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Reduced allergic lung inflammation and airway responsiveness in mice lacking the cytoskeletal protein gelsolin

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Abstract

Airway smooth muscle hyperresponsiveness associated with chronic airway inflammation leads to the typical symptoms of asthma including bronchoconstriction and wheezing. Asthma severity is associated with airway inflammation; therefore, reducing airway inflammation is an important therapeutic target. Gelsolin is an actin capping and severing protein that has been reported to be involved in modulation of the inflammatory response. Using mice genetically lacking gelsolin, we evaluated the role of gelsolin in the establishment of house dust mite (HDM) antigen-induced allergic lung inflammation. The genetic absence of gelsolin was found to be protective against HDM sensitization, resulting in reduced lung inflammation, inflammatory cytokines, and Muc5AC protein in bronchoalveolar lavage (BAL) fluid. The number of eosinophils, lymphocytes, and interstitial macrophages in the BAL were increased after HDM sensitization in wild-type mice but were attenuated in gelsolin-null mice. The observed attenuation of inflammation may be partly due to delayed migration of immune cells, because the reduced eosinophils in the BALs from gelsolin-null mice compared with controls occurred despite similar amounts of the chemoattractant eotaxin. Splenic T cells demonstrated similar proliferation rates, but ex vivo alveolar macrophage migration was delayed in gelsolin-null mice. In vivo, the reduced lung inflammation after HDM sensitization in gelsolin-null mice was associated with significantly diminished airway resistance to inhaled methacholine compared with HDM-treated wild-type mice. Our results suggest that modulation of gelsolin expression or function in selective inflammatory cell types that modulate allergic lung inflammation could be a therapeutic approach for asthma.

Keywords: cytokine, flexiVent, house dust mite, macrophage

INTRODUCTION

Asthma is a chronic respiratory disease characterized by lung inflammation and airway smooth muscle hyperresponsiveness. It affects over 8% of the US population, with an increased incidence in children and those from disadvantaged backgrounds. Acute relaxation of contracted airways is important for alleviating the acute symptoms of asthma such as wheezing and shortness of breath, but controlling chronic airway inflammation can prevent the occurrence of these acute exacerbations. Persistent inflammation modulated by immune responses results in structural airway changes, including epithelial cell denudation, epithelial cell mucus hyper/metaplasia and smooth muscle hypertrophy, hyperplasia, and hyperresponsiveness (15). We (28) have previously reported that the global genetic deletion of the actin capping and severing protein

gelsolin results in impaired airway smooth muscle relaxation. These observations suggest that a modification of the expression or function of gelsolin in airway smooth muscle cells could be therapeutic approach for bronchoconstrictive diseases such as asthma. In the current study, we sought to investigate the role of gelsolin in another pathophysiologic component of asthma: allergic lung inflammation induced by house dust mite (HDM) intranasal sensitization in mice.

In addition to its well-known actin capping and severing activity, gelsolin is reported to be involved in modulation of the inflammatory response. Gelsolin is functionally expressed intracellularly, but an isoform arising from the same gene contains an additional 25 amino acids at the NH_2 terminus and is found extracellularly as plasma gelsolin in serum, cerebrospinal fluid, and urine (23). Plasma gelsolin binds to lipoteichoic acid (LTA) and lipopolysaccharide (LPS) from various bacteria strains with high affinity and neutralizes their effects on cellular functions (6). Plasma gelsolin prevented interleukin (IL)-8 release from human neutrophils if added before LPS or LTA treatment, presumably acting as an LPS/LTA buffer (4). Plasma gelsolin also acts as "inflammation dampener" by binding to platelet-activating factor and interferes with downstream platelet activation and oxygen radical generation in neutrophils in vitro (32). Exogenous administration of recombinant gelsolin in vivo was also reported to exert anti-inflammatory activities by reducing tumor necrosis factor-α (TNF-α) and IL-6 levels in plasma after carrageenan injection to mice paws (18). These anti-inflammatory properties of gelsolin were demonstrated by administering exogenous plasma gelsolin and not by modifying endogenous gelsolin (intracellular and/or plasma gelsolin) expression. Using gelsolin-null mice, which lack both intracellular and plasma gelsolin proteins, delayed in vivo neutrophil migration to an intraperitoneal inflammatory site was observed (48). Gelsolin-null mice also showed reduced lung injury and diminished cytokine expression in ventilator-induced lung injury (24), suggesting gelsolin's possible role in other types of lung inflammation.

Gelsolin's role in allergic lung inflammatory diseases such as asthma has not been well characterized. It was shown that IL-4-treated human airway epithelial cells secrete gelsolin and increased gelsolin protein was demonstrated in the bronchoalveolar lavage (BAL) from patients with asthma (7); however, the possible role of gelsolin in the pathogenesis of allergic lung inflammation needs to be elucidated. We utilized gelsolin-null mice and control wild-type mice that were subjected to a well-established mouse model of allergic lung inflammation by intranasal HDM antigen administration, and tested our hypothesis that the actin capping and severing protein gelsolin modulates the establishment of allergic lung inflammation.

METHODS

Animals. All animal studies were approved by the Columbia University Institutional Animal Care and Use Committee. Wild-type C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). An established colony of mice on a mixed background (C57BL/6 and BALB/c) globally genetically deficient in gelsolin protein expression (48) were originally developed and kindly shared by Dr. David J. Kwiatkowski (Harvard Medical School, Boston, MA). The genetic background of our existing colony was confirmed to be predominantly C57BL/6 (28). Both male and female mice between 10 and 16 wk of age were used for experiments.

Allergic lung inflammation. A mouse asthma model of house dust mite (HDM) sensitization was utilized to induce allergic lung inflammation. Under brief 2–5% isoflurane anesthesia, mice received either intranasal HDM extract (30 μg) (Stallergenes Greer; Greer Laboratories, Lenoir, NC) dissolved in PBS (25 μL) or PBS alone, once a day for 5 days over either a 2- or 3-wk period.

Collection of BAL fluid. Mice were euthanized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg), and a tracheostomy was performed. After placement of an 18-gauge metal tracheal cannula, BAL was performed by slow instillation and withdrawal of 1 mL of PBS to the lungs twice. The two aliquots of retrieved BAL were combined and centrifuged at 200 *g* for 5 min, and the supernatant was transferred to new tubes and stored at −80°C for later use. The remaining cell pellet from the BAL fluid was resuspended in ice-cold PBS, stained with trypan blue, and counted using a standard hemocytometer. For the macrophage migration and polarization assays, BAL cells from nonsensitized mice were used. BAL was performed by instillation and withdrawal of 1 mL of PBS with 5 mM EDTA to mouse lungs five times, and the approximate 5 mL of collected BAL fluid was pooled and centrifuged at 200 *g* for 5 min.

