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Experimental progressive emphysema in BALB/cJ mice as a model for chronic alveolar destruction in humans

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Emphysema, one of the major components of chronic obstructive pulmonary disease (COPD), is characterized by the progressive and irreversible loss of alveolar lung tissue. Even though >80% of COPD cases are associated with cigarette smoking, only a relatively small proportion of smokers develop emphysema, suggesting a potential role for genetic factors in determining individual susceptibility to emphysema. Although strain-dependent effects have been shown in animal models of emphysema, the molecular basis underlying this intrinsic susceptibility is not fully understood. In this present study, we investigated emphysema development using the elastase-induced experimental emphysema model in two commonly used mouse strains, C57BL/6J and BALB/cJ. The results demonstrate that mice with different genetic backgrounds show disparate susceptibility to the development of emphysema. BALB/cJ mice were found to be much more sensitive than C57BL/6J to elastase injury in both a dose-dependent and time-dependent manner, as measured by significantly higher mortality, greater body weight loss, greater decline in lung function, and a greater loss of alveolar tissue. The more susceptible BALB/cJ strain also showed the persistence of inflammatory cells in the lung, especially macrophages and lymphocytes. A comparative gene expression analysis following elastase-induced injury showed BALB/cJ mice had elevated levels of il17a mRNA and a number of classically (M1) and alternatively (M2) activated macrophage genes, whereas the C57BL/6J mice demonstrated augmented levels of interferon-γ. These findings suggest a possible role for these cellular and molecular mediators in modulating cytokine-mediated inflammation between C57BL/6J and BALB/cJ. The molecular basis underlying this intrinsic susceptibility is not fully understood, as measured by significantly higher mortality, greater body weight loss, greater decline in lung function, and a greater loss of alveolar tissue. The more susceptible BALB/cJ strain also showed the persistence of inflammatory cells in the lung, especially macrophages and lymphocytes. A comparative gene expression analysis following elastase-induced injury showed BALB/cJ mice had elevated levels of il17a mRNA and a number of classically (M1) and alternatively (M2) activated macrophage genes, whereas the C57BL/6J mice demonstrated augmented levels of interferon-γ. These findings suggest a possible role for these cellular and molecular mediators in modulating the severity of emphysema and highlight the possibility that they might contribute to the heterogeneity observed in clinical emphysema outcomes.

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Chronic obstructive pulmonary disease (COPD) is the third leading cause of death in the United States, and its prevalence and mortality is also steadily increasing globally (34, 85). COPD is comprised of two key components, chronic bronchitis and emphysema, both of which contribute to persistent airflow limitation (83, 85). Emphysema is anatomically defined as a condition marked by irreversible airspace enlargement that derives from progressive alveolar wall destruction (70). Currently, beyond smoking cessation to prevent the onset of emphysema in the first place, there exist very limited options for therapeutic intervention to stem disease progression once a diagnosis has been made. The mechanistic basis underlying the pathogenesis of emphysema is very complex, involving a combination of recurrent inflammation, oxidative stress, excess protease activity, and cell death, all of which can be modulated by environmental exposures and host genetics (22, 91). Cigarette smoking has been implicated as the most important environmental risk factor in developing emphysema, since smokers account for >80% of cases (6, 68, 79a). Although clinical studies have shown that all smokers demonstrate some degree of pulmonary inflammation, only 15–20% of smokers develop severe progressive emphysema (23). This disparity underscores the importance of susceptibility factors, a subset of which are almost certainly controlled by host genetics.
activation (M2 activation) of macrophages by the Th2 cytokine milieu in BALB/cJ mice with the synthesis of arginase 1, RELMα, and YM1 (54, 78). Interestingly, it has been documented that the development of emphysema in cigarette smoke-exposed mice is strain dependent (28, 50).

Although an appropriate model for studying the initiation of emphysema, experimental cigarette smoke exposure suffers from a number of limitations with regard to identifying the mechanisms that contribute to emphysema progression. Even after 6 mo of chronic cigarette exposure, mice develop very mild emphysema with only marginal airspace enlargement and pulmonary function decline (28, 75, 86). Furthermore, unlike in human populations where many habitual smokers who quit continue to develop worsening lung function, in nearly all cases smoking cessation in mice halts disease progression (5, 87, 88). A long-standing alternative approach to establish experimental emphysema in mice is through the use of elastolytic proteases instilled in the lung. This model provides a much more tractable system with which to study emphysema progression, since not only is emphysema readily apparent 2–3 wk after the acute proteolytic insult, but the damage continues to progress over time. Such a model is clinically relevant, since therapeutics to halt the progression of emphysema can be studied in this system.

Whether there are strain-dependent sensitivities to progressive protease-induced emphysema for which an underlying genetic basis may exist has not been studied. In the present work we investigated whether two common inbred mouse strains, C57BL/6J and BALB/cJ, show differential susceptibility to progressive emphysema induced by elastase instillation. The results of this study could help to identify candidate genes and pathways contributing to discordant emphysema phenotypes, and as such may have implications for the design of novel therapeutic interventions or screening tools to stem the progression of human emphysema.

**MATERIALS AND METHODS**

**Animals.** Male, 8–wk-old, wild-type BALB/cJ and C57BL/6J mice were purchased directly from The Jackson Laboratory (Bar Harbor, ME). All mice were housed under specific pathogen-free conditions at the Johns Hopkins School of Public Health. The animals were provided filtered air (60–70% relative humidity) at 22–26°C and maintained on a 12:12-h light-dark cycle with ad libitum access to autoclaved food and filtered water. All experiments and procedures were approved and performed according to guidelines set forth by the Institutional Animal Care and Use Committee of the Johns Hopkins University.

