

## Miniaturized Bronchoscopy Enables Unilateral Investigation, Application, and Sampling in Mice

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### Abstract

Lung diseases, including pneumonia and asthma, are among the most prevalent human disorders, and murine models have been established to investigate their pathobiology and develop novel treatment approaches. Whereas bronchoscopy is valuable for diagnostic and therapeutic procedures in patients, no equivalent for small rodents has been established. Here, we introduce a miniaturized video-bronchoscopy system offering new opportunities in experimental lung research. With an outer diameter of 0.75 mm, it is possible to advance the optics into the main bronchi of mice. An irrigation channel allows bronchoalveolar lavage and unilateral application of substances to one lung. Even a unilateral infection is

possible, enabling researchers to use the contralateral lung as internal control.

**Keywords:** bronchoscopy; mouse; unilateral application in lung

### Clinical Relevance

Miniaturized bronchoscopy enables researchers to conduct repetitive sampling from specific lung regions and allows side-selective pulmonary drug administration in living mice, whereas other lung regions may serve as control. Consequently, the quality of current protocols may be improved and the number of animals needed for specific experiments may be reduced.

In line with the tremendous impact of pulmonary diseases, a large number of experimental models have been established. To date, animal models are still not replaceable in preclinical research, both to answer basic research questions and to test novel drugs. Despite several differences between murine and human lungs (1), the mouse is the most commonly used laboratory animal in respiratory research. However, considering the small size of the murine

respiratory system, it remains a challenge to reproducibly apply substances into the main bronchi of the mouse lung with an inner diameter of 1.5–2.5 mm (2) and to perform noninvasive analyses of the bronchoalveolar compartment. Furthermore, individual differences in immune responses necessitate large numbers of animals, hence, performing longitudinal studies or using one lung for control and the contralateral lung for treatment might be attractive approaches

to reducing variance and, subsequently, the number of animals. Thus, bronchoscopy-guided delivery of agents into mouse lungs, localized examination of pulmonary responses, and the ability to perform intraindividual comparisons of different anatomical sites of the lungs *in vivo* is highly desirable.

*In vivo* examination of murine lungs is facilitated by tremendous progress in lung imaging using magnetic resonance imaging (MRI), computed tomography,

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or positron emission tomography (3), which, however, provide only indirect information on pulmonary immune responses. Bronchoalveolar lavage (BAL) via bronchoscopy is a standard procedure in pulmonary care for examination of the lung, and is valuable for experimental assessment of bronchoalveolar immune responses in models of inflammatory lung diseases. However, in most experimental studies, BAL is performed in mice only *post mortem*, thereby increasing animal numbers and precluding longitudinal assessment of disease course. To examine bacterial burden, cellularity, cellular functions, mediators and other determinants of inflammation, protocols for BAL via oral tubes have been applied (4). Although unilateral lung flushing via tubes has been described to be feasible, a direct proof for a unilateral BAL is missing, and flushing could not be restricted to specific sites within the lungs (5). Unilateral application of substances to one lung is a challenging issue in mice. Different protocols for noninvasive orotracheal intubation have been proposed (6, 7). However, the site of application is uncontrollable, even when differences in anatomy between strains are taken into account (2). After unilateral inflammatory challenge of lungs, ipsilateral, but not contralateral, responses have been observed in humans and rats (8–10), but this observation has not yet been confirmed in mice.

To overcome these shortcomings, we designed a bronchoscope system for mouse models of pulmonary diseases. The aim of this study was to show the feasibility of bronchoscopic examination and BAL in living mice, as well as unilateral application of substances or pathogens. To address this, we developed a miniaturized bronchoscope with an outer diameter of 0.75 mm, small enough for the main bronchi of most commonly used mouse strains and smaller than the smallest previously available bronchoscopes. Moreover, this approach may support our efforts to recapitulate current patient care in mice, which is prerequisite to meaningful preclinical studies in pulmonology aiming at translational research. (11).

## Materials and Methods

Methods are described in detail in the online supplement.

### Animals

Experiments were performed with specific pathogen-free C57Bl/6 mice (Janvier, Saint Berthevin, France) in accordance with applicable European and German legislation and approved by the relevant authority (LAGeSo, Berlin, Germany).

