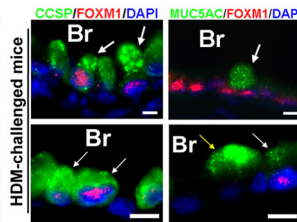
C.



D. Foxm1 in human lungs



E. Mouse airway epithelial cells



F. Human airway epithelial cells

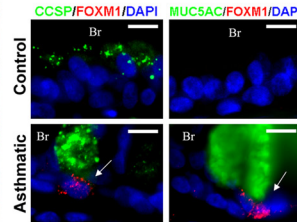


FIG 1 FOXM1 is increased after allergen stimulation. (A) FOXM1 is increased in mouse models of asthma. Paraffin sections were prepared from lungs of WT BALB/c mice 24 and 72 h after the last HDM challenge or 48 h after the last OVA challenge. Five mice were used in each group. Lung sections were stained with FOXM1 Abs (dark brown) and counterstained with nuclear fast red (red). FOXM1 staining is increased in airway epithelial cells and inflammatory cells after OVA or HDM treatment. FOXM1 was not found in WT mice treated with saline (left panels). (B) qRT-PCR of total lung RNAs shows increased expression of *Foxm1* at different time points. Data are means \pm standard errors of the means (SEM) ($n = 5$ mice/group; *, $P < 0.05$; **, $P < 0.01$). (C) HDM induces FOXM1 in myeloid cells. Lung tissue was harvested from WT mice 48 h after the last HDM challenge. Cells were purified using flow cytometry-based cell sorting. Cell surface markers are shown in Fig. 2. FOXM1 mRNA was detected in various types of inflammatory cells by qRT-PCR. FOXM1 mRNA was not detected in eosinophils. (D) FOXM1 staining in lungs of patients with asthma and COPD. FOXM1 staining was increased in airway epithelium (arrows), peribronchial regions, and cells located within the lumen (arrowheads) in patients with COPD and asthma but not in control lungs ($n = 5$). (E and F) FOXM1 (red) colocalized with CCSP (green) in HDM-treated airway epithelium of WT mice (E) and asthmatic human lungs (F). FOXM1 is also found in subsets of MUC5AC-positive goblet cells from mouse and human lungs. Abbreviations: Br, bronchiole; Ve, vessel. Bars, 50 μ m (A and D) and 10 μ m (E and F).

reduction in *Foxm1* mRNA was observed in monocytes, macrophages, mDCs, and neutrophils obtained from *LysM-Cre Foxm1^{fl/fl}* mice, whereas *Foxm1* mRNA in lymphocytes was unchanged (Fig. 3D), a finding consistent with efficient deletion of *Foxm1* from myeloid cells (37). FOXM1 was present in a subset of Clara cells but absent from alveolar type II cells of HDM-treated *LysM-Cre Foxm1^{fl/fl}* and control lungs (Fig. 3E).

Deletion of FOXM1 from myeloid cells reduced pulmonary inflammation and airway resistance after HDM challenge. Consistent with previous studies (50, 51), HDM exposure induced

goblet cell metaplasia in pulmonary airways and caused inflammation in interstitial tissue surrounding airways and pulmonary blood vessels (Fig. 3A and B). While goblet cell metaplasia was similar in control *Foxm1^{fl/fl}* and *LysM-Cre Foxm1^{fl/fl}* mice (Fig. 3B), total number of inflammatory cells was lower in *LysM-Cre Foxm1^{fl/fl}* lungs (Fig. 3A and C). Numbers of eosinophils and macrophages were decreased in lung tissue of HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice, whereas numbers of neutrophils and mDCs were unchanged (Fig. 3G, left). In peripheral blood of HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice, percentages of eosino-

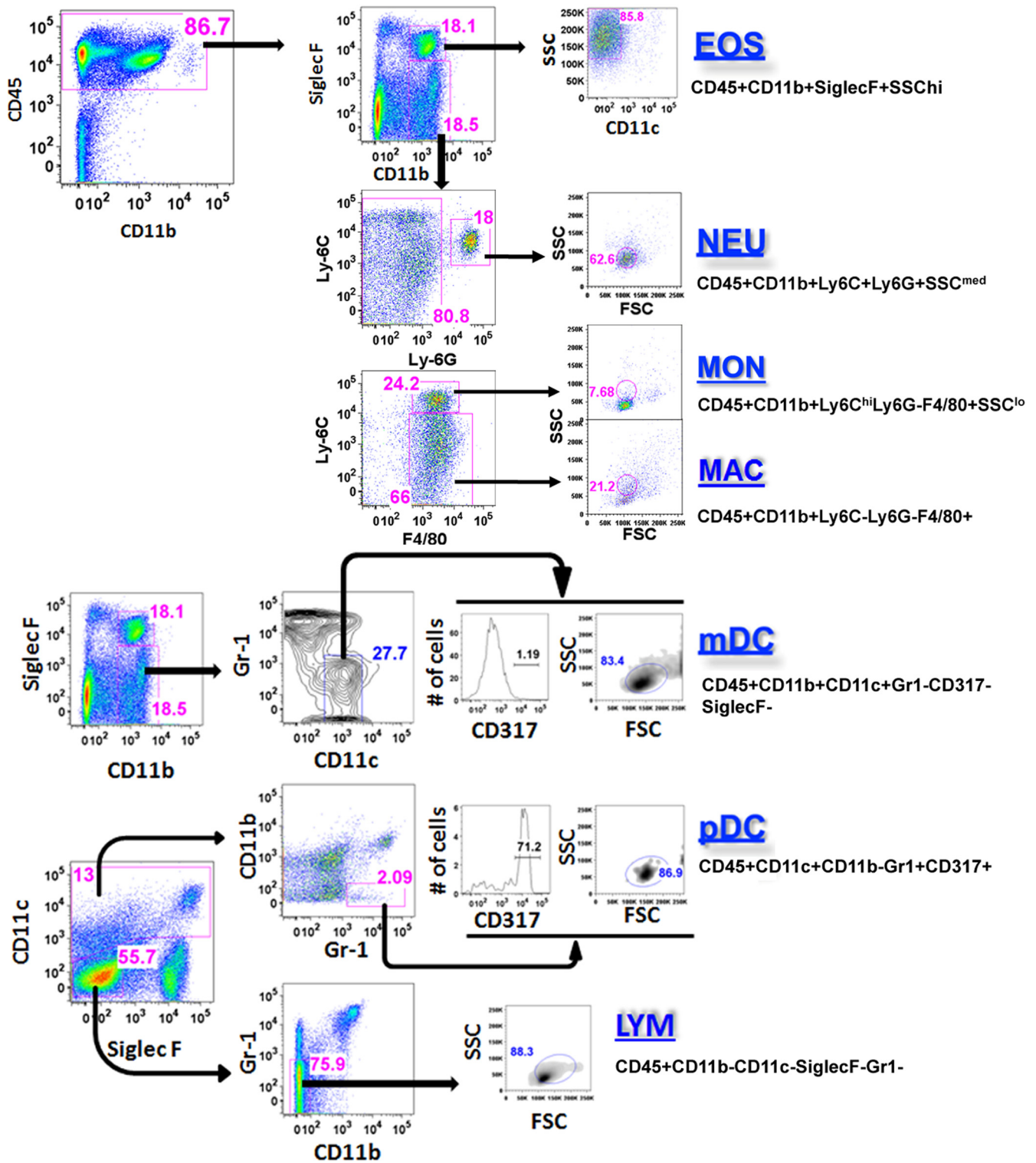


FIG 2 Identification and purification of inflammatory cells from HDM-challenged lung tissue. Inflammatory cells were isolated from either *LysM-Cre Foxm1^{fl/fl}* or control *Foxm1^{fl/fl}* lungs. Flow cytometry was used to identify myeloid inflammatory cells. The cell surface marker phenotypes used were as follows: eosinophils, CD45⁺ CD11b⁺ CD11c⁻ SiglecF⁺ SSC^{hi}; neutrophils, CD45⁺ CD11b⁺ Ly6G⁺ Ly6C⁺ SSC^{med}; monocytes, CD45⁺ CD11b⁺ Ly6G⁻ Ly6C^{hi} F4 80⁺ SSC^{lo}; macrophages, CD45⁺ CD11b⁺ Ly6G⁻ Ly6C⁻ F4 80⁺; myeloid dendritic cells (mDCs), autofluorescence^{lo} CD45⁺ CD11b⁺ CD11c⁺ Gr1⁻ CD317⁻; plasmacytoid dendritic cells (pDCs), autofluorescence^{lo} CD45⁺ CD11b⁻ CD11c⁺ Gr1⁺ CD317⁺; lymphocytes, CD45⁺ CD11b⁻ CD11c⁻ Gr1⁻ SiglecF⁻. Cells were used for either RNA isolation or cell culture experiments.