The cell pellet was resuspended in 1 mL RPMI 1640 medium, supplemented with 10% FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, 10 μM sodium pyruvate, and 50 μM 2-mercaptoethanol, and plated on a 24-well plate. After a 4 h incubation, floating cells were removed by aspirating and adding 0.5 mL RPMI medium twice, and the adherent cells were used for assays. Typically 0.3–0.4 million cells were collected in BAL from a nonsensitized mouse.

Flow cytometry analysis of BAL cells for macrophage cell count. After 10 days of intranasal PBS or HDM treatment, the collected BAL cells were stained overnight at 4°C in 0.2% bovine serum albumin in PBS containing 1:100 dilution of the following antibodies (BD Biosciences): PE-CF594 rat anti-mouse CD45, PerCP-Cy5.5 rat anti-mouse CD11b, FITC hamster anti-mouse CD11c, and PE rat anti-mouse Siglec-F. A BD LSR II flow cytometer was used for analytical cell counts, and FlowJo software (FlowJo LLC, Ashland, OR) was used for data analysis.

Measurement of total and Muc5AC protein in BAL fluid. Protein concentration in the BAL supernatant was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA), following the manufacturer's instructions. Muc5AC concentration was measured using a mouse Muc5AC ELISA kit (LS-F26592; LifeSpan BioSciences, Inc., Seattle, WA).

Lung cytokine ELISA. Whole left lungs were dissected out and rinsed briefly in PBS and homogenized using an Ika Ultra-turrax disperser T-25 (top speed 20 s, in ice) in 1 mL lysis buffer (Cell Signaling Technology, Inc., Danvers, MA) containing 1% Triton, 20 mM Tris (pH7.5), 150 mM NaCl, 1 mM Na_2 EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 μg/mL leupeptin, and freshly added protease inhibitor cocktail (Complete; Millipore Sigma, Burlington, MA). The lung homogenate samples were centrifuged at 14,000 *g* for 10 min at 4°C, and 50 μL of the resulting lung digest supernatant was used to measure 23 cytokines using a Bio-Plex Pro Mouse Cytokine 23-plex Assay kit (Bio-Rad Laboratories, Inc., Hercules, CA) according to the manufacturer's instructions. The sample data were acquired using the Bio-Plex MAGPIX Multiplex Reader and analyzed using Bio-Plex Manager Software (Bio-Rad Laboratories, Inc.).

Lung histology. The dissected right lung was fixed in 10% formalin (Fisher Diagnostic, Thermo Fisher Scientific, Waltham, MA) for 24 h, dehydrated through serial ethanol concentrations, and embedded in paraffin. Slices (6-μm) were stained with hematoxylin and eosin or underwent the Periodic Acid-Schiff (PAS) staining procedure. Images were obtained with an upright microscope (Nikon, Tokyo, Japan). The treatment group for each section was masked, and histopathological scoring of allergic lung inflammation was performed by two blinded observers separately and averaged to obtain the final scores. The score was determined from cellular infiltration around both blood vessels and airways as described previously (35), with 0 being lowest and 8 being highest scores.

Cell proliferation assay. C57BL/6J or gelsolin-null mice were euthanized with an intraperitoneal injection of pentobarbital sodium (100 mg/kg), and the spleens were collected in PBS containing 2× antibiotic and antimycotic (GIBCO, Thermo Fisher Scientific). The spleen tissue was minced with scissors and pushed through a 40-μm sterile cell strainer. The resulting splenocyte suspensions in 3 mL of PBS were layered on 3 mL of Histopaque 1083 (Millipore Sigma) and centrifuged at 400 *g* for 30 min at room temperature. The cloudy middle layer was transferred to a new conical tube and was washed twice with 10 mL of PBS and centrifuged at 250 *g* for 10 min. Collected splenocyte numbers were manually counted using a standard hemocytometer. Splenocytes were resuspended as single-cell suspensions in PBS (20×10^6 cells/mL), and Violet Proliferation Dye 450 (BD Biosciences, San Jose, CA) was added at a final concentration of 1 μM. The dye-cell suspension was incubated at 37°C for 15 min in the dark, washed with PBS and then with RPMI 1640 medium (GIBCO, Thermo Fisher Scientific) with 10% FBS, and resuspended in RPMI 1640 medium supplemented with 10% FBS, 30 U/mL rIL-2, and 15 μM 2-mercaptoethanol, at 1×10^6 cells/mL. The splenocytes were activated by incubation with Dynabeads Mouse T-Activator CD3/CD28 (GIBCO, Thermo Fisher Scientific) according to the manufacturer's instructions. Briefly, 12.5 μL of magnetic beads were added to a 1-mL cell suspension in medium (1×10^6 cells/mL) and incubated in 24-well culture dishes for 72 h. The culture dish was rotated every 24 h for even mixing. At the end of the incubation, cells were labeled with FITC rat anti-mouse CD4 and APC rat anti-mouse CD8a (BD Biosciences). Cell labeling was analyzed using the BD LSR II flow cytometer and FlowJo software (FlowJo LLC, Ashland, OR) was used for data analysis.

Macrophage migration assay. After overnight culture in a 37°C humidified incubator (95% air-5% CO_2), adherent BAL cells were washed with warm PBS followed by incubation in 0.5 mL cold PBS for 10 min on ice, detached, and collected by vigorous pipetting. Our preliminary experiments showed that ~99% of the resulting cells were CD11b and F4/80 double positive and CD68 positive by flow cytometry analysis confirming a macrophage phenotype (data not shown). Chemotaxis of the isolated macrophages in BAL was measured using ChemoTx Chemotaxis System (pore size 5 μm; Neuro Probe, Inc., Gaithersburg, MD). The lower chamber was filled with RPMI 1640 with 10 ng/mL macrophage inflammatory protein-1α (MIP-1α), and 5×10^4 macrophages per well in RPMI 1640 were placed into the upper chamber, and cells were incubated at 37°C in 5% CO_2 for 1 h. Migrated macrophages were stained using a Diff-Stain kit (IMBE Inc., San Marcos, CA) and manually counted, and the chemokine-induced migration ratio was calculated.

Macrophage polarization. Adherent BAL cells were cultured in supplemented RPMI medium with 100 ng/mL lipopolysaccharide (LPS from *Escherichia coli* O55:B5, Millipore Sigma) plus 20 ng/mL interferon-γ (INF-γ, eBioscience) for M1 or with 20 ng/mL IL-4 (eBioscience) for M2 polarization for 16 h (25). Polarization of cells was assessed by flow cytometry for expression of the M1 marker CD38, or M2 marker early growth response protein 2 (Egr2) (20).

