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ACUTE LUNG INJURY

Deletion of LysM in LysMCre Recombinase Homozygous Mice is Non-contributory in LPS-Induced Acute Lung Injury

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Abstract

Lysozyme is an important component of the innate immune system and has roles in peptidoglycan cleavage of gram-positive organisms. Myeloid cells highly express the isoform, lysozyme M, and its promoter has been used to direct Cre recombinase expression to target deletion of floxed genes in myeloid cells. However, generation of the LysMCre mouse effectively disrupts the LysM gene, and mice homozygous for the Cre allele lack the LysM gene product. To test the contribution of LysM in sterile acute lung injury, we generated LysMCre mice homozygous for the Cre allele (+/+) or wild-type allele (−/−). These mice were challenged with LPS delivered via oropharygneal aspiration. Mice were monitored and weighed daily, and BAL cell counts, differential, protein, and cytokine levels were assessed at days 2 and 4. LysMCre+/+ and LysMCre−/− had similar weight loss and recovery, and similar inflammatory responses to LPS at days 2 and 4. These findings indicate that loss of LysM and expression of Cre recombinase are non-contributory in sterile acute lung injury.

Keywords Murine · Lysozyme · LysMCre · ALI · Acute lung injury

Introduction

Lysozyme is a glycoside hydrolase that catalyzes the hydrolysis the β-1,4 glycosidic bond between *N*-acetyl muramic acid and *N*-acetyl glucosamine [1]. Its main substrates are predominantly found in the cell wall of gram-positive bacteria, but shorter saccharides and chitin have also been shown to be substrates of this enzyme [2, 3]. Beyond its catalytic activity, non-catalytic antimicrobial roles have also been ascribed to lysozyme [4], as well as roles in monocyte-mediated tumoricidal activity [5]. Lysozyme M (LysM or Lyz2), one of two isoforms, is abundant in the airway and functions as a critical antimicrobial in the lung. Augmentation of its expression in lung increases antimicrobial killing of *P. aeruginosa* and group B *Streptococcus* [6], and LysM deletion in mice leads to increased susceptibility to *Klebsiella pneumoniae* [7].

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Lysozyme M is expressed by myeloid cells, and its promoter has been utilized to direct Cre recombinase to target deletion of floxed genes in these cells. LysMcre mice have a nuclear-localized Cre recombinase inserted into the first coding ATG of the lysozyme M gene, abolishing endogenous LyzM gene function, and placing the Cre recombinase under the control of the LyzM promoter [8]. When crossed with reporter mice (ROSA-EYFP) to characterize efficiency and specificity, use of this strain targets monocytes, neutrophils, and macrophages [9]. In addition to myeloid cells, a portion of type II alveolar epithelial cells also express Cre recombinase [10].

Given the growing interest of assessing myeloid-specific genes in acute lung injury, LysMcre mice are frequently employed in these models [11, 12]. However, there is an under appreciation of loss of LysM expression in homozygous LysMcre mice, with occasional lack of reporting of the zygosity for LysMcre alleles or use of appropriate controls to account for LysM contribution. In this study, we tested the contribution of lysozyme M in LPS-induced lung injury. Mice homozygous for the LysMcre allele (and thus, LysM deficient) and littermate wild-type controls were challenged with oropharyngeal aspiration of *E. coli* LPS and assessed at days 2 and 4 for BAL cell count, differential, protein, and cytokines. We observed similar changes in total body

weight in response to LPS and a similar neutrophilic predominant inflammatory influx at days 2 and 4. These findings indicate that deletion of LysM and expression of Cre recombinase does not modulate the inflammatory response to LPS-induced ALI.

Materials and Methods

Animals

LysMcre mice (B6.129P2-Lyz2$^{tm1(cre)Ifo}$/J) were obtained from Jackson Laboratory (Bar Harbor, ME) and crossed with C57BL/6J mice. Heterozygous LysMcre (+/−) mice from these crosses were established as breeders. Offsprings were genotyped, and homozygous Cre+/+ mice and Cre−/− mice were used for these studies. Mice were age and gender matched, and LPS doses (1.5 µg/g) were administered by oropharyngeal aspiration as described [13]. Mice were euthanized at days 2 and 4. BAL and cytospins were performed as described [14]. The University of Washington Office of Animal Welfare approved all animal protocols.