### Bronchoscope

The micro-endoscopy-system (Polydiagnost, Pfaffenhofen, Germany) consists of an image processing unit with built-in monitor, xenon light source, and camera (Endognost RA100), an ocular (fixed focus, PD-FS-4004), and a modular endoscope. This includes a handle with three Luer connections (PD-DS-1315) and a semirigid optic (3,000-pixel resolution, viewing direction 0°; field of vision 70°; outer diameter 0.4 mm) connected to one of the Luer connections via a special adapter (PD-DS-1210). In addition, a cannula (outer diameter, 0.75 mm; length, 50 mm; PolyShaft PD-DS-1030) covers the optic and provides a lumen for flushing.

### Final Dissection and Organ Sampling

Mice were anesthetized intraperitoneally with ketamin (160 mg/kg; Ketavet, Pfizer

GmbH, Berlin, Germany) and xylazin (75 mg/kg; Rompun; Bayer AG, Leverkusen, Germany), killed by exsanguination, dissected, and *post mortem* BAL was performed.

### Unilateral Application of Marking Agents

Green dye (Histological Marking Colors; WAK-Chemie, Steinbach, Germany) or gadolinium (Magnevist; Bayer Healthcare, Berlin, Germany) was applied unilaterally (20  $\mu$ l) in *post mortem* bronchoscopy, and lungs were examined histologically or by MRI, respectively.

### Unilateral Application of Macrophage-Activating Lipopeptide of 2 kD

Macrophage-activating lipopeptide of 2 kD (MALP-2; Alexis Biochemicals, San Diego, CA), a specific synthetic agonist of the pattern-recognition receptor, Toll-like receptor 2/6 (TLR-2/6), was used at a dose of 0.25  $\mu$ g in 20  $\mu$ l, half of the dosage established for pulmonary inflammation models in whole murine lungs. Organ sampling

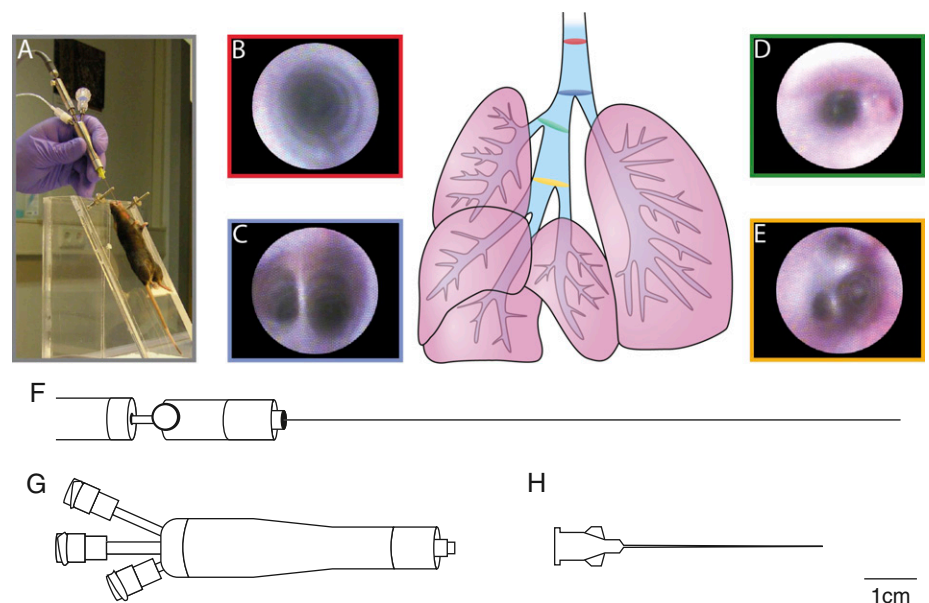


**Figure 1.** Miniaturized bronchoscopy in mice. (A) Mouse during intubation and use of the handle with three Luer connections. Different sections of the mouse lung and images of the trachea (indicated in red [B]), carina (blue [C]), right main bronchus at the branching of apical lobe bronchus (green [D]), and the branching into bronchi of right diaphragmatic, cardiac, and azygous lobe (yellow [E]) as potential sites of examination or application. Schematic drawing of (F) the optics with fiber optics for light and image transmission, which are introduced into the middle of the three Luer connections at the handle (G), and the connected PolyShaft cannula (H).

was performed 24 hours after application (13).