**Intratracheal elastase administration.** Mice were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (15 mg/kg) via intraperitoneal injection and suspended supine on a 15° sloped platform by their upper teeth using silk thread. A small incision was made to the skin on the neck to visualize the trachea. A 20-gauge intravenous catheter (Jelco Optiva; Smith Medical, Dublin, OH) with a manually bent tip was inserted in the trachea by gently pulling out the tongue and inserting the cannula toward the ventral surface of the mouse. A single dose of either 1.5, 3, or 6 enzymatic activity units (U) of porcine pancreatic elastase (EC-134; Elastin Products, Owensville, MO) dissolved in 50 μl of sterile 1× PBS were injected with a standard gel-loading pipet tip in the lung through the catheter. To promote the distribution of the solution, the mice were ventilated through the cannula for 10 s with room air at 0.1 ml tidal volume and 150 strokes/min using a MicroVent Ventilator (model 848; Harvard Apparatus, Holliston, MA). Control animals received 50 μl of sterile PBS only. After the cannula was removed, the incision was closed by applying a small amount of cyanoacrylate tissue adhesive (Vetbond 3M), and the mice were allowed to recover for 2 days.

**Diffusion factor for carbon monoxide measurement.** The procedure for measuring the diffusion factor for carbon monoxide (DFCO) in animals was based on our previously published protocol (47). Briefly, a tracheostomy was performed with an 18-G stab cannula to anesthetize animals, and the lungs were quickly inflated with 0.8 ml of a gas mixture containing ~0.5% carbon monoxide (CO), 0.5% neon (Ne, an insoluble inert tracer gas), and 99% room air. After a 9-s breath hold, the 0.8 ml was quickly withdrawn from the lung and diluted to 2 ml with room air. The diluted exhaled gas was allowed to mix for 15 s and then injected in a Micro GC gas chromatograph (Micro GC Model 3000A; INFINIC, East Syracuse, NY) to measure the concentrations of Ne and CO. Two repeated measures were used to derive average Ne and CO concentrations for each mouse. The DFCO was calculated as 1 – (CO9/CO)(Ne9/NeC), where the “9” subscript refers to gases after the 9 s of being held by the animal, and the “C” subscript refers to the calibration gases (20).

**Pulmonary mechanics and quasistatic pressure-volume relationships.** After DFCO measurements, mice were paralyzed with an intramuscular injection of succinylcholine at a dose of 75 mg/kg body wt and immediately connected to a flexiVent ventilator (Scireq, Montreal, QC, Canada) set to provide a constant-volume ventilation with 100% oxygen at a tidal volume of 10 ml/kg, a rate of 150 breaths/min, and a positive end-expiratory pressure of 3 cmH2O. Following at least 3 min of ventilation, the lungs were inflated to 30 cmH2O for 5 s and returned to normal ventilation for 1 min. Next, the system was sealed for 4 min to allow the animal to absorb all gas from the lung. After complete degassing, the lung volume was zero, and quasistatic pressure-volume (P-V) curves were immediately generated with a system detailed previously (46). Briefly, a 5-ml air-filled glass syringe mounted on a dual infusion-withdrawal syringe pump (model 55–2226; Harvard Apparatus) delivered air to the lungs, and the P-V were measured with a linear displacement transformer (model 244-000; Transtek, Ellington, CT) and a differential pressure transducer (PX-137, Omega Engineering, Stamford, CT), respectively. The P-V curve was recorded on a PowerLab digital data acquisition system (ADInstruments, Colorado Springs, CO). The lung was inflated and deflated at 1 ml/min between −10 and 35 cmH2O. At each of these pressures, the pump was manually switched, thereby generating three sequential P-V loops between 0 and 35 cmH2O. Total lung capacity (TLC) and residual volume (RV) of each mouse were defined as the volume at 35 and −10 cmH2O, respectively, and quasistatic compliance of the respiratory system was defined as the slope of the most linear part of the second deflation limb (between 3 and 8 cmH2O).

**Lung harvesting and fixed left lung volume measurement.** After pulmonary mechanics were measured, the chest wall and diaphragm were exposed, and the right main stem bronchus was tied off with a suture before removing and snap-freezing the right lobes of the lung in liquid nitrogen. The right lung was stored at −80°C for RNA and protein analysis. The left lobe was inflated with zinc-buffered formalin (Z-Fix, 174; Anatech, Battle Creek, MI) at a constant pressure of 25 cmH2O for at least 20 min. The trachea was tied off, and the heart and left lung were excised en bloc and submerged in Z-fix for at least 20 min. The heart was decalcified, and the left lung was cut transversely at 0 and 20 μm. Histology and morphometry. To quantify the changes in lung structure, we used the nonlaved left lung for quantitative histological analysis. As previously described, this lung was cut transversely to the long axis (circular to caudal) into three sections that were then embedded in paraffin (16). Photos of sections (5 μm) stained with hematoxylin and eosin were taken using a systematic uniform random sampling method (35) with a Nikon Eclipse 80i (Nikon, Tokyo, Japan) at ×200 magnification. Fifteen to 20 images were obtained from each of the 3 lung sections. Each image was analyzed by