Genotyping

The following primers were used to detect Cre recombinase or wild-type alleles: Cre 5′ CCC AGA AAT GCC AGA TTA CG, Common 5′ CTT GGG CTG CCA GAA TTT CTC, and Wild type 5′ TTA CAG TCG GCC AGG CTG AC.

Broncho-Alveolar Lavage

In euthanized mice, the trachea was cannulated with an angiocatheter. Broncho-alveolar lavage was performed with three serial instillations of lavage buffer (PBS, 0.5% EDTA) (total volume 2.5 ml). BAL cells were pelleted and re-suspended in RPMI + 10% FBS, and approximately 50,000 cells placed on slides using a cytospin. Cells were stained with Differential quik (VWR, Radnor, PA), and a manual differential was performed on 100 cells.

Lung Histolopathology

Lungs were inflated and fixed with 10% formalin, dehydrated with serial dilutions of ethanol, and embedded in paraffin. Lung sections (5 µm) were stained with hematoxylin and eosin. Lungs were scored on a 5-point scale (0 = no abnormality; 1 = minimal inflammation involving < 10% of lung; 2 = mild involving 10–25%; 3 = moderate involving 26–50%; 4 = moderate-severe involving 51–75%; 5 = severe involving > 75%).

Quantitative RT-PCR (qRT-PCR)

Total RNA from cells was isolated using RNeasy Mini kit (Qiagen, Valencia, CA). The quantity and quality of RNA were determined using a NanoDrop spectrophotometer (NanoDrop Inc., Wilmington DE). Primers and TaqMan probes (FAM dye-labeled) for *Lyz2* (Mm01612741; ThermoFisher Scientific, Waltham, MA) *and Hprt* were added to cDNA synthesized from total RNA with a High Capacity cDNA Archive kit (Applied Biosystems, Carlsbad, CA). Product amplification was measured with an ABI HT7900 Fast real-time PCR system (Applied Biosystems). The threshold cycle (Ct) was obtained from duplicate samples and averaged. The ΔCt was the difference between the average Ct for the target gene and the housekeeping gene, *Hprt*. The ΔΔCt was the average ΔCt for a given sample point minus the average ΔCt of the control samples; relative quantification was calculated as $2^{-\Delta\Delta Ct}$.

Cytokine Measurements

Cytokines and chemokines concentrations for CXCL1/KC and CCL2/MCP-1 were measured in broncho-alveolar lavage (BAL) samples using ELISA as per the manufacturer's instructions (R&D Systems, Minneapolis, MN) and analyzed using a Synergy 4 plate reader (BioTeck, Winooski, VT).

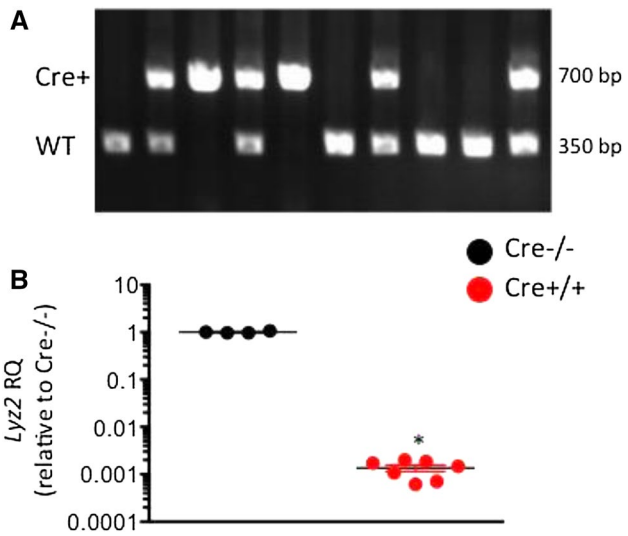

Fig. 1 Loss of LysM expression in Cre+/+ mice. **a** Representative PCR demonstrating amplification of the Cre gene (700 bp band) or Wild-type allele (350 bp band) used to identify Cre+/+ (single 700 bp band) or Cre−/− (single 350 bp band) mice. **b** RT-PCR for LysM (*Lyz2*) mRNA expression. Results shown as relative quantification (RQ) to the average of Cre−/− samples (black circle). *p value < 0.05