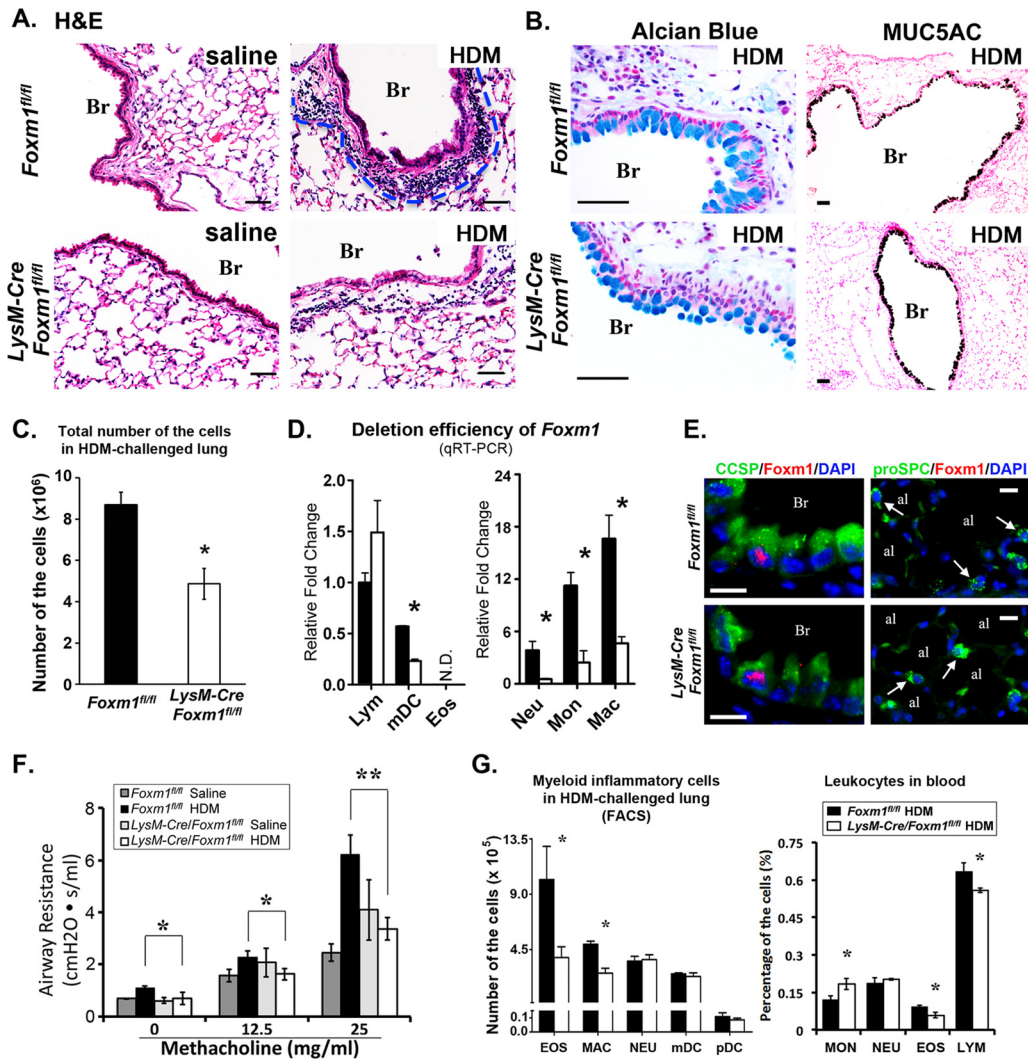


FIG 3 Deletion of FOXM1 from myeloid cells decreased airway resistance and lung inflammation after HDM challenge. (A and B) Pulmonary inflammation was assessed in *LysM-Cre Foxm1^{fl/fl}* mice after HDM treatment. Control *Foxm1^{fl/fl}* and *LysM-Cre Foxm1^{fl/fl}* mice were treated with HDM or saline and harvested 72 h after the last challenge. H&E staining shows decreased peribronchial inflammation in HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice (A). The number of inflammatory cells was counted in 10 random sections using 5 mice in each group (C). A *P* value of <0.05 is shown with an asterisk. Alcian blue staining and immunostaining for MUC5AC were similar in HDM-treated *Foxm1^{fl/fl}* and *LysM-Cre Foxm1^{fl/fl}* lungs (B). (D) Efficiency of *Foxm1* deletion in myeloid cells. Cells were purified from lungs of HDM-treated *Foxm1^{fl/fl}* and *LysM-Cre Foxm1^{fl/fl}* mice by flow cytometry (see Fig. 2 for cell surface markers) and used for qRT-PCR. *Foxm1* mRNA was decreased in macrophages, monocytes, neutrophils, and mDCs of HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice. Six mice were used in each group. (E) Colocalization experiments show the presence of FOXM1 in a subset of CCSP-positive Clara cells (left panels). FOXM1 is not expressed in proSPC-positive type II cells (right panels). (F) Decreased airway resistance in HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice. FlexiVent was used to measure airway resistance in 8 to 10 mice in each group. (G) Leukocyte counts in HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice. Flow cytometry was used to determine the number of various inflammatory subsets using cell surface markers described in Fig. 2. Eosinophils and macrophages were decreased in lung tissue of HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice (left). In peripheral blood, percentages of eosinophils and lymphocytes were significantly reduced, but the percentage of circulating monocytes was increased after deletion of FOXM1 (right). Five mice were used in each group. Abbreviations: Br, bronchiole; al, alveoli. Bars, 50 μ m (A and B) and 10 μ m (E).

phils and lymphocytes were significantly reduced but the percentage of circulating monocytes was increased (Fig. 3G, right), a finding consistent with decreased migration and maturation of FOXM1-deficient monocytes from peripheral blood into injured liver (35). Consistent with decreased pulmonary inflammation in HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice, airway resistance after methacholine treatment was significantly decreased (Fig. 3F).

Total lung RNA was used to identify genes regulated by FOXM1 in response to HDM stimulation. *Foxm1* mRNA was increased in HDM-treated *Foxm1^{fl/fl}* lungs but not in *LysM-Cre Foxm1^{fl/fl}* lungs (Fig. 4A). Consistent with the loss of *Foxm1*, *Ccr2*

mRNA, a known FOXM1 target (35), was significantly decreased (Fig. 4D). While granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA was not changed, mRNAs of IL-4, IL-13, IL-12, IL-5, *Ccl11* (eotaxin-1), *Ccl24* (eotaxin-2), *Ccl2*, *Cx3cl1*, *Ptgs2*, and *Ltc4s*, all critical for recruitment of inflammatory cells and airway hyperreactivity after allergen stimulation (52–55), were reduced in HDM-treated *LysM-Cre Foxm1^{fl/fl}* lungs (Fig. 4B and D). Protein levels of IL-4, IL-5, CCL2, and IL-12p70 were decreased in BALF of HDM-treated *LysM-Cre Foxm1^{fl/fl}* mice, as demonstrated by ELISA (Fig. 4C). Expression of proinflammatory genes was also examined in purified neutrophils and