**Pulmonary diffusion capacity for carbon monoxide measurement.** The procedure for measuring the diffusion factor for carbon monoxide (DFCO) in animals was based on our previously published protocol (47). Briefly, a tracheostomy was performed with an 18-G stab cannula to anesthetize animals, and the lungs were quickly inflated with 0.8 ml of a gas mixture containing ~0.5% carbon monoxide (CO), 0.5% neon (Ne, an insoluble inert tracer gas), and 99% room air. After a 9-s breath hold, the 0.8 ml was quickly withdrawn from the lung and diluted to 2 ml with room air. The diluted exhaled gas was allowed to mix for 15 s and then injected in a Micro GC gas chromatograph (Micro GC Model 3000A; INFINIC, East Syracuse, NY) to measure the concentrations of Ne and CO. Two repeated measures were used to derive average Ne and CO concentrations for each mouse. The DFCO was calculated as 1 – (CO/CO)(Ne/NeC), where the “9” subscript refers to gases after the 9 s of being held by the animal, and the “C” subscript refers to the calibration gases (20).
overlying a sample grid line of 12 short line segments. The quantitative assessments of emphysematous changes in the lung were performed with a traditional system of quantification (41) using manual identification of where the end points of each line segment fell and counts of the number of line segment intercepts with alveolar walls. The mean linear intercept (Lm) and was then calculated as:

\[ L_m = \frac{V_p}{P \times L_{alv}} / (d \times P_{air}) \]

where \( V_p \) is parenchymal volume (calculated from parenchymal fraction \( \times \) fixed lung volume from the water displacement method), \( L_{alv} \) is the number of intercepts with alveolar septal walls, \( d \) is the length of a single test line (67 \( \mu \)m), and \( P_{air} \) is the number of line end points hitting air spaces in alveoli and ducts.

**Bronchoalveolar lavage.** Lungs were lavaged three times with three fresh 0.8-ml syringes of cold PBS containing complete protease cocktail inhibitor tablets (Roche Applied Science, Indianapolis, IN). The first recovered bronchoalveolar lavage (BAL) fluids from each sample were centrifuged at 300 g at 4°C for 10 min, and supernatant was collected and stored at -80°C to assess total protein levels. The cell pellets from the lavages were pooled, washed, and suspended in 1 ml of cold PBS and aliquoted into two separate tubes (0.5 ml each). One aliquot was used to determine the hemoglobin content, whereas another was processed for total and differential cell counts. For hemoglobin determination, an aliquot of cells was lysed with 0.5 ml hypotonic distilled water for 5 min, and absorbance was measured at 405 nm using an Epoch Micro-Volume Spectrophotometer (BioTek, Winooski, VT). For differential cell counts, the pelleted cells were treated with ammonium-chloride-potassium lysin buffer (Quality Biological, Gaithersburg, MD) for 5 min. After 0.4 ml of cold 1× PBS was added, a 10-µl aliquot of cells was stained with Turk’s solution (EMD Millipore, EMD Millipore) before counting on a hemacytometer (Cambridge Scientific, Rockford, IL) according to the manufacturer’s protocol.

**RNA extraction, reverse transcription, and real-time PCR.** Lung tissue was placed in 1 ml of Trizol Reagent (Ambion RNA Life Technologies, Carlsbad, CA) and homogenized using a Bead Bug microtube homogenizer (Benchmark Scientific, Edison, NJ). RNA was precipitated with isopropanol, washed, and suspended in 100 µl of diethylpyrocarbonate (DEPC)-treated water. Concentration and purity were assessed at 260 and 280 nm, and the RNA was stored at -80°C. Total RNA (1 µg) was reverse transcribed into cDNA with oligo(dT) and random primers using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) following the manufacturer’s instructions. The cDNA was analyzed in a 96-well format using the Applied Biosystems 7500 real-time PCR system with TaqMan Gene Expression Assays-on-Demand and TaqMan Universal Master Mix (Life Technologies, Grand Island, NY) following the manufacturer’s recommendations. Briefly, 15-µl reactions were used containing 2 µl of cDNA, 0.5 µl commercially available gene expression Taqman fluorogenic primer/probe sets as mentioned in Table 1, 7.5 µl of Taqman Universal Master Mix (Life Technologies), and 5 µl of DEPC-treated water. The PCR reaction was performed with the following thermal profile: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C (15 s), followed by 60°C (1 min). Analysis of gene expression was performed using the Applied Biosystems 7500 system SDS software package (Life Technologies). The relative expression ratio of the real-time PCR products was calculated by the 2^-ΔΔCt method (48), which represents the fold difference in gene expression normalized to a housekeeping gene control from the same sample. Samples were initially run in parallel with the housekeeping genes β-actin (actb) and hypoxantine-guanine phosphoribosyltransferase 1, but expression data are shown relative to actb, since it was found to be more stably expressed across treatment groups.

**Flow cytometric analysis.** Single-cell suspensions from whole lungs were generated as previously described (69). The cells were washed two times in FACS staining buffer (1× PBS with 2% heated-inactivated FCS; Mediatech, Manassas, VA) and treated with antisea class Cd16/Cd32 Fe Block (BD Biosciences, San Jose, CA) for 10 min before incubation in the dark with appropriate fluorochrome-conjugated surface marker staining antibodies on ice for 20 min. CD4-FITC, CD8-PE, CD3-PerCP-Cy5.5, and NKp46-FITC antibodies were purchased from eBioscience (San Diego, CA), and an anti-CD49b-PE antibody was obtained from BD Bioscience. All antibodies were heated-inactivated FCS; Mediatech, Manassas, VA) and treated with heated-inactivated FCS; Mediatech, Manassas, VA) and treated with

**Table 1. List of Taqman gene expression Assays-on-Demand primer/probe sets used in this study**

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<th>Gene Name</th>
<th>Symbol</th>
<th>Entrez Gene ID</th>
<th>Taqman Assay ID</th>
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<td>Ilng</td>
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**RESULTS**

