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Relationship between neutrophil influx and oxidative stress in alveolar space in lipopolysaccharide-induced lung injury

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Abstract

We intratracheally administered lipopolysaccharide (LPS) to ICR mice and then collected BAL fluid and lung tissue to determine whether levels of neutrophils and/or myeloperoxidase (MPO) in bronchoalveolar lavage (BAL) fluid reflect lung tissue damage. Robust neutrophil accumulation into the alveolar space and lung tissue were almost completely abolished at seven days along with oxidative stress markers in the lung. However, lung injury scores and lung wet/dry ratios, as well as MPO and oxidative stress markers in BAL fluid were significantly increased at five and seven days after LPS administration. At later time points, BAL neutrophils generated more MPO activity and ROS than those harvested sooner after LPS administration. Although elevated neutrophil levels in BAL fluid reflected oxidative stress in the lungs, MPO might serve as a useful marker to evaluate damage sustained by epithelial cells over the long term.

Key words: oxidative stress marker, neutrophil recruitment, myeloperoxidase, reactive

oxygen species, lung injury model

1. Introduction

Acute respiratory distress syndrome (ARDS) is a type of acute diffuse, inflammatory lung injury that leads to increased pulmonary vascular permeability, increased lung weight, and loss of aerated lung tissue. The clinical hallmarks are hypoxemia and bilateral radiographic opacities associated with increased venous admixture, increased physiological dead space and decreased lung compliance (Force et al., 2012). Acute respiratory distress syndrome is a frequent complication among critically ill patients and it is responsible for high morbidity and mortality rates (Lesur et al., 1999; Ware et al., 2000). Treatment of the underlying disease and supportive care using the “lung protective” strategies of mechanical ventilation and prone positioning, contribute to successful clinical outcomes (Network, T.A.R.D.S., 2000; Guerin et al., 2013). However, specific therapies have not been established and once the cascade of events leading to ARDS has been initiated, the condition becomes much less amenable to specific treatment.

Reactive oxygen species (ROS) such as superoxide anion radicals (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radicals ($OH^\cdot$) and hypochlorous acid (HOCl) play central roles in the pathogenesis of acute lung injury (Haegens et al., 2009, Tate et al., 1983). Endothelial or epithelial cells express several antioxidants such as superoxide

dismutase, catalase and glutathione peroxidase to neutralize free radicals and counteract the detrimental effects of ROS (Fink et al., 2002). However, ROS generated by phagocytes during the acute inflammatory response overwhelm these antioxidants and lead to cell and lung damage. Neutrophils that have high oxidant-generating capacity migrate into the alveolar space where they degranulate and release proteins from azurophilic granules into phagolysosomes (Nauseef, 2001). Bronchoalveolar lavage (BAL) is a diagnostic method of sampling cells in the airway-alveolar space and soluble substances in the extracellular lining. The number of neutrophils in BAL fluid is robustly increased in ARDS, and the ratios (%) of neutrophils are markers of disease activity (Steinberg et al., 1994). The time course of transpulmonary polymorphonuclear leukocyte migration has been investigated (Hirano, 1997; Reutershan et al., 2005). However, whether or not inflammatory cells, especially neutrophils that presently serve as clinical markers in BAL fluid, reflect the extent of damage in lung tissues remains obscure.

The short biological half-life of ROS renders them difficult to measure directly in biological materials from the lungs of patients with ARDS, and reports describing increased ROS activity in ARDS are scant (Baldwin et al., 1986). Alternatively, the oxidative modification of ROS targets such as proteins, lipids, and antioxidants are

regarded as useful markers with which to indirectly reflect oxidative stress. Levels of protein carbonyls, myeloperoxidase (MPO), thiobarbituric acid-reactive substances (TBARS), lipid oxidation products and oxidized glutathione are elevated in BAL fluid from patients with ARDS (Bunnell et al., 1993; Winterbourn et al., 2000). Whether or not oxidative stress markers exactly reflect lung oxidative stress in patients with ARDS is unknown. These oxidative markers have been evaluated in animal models of ARDS to determine the amount of oxidative stress in the lungs. However, few studies have investigated the same oxidative stress markers both in BAL fluid and in lung tissue (Bergeron et al., 1998).

The present study investigated whether or not neutrophils and MPO in BAL fluid can reflect oxidative stress or epithelial damage in the lungs of a mouse model of LPS-induced lung injury. We compared the kinetics of various oxidative stress markers with neutrophil accumulation and MPO activities in BAL fluid and tissues from mouse lungs with lipopolysaccharide (LPS)-induced lung injury. We also examined the ROS-producing potential of neutrophils harvested from BAL at various intervals after the intratracheal instillation of LPS to produce ROS. Not only a higher ratio of neutrophils but also an increase in MPO activity in BAL fluid suggested the existence of epithelial cell damage and oxidative stress both in BAL fluid and in the lungs with

LPS-induced lung injury. Thus, MPO might be a useful marker to evaluate long term damage sustained by epithelial cells.

2. Materials and Methods

Animals

Nine-week-old male ICR mice purchased from Japan Clea (Tokyo, Japan) were housed in plastic chambers with free access to food and water. None of the mice had gross pathological lesions. The Ethics Committee for Animal Research at Hokkaido University School of Medicine approved the experimental protocols.

2.1 Mouse model of LPS-induced lung injury

Saline (50 μ L) containing 200 μ g of LPS (Sigma Chemical Co., St. Louis, MO, USA) was intratracheally administered to mice anesthetized with a mixture of ketamine and xylazine as described (Betsuyaku et al., 1999; Ito et al., 2009). Age-matched, untreated healthy mice served as controls.

2.2 BAL and tissue measurements

2.2.1 Wet/dry weight ratio

The wet lungs of mice from which BAL had not been collected were weighed immediately after dissection, dried at 37°C for 72 h, and then weighed once again to determine the wet/dry (W/D) weight ratio.

2.2.2 Lung histopathology

Paraffin-embedded lung sections were stained with hematoxylin and eosin for assessment by light microscopy. Lung damage was graded from 0 (normal) to 4 (severe) based on the criteria of interstitial inflammation, neutrophil infiltration, congestion and edema (Michetti et al., 2003). Lung damage was scored by adding the individual scores for each category and the score for each mouse was calculated as the mean of four lung sections.

2.2.3 BAL and sampling of mouse lung tissues

Mice were killed by CO₂ narcosis at 1, 3, 5, 7 and 14 days after LPS injection (n = 5 - 6 per time point) and BAL was collected using three 0.6-mL injections of saline through a tracheal cannula. Red blood cells in BAL fluid samples were disrupted using red blood cell lysis buffer (Sigma) and then total numbers of cells were counted using a hemocytometer. Cell differentials in BAL fluid were examined in Cytospin preparations stained with Diff-Quik reagent (Sysmex International Reagents, Kobe, Japan). After BAL fluid was collected, the lungs were inflated with 50% (vol/vol) Tissue-Tek OCT (Sakura Finetek USA, Torrance, CA, USA) in RNase-free phosphate-buffered saline

(PBS) containing 10% sucrose and stored at -80°C as described (Suzuki et al., 2008).

2.2.4 Immunohistochemical evaluation of neutrophils in lungs

Lung sections were immunostained for Gr-1 as described (Moriyama et al., 2010).

Briefly, non-specific binding was blocked for 30 min using 5% (v/v) normal rabbit serum in PBS. Neutrophils were detected using a polyclonal rat anti Ly-6G (Gr-1) monoclonal antibody (BD Biosciences, San Jose, CA, USA) followed by anti-rat IgG-horseradish peroxidase-conjugated secondary antibody (DakoCytomation, Glostrup, Denmark). Labeling was visualized using diaminobenzidine as the chromogen (Vector Laboratories). Gr-1-positive cells were counted in five random fields per section of 5 – 6 grafts per group, and then the ratio (%) of total cells per high-power field was calculated.

2.2.5 Assay of MPO activity

We spectrophotometrically assayed MPO activity in BAL fluid and lung tissues as described (Haslam et al., 1999). Briefly, BAL fluid (25 μL) or lung homogenate was reacted with H_2O_2 (0.0005%) in the presence of o-dianisidine dihydrochloride (0.167 mg/mL) for 30 min and changes in absorbance at 450 nm were measured. Protein

concentrations of tissue extracts were determined using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA).

2.2.6 Assessment of carbonylated protein in BAL fluid

The carbonylation of proteins in BAL fluid was measured by Western blotting as described (Nagai et al., 2006; Nagai et al., 2008). Briefly, raw BAL fluid (16 μ L) was derivatized with dinitrophenylhydrazine (DNP) using the OxyBlot Protein Oxidation Detection Kit (Chemicon International, Temecula, CA, USA) and resolved by electrophoresis on 10% SDS-polyacrylamide gels. Proteins were Western blotted with anti-DNP antibody and band intensity was calculated using NIH Image software (version 1.62). The intensity of the 68-kDa band corresponding to carbonylated albumin on each blot is shown as arbitrary units (AU).

2.2.7 Total protein assay

Total protein concentration in BAL fluid was quantified using the bicinchoninic acid microassay method (Pierce Chemical).

2.2.8 Measurement of LPO, GSH and GSSG in BAL fluid

Levels of LPO, GSH and GSSG in BAL fluid were measured using kits according to the manufacturer's protocols (Cayman Chemical, Ann Arbor, MI, USA).

2.2.9 Measurement of protein carbonyl contents of the lung

Protein carbonyl contents in lung homogenates were determined using a protein carbonyl assay kit (Cayman Chemical), according to the manufacturer's instructions.

2.2.10 Immunohistochemical evaluation of 4 hydroxy-2-nonenal modified proteins (4-HNE)

Frozen sections cut at 4 μ m were fixed in 4% paraformaldehyde for 10 min and then immunostained using the Vectastain ABC-AP Kit (Vector Laboratories, Burlingame, CA, USA) with rabbit anti-4-HNE (Alpha Diagnostic, San Antonio, TX, USA) antibody. Non-specific binding was blocked for 1 h using 5% goat serum diluted in PBS at room temperature, and then the sections were incubated in primary antibody (diluted 1:3000) at room temperature for 30 min. Biotinylated universal secondary antibody and Elite ABC reagent were applied at room temperature for 30 min. The sections were washed with Tris-buffered saline containing 0.05% Tween 20 (Sigma) and then alkaline phosphatase substrate was used as chromogen (Vector Laboratories). Staining of 4-HNE

was quantified in images captured in a blinded fashion using NIH Image software. The lower threshold of detection was established using lungs that had not been exposed to LPS and then the overall area of the alveolar wall was measured. The intensity of 4-HNE positive areas was captured from five random fields per graft section and calculated as the ratio (%) of all alveolar wall areas per high-power field.

2.2.11 Evaluation of intracellular ROS generation in alveolar neutrophils from lungs of mice instilled with LPS

Cells from BAL fluid were resuspended in PBS and loaded with 10 μ M aminophenyl fluorescein (APF) (Sekisui Medical, Tokyo, Japan) by incubation at room temperature in a humidified atmosphere with 5% CO₂ in 96-well black tissue culture plates (BD BioCoat, Tokyo, Japan). Fluorescence intensity per neutrophil was measured using a microplate fluorescence reader at 495 (excitation) and 520 (emission) nm after incubation for 30 min and MPO activity in lysed cells from BAL fluid was also evaluated as described above.

2.2.12 Flow cytometry

Cells collected from BAL fluid were resuspended in Hanks' balanced salt solution

(HBSS) containing 0.1% BSA. Nonspecific binding was blocked by incubating the cells in HBSS supplemented with anti-mouse CD16/32 antibodies (BD Biosciences) for 20 min at 4°C. The cells were stained with 10 μ M APF and anti-mouse Ly-6G and Ly-6C (Gr-1) conjugated to phycoerythrin (BD Biosciences) for 20 min, at 4°C, washed twice and then analyzed by flow cytometry using a FACSCalibur (BD Biosciences). Ten minutes before FACS analysis, 7-AAD (BD Biosciences) was added to exclude dead cells. The ratio of neutrophils expressing ROS to total neutrophils was calculated as the ratio of double positive to Gr-1-positive cells.

2.3 Data presentation and statistical analysis

All data are shown as means $\pm$ standard error (SE). Differences between groups were analyzed using an unpaired t-test. More than two means were compared using Dunnett's method. All data were analyzed using StatView J 5.0 software (SAS Institute, Inc., Cary, NC, USA). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Neutrophilic inflammation after intratracheal LPS instillation

We assessed the kinetics of inflammatory cells in BAL fluid after LPS

instillation. Inflammatory cells consisting mainly of neutrophils accumulated in the lungs of mice that were intratracheally administered with LPS (Fig. 1A and B). Neutrophilia was evident in BAL fluid at day one, peaked on day five and then significantly decreased at seven days after LPS administration. Macrophage counts gradually increased for up to 5 days after LPS administration and returned to baseline levels by 14 days thereafter (Fig. 1C).