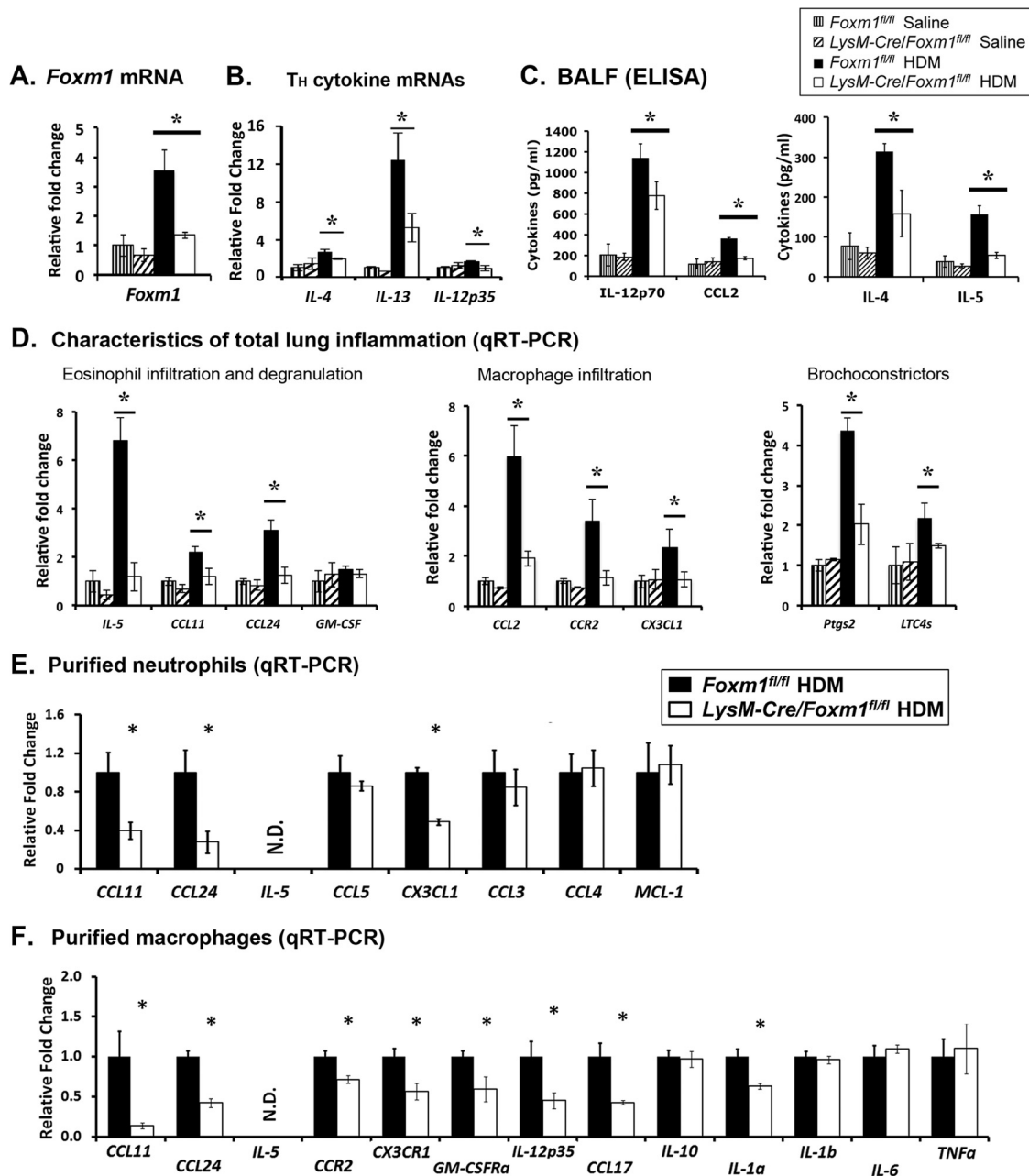


FIG 4 Deletion of *Foxm1* in myeloid cells reduced lung inflammation and decreased expression of proinflammatory genes after HDM treatment. Lung tissue and BALF were obtained from *Foxm1*^{fl/fl} and *LysM-Cre Foxm1*^{fl/fl} mice 72 h after the last HDM challenge. (A and B) qRT-PCR shows decreased mRNAs of *Foxm1*, *IL-4*, *IL-13*, and *IL-12p32* in whole lung tissue from HDM-treated *LysM-Cre Foxm1*^{fl/fl} mice. (C) ELISA shows decreased *IL-4*, *IL-5*, *CCL2*, and *IL-12p70* proteins in BALF of HDM-treated *LysM-Cre Foxm1*^{fl/fl} mice. (D) Gene expression profile in *LysM-Cre Foxm1*^{fl/fl} lungs. qRT-PCR was used to quantitate mRNAs of various inflammatory mediators in total lung tissue (5 mice in each group). (E and F) qRT-PCR shows mRNAs of inflammatory mediators in purified neutrophils (E) and macrophages (F). Cells were isolated from HDM-treated lungs ($n = 5$) by cell sorting (see Fig. 2 for cell surface markers). *, $P < 0.05$. N.D., not detected.

macrophages isolated from HDM-treated lungs. In both cell types, decreased *Foxm1* mRNA was associated with reduced *Ccl11* and *Ccl24* (Fig. 4E and F), findings consistent with decreased numbers of eosinophils (Fig. 3G). *Cx3cl1* mRNA was decreased in FOXM1-deficient neutrophils (Fig. 4E), whereas *Cx3cr1*, *Ccr2*, *GM-CSFR α* , *IL-1 α* , *IL-12p35* and *Ccl17* mRNAs were reduced in FOXM1-deficient macrophages (Fig. 4F). The loss of FOXM1 did not influence *IL-1 β* , *IL-6*, tumor necrosis factor alpha (TNF- α), *IL-10*, *CCL3*, *CCL4*, or *Mcl-1* mRNAs

(Fig. 4E and F). Thus, deletion of FOXM1 altered expression of proinflammatory genes in neutrophils and macrophages, possibly contributing to decreased lung inflammation and reduced airway resistance after HDM challenge.

Impaired T cell activation in FOXM1-deficient mDCs. Total numbers of myeloid and plasmacytoid DCs were not altered in HDM-treated *LysM-Cre Foxm1*^{fl/fl} lungs (Fig. 3G). However, reduced Th₂ cytokines *IL-5*, *IL-4*, and *IL-13* in the lungs and BALF of these mice (Fig. 4B and C) are consistent with impaired func-