Respiratory system mechanics measurement. After being anesthetized with 50 mg/kg intraperitoneal pentobarbital sodium, mice were tracheotomized with an 18-gauge metal cannula and mechanically ventilated with room air at 150 breaths/min with a tidal volume of 10 mL/kg and a positive end-expiratory pressure of 3 cmH_2O using a flexiVent FX1 system (SCIREQ, Montreal, QC, Canada). After baseline measurements, pressure-volume loops were obtained using a ramp-style pressure-driven maneuver. Then, airway responsiveness to nebulized methacholine (MCh) was obtained using the forced oscillation technique. Increasing concentrations of MCh (0, 6.25, 12.5, 25, 37.5, and 50 mg/mL) in PBS were delivered using an ultrasonic nebulizer (Aeroneb Laboratory ANP-1000, pore size 4.0–6.0 μm) with an administration time of 10 s and a 50% duty cycle.

Statistics. Statistical analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, CA). Differences between two groups were tested using Mann–Whitney analyses. Multiple comparisons were tested using ANOVA with the Bonferroni multiple comparisons test. Data are expressed as means with SD, or individual data points with group means, and two-sided P values of <0.05 were considered significant.

RESULTS

Histological examination of lung sections from gelsolin-null mice demonstrated reduced infiltration of mononuclear cells surrounding bronchovascular bundles compared with wild-type mice following 3 wk of intranasal HDM antigen (Fig. 1, *A* and *B*). The total number of cells collected in BALs were significantly increased in HDM-treated wild-type mice compared with vehicle-treated mice, but BAL cell counts were not significantly increased in HDM-treated gelsolin-null mice (Fig. 1*C*). Total protein concentrations in BAL were also increased in wild-type mice after HDM compared with vehicle-treated controls, but BAL protein was not increased by HDM in gelsolin-null mice, and gelsolin-null mice exhibited significantly lower protein concentrations after HDM compared with wild-type mice (Fig. 1*D*). Among various proteins secreted into BAL fluid, polymeric mucins are a component of mucus that contributes to airway obstruction in allergic lung inflammation (3). Mucin 5, subtypes A and C (Muc5AC protein), one of the secreted polymeric mucins and the chief glycoprotein component of airway mucus, is a central effector of allergic lung inflammation and plays a role in airway responsiveness to methacholine (14). In our mouse model of allergic lung inflammation induced by intranasal HDM administration, wild-type mice displayed significantly higher Muc5AC protein concentrations in BALs compared with control mice treated with intranasal PBS. These increases in BAL Muc5AC protein concentrations were not observed in HDM-sensitized gelsolin-null mice (Fig. 1*E*). Consistent with these quantitative findings, Periodic Acid-Schiff (PAS) staining of airways from HDM-sensitized gelsolin-null mice demonstrated less mucus staining compared with PAS-stained airways from HDM-sensitized wild-type mice (Fig. 1*F*).

Allergic lung inflammation is mediated by the secretion of an array of cytokines, with their concentrations associated with the severity of asthma (19). We compared the quantity of various cytokines in lung digests from wild-type mice with gelsolin-null mice treated with intranasal HDM or vehicle (PBS). The T helper cell type 2 (Th2) cytokines IL-4, -5, -9, and -13 concentrations were significantly higher in HDM-sensitized wild-type mice compared with vehicle-treated wild-type controls, and the concentrations of IL-5, -9, and -13 were significantly reduced in HDM-sensitized gelsolin-null mice compared with HDM-sensitized wild-type mice (Fig. 2, *A–D*). IL-17A, which is associated with severe refractory asthma (19), was significantly decreased in HDM-treated gelsolin-null mice compared with HDM-treated wild-type mice (Fig. 2*E*). Other proinflammatory cytokines, including TNF-α and IL-1β and the chemokine monocyte chemoattractant protein (MCP)-1, which is increased in patients with asthma (21), were also significantly decreased in HDM-sensitized gelsolin-null mice compared with HDM-treated wild-type mice (Fig. 2, *F–H*). IL-1β was significantly increased in gelsolin-null mice after HDM sensitization compared with vehicle-treated gelsolin-null controls, but the concentration of IL-1β was still significantly attenuated compared with HDM-treated wild-type mice (Fig. 2*G*). Eotaxin, a potent and eosinophil-specific chemoattractant that is produced mainly by epithelial cells of the lung and gastrointestinal tract, was significantly increased to similar levels after HDM sensitization in both wild-type and gelsolin-null mice (Fig. 2*I*).

Given the similar amount of eotaxin in the lungs of wild-type and gelsolin-null mice after HDM sensitization, we sought to measure BAL differential cell counts to determine the cell types collected in the BAL. The total number of cells in BALs after 2 wk of intranasal HDM were again significantly increased in wild-type but not in gelsolin-null mice compared with their intranasal PBS controls (Fig. 3*A*). Lymphocytes in BALs were also significantly increased in HDM-treated wild-type mice compared with vehicle wild-type controls, but HDM-treated gelsolin-null mice showed less increase compared with HDM-treated wild-type controls (Fig. 3*B*). Despite the similar amount of eotaxin detected in the lungs of wild-type and gelsolin-null mice after intranasal HDM treatment, eosinophils were significantly increased in the BALs of wild-type mice but not in the BALs of gelsolin-null mice after HDM sensitization (Fig. 3*C*). Macrophages expressing interstitial macrophage marker profiles (CD11b high expression, CD11c positive, and Siglec F negative) were significantly increased in the BALs from both gelsolin-null and wild-type mice treated with HDM compared with PBS controls (Fig. 3*D*), but interstitial macrophages were significantly decreased in the BALs of HDM-treated gelsolin-null compared with HDM-treated wild-type mice. In contrast, while HDM treatment increased alveolar macrophages (CD11b negative, CD11c positive, and Siglec F positive) in both gelsolin-null and wild-type mice compared with PBS controls, alveolar macrophages were not significantly different in HDM-treated wild-type compared with HDM-treated gelsolin-null mice (Fig. 3*E*).

Gelsolin is an actin-binding protein, and its absence may affect cellular functions such as cell locomotion (22, 40) and cell proliferation (9, 10) by altering the assembly and/or disassembly of actin filaments. The observed reduced lung inflammation and reduction in the number of BAL cells in gelsolin-null mice following HDM sensitization could be the result of either reduced immune cell proliferation and/or migration. CD4-positive or CD8-positive T cells collected from the spleens of wild-type or gelsolin-null mice showed similar cell proliferation profiles in vitro when stimulated with anti-CD3 and anti-CD28 antibodies (Fig. 4, *A* and *B*). However, ex vivo migration of alveolar macrophages isolated from nonsensitized gelsolin-null mice toward a chemoattractant (i.e., MIP-1α) was significantly decreased compared with alveolar macrophages isolated from wild-type mice (Fig. 4*C*). We next evaluated macrophage polarization, as actin/myosin contractility was reported to influence macrophage polarization in vitro (27). We reasoned that the absence of gelsolin may affect macrophage polarization and its phenotypic change, which could affect the progression of the inflammatory state. We observed that alveolar macrophages collected from gelsolin-null mice and stimulated in culture with LPS and INF-γ showed a reduced polarization to the M1 phenotype compared with alveolar macrophages isolated from wild-type mice (Fig. 4*D*). In contrast, the amount of M2 polarized macrophages after stimulation with IL-4 was not different between wild-type and gelsolin-null mice (Fig. 4*E*).