We then immunohistochemically investigated neutrophil accumulation by staining lung sections for Gr-1. The lungs of mice that did not receive LPS contained a few neutrophils. Immunohistochemical staining revealed a remarkable increase in neutrophil influx into the pulmonary interstitium after LPS instillation (Fig. 2A and B). The number of neutrophils in lung tissues peaked at 5 days after LPS administration and returned to control levels two days later. The ratio of neutrophils in BAL fluid significantly correlated with the ratio of Gr-1-positive cells in lung tissue ($r = 0.831$, $p < 0.001$; Fig.2C). Neutrophils accumulated at equal rates in lung tissues and in BAL fluid.

3.2 Oxidative stress markers in lung tissues from mice with LPS-induced lung injury

We investigated whether neutrophils in BAL fluid reflect oxidative stress in lung tissues. The carbonylated protein content in lung tissue continued to increase for

five days, and then fell to almost baseline levels at seven days after LPS administration (Fig. 3A). We immunohistochemically stained 4-hydroxy-2-nonenal modified proteins (4-HNE) to identify lipid modification caused by oxidative stress. After LPS administration, 4-HNE staining was prominently localized in the alveolar walls (Fig. 3B). Areas of 4-HNE-staining were significantly increased at three and five days after LPS administration and dropped to the control level at seven days thereafter (Fig. 3C). The ratio of neutrophils in BAL fluid closely correlated with levels of oxidative stress markers in the lung (carbonylated protein: $r = 0.830$, $p < 0.001$; 4-HNE: $r = 0.703$, $p < 0.001$; Fig. 3D and E).

3.3 Development of lung injury after intratracheal LPS instillation

We evaluated the degree and duration of lung damage after LPS instillation. An increase in lung injury scores and W/D ratios indicated that lipopolysaccharide caused significant pulmonary damage and edema. Lung injury scores and W/D ratios were both significantly increased at five and seven days after LPS administration and returned to control levels at 14 days thereafter (Fig. 4A - C). These kinetic profiles differed from those in neutrophils in BAL fluid.

3.4 Myeloperoxidase activity in lung tissue and BAL fluid

We evaluated the activity of MPO because it is the most popular marker of neutrophil activation (Chooklin et al., 2009). We found that MPO activity in BAL fluid gradually increased for up to seven days after LPS administration, although neutrophils were almost completely undetectable by that time (Fig. 5A). The kinetics of MPO activity in BAL fluid and the process of lung epithelial damage notably occurred in parallel. In contrast, MPO activity in lung homogenates was significantly elevated for one to five days after LPS administration and returned to baseline levels at seven days thereafter in accordance with neutrophil recruitment in lung tissue (Fig. 5B). The relationship between the ratio of neutrophils and MPO activity was more evident in lung tissue ($r = 0.937$, $p < 0.001$; Fig. 5D) than in BAL fluid ($r = 0.564$, $p = 0.0018$; Fig. 5C). These data suggest that MPO activity does not simply reflect the number of neutrophils in BAL fluid.

3.5 Total protein concentration in BAL fluid

We estimated alveolar-capillary injury by measuring the total protein content in BAL fluid (Holter et al., 1986). Levels of total protein were significantly elevated at three and seven days after LPS administration (Fig. 6A). The activity of MPO in BAL fluid

significantly correlated with levels of total protein ($r = 0.682$, $p < 0.001$; Fig. 6B).

3.5 Oxidative stress markers in BAL fluid

We compared levels of various oxidative stress markers with neutrophil accumulation in BAL fluid. Levels of carbonylated albumin were significantly increased at five and seven days after LPS administration (Fig. 7A) and closely correlated with those of carbonylated albumin ($r = 0.660$, $p < 0.001$; Fig. 7B). Levels of LPO (a marker of lipid peroxidation), total glutathione (GSH), a major intracellular antioxidant, and its oxidized form, glutathione disulfide (GSSG), in BAL fluid gradually increased for up to seven days after LPS administration (Fig. 7C - E). These results showed that levels of various oxidative stress markers remained elevated for 7 days in BAL fluid even though the number of neutrophils had decreased by that time in mouse lungs with LPS-induced lung injury. The trends for markers of oxidative stress in BAL fluid and MPO levels were similar to that of lung damage in LPS-induced lung injury. The activity of MPO in BAL fluid also correlated with levels of total GSH ($r = 0.615$, $p < 0.001$), and LPO ($r = 0.550$, $p = 0.002$) in BAL fluid. However, relationships between ratios of neutrophils and oxidative stress markers in BAL fluid were less evident (carbonylated albumin: $r = 0.403$, $p = 0.033$; total GSH: $r = 0.233$, $p = 0.232$; LPO: $r = 0.410$, $p = 0.030$). These

results indicated that levels of oxidative stress markers in BAL fluid are linked to neutrophil activation.

3.6 Myeloperoxidase activity and ROS generation in alveolar neutrophils at one and five days after LPS administration

We speculated that activated neutrophils produce more MPO in the alveolar space at later, than at earlier time points and thus increase the oxidative stress load on epithelial cells and lining fluid. Inflammatory cells were retrieved from the BAL fluid of mice at one and five days after LPS instillation. Cells retrieved from BAL fluid at various time points were incubated with the probe aminophenyl fluorescein (APF) to selectively detect ROS including HOCl that is specifically produced in neutrophils from hydrogen peroxide via the action of MPO. Neutrophils in BAL fluid recovered from mice produced more ROS at five days than at one day after LPS administration when corrected for the number of neutrophils (Fig. 8A). Furthermore, intracellular MPO activity was higher in neutrophils at five days than at one day after LPS administration (Fig. 8B). The expression of ROS probed with APF in neutrophils was further assessed using FACS analysis. Cells isolated from BAL fluid were double-stained with APF and

for Gr-1. The ratio of APF-positive cells among Gr-1-positive neutrophils was significantly increased at five days compared with one day after LPS administration (Fig. 8C).

Discussion

Calculating the ratios of neutrophils in BAL fluid is a popular method of clinically evaluating lung injury. The present study found that the ratio of neutrophils in BAL fluid reflected elevated oxidative stress markers in damaged mouse lungs. However, the ratio of neutrophils was not always the optimal marker of damage in LPS-induced lung injury because MPO activity in BAL fluid was not always associated with neutrophil accumulation. We also identified a close correlation between carbonylated albumin and MPO in BAL fluid, implying that they interact in the alveolar space. Neutrophils that had accumulated in the alveolar space tended to release far more MPO and ROS at five days after LPS administration than at one day thereafter, which might partly explain the lack of an association among the numbers of neutrophils, MPO activity and oxidative stress, particularly in the alveolar space, at the later stage of inflammation.

Azurophilic granules in neutrophils contain MPO that catalyzes the reaction between H_2O_2 and chloride to yield HOCl, an oxidant that is ~100-fold more reactive than H_2O_2 (Grisham et al., 1990). Levels of MPO activity and of chlorotyrosine formed by the HOCl-dependent chlorination of para-tyrosine are increased in BAL fluid from patients with ARDS (Lamb et al., 1999). Neutrophilic inflammation is prolonged, MPO

activity is enhanced and pro-inflammatory cytokines are up-regulated at three days after LPS administration in BAL fluid from elderly compared with young mice (Ito et al., 2007). In addition, neutrophil influx and inflammatory cytokines levels are decreased in BAL fluid collected from MPO knockout mice after LPS administration (Haegens et al., 2009). Although neutrophils produce ROS via other mechanisms including NADPH oxidase, MPO activity itself in the alveolar space might play a potential role in the generation of oxidative stress. Our findings of a significant correlation between MPO activity and carbonylated albumin in BAL fluid are consistent with previous findings (Chooklin et al., 2009).

Protein carbonylation is a popular oxidative modification marker of protein (Chevion et al., 2000). Since levels of carbonylated proteins in BAL fluid from patients with ARDS are elevated (Baldwin et al., 1986; Lenz et al., 1999), we evaluated the major 68-kDa carbonylated protein, albumin, in BAL fluid (Bunnell et al., 1998; Ito et al., 2009). Albumin is important because it has antioxidative properties and thus participates in the first defense against free-radical attack derived from phagocytes to protect epithelial cells, endothelial cells and basement membrane from excessive damage in the alveolar space (Soriani et al., 1994). The antioxidative properties of albumin are attenuated by oxidative modification (Bourdon et al., 1999). The clearance

of carbonylated protein could be delayed in the alveolar space due to impaired protein degradation. Proteasomal activity and albumin degradation rates are lower among patients with ARDS than among healthy individuals (Sixt et al., 2009), but the mechanism remains unknown.

Although regarded as a marker of oxidative processes, increases in oxidative stress markers indicate cellular damage and/or impaired functions of target molecules, resulting in subsequent damage (Nys et al., 2005, Song et al., 2010). The oxidation of lipid components of the endothelial or epithelial plasma membrane could facilitate neutrophil recruitment into the lungs, thus facilitating the leakage of chemokines and other chemoattractant molecules into the vascular space (Chow et al., 2003). The highly reactive and specific diffusible end product of lipid peroxidation, 4-HNE, is a second messenger that might function in regulating the expression of protective γ -glutamylcysteine synthetase (γ -GCS) (Rahman et al., 2000). Protein carbonyls are generated on proteins by the addition of lipid peroxidation products (Blakeman et al., 1995). A marker of lipid peroxidation closely correlates with the protein carbonyl content and MPO activity in BAL fluids from patients with ARDS at risk (Baldwin et al., 1986). Furthermore, the time courses of indicators of oxidative stress and of neutrophil accumulation with a significant decrease in oxygenation index ($\text{PaO}_2/\text{FiO}_2$ ratio) have

been investigated in an ovine model of acute lung injury and sepsis (Lange et al., 2012). The findings of that study indicated that neutrophil accumulation, oxidative stress in the lung and deteriorated lung function are linked.

The predominant non-protein thiol in cells is GSH and it is vital in the lungs. Increased levels of GSSG in BAL fluid are normally regarded as a marker of oxidative stress in the lungs (Biswas and Rahman, 2009). Total GSH and GSSG were both elevated in BAL fluid from our mouse model. The BAL fluid from patients with ARDS is deficient in total GSH and GSSG is elevated compared with that from normal individuals. This is probably due to the rapid extracellular oxidation of GSH (Winterbourn et al., 2000). The increase of total GSH in BAL fluid, despite being reduced in lung tissues in a mouse model of lung damage caused by oxidative stress, is probably associated with alveolar cell rupture (Sciuto, 1998).

Taken together, these findings indicate that the sustained elevation of these oxidative stress markers might reflect lung damage at a specific time. In fact, our assessment of the W/D ratio and lung histopathology revealed that lung damage was sustained for seven days after LPS administration and that the kinetics of oxidative stress markers were the same in BAL fluid. Our findings are consistent with a recent finding that histological lung damage is not restored while neutrophils are scant in the

alveolar space of a mouse model of LPS-induced lung injury (Janssen et al., 2011). Neutrophil influx into BAL collected from patients with lung disease is often regarded as the most sensitive indicator of an inflammatory response (Haslam et al, 1999; Trisolini et al., 2004). We previously reported that levels of the oxidative stress markers, oxidized glutathione and carbonylated albumin in humans do not correlate with the number of inflammatory cells in BAL fluid obtained from older individuals with a long history of smoking cigarettes (Nagai et al., 2006). Our results indicate that oxidative stress markers in BAL fluid might be more a useful marker of ARDS severity than the number of neutrophils.

We compared levels of ROS production and MPO activities of neutrophils harvested from BAL at various intervals after the induction of lung injury by LPS to investigate the mechanism underlying the dissociation between the number of neutrophils and MPO activity in BAL fluid at the resolution stage of the lung injury. Our results implied that accumulated neutrophils in the alveolar space at the later stages are more activated and generate more ROS via the MPO-HOCl pathway (Fig. 8). This might partly explain the sustained elevation of MPO activity and oxidative stress markers in BAL fluid. The mechanism of the chronological upregulation of MPO activity and ROS in neutrophils remains unclear. However, MPO modulates neutrophil

intracellular signaling in the vasculature, affects the activation state of neutrophils (El Kebir et al., 2008; Johansson et al., 1997), upregulates surface CD11b expression and evokes MPO release from neutrophils, implying the involvement of autocrine and paracrine mechanisms (Lau et al., 2005).