### ***Streptococcus pneumoniae* Infection**

*Streptococcus pneumoniae* (serotype D39 capsular type 2 s; Rockefeller University, New York, NY) doses were lower than in transnasal infection models (14) and were based on laboratory experience, choosing 2,000 CFU for evaluation of bacterial burden 6 hours after infection and 200,000 CFU to elicit histopathological responses.

### **Influenza-A Virus Infection**

Mice were infected with 50 plaque-forming units of influenza A virus/H1N1/PR/8/34 in 20  $\mu$ l PBS, representing half of the dosage established for whole murine lungs (15). Final analysis was performed 6 hours after infection to examine direct effects of unilateral instillation of viruses, but excluding secondary limitations (e.g., viral spread).

### **Allergic Airway Inflammation**

On Days 0 and 14, mice were sensitized by 20  $\mu$ g ovalbumin (OVA; Grade V; Sigma-Aldrich, Deisenhofen, Germany)

intraperitoneally in 100  $\mu$ l alum suspension (1.3%; Serva, Heidelberg, Germany) and 10  $\mu$ l PBS. At 14 days after the last sensitization, mice were unilaterally challenged with 40  $\mu$ g OVA in 20  $\mu$ l PBS (16). Analyses were performed 48 hours after the challenge.

### **Immunological Examination**

Leukocytes of lung and bilateral lavage were analyzed by fluorescence-activated cell sorting (FACS LSRII; BD Bioscience, Heidelberg, Germany) and FlowJo software (Tree Star Inc., Ashland, OR). BAL fluid cytokine levels were measured using cytokine protein multiplex assay (BioPlex; Bio-Rad, Hercules, CA). Leukocytes of unilateral BAL were analyzed on cytospin slides stained with May-Grünwald-Giemsa.

### **Histology**

Lungs were immersion fixed in formalin, embedded in paraffin, cut, and stained with hematoxylin and eosin.

### **Statistical Analysis**

Data are expressed as means ( $\pm$  SD) or scatter plots, and *P* values of less than or

equal to 0.05 were considered to indicate statistical significance.

## **Results**

### **Bronchoscopy Is Technically Feasible in Mice**

We established a bronchoscopic examination procedure for mice with a newly developed endoscope, having an outer diameter of 0.75 mm and a resolution of 3,000 pixels. Whereas, in humans, flexible bronchoscopes are usually used, a semirigid bronchoscope was applied in mice. The modular part of the semirigid bronchoscope consists of three components: optics, handle, and cannula (Figures 1F–1H). To remove mucus from the lens during the examination, a 5-ml syringe filled with air was connected via an infusion line to one of the Luer connections. If application of substances or BAL was intended, we connected a 1-ml syringe with the respective amount of fluid and some additional air to the other connector.

Mice were anesthetized with midazolam (5.0 mg/kg body weight [BW]; Dormicum; Roche Pharma AG,