We measured airway mechanics in vivo using flexiVent to determine whether the reduced inflammation in gelsolin-null mice correlated with reduced airway hyperreactivity. Baseline respiratory mechanics of unsensitized naïve wild-type and gelsolin-null mice are shown in Fig. 5, *A–D*. There was no difference in

baseline central airway resistance or tissue resistance between wild-type and gelsolin-null mice (Fig. 5, *A* and *B*). Baseline dynamic compliance was higher in gelsolin-null mice and tissue elastance was lower in gelsolin-null mice (Fig. 5, *C* and *D*). After 3 wk of intranasal PBS control or HDM sensitization, pressure-volume (PV) curves of wild-type and gelsolin-null mice were similar (Fig. 5, *E* and *F*). Baseline area enclosed by the PV loop, which provides an estimate of the amount of atelectasis, was also similar in all groups (Fig. 5*G*). Static compliances calculated from PV loops showed increased compliance in HDM-treated gelsolin-null mice compared with HDM-treated wild-type mice (Fig. 5*H*). Following the acquisition of these baseline pulmonary mechanics measurements, mice were challenged with nebulized MCh to determine whether lung resistances in the presence of a bronchoconstrictive challenge were different. After HDM sensitization, gelsolin-null mice exhibited significantly less central airway resistance in response to increasing concentrations of nebulized methacholine compared with wild-type mice (Fig. 5*I*).

DISCUSSION

The primary finding of the current study is that mice globally deficient in gelsolin demonstrate reduced airway responsiveness to inhaled methacholine, after lung sensitization with intranasal HDM antigen. Gelsolin-null mice showed reduced lung inflammation induced by intranasal chronic application of HDM compared with wild-type mice, as assessed by lung histology, BAL cell counts, BAL protein and Muc5AC concentrations, PAS staining, and cytokine concentrations in the lung. Alveolar macrophages isolated from nonsensitized gelsolin-null mice demonstrated reduced ex vivo migration and M1 polarization compared with alveolar macrophages isolated from nonsensitized wild-type mice suggesting a critical role for intracellular gelsolin in macrophage functions.

Gelsolin's role in lung inflammation has not been fully investigated and is complex due to intracellular and extracellular effects on actin. Extracellular gelsolin, present in extracellular fluid, is considered a therapeutic mucolytic agent to decrease viscoelasticity of cystic fibrosis sputum (5, 34). Intracellular gelsolin caps and severs actin filaments, thus affecting a host of cellular functions modulated by the cytoskeleton. The current study is the first demonstrating gelsolin's role in the modulation of allergic lung inflammation. After HDM sensitization, wild-type mice developed lung inflammation with increased T helper cell type 2 (Th2) cytokines in the lung, which play critical roles in asthma (16). The concentrations of IL-4 in the lungs were lower than other Th2 cytokines, as previously reported in murine allergic airway disease (29). IL-13 is considered more important than IL-4 for induction of airway hyperreactivity and chronic remodeling changes (46), and it was significantly decreased in gelsolin-null compared with wild-type mice following HDM sensitization. In addition, IL-4 may signal more effectively at low concentrations (16); therefore, small changes in IL-4, which did not reach statistical significance between HDM-treated gelsolin-null and wild-type mice in the current study, might still have resulted in altered lung inflammation. Nonetheless, overall, Th2 cytokines were not significantly increased in gelsolin-null mice after HDM lung sensitization and reduced in gelsolin-null mice compared with wild-type mice after HDM lung sensitization. IL-17A, which has been reported to exacerbate Th2 cell-mediated eosinophilia in the airway (44) and hyperreactivity (47) in mice, was significantly increased in HDM-sensitized wild-type mouse lungs but not in gelsolin-null mice. In addition, monocyte chemoattractant protein (MCP)-1, which is primarily secreted by monocytes, macrophages, and dendritic cells and recruits monocytes, memory T cells, and dendritic cells to the site of inflammation (26), was elevated in lung digest after HDM sensitization in wild-type but significantly decreased in gelsolin-null mice compared with the wild-type. Interestingly, there was no difference in the amount of eotaxin in the lungs between the two groups after HDM treatment; however, the number of eosinophils collected in BALs were significantly increased in wild-type but not in gelsolin-null mice. This result suggests that, even in the presence of the same amount of chemoattractant, eosinophil recruitment to the lungs was reduced in gelsolin-null mice. Our findings of reduced lung inflammation in gelsolin-null mice during allergic sensitization agree with a prior study that showed reduced lung injury and BAL cytokine expression in a ventilator-induced lung injury model using gelsolin-null mice (24).

The mechanisms underlying the observed reduction of HDM-induced lung inflammation in gelsolin-null mice could be multifactorial. Rearrangement of the cytoskeleton is a prerequisite for a variety of cellular processes, and gelsolin is one of many cytoskeletal regulatory proteins that play roles in these cellular events. Gelsolin belongs in the gelsolin superfamily of proteins that are known to control actin

organization by severing actin filaments and capping the filament's end (38). Dynamic actin assembly and disassembly is important for cellular functions related to cell shape and movement including contraction and migration. The actin-binding protein gelsolin and several other actin-binding and -severing/depolymerizing proteins can enhance disassembly of actin filaments and promote actin cytoskeletal reorganization (30). In the absence of gelsolin, modified dynamic disassembly of actin can alter cell movements, as previously shown in neutrophils and gingival fibroblasts (2).

In the current study, we observed delayed migration of lung alveolar macrophages isolated from gelsolin-null versus wild-type mice. After HDM sensitization, the number of alveolar and interstitial macrophages collected in BALs was significantly increased in both wild-type and gelsolin-null mice compared with unsensitized mice. There was no difference between wild-type and gelsolin-null mice in the number of alveolar macrophages in alveolar space, possibly due to slow and minimal replenishment of alveolar macrophage by circulating monocytes (17, 41) and unchanged local proliferation of resident macrophages in gelsolin-null mice. In contrast, the number of interstitial macrophages after HDM sensitization was significantly higher in wild-type compared with gelsolin-null mice. This could be due to delayed recruitment of circulating monocytes to the site of lung inflammation in gelsolin-null mice, as subsets of interstitial macrophages originate from circulating monocytes (17). Although we did not test migration of interstitial macrophages or circulating monocytes, based on previous report on neutrophils (48), bone marrow-derived osteoclasts (8), and our ex vivo alveolar macrophage migration study, it is likely that impaired migration of inflammatory cells plays a role in the attenuated inflammation observed in gelsolin-null mice.