Another possible explanation for the sustained MPO activity in BAL fluid is that oxidative stress impairs alveolar macrophage function, thus leading to delayed clearance of MPO. Extracts of cigarette smoke induces defective pathogen clearance in murine macrophages via the carbonylation of pseudopodia (Bozinovski et al., 2011) and MPO released from activated neutrophils in the alveolar space is cleared from epithelial lining fluid by alveolar macrophages through a reaction with mannose receptors (Klebanoff, 2005). Reactive oxygen species compartmentalized in the alveolar space might mediate the functional impairment of alveolar macrophages and result in prolonged MPO activation. An MPO inhibitor prevents the progression of emphysema induced by cigarette smoke along with the downregulation of MPO-generated oxidative stress markers (Churg et al., 2011). We speculate that MPO inhibition could also be a useful therapeutic treatment for ARDS.

Although LPS-induced pulmonary inflammation in mice is a popular model of ARDS that is characterized by neutrophil accumulation in the lungs, it does not fully

manifest the features of ARDS in humans (Matute-Bello et al., 2011). Nevertheless, intratracheal LPS delivery has led to significant advances in understanding of the fundamental mechanisms that regulate lung injury and resolution. The manifestation of ARDS includes a massive sequestration of neutrophils within the pulmonary microvasculature in response to various stimuli, and a decrease in the number of neutrophils in the lung is considered to play a major role in the resolution of this pathophysiological process (Tsushima et al., 2009). The present study is the first to demonstrate that the association between the number of neutrophils in alveolar space or lung interstitium and the degree of lung damage disappears during the resolution of acute lung injury.

In conclusion, the ratio (%) of neutrophils in BAL fluid reflects oxidative stress in the lung, whereas MPO activity in BAL fluid indicates oxidative stress in BAL fluid as well as epithelial damage in lung tissue. The potential of neutrophils to release ROS and MPO into the alveolar space time-dependently differed from that of the lungs of mice with LPS-induced lung injury. Evaluating not only inflammatory cell differentials, but also MPO activity in BAL fluid from patients with ARDS should help to understand disease status.

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Figure legends

Figure 1. Kinetics of LPS-induced inflammatory cell accumulation in bronchoalveolar lavage (BAL) fluid.

Mice were intratracheally administered with LPS and the cell content in BAL fluid was determined as described in the Materials and Methods. Data represent average numbers of total cells (A), neutrophils (B), and macrophages (C) per mL of BAL fluid ($\pm$ SE) from five to six mice.

Figure 2. Kinetics of LPS-induced neutrophil accumulation in lung tissue.

(A) Immunohistochemically identified neutrophils in parenchyma of untreated mice (day 0) and at one, five and seven days after LPS administration. Original magnification, $\times 200$. (B) Gr-1-positive cells presented as ratios (%) of total cells per high-power field. (C) Number of neutrophils in BAL fluid correlates with ratio of Gr-1 positive cells in damaged lung tissue ($r = 0.729$, $p < 0.001$).

Figure 3. Oxidative stress markers in lung tissue after LPS administration.

Carbonylated protein contents in lung tissues after LPS instillation evaluated for up to 14 days using ELISA (A). Sections of lungs from untreated mice (Day 0) and at one,

five and seven days after intratracheal administration of LPS stained for 4-HNE (B). Original magnification, $\times 200$ (large panels) and $\times 400$ (insets). Staining intensity was determined as ratio (%) of 4-HNE positive areas (C; $n = 5 - 6$ per group). $*p < 0.05$ compared with untreated mice. Correlation between ratio of neutrophils in BAL fluid and carbonylated protein content (D; $r = 0.830$, $p < 0.001$) and ratio of 4HNE positive areas (E; $r = 0.703$, $p < 0.001$) in lung tissue.

Figure 4. Lung injury after LPS administration.

(A) Representative sections of untreated lungs (day 0) and at one, five and seven days after LPS administration. (B) Histological lung injury scores from tissue sections from mice with (LPS)-induced lung injury stained with hematoxylin and eosin. (C) Lung wet/dry (W/D) ratios. ($n = 5 - 6$ per group). $*p < 0.05$ compared with untreated mice.

Figure 5. MPO activity in BAL fluid and lung tissue after LPS administration.

Activity of MPO in BAL fluid from mice evaluated for up to 14 days after intratracheal LPS administration (A). Time course of MPO activity in lung tissue (B) ($n = 5 - 6$ per group). $*p < 0.05$ compared with untreated mice. Correlation between ratio of neutrophils and MPO activity in BAL fluid (C: $r = 0.564$, $p = 0.0018$) and in damaged

lung tissue (D: $r = 0.937$, $p < 0.001$).

Figure 6. Total protein concentration in BAL fluid and correlation with MPO activity.

Total protein concentration in BAL fluid after intratracheal LPS administration (A).

Correlation between MPO activity and total protein concentration in BAL fluid (B; $r = 0.682$, $p < 0.001$)

Figure 7. Oxidative stress markers in BAL fluid after LPS administration.

Time course of other oxidative stress markers in BAL fluid after LPS administration for up to 14 days. (A) Representative Western blot of 68-kDa carbonylated albumin in BAL fluid and time course of carbonylated albumin in BAL fluid after LPS administration.

(B) Activity of MPO correlates with carbonylated albumin in BAL fluid ($r = 0.660$, $p < 0.001$). Levels of LPO (C), GSH (D) and GSSG (E) at various times up to 14 days measured in BAL fluid ($n = 5 - 6$ per group). (F) Relationship between MPO activity and carbonylated albumin in BAL fluid ($r = 0.703$, $p < 0.0001$). * $p < 0.05$ compared with untreated mice.

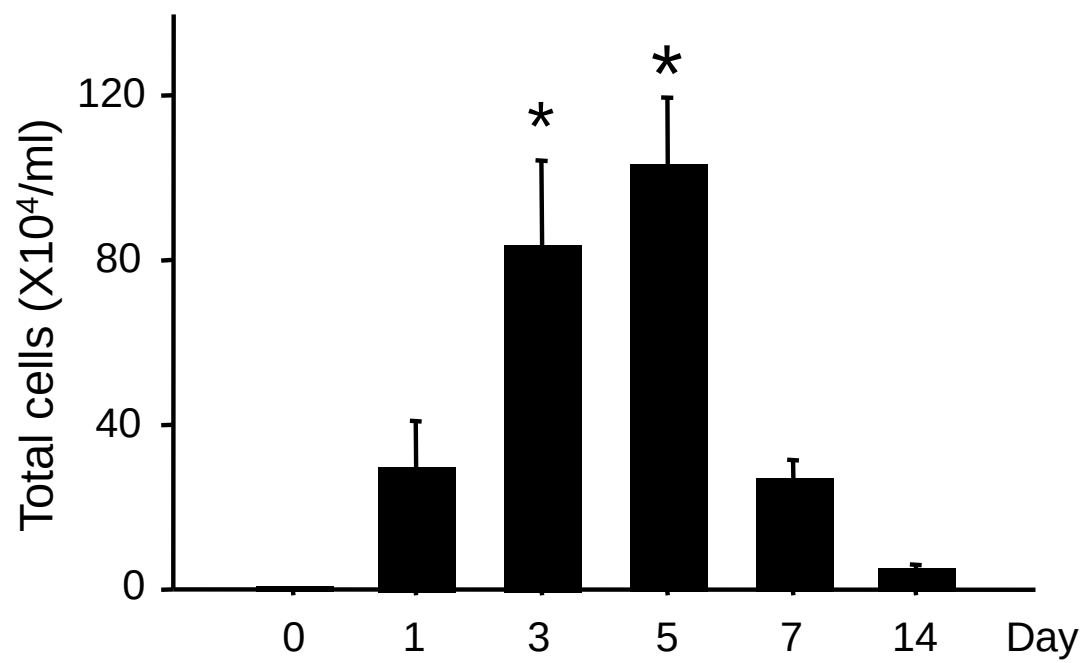
Figure 8. Comparison of ROS generation and MPO activity in activated neutrophils

from mouse lungs at 1 and 5 days after LPS administration.

Mice were administered with intratracheal LPS and BAL was collected at one and five days thereafter. Cells from BAL fluid were incubated with APF for 30 min at room temperature and then fluorescence intensity measured at 495/520 nm was calculated per neutrophil (A). Intracellular MPO activity in BAL fluid cells at same time points (B; n = 5 per group). Cells in BAL fluid incubated with APF, stained with phycoerythrin (PE)-labeled anti Gr-1 antibody and analyzed by flow cytometry. APF^{high}Gr-1^{high} cells/Gr-1^{high} cells were calculated as ratios of neutrophils expressing ROS to total neutrophils (C; n = 5 per group). *p < 0.05 compared with cells from BAL fluid collected from mice at one day after LPS instillation.