**Morbidity and mortality in C57BL/6J and BALB/cJ mice following elastase challenge.** Previous studies from our laboratory and others have indicated that a single oropharyngeal or intratracheal administration of porcine pancreatic elastase is...
sufficient to induce emphysematous lung damage in experimental models (14, 20, 29, 79). To measure strain-dependent responses in C57BL/6J and BALB/cJ mice, we administered either PBS alone or 1.5, 3, 6, or 9 U of elastase suspended in PBS and documented survival and changes in body weight. In addition to the PBS control, 1.5- and 3-U challenge doses of elastase resulted in no mortality in either strain of mouse (Fig. 1A). In contrast, at doses of 6 and 9 U of elastase, both C57BL/6J and BALB/cJ mice experienced mortality, with the percentage of BALB/cJ mice surviving at each dose being significantly lower than their C57BL/6J counterparts (Fig. 1A). Of note, all mortality occurred during the first 3 days following elastase challenge, suggesting that death was associated with elastase-induced acute lung injury.

Even though 3 U of elastase did not result in mortality in either strain, this challenge dose did cause both strains of mice to transiently lose a significant amount of body weight (Fig. 1B). Both strains proceeded to recover over the 1st wk following elastase exposure, albeit slightly delayed in the BALB/cJ strain, and by day 8 postchallenge the weights were nearly equivalent to the weights of PBS control animals. A more pronounced and sustained reduction in body weight was observed for BALB/cJ mice receiving the 6-U dose of elastase such that they had not returned to their baseline body weight even by day 21 postchallenge. However, in C57BL/6J animals, the significant and transient drop in body weight that occurred in the days immediately after the 6-U challenge was largely resolved by day 9, and the mice continued to gain weight at a rate similar to controls. Combined, these survival and weight loss data clearly indicate that BALB/cJ mice are much more sensitive to the consequences of acute lung injury induced by elastase compared with C57BL/6J mice.

Dose-dependent development of elastase-induced emphysema in C57BL/6J and BALB/cJ mice. To address the dose responsiveness of elastase on the lungs of these two strains of mice, we assessed the development of emphysema through pulmonary function testing and morphometry. Both strains of mice had similar baseline DFCO values and experienced a dose-dependent decline in DFCO after instillation of 1.5, 3, or 6 U of elastase (Fig. 2A). However, the fall in DFCO was significantly more pronounced at each dose in the BALB/cJ compared with C57BL/6J mice (Fig. 2A). These drops in gas exchange were paralleled by changes in lung structure and function, as assessed by air-filled P-V measures of total lung capacity, RV, and quasistatic compliance (Fig. 2, B–D). Notably, BALB/cJ mice had larger baseline lung volumes and compliance compared with C57BL/6J mice. Moreover, increasing doses of elastase resulted in a dose-dependent increase in TLC, RV, and lung compliance in both strains, but the changes were much more pronounced in BALB/cJ mice.

Microscopic examination of histological sections of lungs at day 21 postchallenge with 1.5, 3, or 6 U of elastase showed substantial enlargement of the alveoli in both strains of mice, which was reflected by higher Lm, with increasing elastase doses (Fig. 2, E and F). However, at each dose of elastase, the progressive changes in the BALB/cJ lungs were much greater than those in C57BL/6J lungs.

Temporal progression of experimental emphysema in C57BL/6J and BALB/cJ mice. Based on the dose-dependent survival results (Fig. 1) and measures of lung function (Fig. 2), we selected 3 U of enzymatic activity as the dose for the remainder of our experiments. Gross examination of the lungs at day 2 postadministration revealed prominent hemorrhage in both C57BL/6J and BALB/cJ mice, consistent with overt acute lung injury (Fig. 3A). While much of the hemorrhage was resolved by day 4 in C57BL/6J mice, signs of hemorrhage remained until day 7 in BALB/cJ mice. The differences in the extent and duration of the hemorrhage between the mouse strains were quantified by a colorimetric hemoglobin assay performed on BAL fluid recovered from these mice (Fig. 3B). In addition to the pattern of hemorrhage, starting at day 14, there was a visually obvious increase in the overall size of the lungs from BALB/cJ mice compared with those from the C57BL/6J mice (Fig. 3A). Using stereological point counting, we found significant increases in Lm as early as day 2, and the Lm continued to progress rapidly, reaching a more than threefold increase by day 21 in BALB/cJ mice (Fig. 3C). In contrast, C57BL/6J mice had very little change in Lm with only a 30% increase above baseline at day 21.
Both C57BL/6J and BALB/cJ mice treated with 3 U of elastase exhibited a significant decrease in DFCO as early as day 4 (Fig. 4A). However, the decrease in DFCO in BALB/cJ mice was much greater than in C57BL/6J mice beginning on day 7, and a further gradual decline of DFCO after day 21 was found only in BALB/cJ mice. These changes in DFCO were also accompanied by greater increases in TLC and RV in BALB/cJ mice (Fig. 4B and C). BALB/cJ mice experienced a >40 and 100% increase in total lung capacity on days 7 and 96, respectively, compared with only ~10 and 20% increases in C57BL/6J mice at those same time points (Fig. 4B). Similar trends were also observed for changes in RV between the two strains (Fig. 4C). Although BALB/cJ mice had significantly reduced quasistatic compliance during the first few days after elastase challenge (likely because of the acute edema and inflammation in the lung), the compliance had recovered to baseline by day 7 (Fig. 4D). After this recovery, compliance was increased over control to levels significantly higher than what was observed in the C57BL/6J mice. Cumulatively, these structural and functional changes clearly show much greater emphysematous damage in the lungs of BALB/cJ compared with C57BL/6J mice.