We next questioned whether the reduced numbers of inflammatory cells and cytokines after HDM sensitization in gelsolin mice could be related to alterations in cell proliferation modulated by gelsolin. We did not detect any difference in CD4+ or CD8+ T cell CD3/CD28-induced proliferation among splenocytes recovered from wild-type versus gelsolin-null mice. We interpret these results to indicate that the reduction of inflammatory cells in the lungs is not due to impaired resident immune cell proliferation but rather is due to impaired migration of inflammatory cells into the lung during allergic sensitization. However, increased cell proliferation was reported when gelsolin protein was overexpressed by transfection (9, 10) using cancer cell lines. In our study, we measured proliferation of primary lymphocytes freshly isolated from mice, which may possess significantly different phenotypes and cell signaling events compared with cancer cell lines overexpressing the gelsolin protein.

In addition to delayed cell migration, the observed attenuation of HDM-induced lung inflammation in gelsolin-null mice could also be due to reduced cytokine production and/or secretion, because actin assembly is also involved in immune cell activation, membrane transport, and cytokinesis (37, 45). While gelsolin is the founding member of the gelsolin superfamily of cytoskeletal regulatory proteins and known to be involved in cell motility in several types of cells (38), its role in cytokine production and/or secretion has not been fully explored. In contrast, adseverin, another actin-capping and -severing protein, has a demonstrated role in exocytosis. It was shown that adseverin knockdown osteoclasts display defects in enzyme (cathepsin K and tartrate resistance acid phosphatase) secretion (39). Gelsolin's regulatory role on insulin granule exocytosis in a mouse pancreatic β-cell line (42) has been reported, but the role of gelsolin in cytokinesis of immune cells has not been investigated. In our cytokine measurements from lung digests, the amounts of almost all cytokines were reduced in gelsolin-null mice compared with the wild-type, after HDM sensitization. In vitro cytokine measurements from different types of immune cells could determine whether gelsolin is involved in cytokinesis and/or exocytosis and is a fertile area for further research.

Actin dynamics are involved in acute lung injury (ALI) induced by a systemic inflammatory response syndrome. When actin reorganization was blocked with cytochalasin B, reduced inflammation and improved oxygenation were observed in a rat model of ALI, and their pulmonary monocytes/macrophages showed reduced production of inflammatory cytokines and altered polarization when treated with cytochalasin B (13). During lung inflammation, macrophage subpopulations in the lungs changed (1), and the phenotypic change was modulated by cell shape (27) and a change in actin dynamics (33). Phenotypic changes in lung macrophages have been implicated in chronic inflammatory responses (49). Therefore, we sought to investigate the effect of gelsolin on macrophage phenotype. Our ex vivo alveolar macrophage phenotypic polarization measurements revealed a reduced number of the M1 phenotype in gelsolin-null mice after M1-polarizing stimuli (endotoxin and INF-γ) compared with wild-type. The M1 phenotype is

associated with a proinflammatory status; therefore, reduced allergic lung inflammation in gelsolin-null mice is possibly due, in part, to altered macrophage polarization in the lungs, and modulation of actin dynamics by the absence of gelsolin might have contributed to the phenotypic change. In contrast, the number of macrophages with M2 polarization induced by IL-4 was not different between wild-type and gelsolin-null mice. A similar observation was previously seen in bone marrow-derived macrophages where IL-4/IL-13-mediated M2 polarization assessed by arginase-1 staining was not affected by cytochalasin B treatment (27), suggesting that actin reorganization is not playing a critical role in at least the expression of a prototypic M2 marker arginase-1. Although M2 macrophages that are typically induced by IL-4 and IL-13 have been suggested to play an important role in allergic asthma, and the HDM-induced allergic mouse asthma model showed M2 polarization with a predominant Th2 cell response (36), M1 macrophages also significantly increased in the HDM model (36). M1 macrophages induced by the local cytokine environment can augment inflammation and tissue injury (12), and M1 macrophages have been linked to severe asthma with poor response to corticosteroids (31). Therefore, reduced M1 polarization in gelsolin-null mice can be one of underlining mechanisms for the observed attenuation of lung inflammation.

In our in vivo lung function measurements, at baseline, tissue elastance was significantly decreased in gelsolin-null mice compared with wild-type mice. Tissue elastance was reported to be reduced in the porcine pancreatic elastase (PPE)-treated mouse model of emphysema (11), where a decrease in elastic recoil was expected. However, unlike PPE-treated mice, gelsolin-null mice did not show decreases in tissue damping or increases in central airway resistance at baseline. The pressure-volume loops of gelsolin-null mice were not different from wild-type mice both in PBS and HDM groups, while those of emphysematous mice were reported to display upward shifts (43). Therefore, baseline pulmonary function of gelsolin-null mice was different from those of emphysematous mice. Central airway resistance in response to inhaled methacholine was not different between wild-type and gelsolin-null mice that had been chronically treated with intranasal PBS. However, gelsolin-null mice that had been chronically treated with intranasal HDM demonstrated central airway resistance in response to inhaled methacholine that was significantly decreased compared with wild-type mice chronically treated with intranasal HDM. In fact, the central airway resistance in HDM-treated gelsolin-null mice was identical to the resistance seen in PBS-treated gelsolin-null or wild-type mice. This was likely a result of the reduction of chronic lung inflammation in the gelsolin-null mice. As previously reported, static compliance was not different in wild-type mice chronically treated with intranasal HDM versus PBS (11), and PBS-treated gelsolin-null mice exhibited the same static compliance. In contrast, HDM-treated gelsolin-null mice showed a significant increase in static compliance. This is a surprising finding that will require further study, since one would speculate that the absence of gelsolin would leave the actin cytoskeleton in a more polymerized and rigid state, as we previously demonstrated in airway smooth muscle cells (28), resulting in a decrease static compliance.

In conclusion, excessive inflammation is a key component in allergic lung inflammation in asthma. Gelsolin is an actin-binding multifunctional protein and is emerging as a player in regulating inflammation in multiple organ systems. Our current study demonstrates that the global genetic deletion of gelsolin results in reduced HDM-induced allergic lung inflammation, decreased Muc5AC production, and PAS staining in the airway indicating reduced mucus plugging and goblet cell hyperplasia, decreased cytokine concentrations in the lung, and reduced in vivo methacholine hyperresponsiveness in a mouse asthma model. Identifying the cell types that are playing key roles in the observed reduction in allergic lung inflammation in gelsolin-null mice may help identify future cellular and cytokine therapeutic targets in asthma.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

M.M. and C.W.E. conceived and designed research; M.M. and G.T.Y. performed experiments; M.M. and G.T.Y. analyzed data; M.M., G.T.Y., and C.W.E. interpreted results of experiments; M.M. prepared figures; M.M. and C.W.E. drafted manuscript; M.M., G.T.Y., N.M.H., and C.W.E. edited and revised manuscript; M.M., G.T.Y., N.M.H., and C.W.E. approved final version of manuscript.