Fig.1

A



B

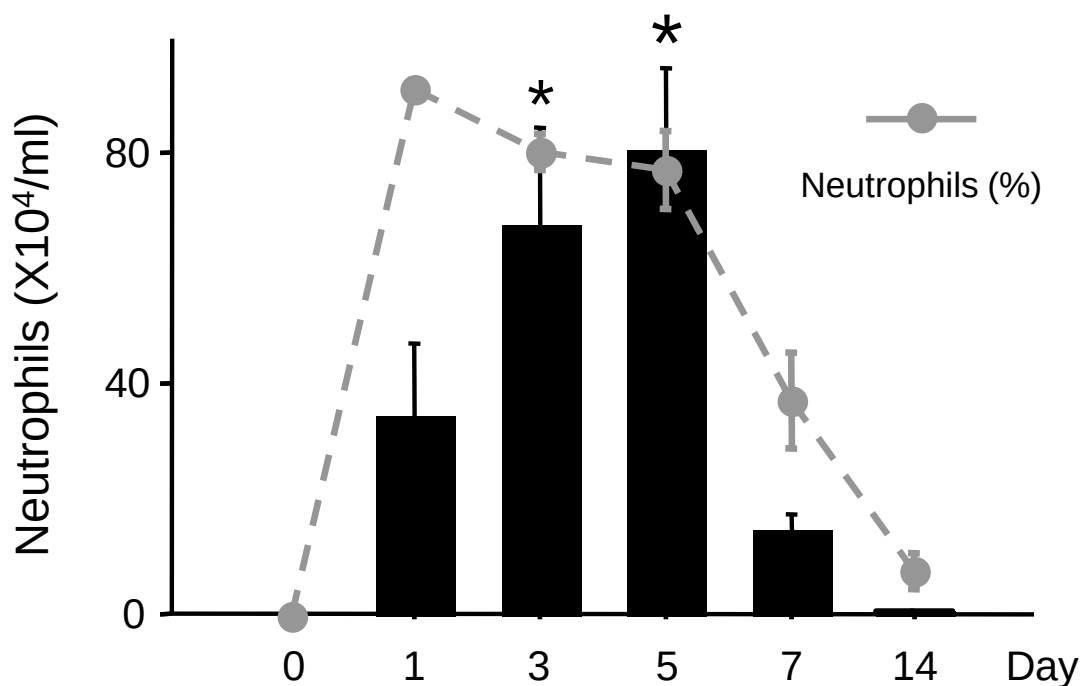


Fig.1

C

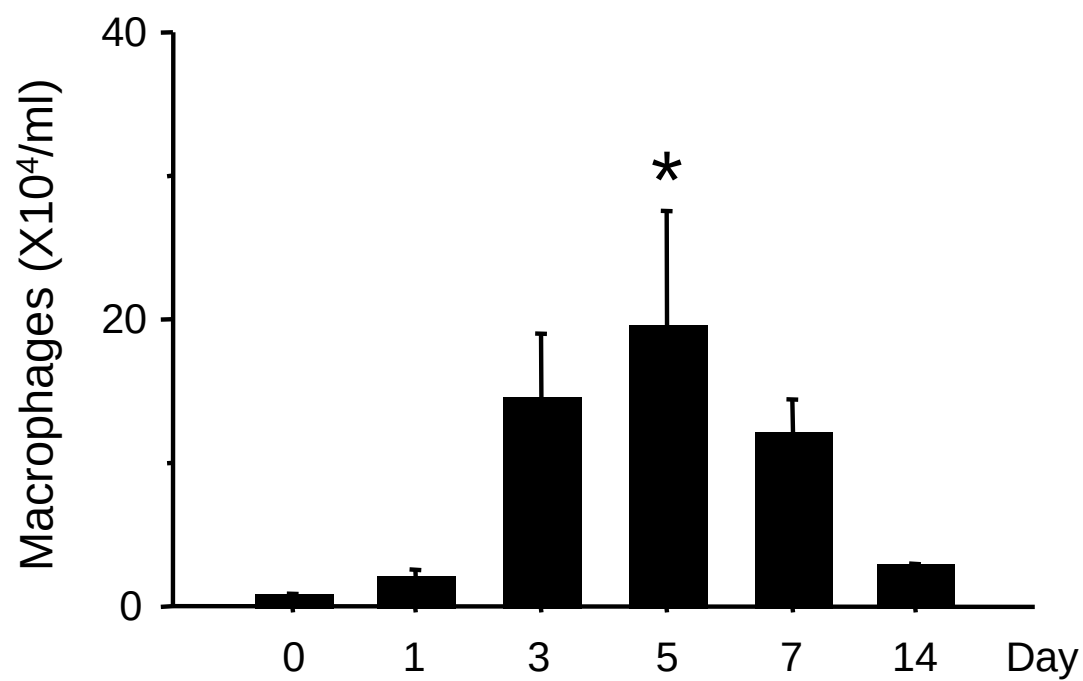


Fig.2

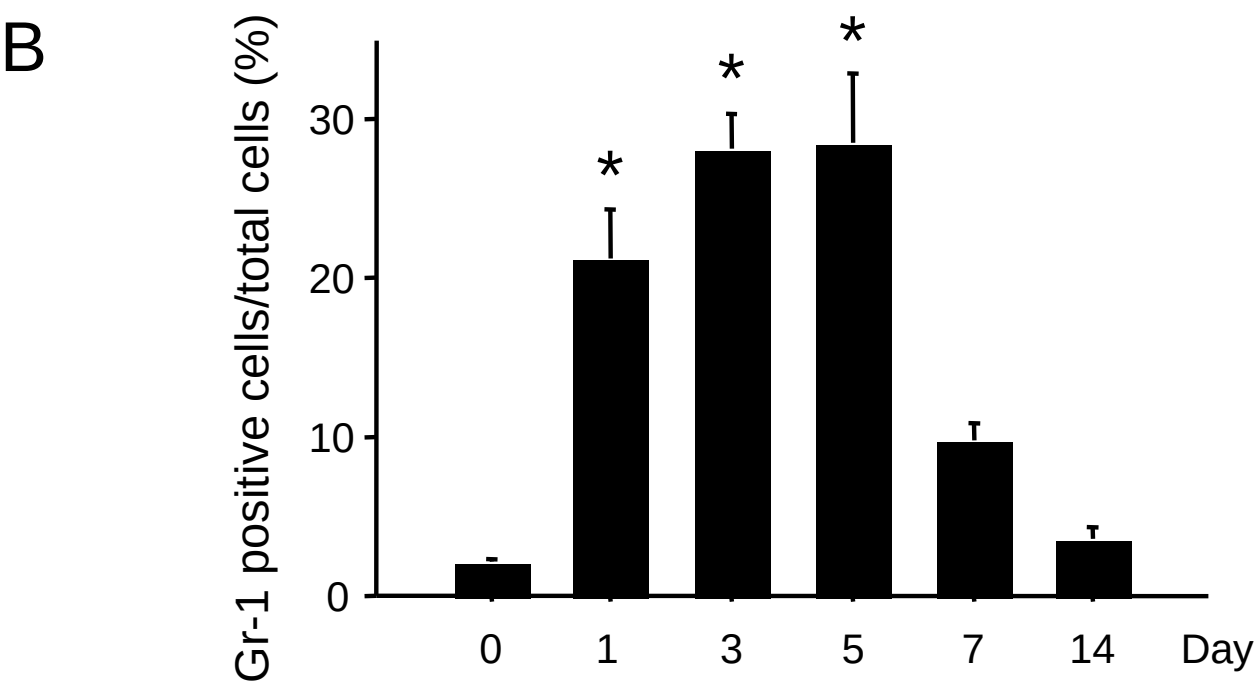
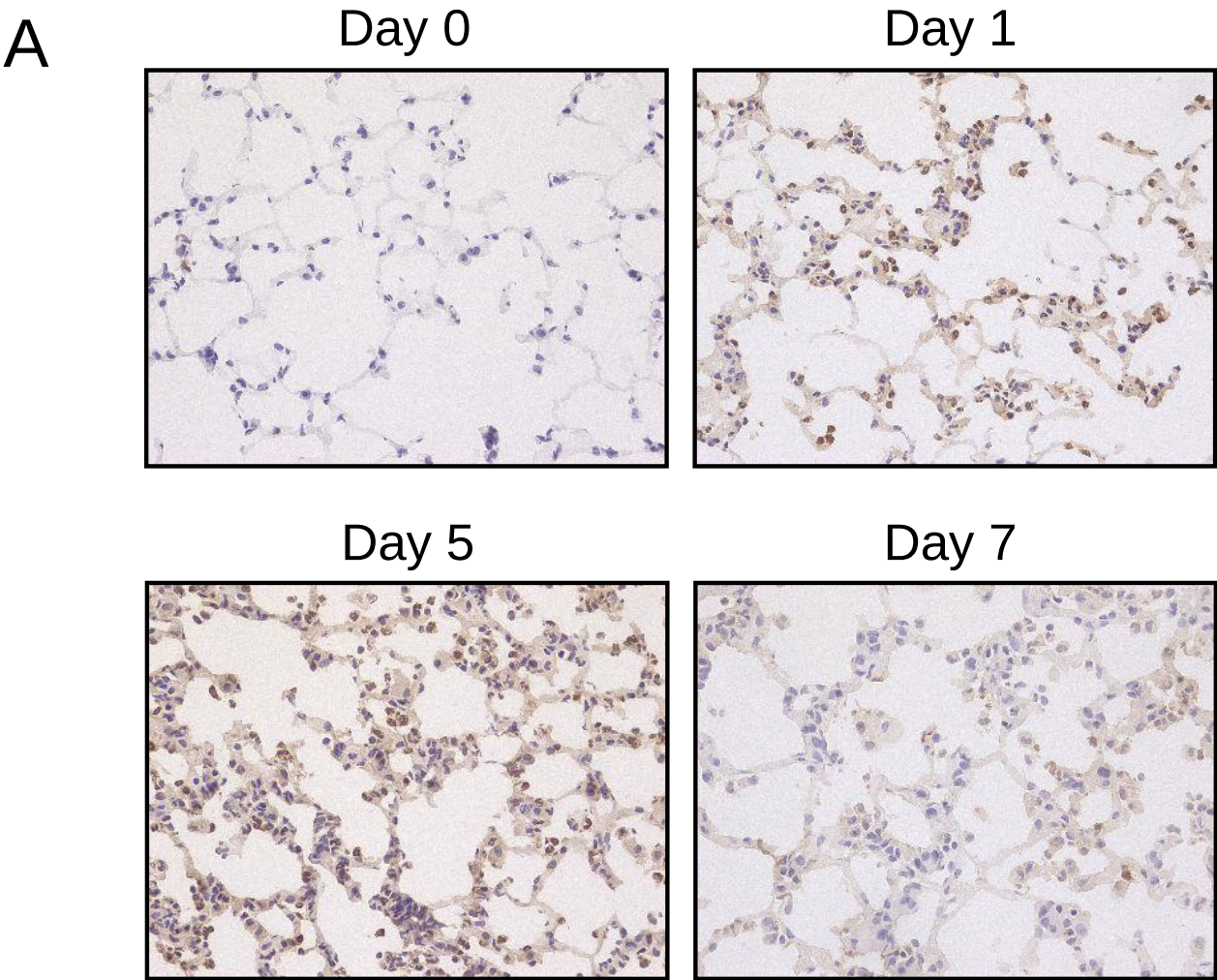