Cellular airway infiltration in C57BL/6J and BALB/cJ lungs in response to elastase. During the first several days after elastase-induced damage, both C57BL/6J and BALB/cJ mice were marked by a cellular infiltration in alveolar spaces (Fig. 5). An assessment of the cells recovered from the BAL fluid showed a comparable increase in the total cell number for both strains at day 2 (Fig. 5A), but the number of cells steadily decreased over time in C57BL/6J lungs while remaining elevated in the BALB/cJ lungs through day 21. The composition of the cellular infiltration changed over time, with a transient neutrophil influx making up a significant proportion of the early day 2 response for both strains (Fig. 5B). Although the number of macrophages and lymphocytes increased in both strains, both the magnitude and persistence of change were greater in the lungs from BALB/cJ mice (Fig. 5, C and D). Modest numbers of eosinophils were found in the BAL from both strains between days 2 and 14 after administration of elastase (Fig. 5E).

The lymphocyte dynamics in the lung tissue were further characterized using flow cytometry to assess the numbers of NK cells (CD3<sup>−</sup>CD49<sup>+</sup>CD4<sup>+</sup>CD49<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>), NKT cells (CD3<sup>+</sup>CD49<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup>), and CD4<sup>+</sup> T cells (CD3<sup>+</sup>CD4<sup>−</sup>CD8<sup>−</sup>), and
CD8+ T cells (CD3+CD4−CD8+) (Fig. 6). Lymphocyte numbers in both strains appeared to peak at day 14 before receding to near baseline levels by day 21. Although not statistically significant, BALB/cJ lungs were found to contain more NK (Fig. 6A), NKT (Fig. 6B), CD4+ (Fig. 6C), and CD8+ (Fig. 6D) lymphocytes at baseline, and trended toward higher cell numbers for all four cell types at each time point following elastase challenge as well.

Inflammatory gene expression profiles in C57BL/6J and BALB/cJ mice during the progression of emphysema. Given that increases in the number of neutrophils, macrophages, cytotoxic CD8+ T cells, and CD4+ Th1 and Th17 cells have all been associated with the development of COPD (12, 26, 32) and were elevated in our model, we proceeded to characterize the expression of several genes associated with the differentiation, activation, and maintenance of these various cell types following elastase exposure. Although there was a gradual upregulation of Th2-associated il4 (Fig. 7A), il5 (Fig. 7B), and il13 (Fig. 7C) gene expression in both C57BL/6J and BALB/cJ mice after instillation of elastase, we did not find any dramatic differences between the two strains. The same profile was also observed for the expression of il10 (Fig. 7D), an immu-
modulatory cytokine made in large part by macrophages/monocytes and regulatory T cells. Interestingly, while both strains showed the same trend over time, C57BL/6J mice expressed significantly higher levels of the gene encoding for the Th1-associated cytokine IFN-γ (Fig. 7E). On the other hand, BALB/c mice had highly elevated levels of il17a, the canonical Th17-derived cytokine (Fig. 7F).

When we extended our transcriptional profiling of lung tissues to include genes associated with classical (M1) and alternative (M2) macrophage activation, we found that BALB/c mice took on a pronounced but mixed M1/M2-associated phenotype. During the peak of acute lung injury at day 2 and through day 4, BALB/c mice upregulated arginase 1 (arg1; Fig. 8A) and found in inflammatory zone 1 (fizz1; Fig. 8B) mRNA to levels 15-fold higher than that of C57BL/6J mice. In addition, BALB/c mice also had significantly higher levels of inducible nitric oxide synthase (inos; Fig. 8C), il6 (Fig. 8D), il18 (Fig. 8E), and il1b (Fig. 8F) compared with C57BL/6J mice at several of the time points analyzed.

Because the imbalance of proteases/antiproteases has long been a proposed mechanism by which the chronic lung destruction seen in emphysema occurs (1), we measured the level of several protease and antiprotease genes. Serpin1a (Fig. 9A), encoding for α1-antitrypsin (α1-AT), was significantly higher in naïve BALB/cJ relative to naïve C57BL/6J mice, but the levels were similarly decreased in both strains after the mice received elastase. Tissue inhibitor of metalloproteinase 1 (Timp1; Fig. 9B) levels also rapidly increased after elastase insult and to a much greater degree in BALB/cJ mice. In contrast, naïve C57BL/6J mice had higher levels of α2-macroglobulin (A2m; Fig. 9C), which encodes for α2-macroglobulin, although the level of A2m was elevated in response to elastase in both strains. Interestingly, at times when the expression of the protease inhibitor genes we measured had largely returned to baseline (days 7–21), matrix metalloproteinase (mmp)-12 (Fig. 9D), and to a lesser extent mmp2 (Fig. 9E), were significantly elevated in BALB/cJ relative to the C57BL/6J mice. Furthermore, at day 4, the gene encoding for the antioxidant protein heme oxygenase-1 was robustly elevated in BALB/cJ lungs but not in C57BL/6J lungs (Fig. 9G), suggesting a minor role for this molecule in protecting from elastase-induced emphysema.