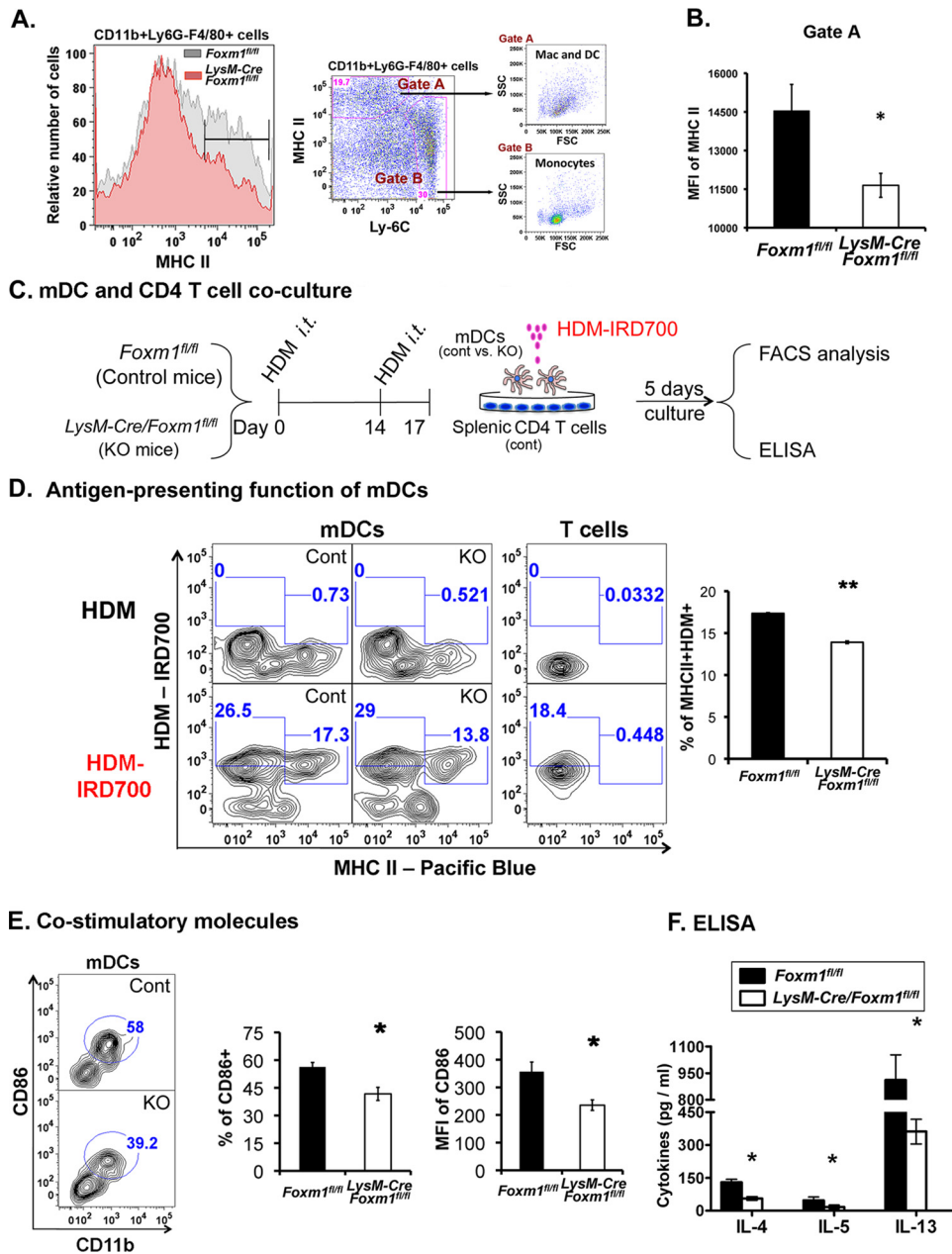


FIG 5 Decreased cell surface expression of MHC II and CD86 in *LysM-Cre Foxm1^{fl/fl}* mDCs. (A and B) Impaired MHC class II expression on the surfaces of FOXM1-deficient antigen-presenting cells after HDM sensitization *in vivo*. Antigen-presenting cells were purified from lung tissue of *Foxm1^{fl/fl}* and *LysM-Cre Foxm1^{fl/fl}* mice 72 h after the last HDM challenge. The mean fluorescence intensity (MFI) of MHC II was determined by flow cytometry. Five mice were used in each group. *, $P < 0.05$. (C to F), Decreased activation of T cells by *LysM-Cre Foxm1^{fl/fl}* mDCs *in vitro*. A schematic shows experimental design of coculture of pulmonary mDCs with splenic CD4⁺ T cells (C). Pulmonary mDCs and splenic *Foxm1^{fl/fl}* CD4⁺ T cells were isolated from HDM-sensitized mice, restimulated with infrared dye 700 (IRD700)-labeled HDM antigens *in vitro*, and cultured for 5 days (5 T cells/1 mDC). Uptake of IRD700-labeled HDM and cell surface expression of MHC II were measured by flow cytometry in 5 independent cell cultures (D). The percentage of MHC II⁺ HDM⁺ mDCs was lower in *LysM-Cre Foxm1^{fl/fl}* mDCs (D, right). The percentage of CD86-positive mDCs and MFI of CD86 were reduced in *LysM-Cre Foxm1^{fl/fl}* mDCs (E). ELISA was used to examine concentrations of IL-4, IL-5 and IL-13 in the culture supernatants (F). *, $P < 0.05$; **, $P < 0.01$.

tion of antigen-presenting cells, resulting in decreased activation of T cells. Cell surface expression of MHC II, a receptor critical for antigen presentation and T-cell activation, was examined in antigen-presenting cells from HDM-treated lungs. Mean fluorescence intensity (MFI) of MHC II was decreased in antigen-presenting myeloid cells (macrophages and DC) from *LysM-Cre Foxm1^{fl/fl}* lungs (Fig. 5A and B). To examine antigen presentation *in vitro*,

mDCs were purified from HDM-sensitized *LysM-Cre Foxm1^{fl/fl}* or *Foxm1^{fl/fl}* lungs, restimulated with fluorescently labeled HDM antigens *in vitro*, and cultured with splenic CD4⁺ T cells from HDM-treated *Foxm1^{fl/fl}* mice (Fig. 5C). The percentage of MHC II⁺ HDM⁺ mDCs was decreased after the loss of FOXM1 (Fig. 5D). FOXM1-deficient mDCs exhibited diminished cell surface expression of CD86 (B7.2) (Fig. 5E), a costimulatory molecule im-

portant for activation of T cells (56, 57). The percentage of CD86⁺ mDCs was also reduced (Fig. 5E). Finally, the T-cell-produced cytokines IL-4, IL-5, and IL-13 were decreased in supernatants from cell cultures containing FOXMI-deficient mDCs (Fig. 5F), a result consistent with decreased activation of T cells. Thus, deletion of FOXMI from mDCs resulted in decreased cell surface expression of MHC II and CD86 and reduced activation of T cells in response to HDM *in vitro* and *in vivo*. Altogether, our results demonstrate that expression of FOXMI in myeloid cells is critical for lung inflammation after allergen stimulation.

FOXMI deletion from Clara cells reduced pulmonary inflammation and decreased airway resistance after HDM challenge. FOXMI is increased in airway epithelial cells of asthma patients and mice treated with OVA and HDM (Fig. 1). To determine the role of FOXMI in airway epithelial cells after allergen exposure, triple-transgenic mice containing *Foxm1*^{fl/fl}, the *Scgbla1* (*CCSP*)-*rtTA*^{tg/-} and the *Otet-Cre*^{tg/-} transgenes (*CCSP-rtTA*^{tg/-}/*Otet-Cre*^{tg/-}/*Foxm1*^{fl/fl} or *CCSP-Foxm1*^{-/-} mice) were produced. To induce Cre expression in airway epithelial cells, doxycycline (Dox) was given 5 days after sensitization with HDM. In the presence of Dox, the reverse tetracycline transactivator (rtTA) binds to the TetO promoter and induces expression of Cre recombinase, deleting DNA binding and transcriptional activation domains of the FOXMI protein (49). Specific loss of FOXMI protein from airway epithelium of *CCSP-Foxm1*^{-/-} mice was shown by immunostaining (Fig. 6A). In response to HDM, airway resistance in *CCSP-Foxm1*^{-/-} mice was reduced compared to controls (Dox-treated *Foxm1*^{fl/fl} mice and *CCSP-Foxm1*^{fl/fl} mice without Dox) (Fig. 6B). FOXMI deletion from Clara cells reduced pulmonary inflammation in response to HDM, as shown by decreased numbers of inflammatory cells in BALF (Fig. 6C), and reduced mRNA and protein levels of eotaxins, IL-4, IL-5, IL-13, IL-33, Ccl2, Ccr2, and Ccr3 in lung tissue (Fig. 6D and E). FOXMI specifically bound to promoter regions of eotaxin genes (*Ccl11* and *Ccl24*) as demonstrated by ChIP assay (Fig. 6F). Reduced expression of proinflammatory mediators can contribute to decreased inflammation in HDM-treated *CCSP-Foxm1*^{-/-} mice. Thus, FOXMI deletion from airway epithelium inhibits pulmonary inflammation and decreases airway resistance after HDM challenge.

FOXMI deletion decreased goblet cell metaplasia in airway epithelium. Decreased numbers of goblet cells were observed in airway epithelium of HDM-treated *CCSP-Foxm1*^{-/-} mice (Fig. 6A). Decreased staining and mRNAs of *Spdef* and *Foxa3*, transcriptional regulators associated with goblet cell metaplasia (14), were consistent with reduced differentiation of goblet cells in *CCSP-Foxm1*^{-/-} lungs (Fig. 7A to C). The number of FOXA2-positive cells was increased in HDM-treated *CCSP-Foxm1*^{-/-} lungs (Fig. 7B). FOXMI colocalized with SPDEF in airway epithelium of HDM-treated mice, whereas the loss of FOXMI prevented SPDEF expression (Fig. 8A). In cotransfection experiments, CMV-FoxM1b expression vector induced promoter activity of *Spdef* (Fig. 8D). ChIP assay demonstrated that endogenous FOXMI protein specifically bound to the *Spdef* promoter (Fig. 8E), indicating that *Spdef* is a direct transcriptional target of FOXMI. Staining for proliferation-specific Ki-67 and PH3 showed no differences in proliferation of airway epithelial cells between *CCSP-Foxm1*^{-/-} and control lungs (Fig. 7D and E), suggesting a cell cycle-independent function of FOXMI in formation of goblet cell metaplasia. Interestingly, time course studies demonstrated that

FOXMI was induced prior to SPDEF but was decreased following upregulation of SPDEF (Fig. 8B), suggesting a negative feedback mechanism by which *Spdef* and *Foxm1* interact. Consistent with these results, *Foxm1* mRNA was increased in airway epithelium obtained by laser capture microdissection from HDM-treated *Spdef*^{-/-} lungs (Fig. 8C). Although the loss of SPDEF did not influence *Sox2* mRNA, expression of *Foxa3* was decreased (Fig. 8C), a result consistent with previous studies (14). Altogether, our results demonstrated an important role for FOXMI in regulation of transcriptional network critical for differentiation of goblet cells in the mouse asthma models.