Fig.2

C

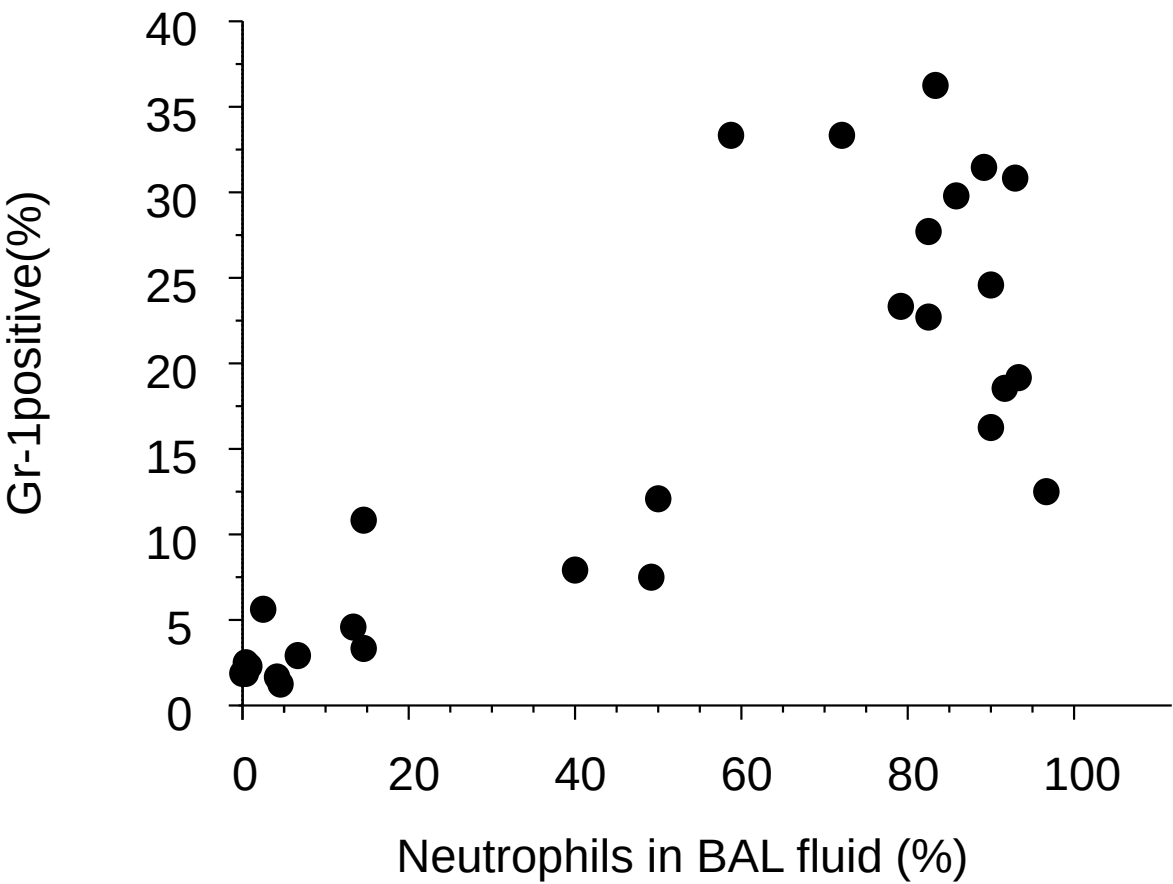


Fig.3

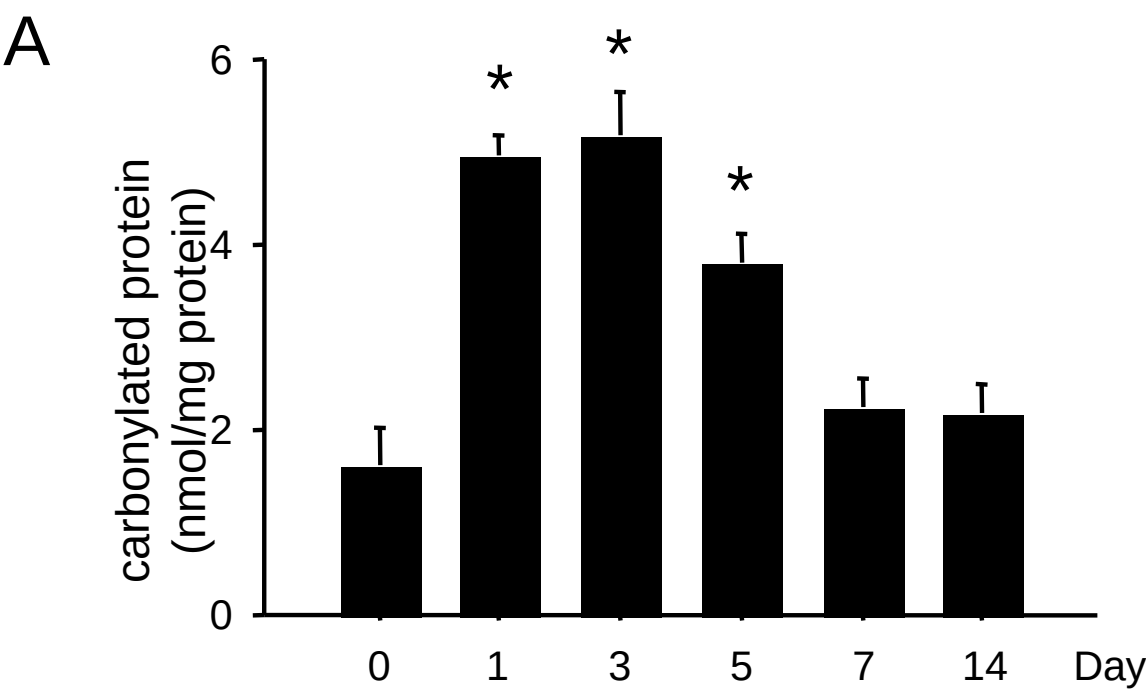
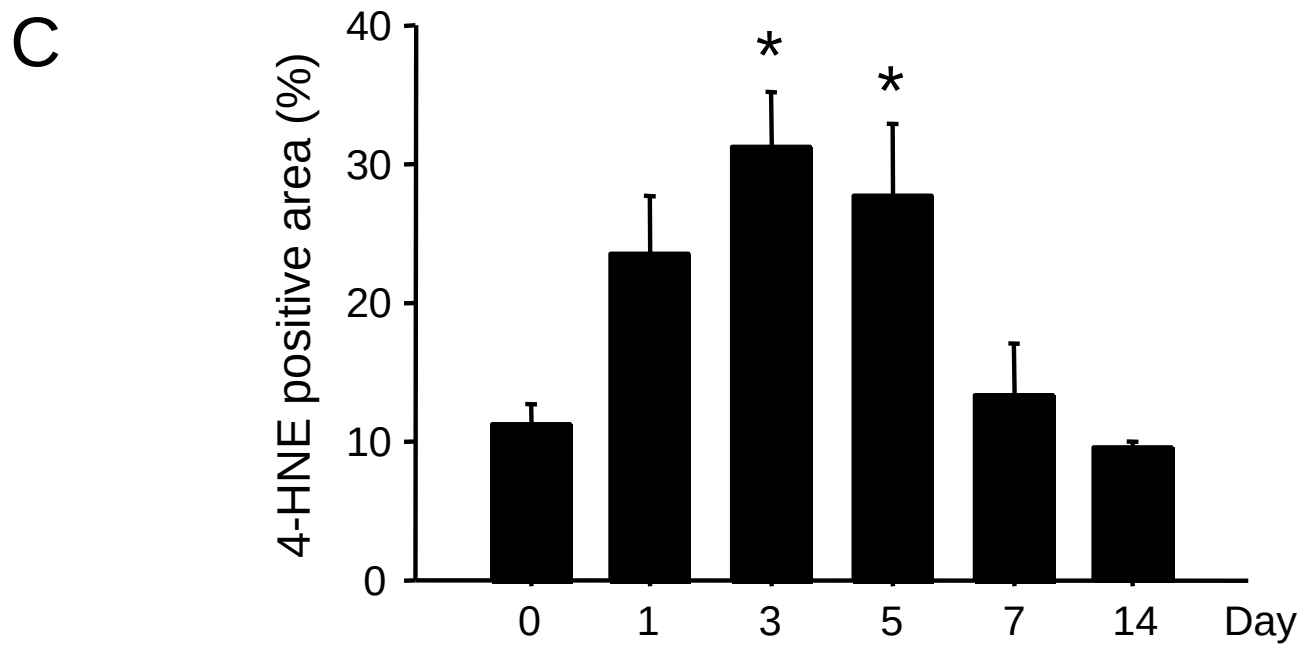
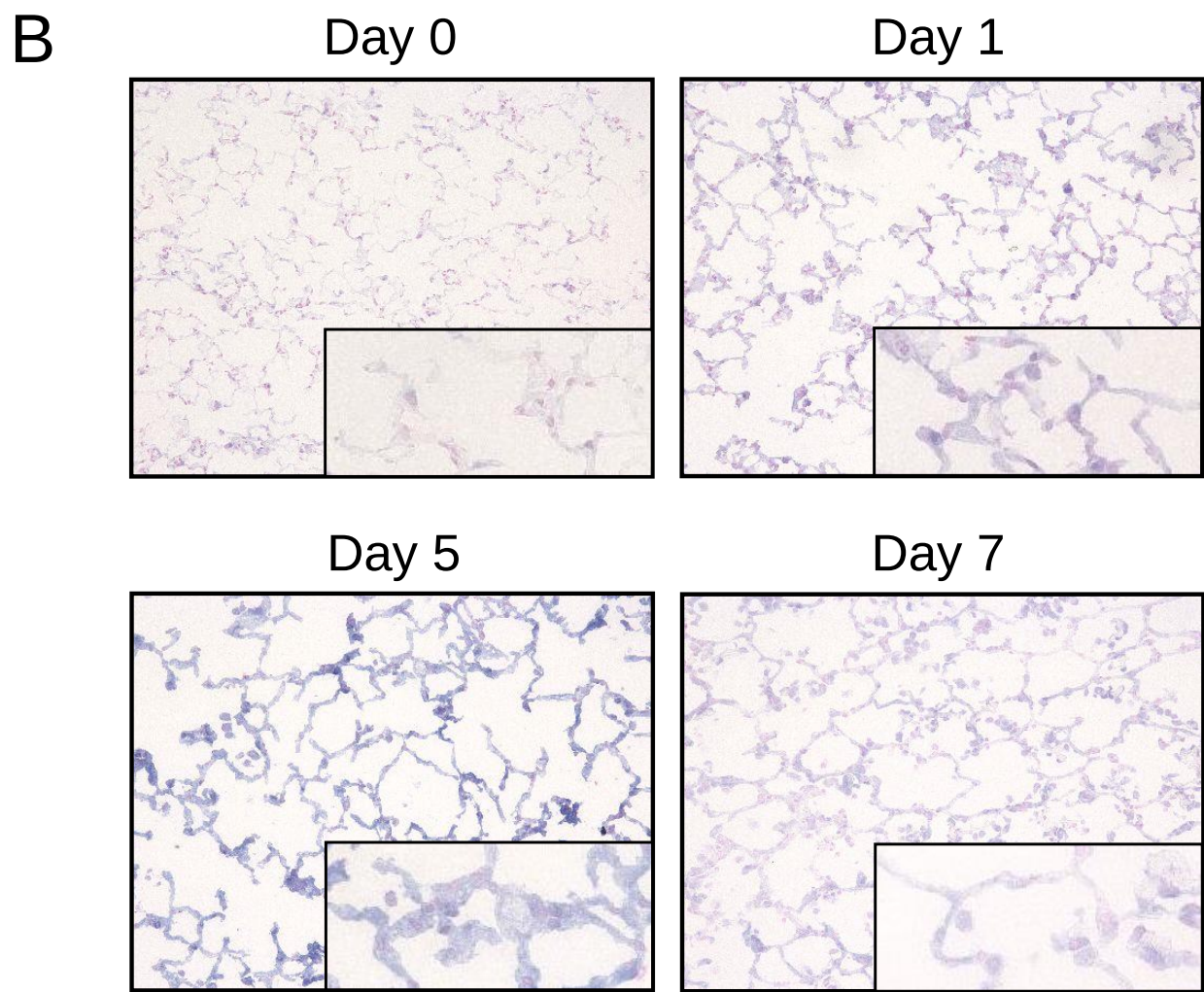
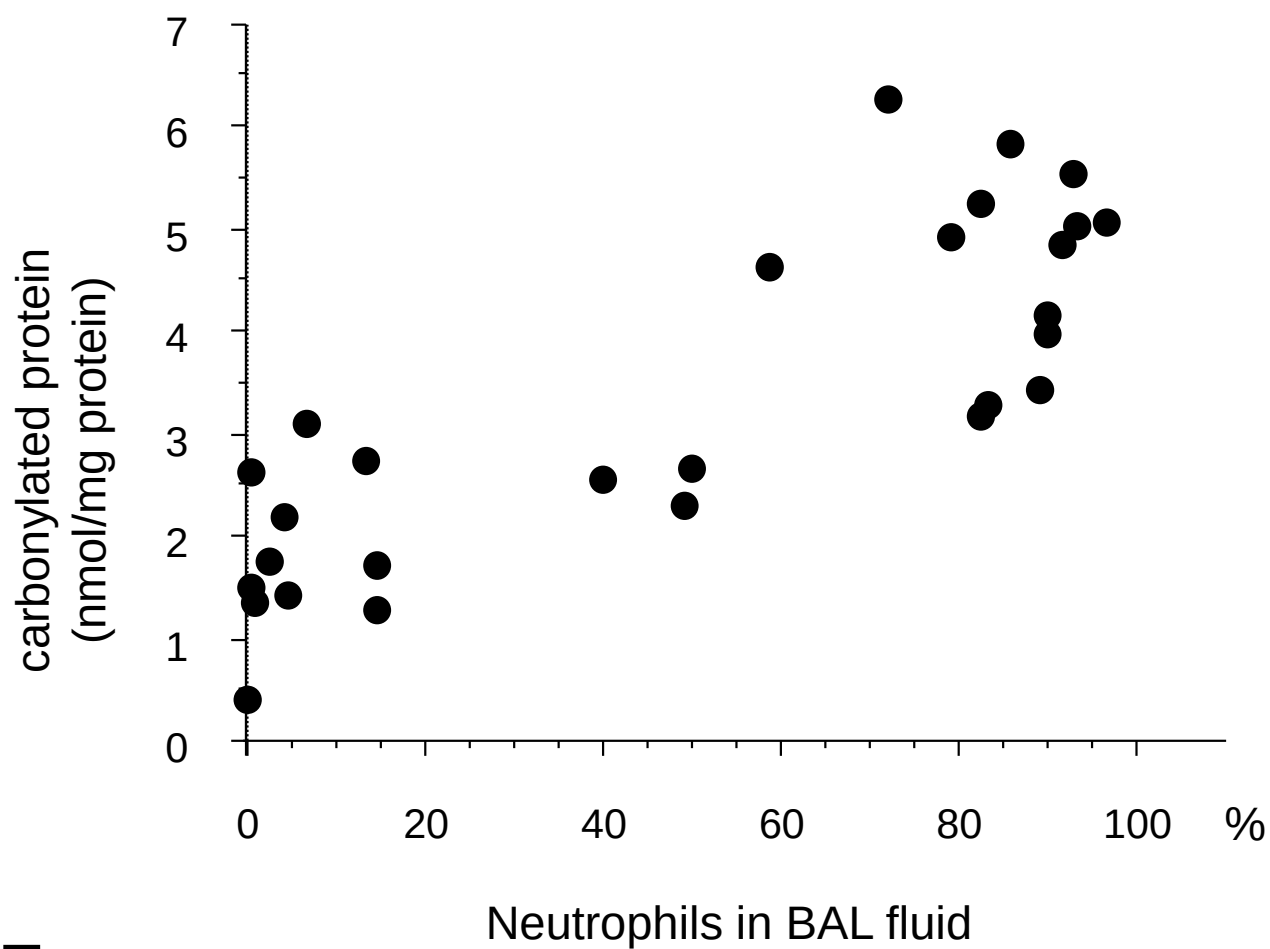