DISCUSSION

A single intratracheal administration or aerosol inhalation of porcine-derived pancreatic elastase has long served as an effective approach to rapidly induce acute lung injury leading to subsequent pulmonary emphysema in a wide variety of experimental animals (9, 31, 37, 80). One proposed mechanism for the development of emphysematous changes involves the concept of a protease/antiprotease imbalance (1). This notion emerged following studies of emphysema patients with severe α1-AT deficiency (44). Much of the initial work studying this in preclinical models was performed in Syrian golden hamsters, and it was thought that this species developed severe emphysema following elastase because of its relatively low α1-AT level, inadequate repair following acute lung injury, and depletion of glutathione-related antioxidant enzymes in the early phase (3, 4, 82). More recently, mice on the C57BL/6 background have been used to model elastase-induced experimental emphysema (14, 29, 79). Widespread use of the C57BL/6 strain stems from the fact that its genome has been fully sequenced, and many transgenic and knockout strains of mice have been produced on this genetic background. However, as noted in the mouse models for allergic asthma (19, 45), there are likely to be important strain differences in the response to elastase as well. In the present work, our aim was to

![Figure 4](http://ajplung.physiology.org/) Changes over time in measures of lung function in C57BL/6J and BALB/c mice following administration of elastase. DFCO (A), total lung capacity (B), residual volume (C), and quasistatic compliance (D) were measured at days 2, 4, 7, 14, 21, 48, and 96 following IT administration of 3 U elastase; n = 5–10 mice/group. Two-way ANOVA was used to compare the elastase-treated group with naïve controls or to compare BALB/cJ with C57BL/6J at the same time point. *P < 0.05, **P < 0.01, and ***P < 0.001.
compare the emphysematous phenotype in C57BL/6J mice and another commonly used mouse strain in immunological studies, BALB/cJ mice. Characterizing differential responses to elastase-induced lung injury could provide new insights into the cellular and molecular processes that contribute to the pathogenesis of the progressive stage of emphysema.

To this end we assessed various phenotypic endpoints, including mortality, body weight, pulmonary function, lung mechanics, lung structure, and immune gene expression, in both strains exposed to intratracheal elastase. Our data clearly show that at a given exposure level BALB/cJ mice are much more susceptible to elastase-induced pulmonary emphysema. Our results are consistent with the differential susceptibility to emphysema resulting from Nippostrongylus brasiliensis hookworm infection that has been previously described in the BALB/cJ and C57BL/6J strains (51). A strain-dependent effect has also been demonstrated in cigarette smoke-induced emphysema (2, 10, 28, 59, 90). In these studies, C57BL/6J mice were considered as a “moderately sensitive” strain to develop emphysematous lesions after smoke exposure because this strain of mouse showed more pathological changes in response to cigarette smoke compared with the less sensitive mouse strains studied, the outbred ICR strain and inbred NZW strain (28). However, cigarette smoke only caused mild pathological emphysema in the C57BL/6J strain as evidenced by only a 13–18% increase in $L_m$ after 5–6 mo of exposure (10, 28, 59). This increase was less than in other more sensitive strains studied, i.e., AKR, SJ/L, A/J, and C3H. Only one study compared the effect of cigarette smoke exposure on the C57BL/6J and BALB/cJ strains we studied here. They found that smoke-exposed BALB/cJ mice did not show a significantly greater $L_m$, but they did find significantly more blood vessel muscularization compared with smoke-exposed C57BL/6J mice (59). Similar levels of emphysematous injury between C57BL/6J and BALB/cJ mice were also observed in a chronic ozone-induced emphysema model (77). Nevertheless, these models of emphysema induced by chronic exposure to cigarette smoke or ozone cause only relatively very mild damage in alveolar tissue, which is likely not enough to magnify the interstrain differences that can be observed with the progression of disease following elastase damage or hookworm infection.

It is notable that the BALB/cJ mice had larger lung volumes and higher lung compliance at baseline than the C57BL/6J strain. These differences would seem to suggest a baseline structural difference may exist in the lungs of these mice. From a simple dose-concentration effect, giving the same amount of elastase to a larger lung should have led to a lower concentration spread over the whole lung. This would have led to perhaps a smaller effect in the BALB/cJ if it were present, but we found just the opposite. However, it is possible that the larger lung might have been associated with thinner alveolar septa (7), which could contribute to increased hemorrhage and an augmented propensity to de-

Fig. 5. Analysis of the changes in the composition and dynamics of the cellular infiltrate induced in the lungs by administration of elastase to C57BL/6J and BALB/cJ mice. Bronchoalveolar lavage was used to estimate the total cell (A), macrophage and monocyte (B), neutrophil (C), lymphocyte (D), and eosinophil (E) counts that had infiltrated in the lungs of C57BL/6J and BALB/cJ mice at days 2, 4, 7, 14, and 21 following IT administration of 3 U of elastase; n = 4–5 mice/group. Two-way ANOVA was used to compare the elastase-treated group with naïve controls or to compare BALB/cJ with C57BL/6J at the same time point. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. 

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velop emphysema in these mice. Therefore, it would be of interest to quantify matrix components such as elastin and collagen in the septal walls, particularly given previous work that has demonstrated the importance of elastin fragment release toward the development of emphysema in the elastase model (33). On a related note, it would be worthwhile to assess the level of cellular apoptosis within the alveolar septa following elastase challenge to determine the relative contributions of alveolar matrix thinning and septal cell death to disease progression in this model.

Fig. 6. The dynamics of NK cells, NK T cells, and T cells in the lungs of C57BL/6J and BALB/cJ mice following administration of elastase. Flow cytometry was used to determine the no. of NK cells (A), NK T cells (B), CD4⁺ T cells (C), and CD8⁺ T cells (D) in whole lung preparations from C57BL/6J and BALB/cJ mice at days 7, 14, and 21 after IT administration of 3 U of elastase; n = 5 mice/group. Two-way ANOVA was used to compare the elastase-treated group with naïve controls or to compare BALB/cJ with C57BL/6J at the same time point. *P < 0.05, **P < 0.01, and ***P < 0.001.