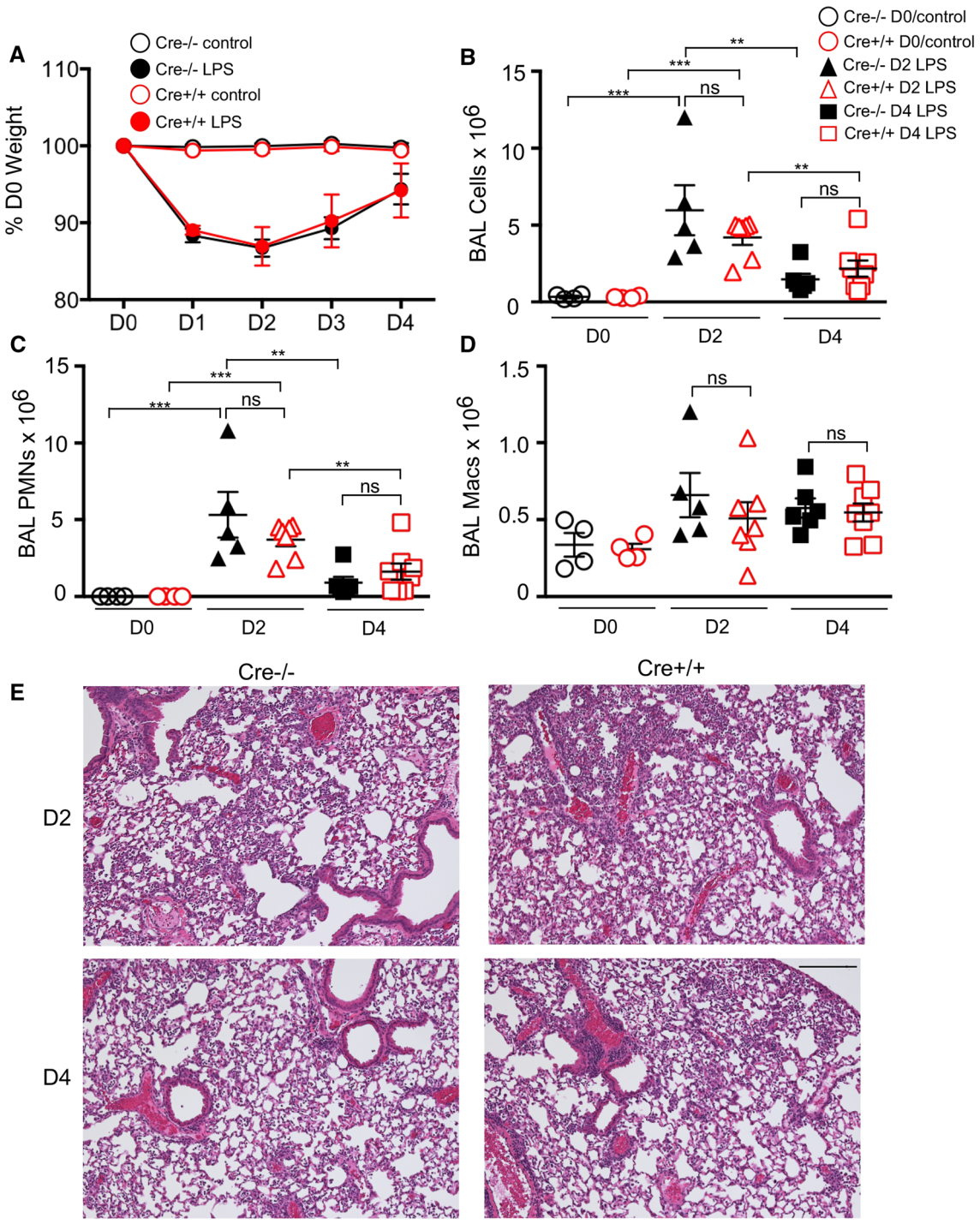

Fig. 2 LPS-induced ALI. LysMCre+/+ or LysMCre−/− mice received LPS via oropharygneal aspiration or were unchallenged (control). **a** Mice were weighed daily. There was a similar decrease in weight in both LPS groups, independent of genotype, with recovery of weights between days 3 and 4. Broncho-alveolar lavage was performed on days 2 and 4 for **b** total cell counts, **c** neutrophil (PMN) cell count, **d** alveolar macrophage (Macs) cell count. There was no difference in BAL cell counts, neutrophils, and macrophages between LPS-treated groups matched for time-point. **e** Representative lung histopathology of D2 (top) and D4 (bottom) post LPS. Scale bar = 200 µm. ***p value < 0.0001; **p value < 0.001, *ns* non-significant