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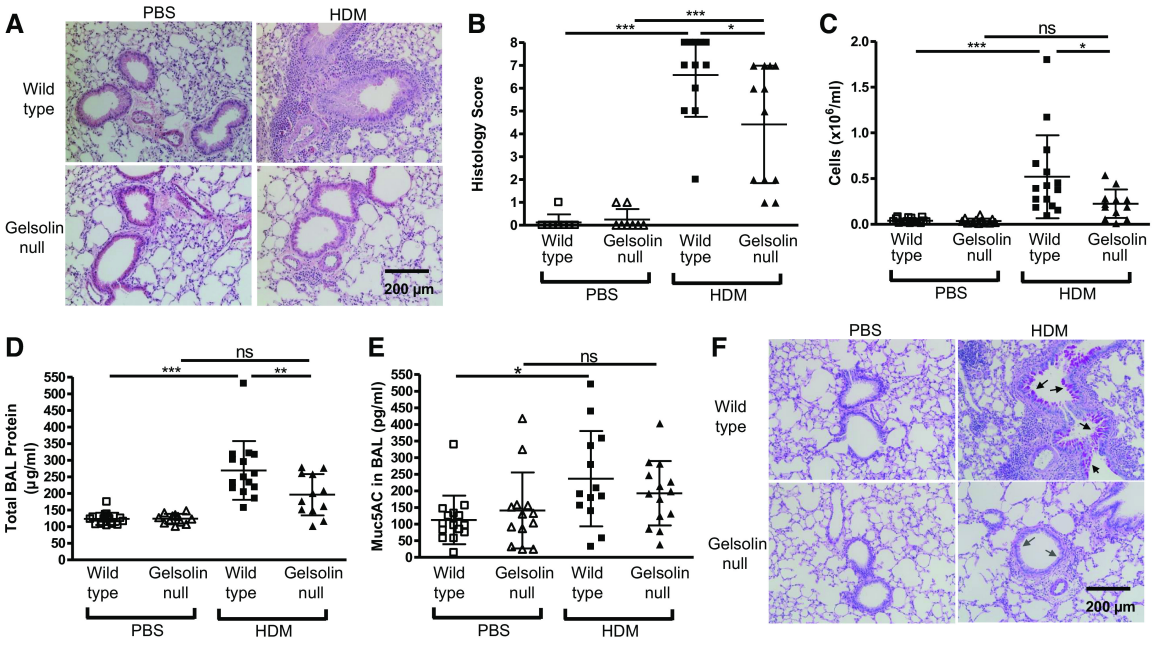
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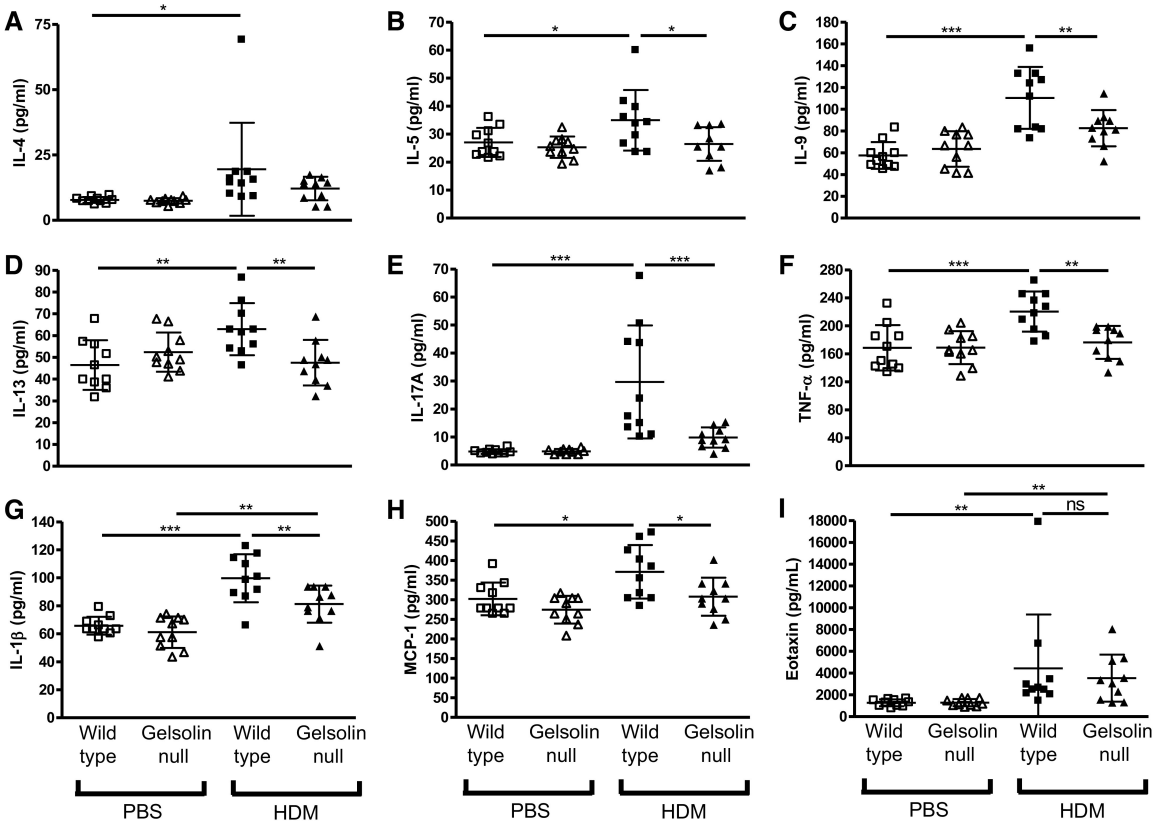
Figures and Tables

Fig. 1.