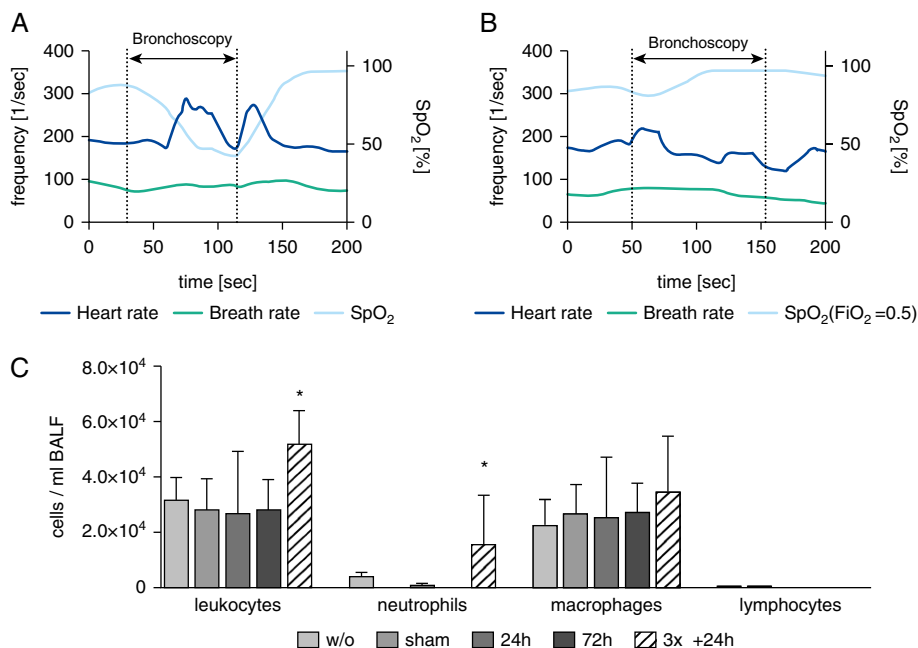


**Figure 2.** Effects of bronchoscopy on physiological parameters and cellularity of lavage. Bronchoscopy without oxygen supply lowered arterial oxygen saturation and increased heart rate (A), whereas, with insufflated oxygen, physiological parameters remained unchanged (B) (representative chart of one mouse). (C) Fluorescence-activated cell sorting analysis was performed from *post mortem* BALF after tracheotomy. No significant changes in leukocyte, neutrophil, macrophage, or lymphocyte numbers were detected 24 and 72 hours after bronchoscopy. However, 24 hours after repetitive lavage (3 $\times$ ), BALF leukocytes were increased, particularly neutrophils. Values are presented as means ( $\pm$  SD; *n* = 6; one-way ANOVA with Dunnett's multiple comparison test with untreated lung as control). \**P* < 0.05 versus untreated. SpO<sub>2</sub>, oxygen saturation as measured by pulse oximetry.

Grenzach-Whylen, Germany) and medetomidin (0.5 mg/kg BW; Domitor; Orion Corp., Espoo, Finland) intraperitoneally, and were then suspended at a 60° angle by the two front upper teeth by a wire attached to a Plexiglas support (12). The tongue was pulled out with a small spatula (custom built, Medizinisch-technische Labore, Charité–Universitätsmedizin Berlin, Berlin, Germany). After clear visualization of the larynx, the assembled bronchoscope was inserted into the trachea at the time of laryngeal opening and advanced to the main bronchi with an inner diameter of around 1 mm (Figure 1A). In the asymmetrical monopodial branching pattern of the mouse lung, it is possible to visualize trachea and main bronchi (Figures 1B–1E). Anesthesia was antagonized by flumazenil (0.5 mg/kg BW; Inresa, Freiburg, Germany) and atipamezol (5.0 mg/kg BW; Antisedan; Orion) subcutaneously, and mice were transferred into a heated cage (30°C) for recovery. For disinfection between examinations, all parts of the bronchoscope that could possibly have been in contact with mouse or sample were washed with 70% ethanol followed by distilled water, and then dried with pressurized air.

Examinations had to be performed carefully to avoid tissue damage. In our hands, arterial oxygen saturation of mice breathing room air spontaneously dropped during examination (Figure 2A). Hence, if prolonged examination times (duration according to our protocol was ~ 2 min) were required, oxygen supplementation could be used to prevent decline in arterial oxygen saturation (Figure 2B). No other adverse events were observed during the study. In addition, histopathological examination did not reveal procedure-related findings, apart from minimal hilus-associated infiltration of macrophages and neutrophils (data not shown).