Pharmacological inhibition of FOXMI decreased pulmonary inflammation and goblet cell metaplasia in response to HDM. Since genetic deletion of *Foxm1* from either airway epithelium or inflammatory cells protected the lung from HDM-induced inflammation (Fig. 3 to 6), we tested whether pharmacological inactivation of FOXMI influenced the pathogenesis of the asthma-like phenotype. Previous studies demonstrated that a membrane-penetrating ARF peptide specifically binds the FOXMI protein and inhibits FOXMI transcriptional activity *in vitro* and *in vivo* (21, 42, 58). The ARF peptide, containing a fluorescent tetramethylrhodamine (TMR) tag and nine N-terminal D-Arg residues to enhance cellular uptake (21, 42), was administered into the tracheas of HDM-treated WT mice on days 13 and 15. TMR-labeled mutant ARF peptide, which does not bind the FOXMI protein (21), was used as a control. TMR fluorescence was detected in airway epithelium (Fig. 9A), inflammatory cells (Fig. 9C), and peribronchial interstitium (Fig. 9C), indicating that the ARF peptide was effectively delivered to pulmonary airways *in vivo*. Flow cytometry demonstrated that 65.4% ± 3.5% of pulmonary macrophages and 39.1% ± 9.7% of mDCs contained TMR-labeled ARF peptide (Fig. 9B). In the alveolar region, TMR fluorescence was detected in a subset of macrophages but not in alveolar type II cells (Fig. 9C). Airway resistance was significantly decreased in ARF-treated mice compared to controls (Fig. 9D). Goblet cell metaplasia was reduced, as indicated by decreased MUC5AC protein and mRNA (Fig. 9A and E) and increased CCSP staining in ARF peptide-treated airways (Fig. 9A). The ARF peptide decreased the number of eosinophils in BALF (Fig. 9F and G) and reduced IL-5, IL-13, and CCL11 proteins and mRNAs (Fig. 9H to J), findings consistent with reduced pulmonary inflammation. Protein levels of CCL2 were unaltered (Fig. 9H). Altogether, inhibition of FOXMI by the ARF peptide protected the lung from goblet cell metaplasia and pulmonary inflammation induced by HDM exposure.

DISCUSSION

Chronic airway diseases, including asthma, COPD, cystic fibrosis (CF), and interstitial lung disease are associated with significant morbidity, mortality, and health care costs worldwide. In the present study, we demonstrated the essential role of FOXMI transcription factor in inducing lung inflammation and goblet cell metaplasia in response to allergen exposure and Th₂ stimulation (Fig. 10). Pharmacological targeting of FOXMI and its effectors provides a novel therapeutic strategy to prevent goblet cell metaplasia and pulmonary inflammation in chronic lung disease.

FOXMI promotes allergen-induced lung inflammation. In this study, we found that FOXMI induces expression of proinflammatory genes in myeloid cells, a finding consistent with an important role of FOXMI in allergen-induced lung inflammation. Previous studies demonstrated that FOXMI induces transcriptional activity of *Ccr2* and *Cx3cr1* (33, 35), encoding chemo-

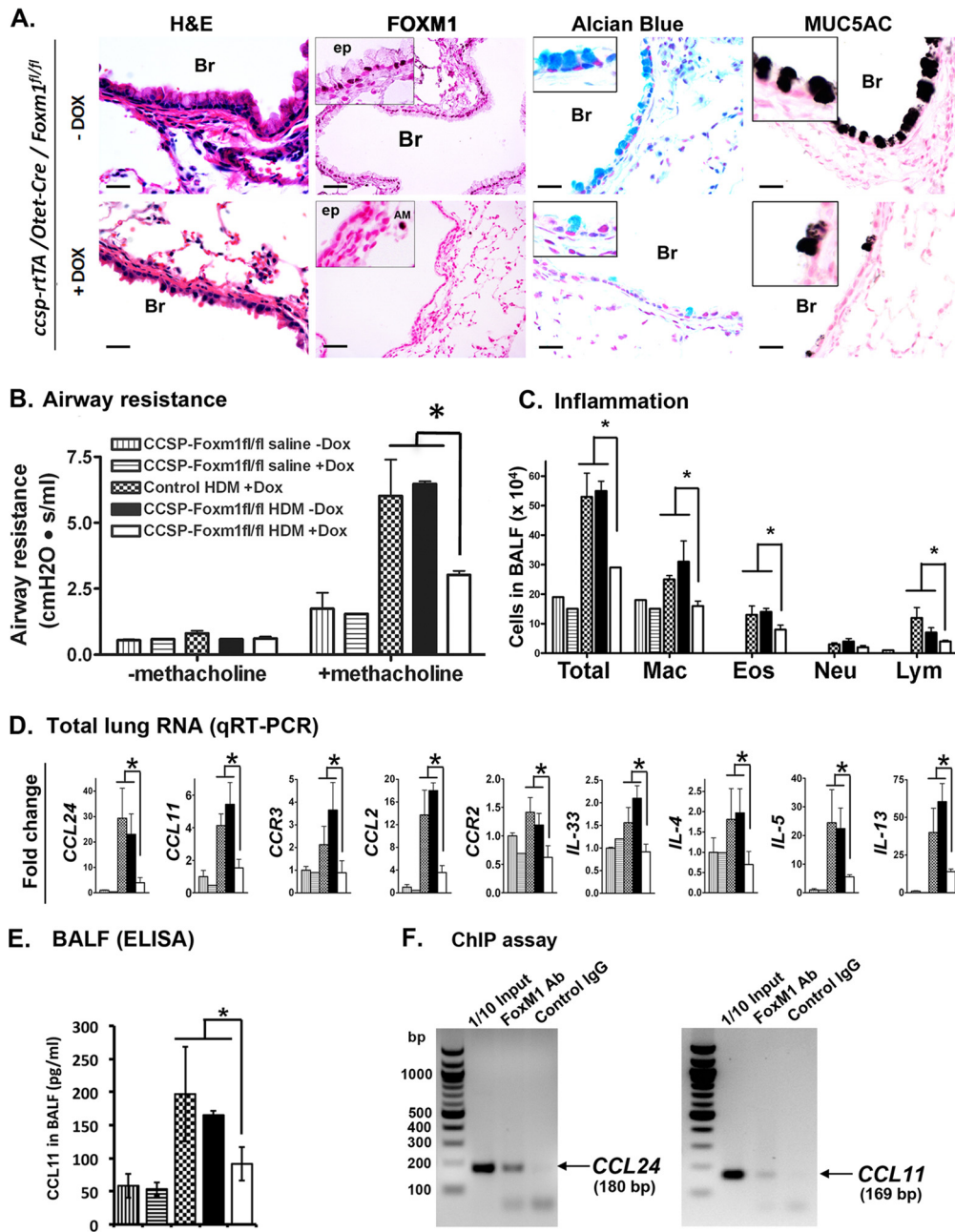


FIG 6 Deletion of *Foxm1* in Clara cells prevented goblet cell metaplasia in response to HDM. (A) Decreased goblet cell metaplasia in HDM-treated *CCSP-Foxm1*^{-/-} mice. Lungs were harvested 72 h after the last HDM challenge. FOXM1 staining is absent in airway epithelium (ep) of Dox-treated *CCSP-Foxm1*^{-/-} mice, while FOXM1 is detectable in alveolar macrophages (AM). FOXM1 is present in airway epithelium of mice without Dox (controls). Decreased numbers of goblet cells in Dox-treated *CCSP-Foxm1*^{-/-} mice are shown by H&E staining, Alcian blue staining, and immunostaining for MUC5AC. Bars, 50 μ m. (B) Decreased airway resistance in HDM-treated *CCSP-Foxm1*^{-/-} mice (7 to 12 mice in each group). The methacholine concentration was 6 mg/ml. (C) The number of inflammatory cells was decreased in BALF of *CCSP-Foxm1*^{-/-} mice compared to controls. BALF was collected 72 h after the last HDM challenge. Five hundred cells were counted to determine the percentage of the cells (5 mice in each group). (D) Decreased mRNAs of proinflammatory genes in *CCSP-Foxm1*^{-/-} mice. Total lung RNA was prepared 48 h after the last HDM challenge and examined by qRT-PCR. *, $P < 0.05$; **, $P < 0.01$. (E) ELISA shows decreased CCL11 protein in BALF of HDM-treated *LysM-Cre Foxm1*^{fl/fl} mice. (F) ChIP assay was performed in BEAS-2B cells. Endogenous FOXM1 protein specifically binds to the promoter regions of *Ccl11* and *Ccl24* genes (F).

kine receptors critical for recruitment of macrophages to injured tissues (59–61). CCR2 and CX3CR1 were decreased in FOXM1-deficient macrophages after CCL₄-mediated liver injury (35) and butylated hydroxytoluene (BHT)-mediated lung injury (33). Consistent with these previous studies, *Ccr2* and *Cx3cr1* mRNAs were reduced in FOXM1-deficient macrophages in an HDM

model, implicating FOXM1 in the regulation of these genes during allergen-mediated lung inflammation. Loss of FOXM1 was associated with decreases in GM-CSFR α and IL-1 α , both of which mediate macrophage maturation, migration, and survival (52, 62). Thus, FOXM1 may play a cell-autonomous role in macrophage recruitment to the lung tissue after allergen exposure (Fig. 10).