Attenuated lung inflammation in gelsolin-null mice after intranasal house dust mite (HDM) antigen sensitization. *A*: marked infiltration of mononuclear cells were observed around bronchovascular bundles in wild-type but less so in gelsolin-null mice after 3 wk of HDM sensitization. Intranasal PBS was given for the same duration in control mice. Representative hematoxylin and eosin stain images are shown. *B*: histology grading score by blinded observers was used to quantify lung inflammation. Gelsolin-null mice showed significantly reduced inflammation compared with the wild-type ($n = 8$ mice for PBS, 12 for HDM, $^{*}P < 0.05$, $^{***}P < 0.001$). *C–E*: bronchoalveolar lavage (BAL) cell count (*C*), protein concentration (*D*), and mucin 5AC (Muc5AC) concentration (*E*) after HDM sensitization. Mouse lungs were lavaged two times with 1 mL of PBS, and supernatants of pooled samples were assayed for protein and Muc5AC, while cell counts were performed on BAL cell pellets. The number of the cells, total protein, and Muc5AC concentrations were increased in HDM-sensitized wild-type mice compared with PBS controls and were significantly lower in HDM-sensitized gelsolin-null mice compared with HDM sensitized wild-type mice ($n = 12–15$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$). *F*: representative Periodic Acid-Schiff (PAS)-stained lung sections from HDM-sensitized wild-type mice showed PAS-positive staining (black arrow), whereas minimal staining was observed in HDM-sensitized gelsolin-null mice (gray arrow).

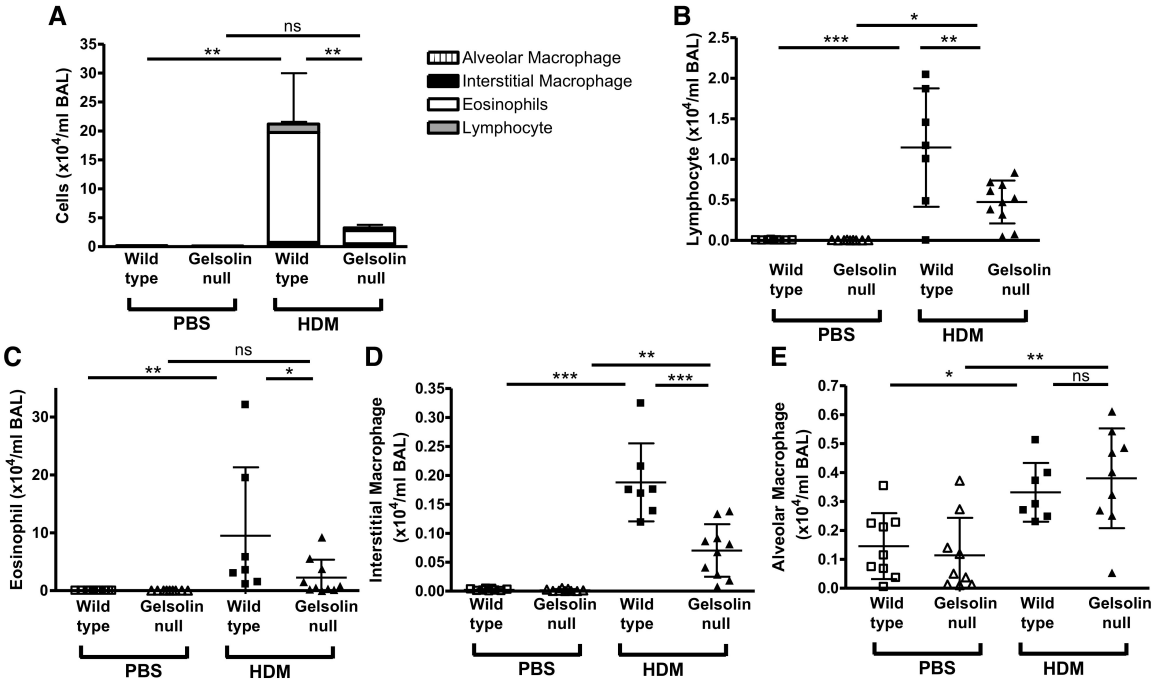
Fig. 2.



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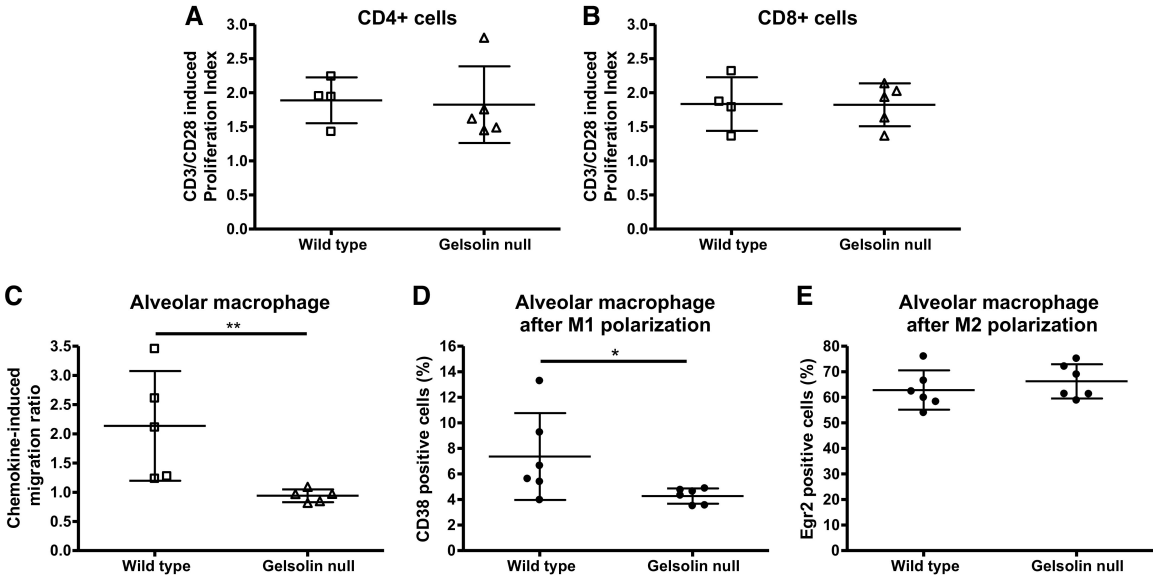
Cytokines and chemokines measured in lung digests. *A–D*: T helper cell type 2 (Th2) cytokines IL-4, IL-5, IL-9, and IL-13 levels were significantly higher in house dust mite (HDM) -sensitized wild-type mice compared with PBS control. IL-5, IL-9, and IL-13 levels in HDM-sensitized gelsolin-null mice were significantly decreased compared with HDM-sensitized wild-type mice. *E–G*: proinflammatory cytokines IL-17A, TNF-α, and IL-1β levels were significantly elevated in HDM-sensitized wild-type mice, and levels were lower in HDM-sensitized gelsolin-null mice compared with wild-type. *H*: the same result was observed with monocyte chemoattractant protein (MCP)-1 level. *I*: eotaxin was increased after HDM sensitization in both wild-type and gelsolin-null mice, and there was no attenuation in gelsolin-null mice compared with wild-type mice ($n = 10$ mice each, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

Fig. 3.