### Intravital BAL

Bilateral intravital BAL was performed at a predefined site after visual confirmation, placing the bronchoscope's tip cranially to the tracheal bifurcation (Figure 1C). First, 300 µl of sterile saline was injected from the 1-ml syringe into the channel of the bronchoscope. Then, air was gently applied from the 5-ml syringe until

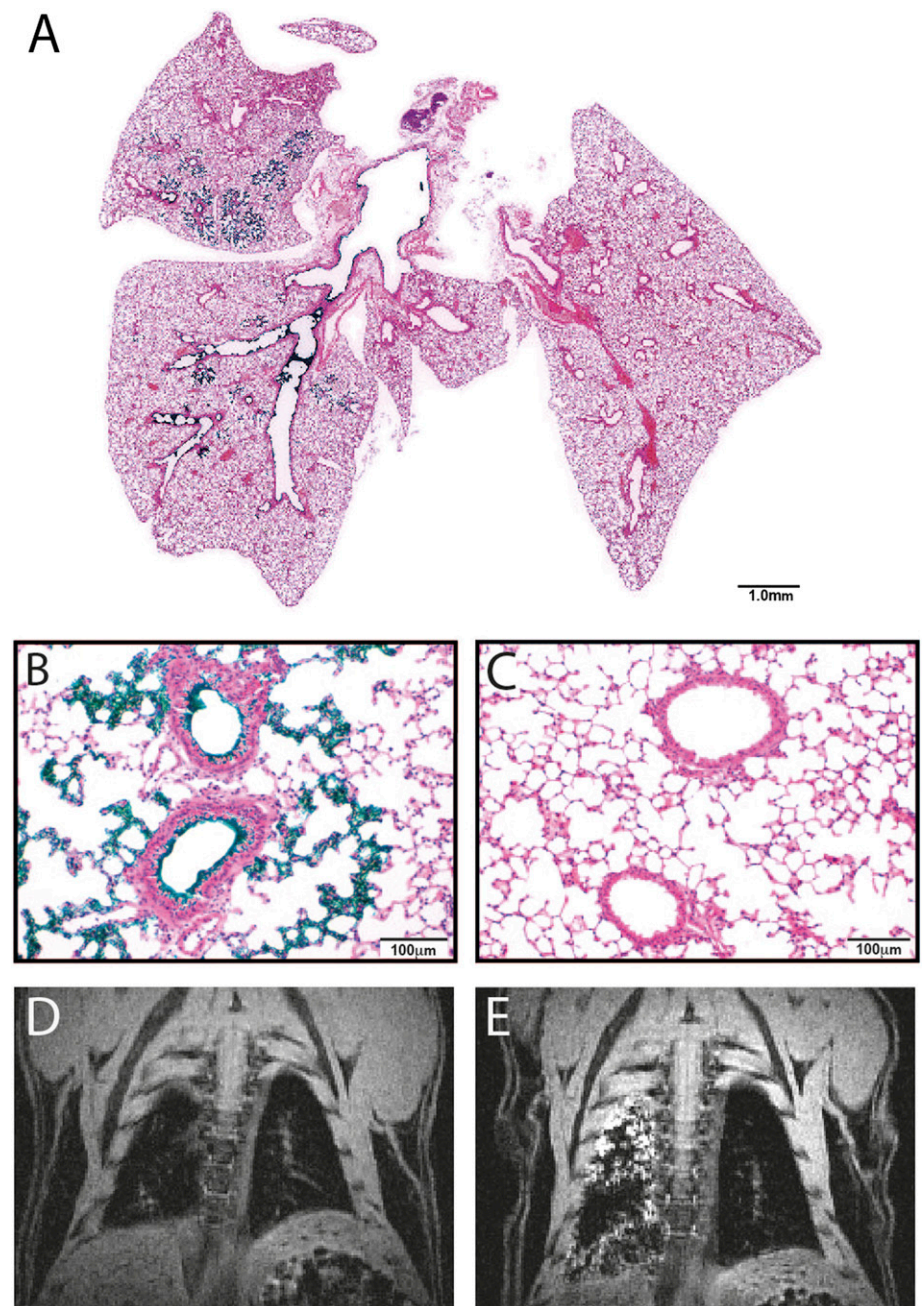


**Figure 3.** Distribution of locally administered green dye or gadolinium. *Post mortem* application of green dye to the right lung showed distribution in all parts of the right lung (A) and in bronchioli and alveolae of the right lung (B), but not in the left lung (C). Magnetic resonance imaging analysis before (D) and after (E) *post mortem* application of gadolinium into the right lung confirmed this finding.

disappearance of all fluid into the designated part of the lung was observed. For BAL, the fluid was withdrawn gently until no more fluid could be seen in the bronchus. The recovery fraction was at least 80% (data not shown).