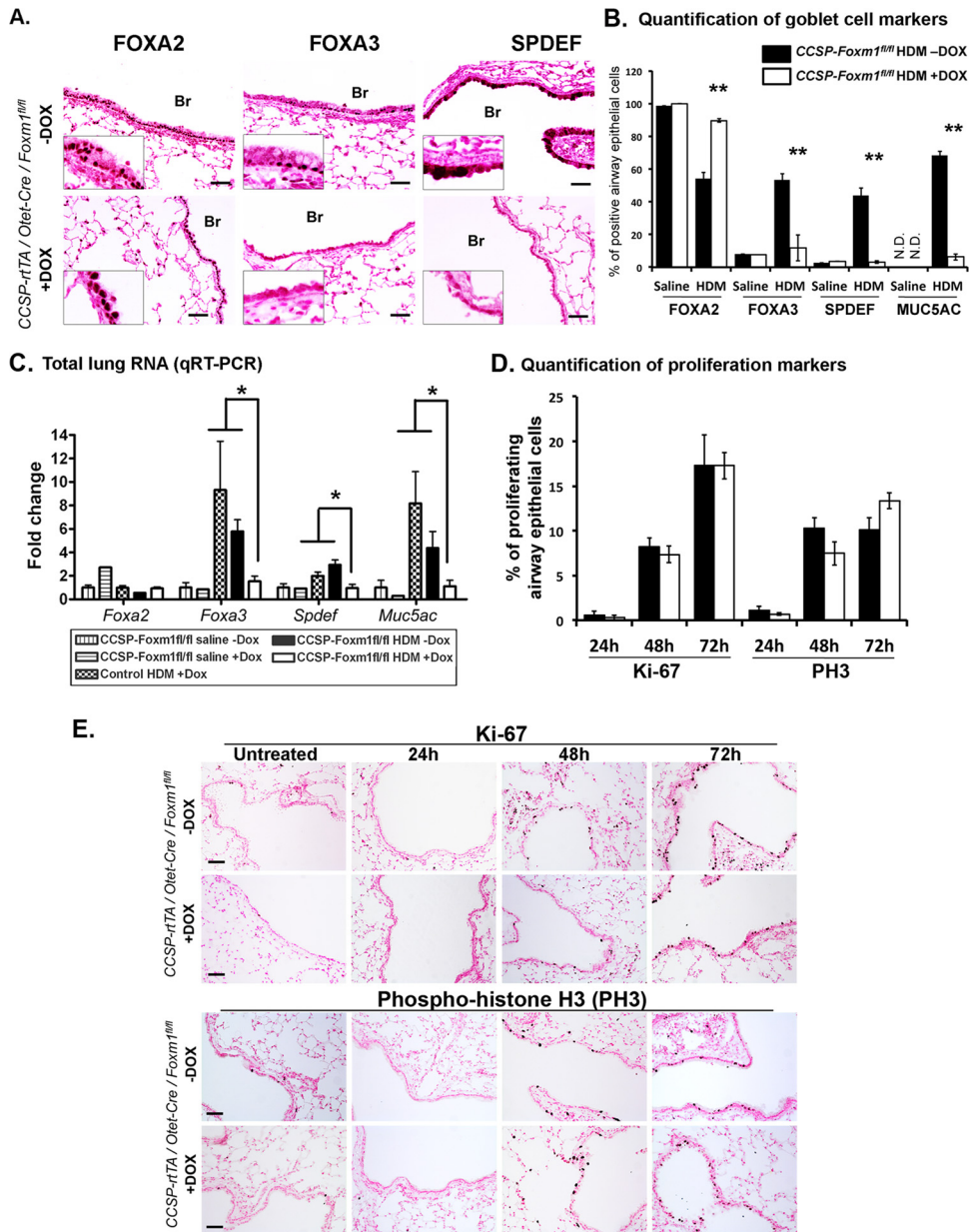


FIG 7 Decreased expression of SPDEF, FOXA3, and MUC5AC in FOXM1-deficient airway epithelium. (A to C) Mice were sacrificed and lungs were harvested 72 h after the last HDM challenge. SPDEF and FOXA3 are decreased in lung sections from HDM-treated *CCSP-Foxm1*^{-/-} mice. The number of goblet cells (B) and levels of mRNAs of Spdef, Foxa3, and Muc5ac are decreased in whole lung tissue of HDM-treated *CCSP-Foxm1*^{-/-} mice (C). mRNA levels were determined by qRT-PCR. (D and E) Cellular proliferation is not affected in HDM-treated *CCSP-Foxm1*^{-/-} mice. Proliferating cells were visualized using Abs against Ki-67 and PH3 (E). The number of proliferating cells is unaltered after deletion of FOXM1 (D). Ten random Ki-67-stained sections from 5 mice were counted for each group. *, *P* < 0.05; **, *P* < 0.01. Br, bronchiole. Bars, 50 μ m.

Specialized populations of DC are critical for antigen presentation and T-cell activation during the pathogenesis of asthma in mice and humans (63, 64). In the present study, FOXM1 was found in mDCs. Reduced Th₂ cytokines in lungs of *LysM-Cre Foxm1*^{fl/fl} mice suggest impaired mDC function in response to allergen stimulation, resulting in decreased activation of T cells. Since MHC II cell surface expression was reduced in FOXM1-deficient mDC, antigen presentation may be impaired by the deletion of FOXM1. In addition to the antigenic signal, FOXM1 may influence T-cell activation by inducing expres-

sion of CD86, a molecule providing second costimulatory signal to T cells (56, 57). Diminished activation of T cells by DCs may contribute to decreased lung inflammation in *LysM-Cre Foxm1*^{fl/fl} mice (Fig. 10).

Although eosinophils do not express FOXM1, reduced numbers of eosinophils were found in HDM-treated *LysM-Cre Foxm1*^{fl/fl} lungs, a finding associated with decreased IL-5 and eotaxins. Since eotaxins are produced by various cells types, FOXM1 may play an indirect or non-cell-autonomous role in eosinophilic recruitment in response to allergen stimulation. The findings that both eotaxin 1 and