Fig. 7. Dynamics of cytokine gene expression in the lungs from C57BL/6J and BALB/cJ mice after administration of elastase. Real-time PCR analysis of whole lung mRNA levels for the genes encoding IL-4 (A), IL-5 (B), IL-13 (C), IL-10 (D), interferon (IFN)-γ (E), and IL-17A (F) in C57BL/6J and BALB/cJ mice at days 2, 4, 7, 14, and 21 after IT administration of 3 U of elastase; n = 4 mice/group. All data were normalized to β-actin transcription levels and expressed as relative expression units. Two-way ANOVA: *P < 0.05, **P < 0.01, and ***P < 0.001 comparing elastase-treated groups with naïve animals and †P < 0.05, ††P < 0.01, and †††P < 0.001 comparing BALB/cJ with C57BL/6J at the same time point.
Beyond these potential structural differences, the differential susceptibility between C57BL/6J and BALB/cJ mice that has been shown in a variety of other disease models has been attributed in part to differences in immune responsiveness. Thus, we sought to identify the differences in the immune milieu in response to elastase injury between the two strains. Our temporal evaluation of BAL cells showed that BALB/cJ mice had more lymphocytes in the first 2 wk after elastase treatment and more persistent macrophages for at least 3 wk after the acute insult. These results suggest a potential role of these two cell types in determining susceptibility to emphysema progression. Therefore, we also measured the alteration of several cytokine and activation marker genes associated with lymphocytes and macrophages. The instillation of elastase in the lungs resulted in higher IFN-γ expression in C57BL/6J mice compared with BALB/cJ mice. This result agrees with a number of published studies showing that C57BL/6J mice are Th1 prone and have the ability to produce elevated IFN-γ (56, 63). However, elastase-treated BALB/cJ mice did not demonstrate a reciprocally greater type 2 cytokine response (IL-4, IL-5, and IL-13; Fig. 7, A, B, and C), an effect that has been shown in allergic or parasitic infection models (21, 27). Interestingly, in BALB/cJ mice there was a 20-fold upregulation of il17a that was not found in C57BL/6J mice. Moreover, there was higher expression of il18 in BALB/cJ relative to C57BL/6J mice, both at baseline and after elastase administration. Mice overexpressing either IFN-γ or IL-18 specifically in the adult lung displayed an emphysema-like phenotype accompanied by high levels of proteases such as MMP-12 and cathepsins in prior studies (40, 84). In addition, it was also reported that IL-17A-deficient mice develop less pulmonary inflammation accompanied by a reduction in the severity of emphysema after elastase exposure (43). Moreover, transgenic IL-18-overexpressing mice had enhanced expression of IFN-γ and IL-17A together with elevated numbers of IFN-γ positive cells in the lung (40). Taken together, these studies suggest that these cytokines derived from innate (NK cells, ILC3, γδT cells) or adaptive (CD4 T cells, CD8 T cells) cells have a potential role to play in the emphysematous damage triggered by elastase, and our work further suggests that they may also be important for dictating the strain-dependent outcome of elastase exposure. In addition, our results suggest that the strain differences are more than simply a result of greater initial injury, since it appears that there are several qualitative differences in the response of these immune factors in BALB/cJ mice.

Another potentially important difference in elastase-treated BALB/cJ mice and C57BL/6J mice is the prolonged increase of genes associated with alternatively activated M2 macrophages. Such M2 macrophages participate in the resolution of inflammation, wound healing, and tissue remodeling by producing a broad array of extracellular matrix proteases such as MMP-2, MMP-9, and MMP-12 (52, 58). To maintain tissue homeostasis, the activation of these macrophages has to be tightly regulated. In chronic inflammatory states, persistent activation of macrophage-derived MMPs is involved in remodeling the extracellular matrix, to the extent that such excessive stimulation can lead to subsequent loss of matrix integrity and...
mechanical failure as seen in periodontal disease or arthritis (57, 61, 71). Several studies have indicated a significant contribution of MMP-12 in degrading the extracellular matrix of alveolar walls, a notion that is supported by a study showing that mice lacking MMP-12 have an attenuation of cigarette smoke-induced emphysema (30). As such, MMP-12 is commonly measured in experimental emphysema models as a correlate for lung damage. Consistent with previous studies, we found that the susceptible BALB/cJ mice had a very marked and prolonged production of MMP-12 in the lung in response to elastase injury than the resistant C57BL/6J strain. The polarization of macrophages toward an M2 phenotype accompanied by MMP-12 elevation has also been observed in the hookworm infection model of emphysema (51), an acute ozone exposure model (74), the mouse cigarette smoke model (24, 92), and possibly in human emphysema patients (42). Interestingly, in addition to the expression of signature M2 markers such as arg1 and fizz1, we also found elevated expression of iNOS, a typical marker for classical activated macrophages (M1 phenotype) both at baseline and after elastase insult in the BALB/cJ strain. These data are similar to several previous human studies showing the increased expression of iNOS in alveolar macrophages and lung tissue from COPD patients (36, 49, 81). This classical activation of macrophages has been linked to the increase in production of reactive oxidation species and nitric oxide as well as the enhanced release of