Applying this protocol, we determined the effect of bronchoscopy

and intravital BAL on pulmonary immunity over time. At different final time points, a second BAL was performed *post mortem* via tracheotomy, and not endoscopically, to enable comparison with previous studies. Subsequently, leukocyte cellularity in BAL fluid and lung tissue was assessed. No significant

changes in cellularity of lavage (Figure 2C) or lung tissue (data not shown) were observed within 24–72 hours after the initial intravital BAL. To investigate the effects of repetitive intravital lavages, lungs were flushed every third day, and cellularity was analyzed in lavage fluid and lung tissue 24 hours after the third BAL. Repetitive flushing of the lungs evoked pulmonary recruitment of leukocytes, mainly neutrophils, whereas the number of macrophages remained unchanged (Figure 2C).

### Unilateral Application into the Right Lung

Next, we investigated the feasibility of applying immunostimulatory agents or pathogens unilaterally to the right lung. The defined place of application was in the right

main bronchus, with the tip being placed cranially to the first lateral bronchus (Figure 1D), and the application volume of 20  $\mu$ l was inserted using the same procedure as for BAL. *Post mortem* unilateral application of green dye into the right main bronchus revealed a strict unilateral distribution (Figure 3A), reaching both bronchi and alveoli only at the right side (Figures 3B and 3C). This finding could be confirmed by MRI measurement after right-sided application of gadolinium (Figures 3D and 3E).

Intravital unilateral treatment with the synthetic TLR-2/6-specific agonist, MALP-2, resulted in recruitment of leukocytes, mainly neutrophils, into the bronchoalveolar space, restricted to the site of application, 24 hours after MALP-2 application (Figure 4A). Leukocyte numbers of untreated left lungs remained

unaltered (Figure 4A) and were comparable to BALF leukocyte levels of PBS-treated right lungs (PBS,  $n = 4$ ; leukocytes,  $58,283 \pm 29,465$ ; neutrophils,  $0 \pm 0$ ; macrophages,  $58,092 \pm 29,420$ ; lymphocytes,  $191 \pm 72$ ). IL-1 $\beta$ , keratinocyte chemoattractant (KC), and macrophage inflammatory protein-1 $\alpha$  production was enhanced in the bronchoalveolar space of MALP-2-treated right lungs compared with untreated left lungs (Figure 4B), whereas cytokine levels of right lungs after unilateral application of PBS were unaffected (PBS,  $n = 4$ ; IL-1 $\beta$ ,  $0.000 \pm 0.000$ ; KC,  $9.235 \pm 4.036$ ; macrophage inflammatory protein-1 $\alpha$ ,  $1.263 \pm 0.343$ ).

Infection with 50 plaque-forming units of influenza A virus (H1N1/PR/8/34) induced influx of leukocytes (mainly macrophages) 6 hours after infection into