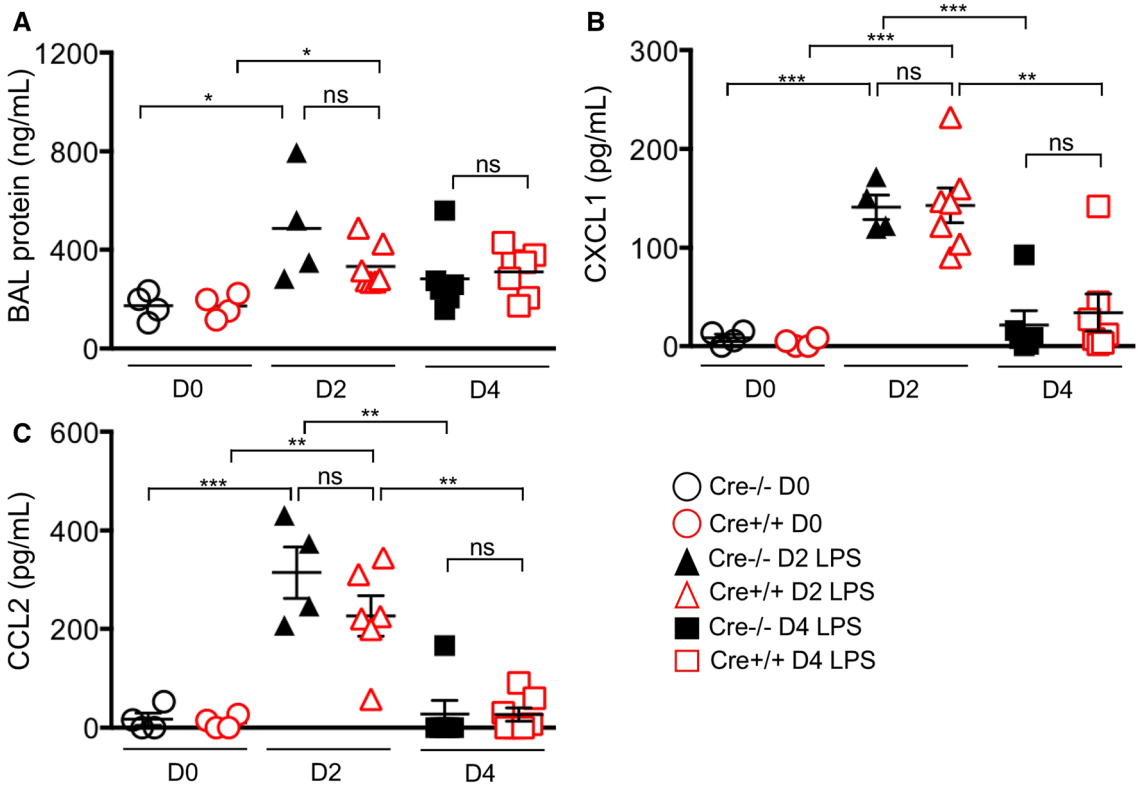

Fig. 3 BAL protein and chemokine response. LysMCre+/+ or LysMCre−/− mice received LPS via oropharyngneal aspiration or were unchallenged (control). Broncho-alveolar lavage was performed on days 2 and 4 for **a** total protein, **b** CXCL1, and **c** CCL2 concentration. There were no differences in these measurements between LPS-treated groups matched for time-point. ***p value < 0.0001; **p value < 0.001; *p value < 0.05, *ns* non-significant

Statistics

Results are expressed as means ± SEM. Statistical significance was determined using one-way ANOVA with multiple comparisons. Differences were considered significant if the P-value was < 0.05.