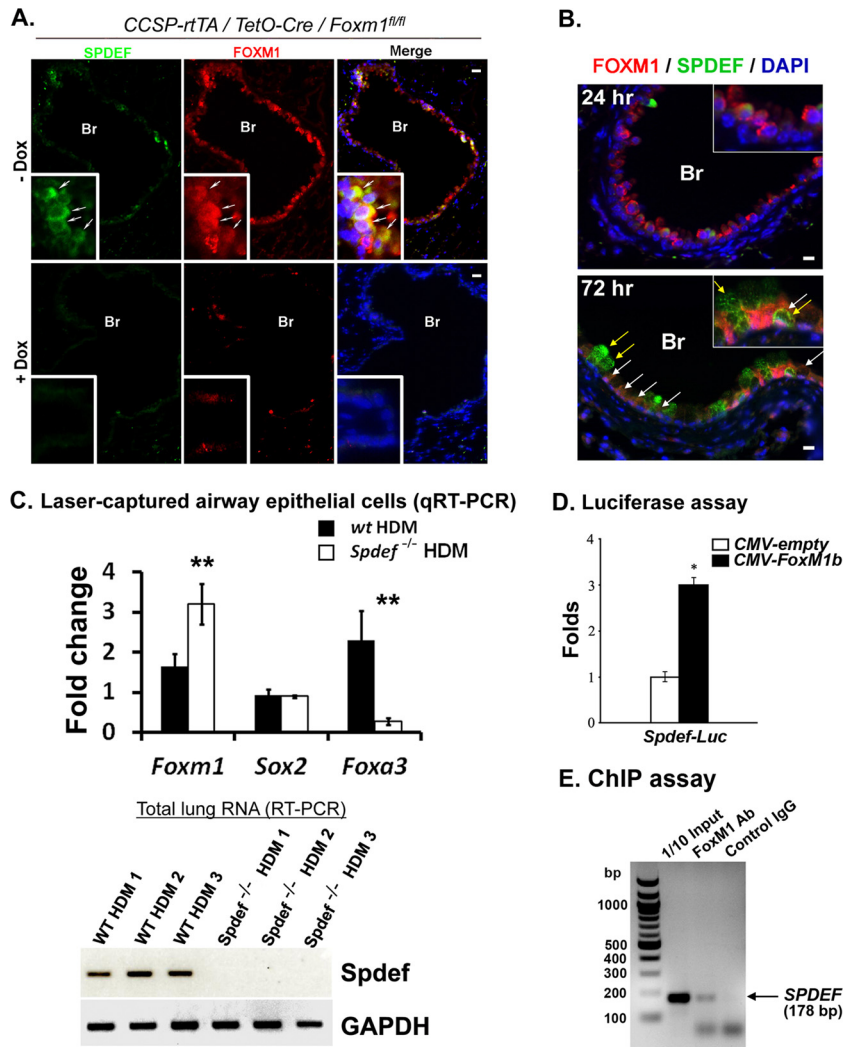


FIG 8 FOXM1 is required for induction of SPDEF. (A) FOXM1 coexpressed with SPDEF in airway epithelial cells of HDM-treated mice. *CCSP-Foxm1^{fl/fl}* and control mice were treated with HDM and harvested 24 h after the last HDM challenge. FOXM1 (red) colocalizes with SPDEF (green) in epithelial cells of control lungs (-Dox; top) but not in *CCSP-Foxm1^{fl/fl}* mice (+Dox; bottom). (B) FOXM1 is decreased in SPDEF-positive goblet cells at 72 h after the last HDM challenge (yellow arrows). FOXM1 expression is maintained in Clara cells (white arrows). HDM-treated WT mice were used for immunostaining. Br, bronchiole. (C) Increased Foxm1 mRNA in airway epithelial cells from *Spdef*^{-/-} mice. RT-PCR shows the absence of *Spdef* mRNA in *Spdef*^{-/-} lungs (bottom). Laser-capture microdissection of airway epithelial cells was performed 24 h after the last HDM treatment. Foxm1, Sox2, and Foxa3 mRNAs were measured in microdissected epithelial cells by qRT-PCR (top). (D and E) SPDEF is a direct transcriptional target of FOXM1. CMV-FoxM1b plasmid induced LUC activity driven by *Spdef* promoter after cotransfection in U2OS cells. CMV-empty plasmid was used as a control. Endogenous FOXM1 protein specifically binds to the promoter region of *Spdef* gene, as demonstrated by a ChIP assay (E). Bars, 20 μ m.

eotaxin 2 are reduced in FOXM1-deficient macrophages and neutrophils and that FOXM1 bound to *eotaxin* promoters suggest a direct regulation of *eotaxin* genes by FOXM1. Since IL-5 is produced by activated T cells (65), reduced T-cell activation by FOXM1-deficient DCs may contribute to reduced IL-5 levels and diminished eosinophilic inflammation in *LysM-Cre Foxm1^{fl/fl}* lungs (Fig. 10). Although HDM stimulation increased FOXM1 expression in neutrophils, their numbers were not altered by the deletion of FOXM1. These data suggest that FOXM1 is dispensable for recruitment of neutrophils to the lung tissue in HDM model, a finding consistent with previous studies showing the FOXM1-independent recruitment of neutrophils after CCL₄-mediated liver injury (35).

Interestingly, inflammatory cells and proinflammatory cytokines were reduced after deletion of FOXM1 from airway epithelial

cells. Published studies demonstrated that in response to allergen stimulation airway epithelial cells produce proinflammatory chemokines, such as CCL2 (MCP1), eotaxin-1 (CCL11) and eotaxin-2 (CCL24), that are critical for recruitment of macrophages and eosinophils to the lung (52, 54, 65). Therefore, decreased expression of these proinflammatory mediators can contribute to decreased inflammation in HDM-treated *CCSP-Foxm1^{fl/fl}* mice. In addition, decreased numbers of macrophages in HDM-treated *CCSP-Foxm1^{fl/fl}* lungs may lead to an impairment in antigen presentation and T cell activation, resulting in decreased production of Th2 cytokines and contributing to diminished airway resistance. Altogether, our results support the concept that FOXM1 induces allergen-mediated lung inflammation through both cell-autonomous and non-cell-autonomous mechanisms.