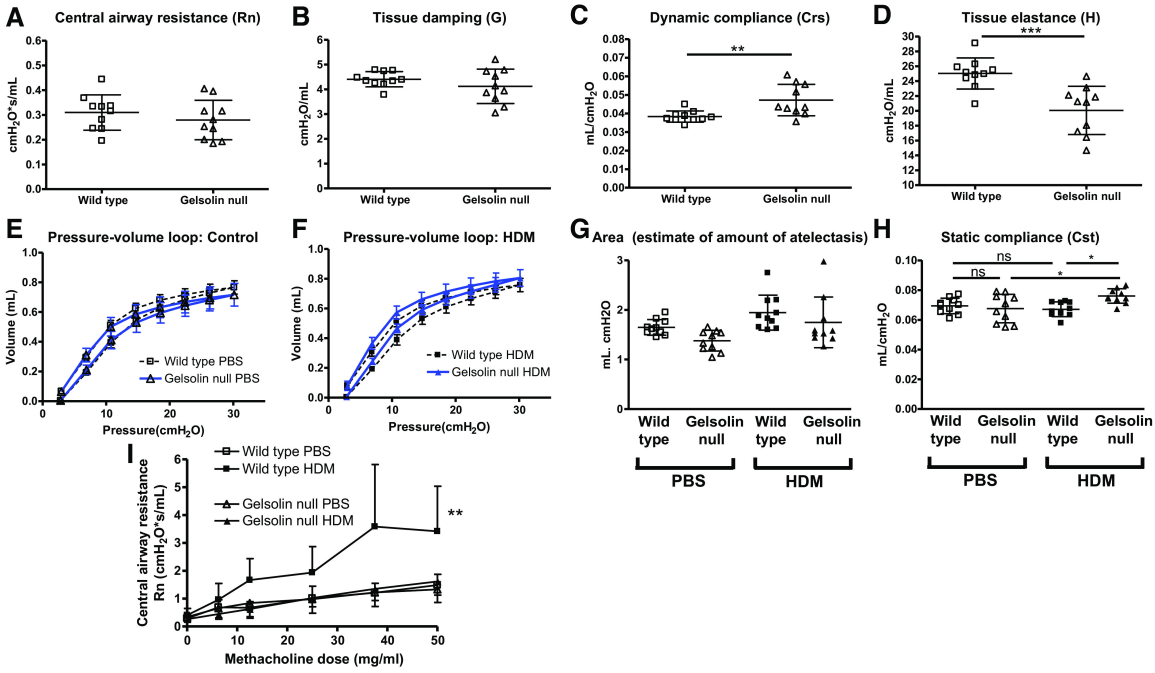
Bronchoalveolar lavage (BAL) cell differentiation. *A*: BAL cell number was significantly increased after 2 wk of house dust mite (HDM) antigen sensitization in wild-type mice but not in gelsolin-null mice. *B–E*: BAL cells were stained for fluorescence-activated cell sorting analysis and lymphocytes, eosinophils, interstitial macrophages, and alveolar macrophages were identified. Lymphocytes, eosinophils, and interstitial macrophages were significantly increased after HDM sensitization in wild-type mice compared with PBS controls; however, HDM-treated gelsolin-null mice showed attenuated increases compared with wild-type. No significant increases in eosinophils were observed in HDM-treated gelsolin-null mice. In contrast, there was no difference in alveolar macrophages between HDM-treated wild-type and gelsolin-null mice (n = 8–10 mice each, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

Fig. 4.



Cell proliferation of splenocytes, chemokine-induced macrophage migration, and macrophage polarization. *A* and *B*: splenocytes from wild-type and gelsolin-null mice were loaded with Violet Proliferation Dye 450 and then stimulated with anti-CD3 and anti-CD28 for 72 h. Cells were stained with APC anti-CD8 and FITC anti-CD4. Cell division was measured using flow cytometry, which provided a fluorescence histogram from which a proliferation index was calculated. Proliferation index, which represents the total number of divisions divided by the number of cells that went into division, was not different between wild-type and gelsolin-null mice both for CD4-positive and CD8-positive cell populations ($n = 4–5$, not significant). *C*: alveolar macrophages from gelsolin-null or wild-type mice that migrated across a transwell system were counted, and a migration ratio (cells with chemokine/without chemokine) was calculated. The migration ratio was reduced in gelsolin-null mice ($n = 5$ mice each, $^{**}P < 0.01$). *D* and *E*: alveolar macrophages were cultured for 16 h in 20 ng/mL INF-γ and 100 ng/mL LPS (for M1 polarization) or 20 ng/mL IL-4 (for M2 polarization). Cells were labeled with FITC anti-CD38 and APC anti-early growth response protein 2 (Egr2), followed by flow cytometry analysis. The percentage of CD38-positive macrophage was reduced in gelsolin-null mice ($n = 6$ from 12 mice each, $^{*}P < 0.05$), but no difference was seen in the percentage of Egr2-positive cells between wild-type and gelsolin-null mice ($n = 6$ from 12 mice, not significant).

Fig. 5.



In vivo respiratory mechanics measured using flexiVent. *A–D*: baseline respiratory mechanics parameters measured from wild-type and gelsolin-null mice. Central airway resistance (*A*) and tissue damping (*B*), which is closely related to tissue resistance, were not different between groups. Dynamic compliance (*C*) and tissue elastance (*D*) were significantly different in gelsolin-null group compared with the wild-type group ($n = 10$ each, parameters from individual mouse and group means are shown, $^{**}P < 0.01$, $^{***}P < 0.001$). *E–H*: pressure-volume (PV) loops and related parameters. *E* and *F*: mean PV loops from intranasal PBS control or intranasal house dust mites (HDM) for wild-type and gelsolin-null groups were not different between groups. *G*: baseline area enclosed by the PV loop, which provides an estimate of the amount of atelectasis, was not significantly different between groups. *H*: static compliance (Cst) calculated from PV loops showed increased Cst in HDM-sensitized gelsolin-null group compared with HDM-sensitized wild-type mice group ($n = 10$ mice per group, $^{*}P < 0.05$). *I*: airway responsiveness to methacholine comparing wild-type and gelsolin-null mice. Central airway resistance (Rn) was measured in response to increasing concentrations of inhaled methacholine. Rn increases in response to increasing concentrations of methacholine challenge were significantly higher in HDM-sensitized wild-type mice compared with PBS control wild-type mice. HDM-sensitized gelsolin-null mice showed a similar degree of airway responsiveness as PBS control mice ($n = 10$, mean with SD for each methacholine doses are plotted. Area under the curve from HDM-sensitized wild-type mice is significantly different from the other three groups, $^{**}P < 0.01$).

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