Fig.3



D



E

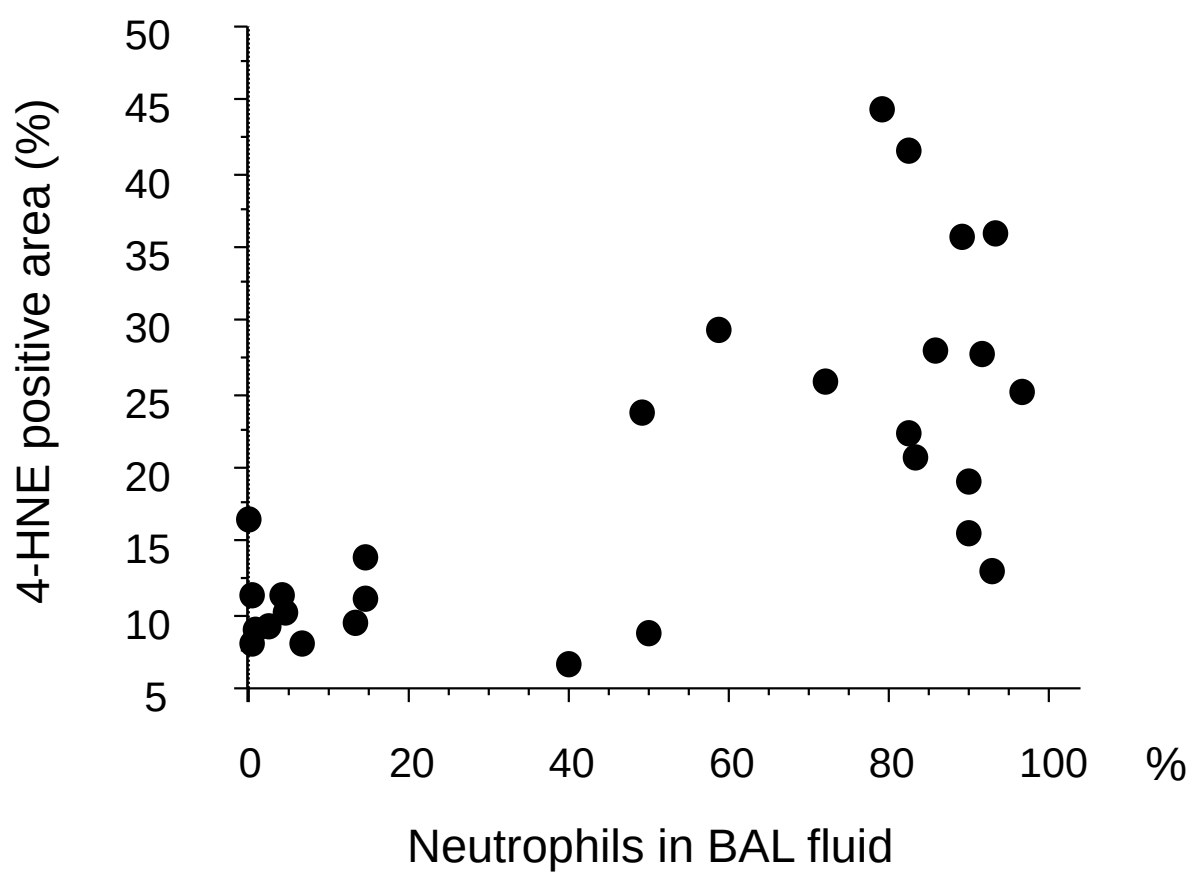
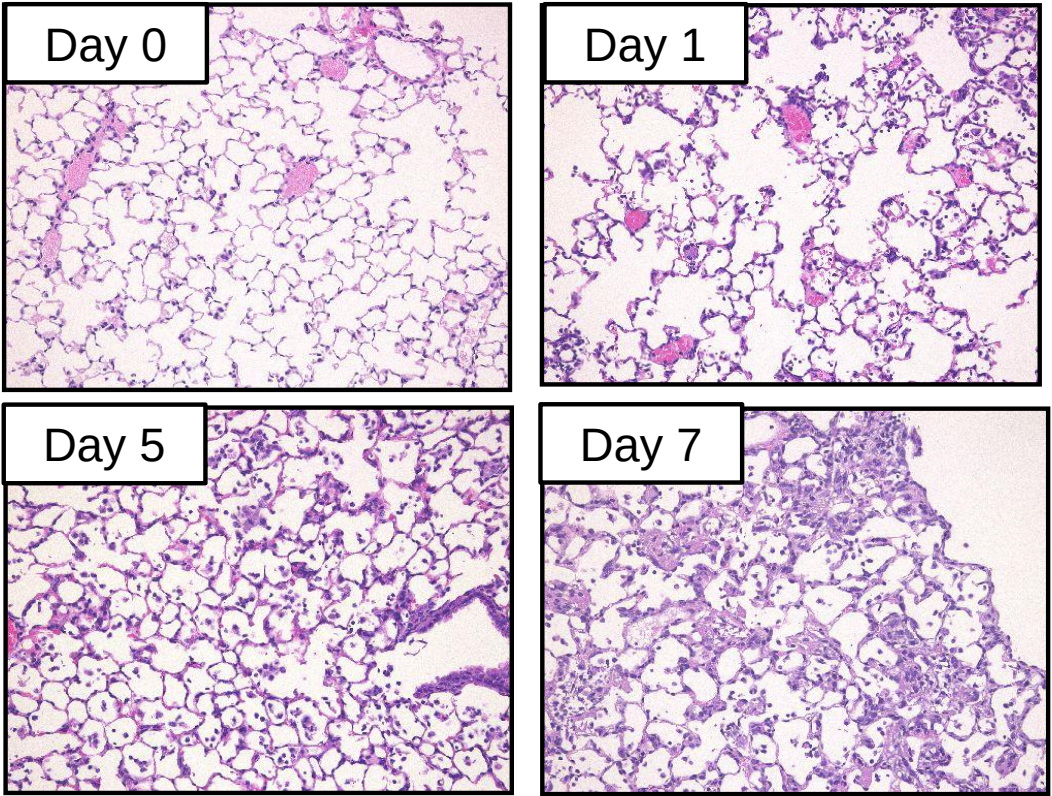
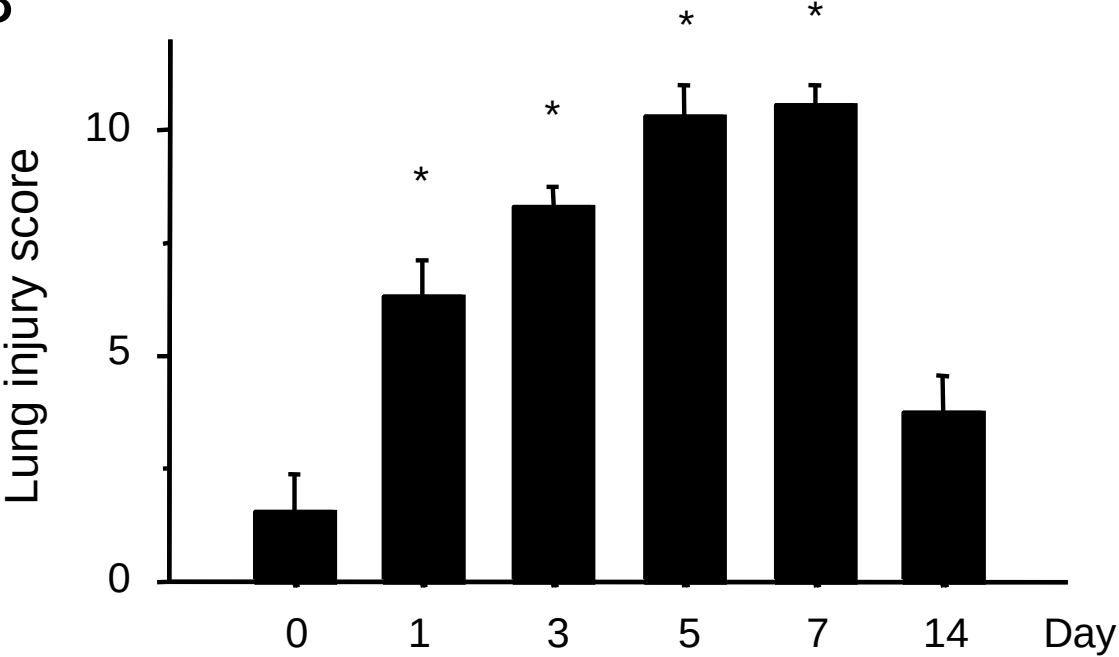


Fig.4

A



B



C

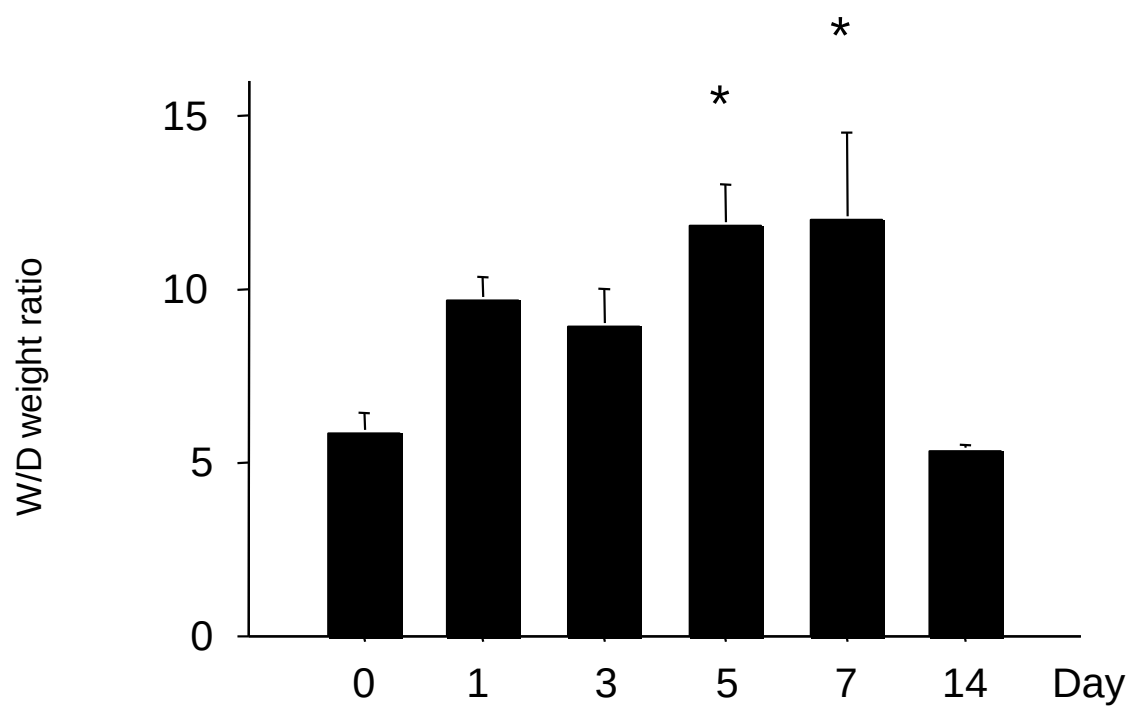
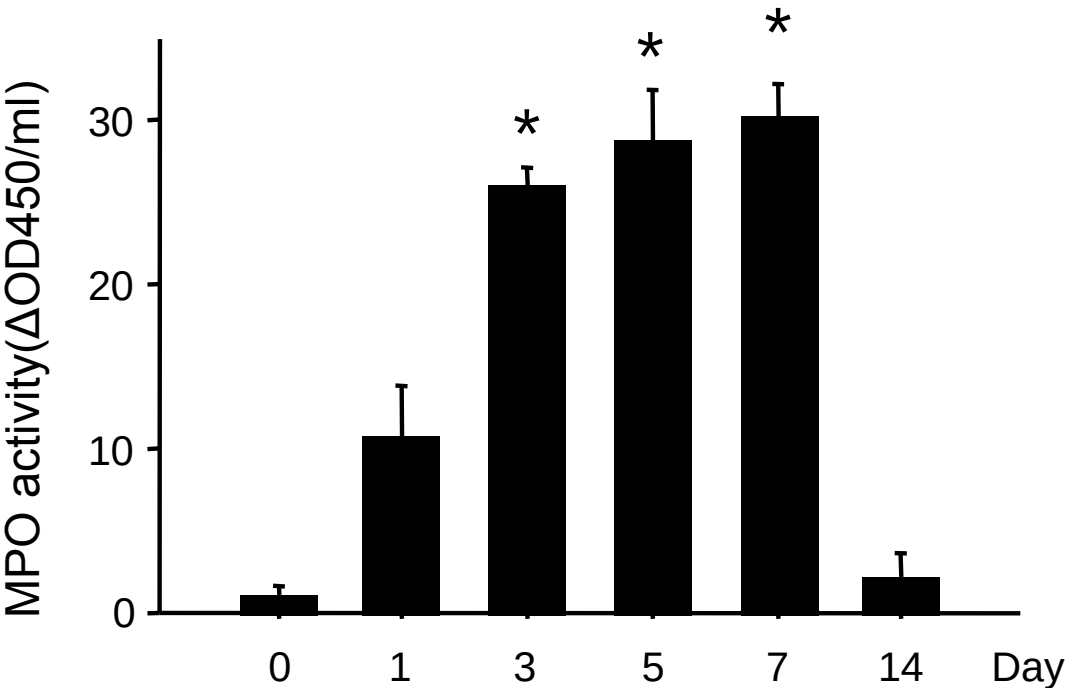