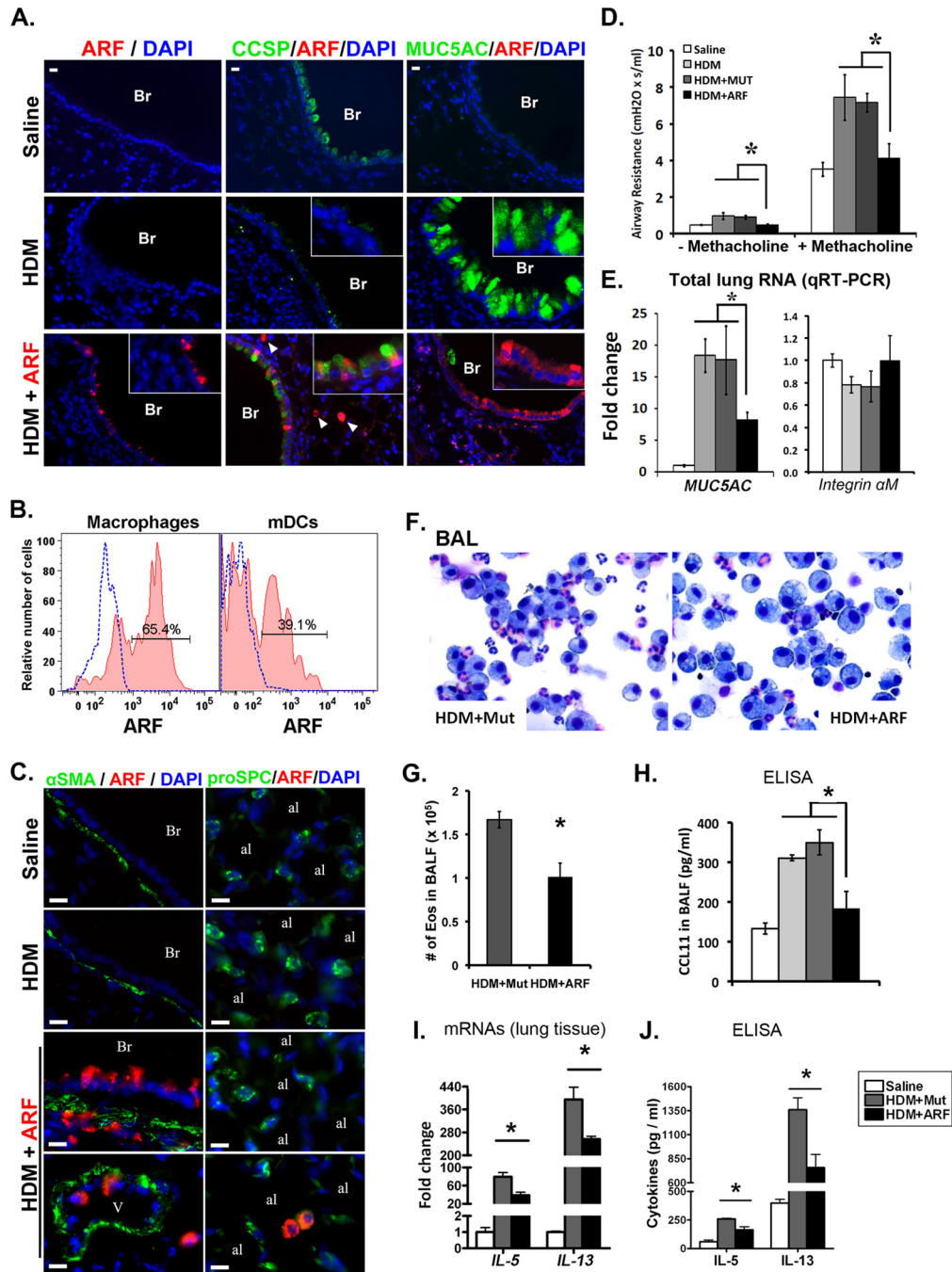


FIG 9 Inhibition of FOXM1 by ARF peptide prevents goblet cell metaplasia and reduced airway resistance in HDM-treated mice. BALB/c mice were subjected to intratracheal administration of HDM on days 0 and 14. ARF peptide or mutated ARF peptide (Mut) was given on days 13 and 15. Mice were sacrificed on day 16. ARF peptide reduced the number of MUC5AC-positive goblet cells and increased the number of CCSP-positive Clara cells (A). TMR fluorescence (red) shows the presence of ARF peptide in airway epithelium (A, insets), inflammatory cells (A, arrowheads; C, bottom) and peribronchial smooth muscle (C) of HDM-treated lungs. Flow cytometry shows TMR fluorescence in pulmonary macrophages and mDCs (B). (D) Airway resistance was decreased in ARF-treated mice compared to controls (7 mice per group). Concentration of methacholine was 6 mg/ml. (E) qRT-PCR shows a reduction in Muc5ac mRNA in ARF-treated lungs. Integrin αM mRNA was not changed. (F and G) ARF peptide decreased the number and the percentage of eosinophils in BALF. Five hundred cells were counted to calculate the percentage of eosinophils (5 mice in each group). (H to J) IL-5, IL-13, and CCL11 were measured in BALF by ELISA (H and J) and in total lung RNA by qRT-PCR (I). *, $P < 0.05$. Br, bronchiole; Ve, vessel; al, alveoli. Bars, 20 μm (A) and 10 μm (C).

FOXM1 induces goblet cell metaplasia after Th₂ stimulation. Goblet cell metaplasia is a prominent feature of asthma, yet there is incomplete knowledge of transcriptional programs that influence airway epithelial cell differentiation leading to the accumu-

lation of goblet cells, and increased mucus production in asthmatic airways. In the present study, we found that FOXM1 influences goblet cell metaplasia through multiple mechanisms. First, FOXM1 increases antigen presentation by DCs, leading to

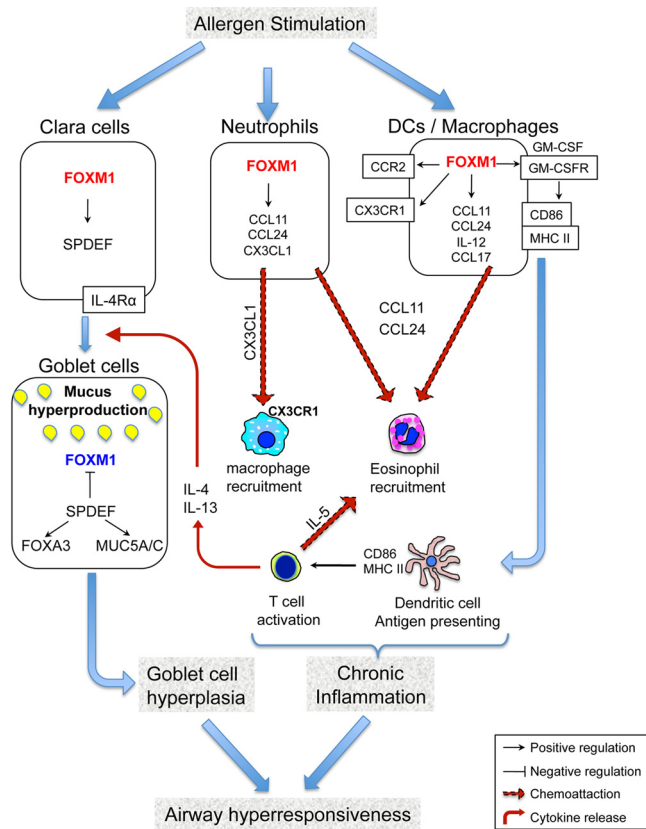


FIG 10 Model of FOXM1-induced goblet cell metaplasia and allergen-mediated lung inflammation. FOXM1 induces differentiation of Clara cells into goblet cells through direct transcriptional activation of *Spdef*. SPDEF inhibits FOXM1 in differentiated goblet cells. FOXM1 increases the cell surface expression of MHC II and CD86 in mDCs, leading to increased activation of T cells and increased production of IL-4, IL-5, and IL-13. FOXM1 recruits eosinophils and macrophages into the lung tissue, at least in part, by activating eotaxins, CCR2, CX3CR1, IL-5, and CX3CL1.

increased production of IL-4 and IL-13 by activated T cells. IL-4 and IL-13, acting through multiple signaling pathways, including IL-4R receptors/Stat-6, induce goblet cell differentiation in response to various allergens (66–69). Thus, diminished T-cell activation and the reduction of IL-4 and IL-13 in HDM model may impair goblet cell differentiation, leading to decreased goblet cell metaplasia in FOXM1-deficient airways. Second, we found that FOXM1 directly induces goblet cell metaplasia through transcriptional activation of *Spdef*, a critical regulator of mucus production and goblet cell differentiation. SPDEF transcription factor is both necessary and sufficient to induce goblet cell differentiation in mice and is induced in goblet cells in lungs of individuals with asthma, COPD, and CF (13, 14). Transgenic expression of SPDEF in Clara cells induced their differentiation into goblet cells, whereas genetic ablation of *Spdef* gene prevented goblet cell metaplasia after Th₂ stimulation (13, 14). SPDEF induces goblet cell differentiation in association with the expression of *Foxa3*, *Muc5ac*, and *Arg2* and reduction of expression of genes associated with Clara cell phenotype, including *Tf1* and *Foxa2* (13). In the present study, genetic deletion of *Foxm1* was sufficient to decrease SPDEF in airway epithelial cells. Thus, reduced levels of SPDEF may contribute to attenuated goblet cell metaplasia in FOXM1-

deficient airways. Interestingly, expression of FOXM1 was significantly decreased after upregulation of SPDEF in airway epithelium, suggesting a negative feedback mechanism by which SPDEF can counterregulate FOXM1. Consistent with the inhibitory effect of SPDEF on FOXM1, airway epithelial cells of *Spdef*^{-/-} mice exhibited increased *Foxm1* mRNA after HDM exposure. Taken together, present findings demonstrate that FOXM1 regulates signaling and transcriptional networks critical for differentiation of goblet cells in response to allergen exposure (Fig. 10).

Pharmacological inhibition of FOXM1 is a promising therapeutic approach in asthma treatment. The present study demonstrated that inhibition of FOXM1 by ARF peptide or genetic inactivation of *Foxm1* gene reduces the severity of lung responses to HDM. The ARF peptide effectively inhibited both goblet cell metaplasia and eosinophilic inflammation, decreasing airway resistance in HDM-treated mice. Previous studies showed that a membrane-penetrating ARF peptide specifically binds to FOXM1 with high affinity, sequestering the ARF peptide/FOXM1 complex inside the cell and inhibiting FOXM1 transcriptional activity (21, 42, 58). In the present study, we found that the ARF peptide decreased IL-5, IL-13, and CCL11 after HDM stimulation, findings consistent with reduced expression of these genes in FOXM1-deficient mice. Inhibition of FOXM1 in inflammatory cells may account for decreased levels of proinflammatory cytokines and reduced airway resistance in response to HDM. These results also suggest that pharmacological inhibition of FOXM1 may be beneficial for treatment of asthma in human patients. Although increased permeability and disruption of alveolar barriers were found in mice with a conditional inactivation of *Foxm1* from either endothelial cells (70) or alveolar type II cells (32), we did not observe pulmonary edema and lung inflammation in the alveolar regions of ARF-treated mice. In the present study, ARF peptide accumulates in airway epithelial cells, peribronchial interstitium, and macrophages, causing local inhibition of FOXM1 in HDM-treated airways.

In summary, FOXM1 was required for the induction of goblet cell metaplasia, mediated in part via direct transcriptional activation of *Spdef*. FOXM1 promoted antigen presentation by increasing the cell surface expression of MHC II and CD86 in dendritic cells, causing activation of T cells and increased production of Th₂ cytokines. FOXM1 induces recruitment of eosinophils and macrophages by activating eotaxins, IL-5, CCR2, and CX3CR1. Altogether, FOXM1 is critical for goblet cell metaplasia and lung inflammation in response to allergen stimulation.

ACKNOWLEDGMENTS

We thank Craig Bolte for helpful comments, Li Yang and J. Snyder for technical assistance, and Ann Maher for secretarial support.

This work was supported by NIH grants HL84151 (to V.V.K.), CA142724 (to T.V.K.) HL095580, and U01-HL110964 and by the Cystic Fibrosis Foundation Research Development Program (to J.A.W.).

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