Results

We generated and bred LysMcre mice heterozygous for the Cre allele (+/−). Littermates homozygous for the Cre allele (Cre+/+) or wild-type allele (Cre−/−) were identified by genotyping (Fig. 1a). Marked reduction of the LysM gene product was confirmed by qPCR using RNA isolated from broncho-alveolar (BAL) macrophages (Fig. 1b).

Next, Cre+/+ and Cre−/− mice were challenged with *E. coli* LPS and assessed daily for changes in their body weight, a surrogate marker for illness severity induced by LPS. The absence of lysozyme M did not alter the initial weight loss, greatest at day 2, or recovery of weights seen at days 3–4 (Fig. 2a). Mice were euthanized at days 2 and 4 for broncho-alveolar lavage, cell count, and differential. At day 2, both genotypes had similar LPS-induced increases in BAL cell count (Fig. 2b) and a neutrophil-predominant influx into the alveolar compartment (Fig. 2c). By day 4, BAL cell counts and BAL PMNs were significantly reduced between days 2 and 4, with no difference by genotype (Fig. 2b, c). BAL macrophages were also similar in LPS-treated groups (Fig. 2d) across genotypes and time points, with a trend for an increase after injury. We assessed lung histopathology to assess degree and localization of the inflammatory influx. There was predominant alveolar and perivascular inflammation with similar extent of patchy lung involvement in LPS-induced groups (Fig. 2e) with a similar assessment of inflammation based on scoring: 2.9 ± 0.2 versus 3.1 ± 0.3, D2 Cre+/+ versus D2 Cre−/− p value = 0.53; and 3.0 ± 0.3 versus 2.8 ± 0.5, D4 Cre+/+ versus D4 Cre−/− p value = 0.64.

BAL protein, a marker of vascular injury was increased at day 2 compared to day 0, with a similar response between LPS-treated genotypes (Fig. 3a). We assessed for differences in inflammatory chemokines, CXCL1 and CCL2. At day 2, both chemokines were increased in LPS-treated groups compared to control, with no difference in responses between genotypes (Fig. 3b, c). By day 4, the chemokine levels were

significantly reduced or below the level of detection in both genotypes.

Discussion

Use of LysMCre recombinase mice to target myeloid cell depletion of floxed genes is commonly employed. However, deletion of LysM gene and its protein product is a confounder when homozygous mice are generated. To develop an understanding of how often the genetics and the use of appropriate controls for LysMCre mice are reported in manuscripts, we examined 12 articles identified using PubMed and the search string, "LysM and Cre and lung." Upon review of these manuscripts, we found that a majority (7/12) did not directly report LysM zygosity and/or use of appropriate controls to account for the loss of the lysozyme 2 gene. Based on the ARRIVE guidelines, it is recommended that authors provide information about the experimental animals used in a study including mouse strain, sex, weight, and genetic modification status [15]. In particular, because the targeted insertion of LysMCre results in the loss of lysozyme 2 gene, it is important to report the zygosity of these mice.

In infectious models, the role of LysM in controlling infection and inflammation is well established [7]; however, in sterile models of ALI, the contribution of LysM has not been reported. Given the broad use of these mice to model myeloid contribution in lung injury, we sought to test if the LysM gene is contributory to LPS-induced acute lung injury. We generated LysCre ± (+/−) mice and studied littermates that were homozygous for the wild-type (Cre−/−) or Cre (Cre+/+) allele. Using commercially available primer probe sets for *Lyz2* mRNA, there was a large reduction in the mRNA detected. However, there was still detectable signal from LysM-deleted mice, possibly a result of detection of its isoform. Importantly, we found that LysM was non-contributory to the inflammatory phenotype of LPS-treated mice as both genotypes had similar inflammatory cell recruitment to the lung, protein leak, and cytokine expression.