Fig.5

A



B

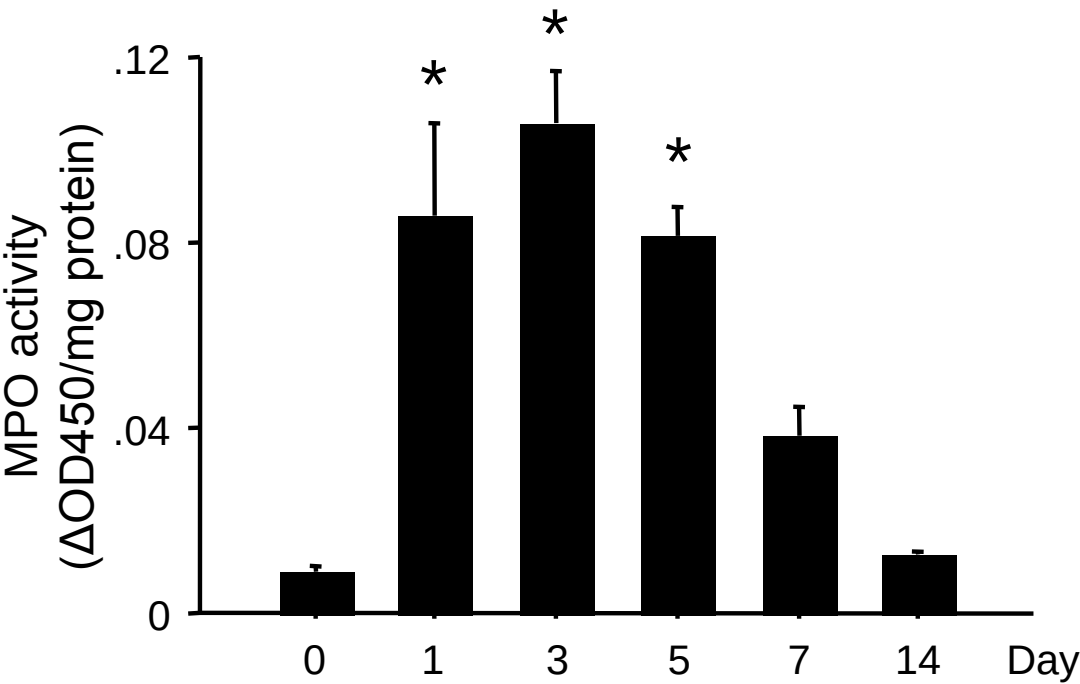


Fig.5

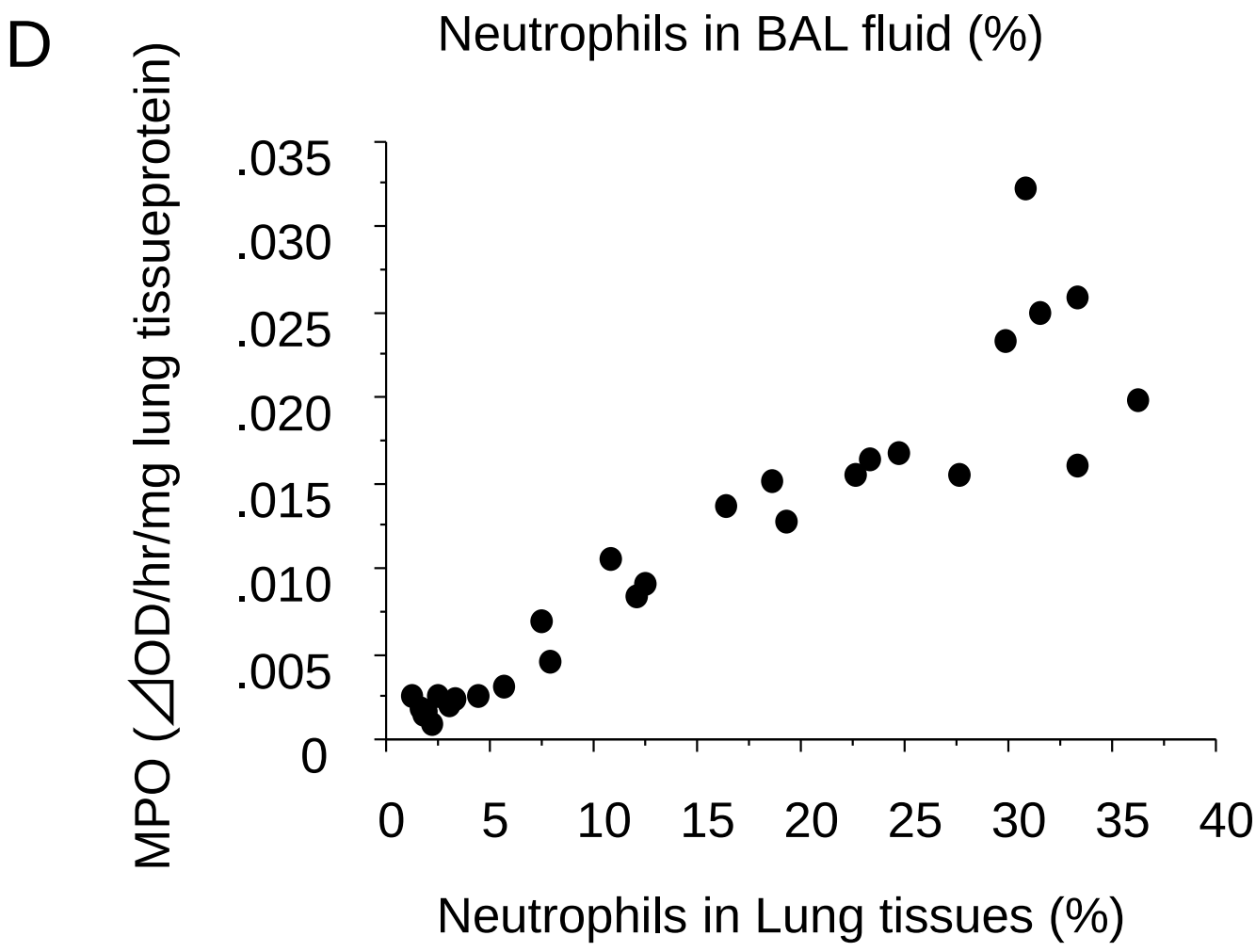
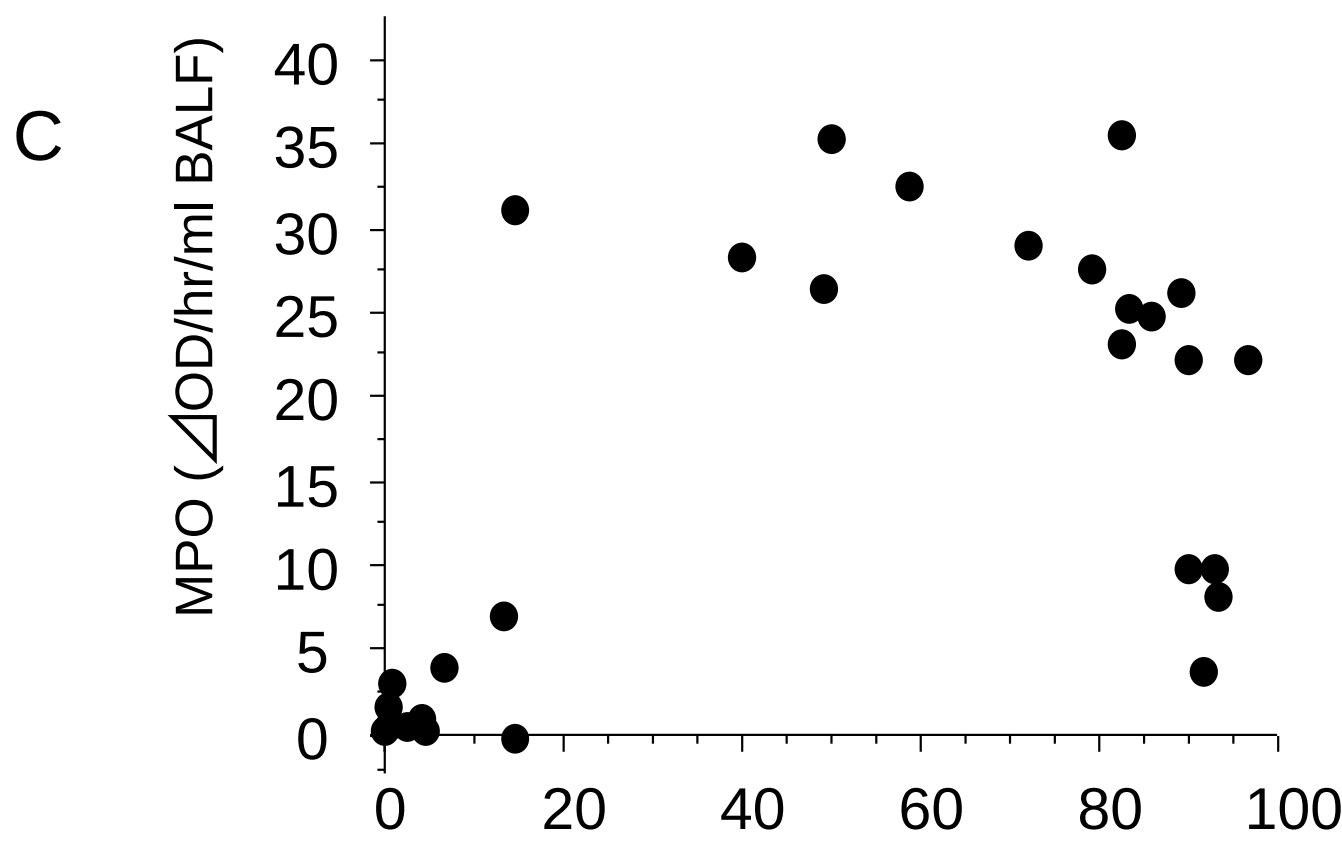
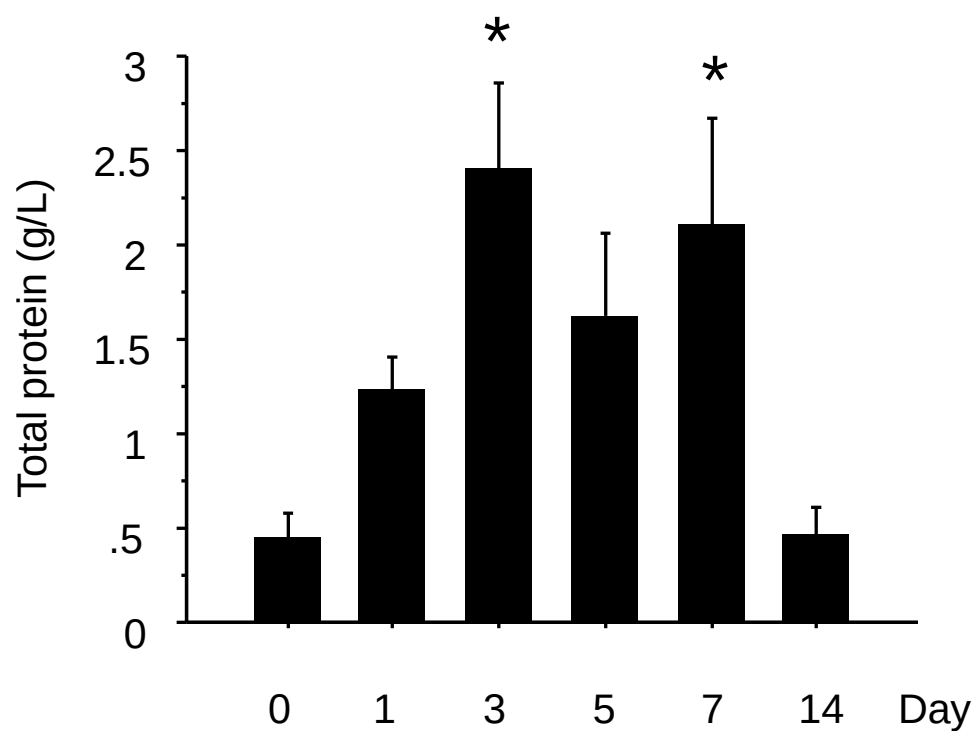