**Fig. 9. Dynamics of protease and antiprotease gene expression in the lungs from C57BL/6J and BALB/cJ mice after administration of elastase.** Real-time PCR analysis of whole lung mRNA levels for the genes encoding α1-antitrypsin (SERPINA1, A), tissue inhibitor of metalloproteinase 1 (TIMP1, B), α2-macroglobulin (A2M, C), matrix metalloproteinase (MMP)-12 (MMP12, D), MMP2 (E), MMP9 (F), and heme oxygenase 1 (HMOX1, G) in C57BL/6J and BALB/cJ mice at days 2, 4, 7, 14, and 21 after IT administration of 3 U of elastase; n = 4 mice/group. All data are compared with day 0 control group and normalized to β-actin expression. Two-way ANOVA: *P < 0.05, **P < 0.01, and ***P < 0.001 comparing elastase-treated groups with naïve animals and †P < 0.05, ††P < 0.01, and †††P < 0.001 comparing BALB/cJ with C57BL/6J at the same time point.
several proinflammatory cytokines (e.g., IL-1β, IL-6, IL-8, and TNF-α) that have been shown to be important contributors to the pathogenesis of COPD (17, 18, 38, 89). Similarly, we also report a higher elevated expression of both IL-1β and IL-6 in BALB/cJ vs. C57BL/6J mice. Moreover, deficiency of either IL-1β or IL-6 has been shown to protect from elastase-induced emphysema in mice (14, 76). This mixed MI/M2 phenotype could represent a unique fixed activation status of a single population of long-lived lung macrophages, a single plastic phenotype that is dependent on changes to the local lung environment, or it could reflect the presence of multiple distinct populations of macrophages. Furthermore, it is possible that genetic factors contribute significantly to the changes in the lung environment that in turn influence a dynamic activation and metabolic phenotype of macrophages that ultimately promote the progression of emphysema. Addressing these possible mechanisms, however, will require further study.

To completely understand the impact that proteases have on the progression of emphysema, it is important to consider the expression dynamics of the endogenous and inducible inhibitors that regulate the overall level of protease activity in the lungs. Our results illustrate that control BALB/cJ mice have lower expression levels of α2-macroglobulin, a major endogenous antiprotease that can bind to porcine pancreatic elastase. Thus, if the upregulation of this inhibitor was lower in BALB/cJ mice relative to C57BL/6J mice, it could account for the increased emphysema we observed. However, α2-macroglobulin is produced mainly by hepatocytes in the liver, and only in small amounts by cells in the lungs (13). Moreover, a primary role of α2-macroglobulin is to rapidly trap excess proteinases, but not to inactivate them, so proteases can still be released after binding α2-macroglobulin (62, 72). Unlike α2-macroglobulin, α1-AT is more likely to be a physiological inactivator of elastase. Interestingly, our data showed a greater level of α1-AT in BALB/cJ mice compared with C57BL/6J mice. These data are consistent with Martorana et al. who reported that there were higher mRNA levels of α1-AT expression in the liver tissue of BALB/cJ than C57BL/6J and pallid mice, corresponding with higher serum α1-AT levels and more elastase inhibitory capacity (53). The pallid mice, which have a marked reduction in α1-AT levels, were shown to spontaneously develop an emphysema-like phenotype in early life and had increased susceptibility to additional emphysematous damage when exposed to the neutrophil chemotactic peptide FMLP (11, 53). The fact that BALB/cJ mice have higher levels of α1-AT suggests that the increased susceptibility of this strain to progressive emphysema is more likely a result of other mechanisms, rather than antiprotease activity.

One of the outstanding issues regarding the pathogenesis of emphysema is the role of persistent chronic inflammation, and how it may lead to the continual alveolar tissue destruction in the lungs. In addition, there is often a gradual decline in lung function in human patients, even in the absence of the risk factors as seen in smokers who have quit but for whom emphysema continues to progress (64). This observation implies that the mechanisms underlying the initiation phase of human emphysema might be different from the mechanisms driving the progression phase. This progression of presumably immune-mediated pathology in humans cannot be reproduced in animals exposed to chronic cigarette smoke. Instead, this aspect of the human disease is perhaps better modeled in the mouse model of elastase-induced experimental emphysema, particularly in BALB/cJ mice as we have shown here. Originally, as mentioned earlier, the hamster was reported to be susceptible to the development of emphysema induced by elastase in part because of its low level of serum α1-AT (3). Nevertheless, the hamster was still shown to have the ability to eliminate the exogenous elastase from the lung within 24 h (72). This situation is mimicked in our mouse model, as evidenced by exogenous elastase-associated acute lung injury (e.g., mortality, hemorrhage, massive neutrophil infiltration) existing only in the first few days postchallenge. Although this is a simple insult to produce, the model is clearly not mechanistically simple. The mechanism requires other endogenous mediators that may have many similarities to the human situation. Due to the fact that many emphysema patients are typically not diagnosed until they are symptomatic and in a relatively late stage of the disease (8, 15), studies using the elastase-induced emphysema in BALB/cJ mice might turn out to be very beneficial for studying the mechanisms that are associated with progression of the disease at early stages, for evaluating potential therapeutic interventions to slow or stop the disease perpetuation, and to identify markers that could be used for early diagnosis.

In conclusion, we provide data in support of the hypothesis that differences in immune responsiveness between BALB/cJ and C57BL/6J mice contribute to a functional disparity in the progression of elastase-induced experimental emphysema. The establishment of this new model provides a novel tool with which to carry out comparative analyses to aid in the identification of genetic, molecular, and cellular pathways that are critical for the development of emphysema, with the ultimate goal of yielding therapeutics that can stem the progression of this disease.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS


REFERENCES


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