Limitations of this study are the assessment of inflammation in a single model of ALI, limiting the generalizability to other models of lung injury. However, recognition of this potential confounder is important in future experimental design in other injury models as is the reporting of zygosity of the LysM allele. Hence, addition of controls using Cre+/+ mice that lack the floxed gene or use of mice with a single copy of the Cre transgene to assure functionality of at least one allele of the LysM gene is necessary.

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Compliance with Ethical Standards

Conflict of interest All authors declare that they have no conflict of interest.

References

1. Cross M, Mangelsdorf I, Wedel A, Renkawitz R (1988) Mouse lysozyme M gene: isolation, characterization, and expression studies. Proc Natl Acad Sci USA 85:6232–6236
2. Ishiguro K, Yoshie N, Sakurai M, Inoue Y (1992) A 1H NMR study of a fragment of partially n-acetylated chitin produced by lysozyme degradation. Carbohydr Res 237:333–338
3. Pangburn SH, Trescony PV, Heller J (1982) Lysozyme degradation of partially deacetylated chitin, its films and hydrogels. Biomaterials 3:105–108
4. Ibrahim HR, Matsuzaki T, Aoki T (2001) Genetic evidence that antibacterial activity of lysozyme is independent of its catalytic function. FEBS Lett 506:27–32
5. LeMarbre P, Rinehart JJ, Kay NE, Vesella R, Jacob HS (1981) Lysozyme enhances monocyte-mediated tumoricidal activity: a potential amplifying mechanism of tumor killing. Blood 58:994–999
6. Akinbi HT, Epaud R, Bhatt H, Weaver TE (2000) Bacterial killing is enhanced by expression of lysozyme in the lungs of transgenic mice. J Immunol 165:5760–5766
7. Markart P, Korfhagen TR, Weaver TE, Akinbi HT (2004) Mouse lysozyme M is important in pulmonary host defense against Klebsiella pneumoniae infection. Am J Respir Crit Care Med 169:454–458
8. Clausen BE, Burkhardt C, Reith W, Renkawitz R, Forster I (1999) Conditional gene targeting in macrophages and granulocytes using LysMcre mice. Transg Res 8:265–277
9. Abram CL, Roberge GL, Hu Y, Lowell CA (2014) Comparative analysis of the efficiency and specificity of myeloid-Cre deleting strains using ROSA-EYFP reporter mice. J Immunol Methods 408:89–100
10. McCubbrey AL, Allison KC, Lee-Sherick AB, Jakubzick CV, Janssen WJ (2017) Promoter specificity and efficacy in conditional and inducible transgenic targeting of lung macrophages. Front Immunol 8:1618
11. Jiang Z, Chen Z, Li L, Zhou W, Zhu L (2017) Lack of SOCS3 increases LPS-induced murine acute lung injury through modulation of Ly6C(+) macrophages. Respir Res 18:217
12. Xia H, Ren X, Bolte CS, Ustiyan V, Zhang Y, Shah TA, Kalin TV, Whitsett JA, Kalinichenko VV (2015) Foxm1 regulates resolution of hyperoxic lung injury in newborns. Am J Respir Cell Mol Biol 52:611–621
13. Eddy WE, Gong KQ, Bell B, Parks WC, Ziegler SF, Manicone AM (2017) Stat5 is required for CD103(+) dendritic cell and alveolar macrophage development and protection from lung injury. J Immunol 198:4813–4822
14. Manicone AM, Gharib SA, Gong KQ, Eddy WE, Long ME, Frevert CW, Altemeier WA, Parks WC, Houghton AM (2017) Matrix metalloproteinase-28 is a key contributor to emphysema pathogenesis. Am J Pathol 187:1288–1300
15. Kilkenny C, Browne W, Cuthill IC, Emerson M, Altman DG, Group NCRRGW (2010) Animal research: reporting in vivo experiments: the ARRIVE guidelines. Br J Pharmacol 160:1577–1579