Fig.6

A



B

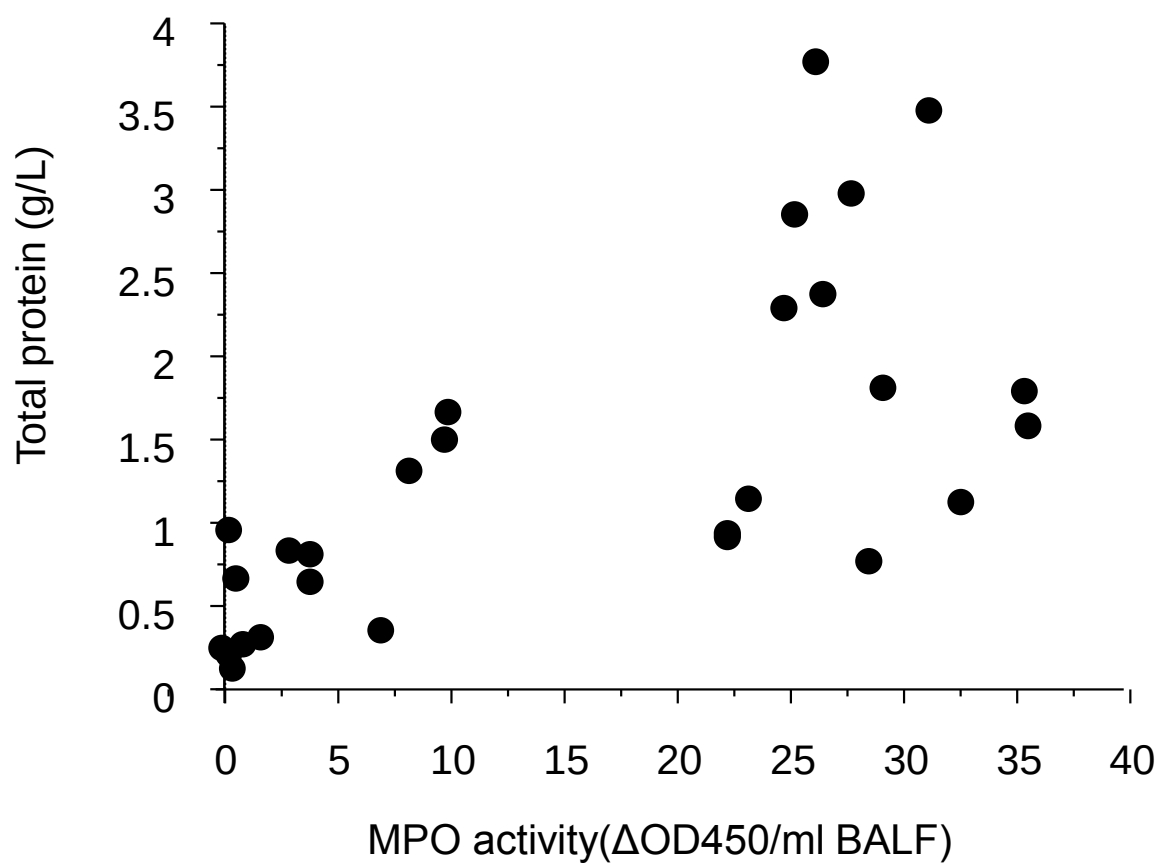


Fig.7

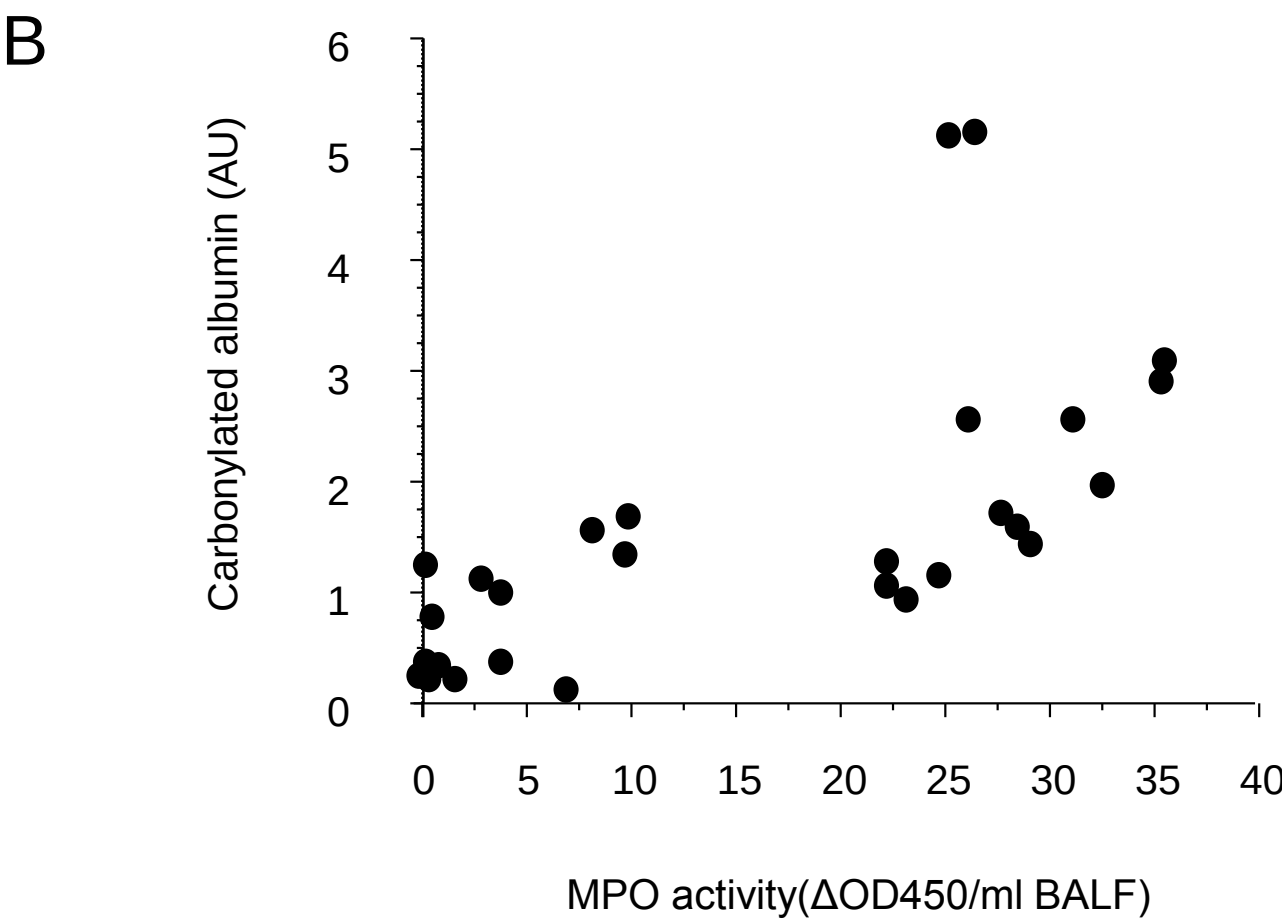
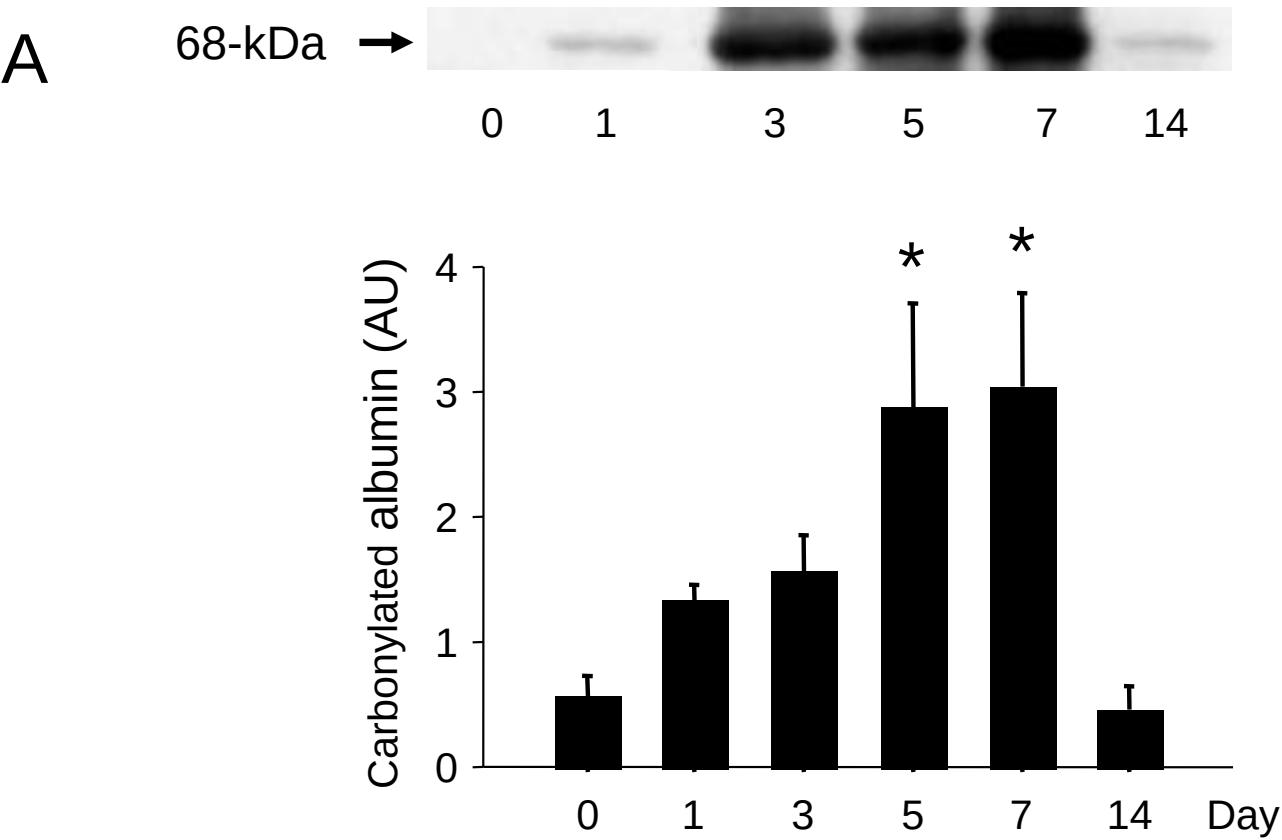
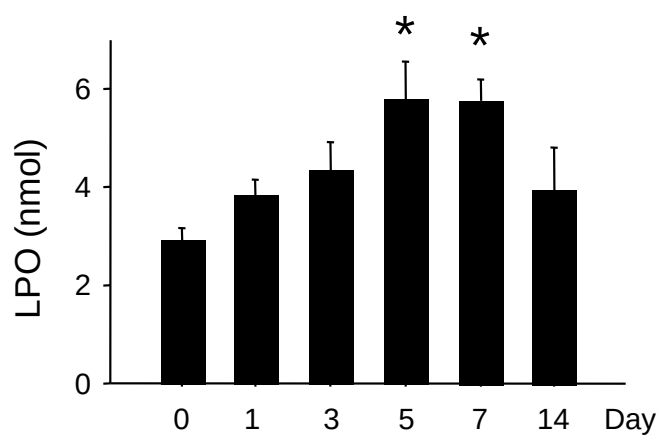
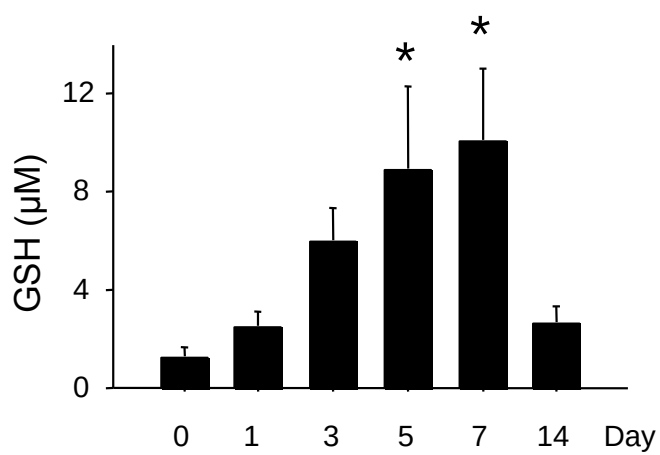


Fig.7

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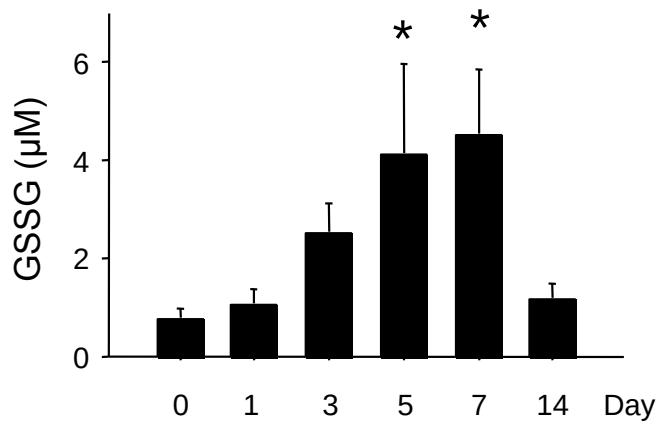


Figure 8