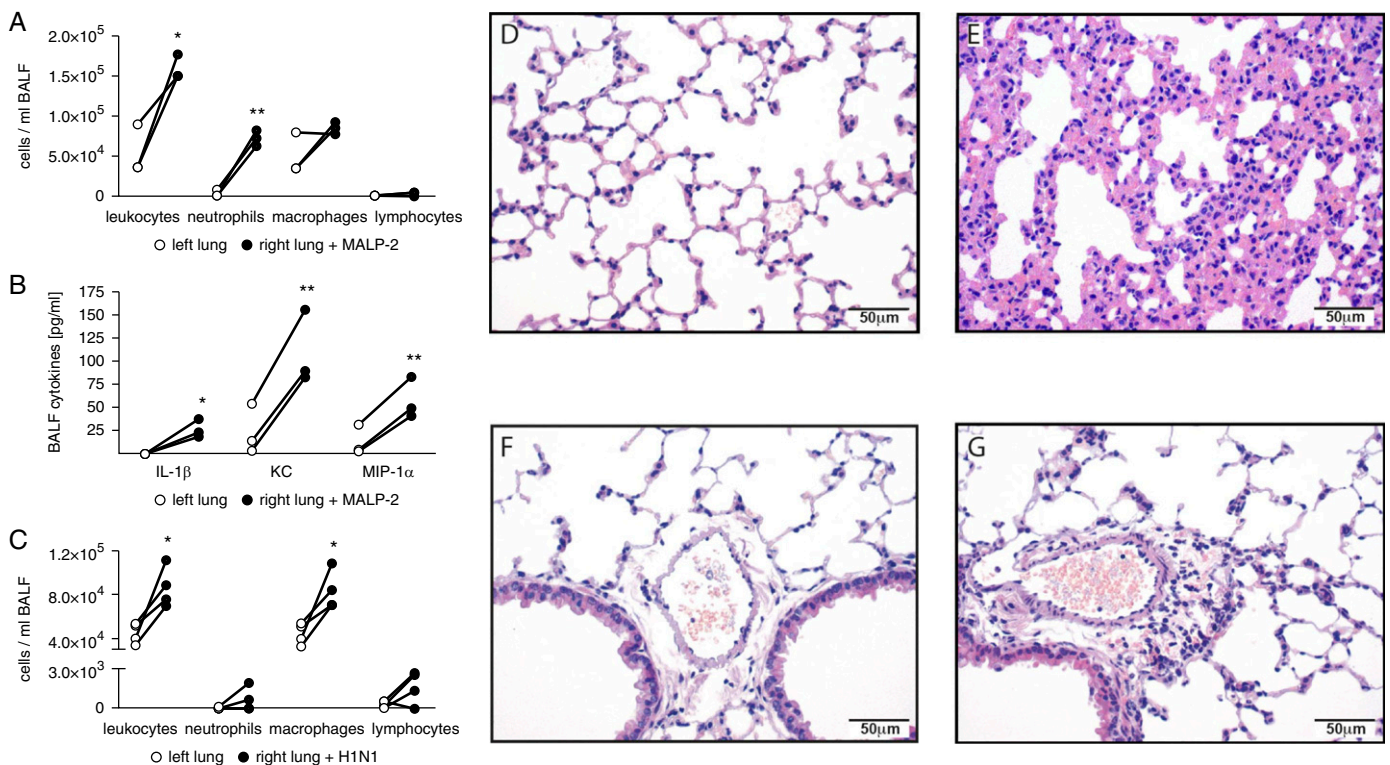


**Figure 4.** Unilateral application of the Toll-like receptor (TLR) 2/6-specific agonist macrophage-activating lipopeptide of 2 kD (MALP-2) and different pathogens. Application of MALP-2 into the right lung induced (A) recruitment of leukocytes, mainly neutrophils, into the bronchoalveolar space of the right lung, accompanied by (B) significantly increased cytokine levels restricted to the right lung. (C) Unilateral infection with 50 plaque-forming units influenza A virus resulted in a significant increase in leukocyte numbers in the bronchoalveolar lavage fluid (BALF) of the right lung compared with the left. In contrast to the noninfected left lung (D), histopathological analysis of right lung infected with influenza virus (E) revealed an enlargement of alveolar septae and interstitial recruitment of macrophages and neutrophils. After unilateral infection of the right lung with 2,000 CFU *Streptococcus pneumoniae*, homogenates of the right lungs exhibited a significantly higher bacterial burden. Accordingly, histologic examination of the left lung (F) did not show pathology, whereas the infected right lung revealed early signs of pneumonia with recruitment of neutrophils and edema formation in the perivascular space (G). Values are given as scatter plots and lines connecting paired values of the same animal ( $n = 3-4$ ).  $*P < 0.05$ ,  $**P < 0.01$  versus untreated left lung using paired  $t$  test (A–C). Representative histopathological details derived from left and right lungs are shown (D–E).

the infected side of the lung, as revealed by BAL (Figure 4C). Histological examination confirmed leukocyte infiltration into the infected right side of the lung (Figure 4E), but not into the uninfected left side (Figure 4D).

Furthermore, for bacterial infection, the bronchoscope was placed into the right main bronchus cranially to the first lateral bronchus branching, and 2,000 CFU *S. pneumoniae* were applied. Significant differences in bacterial burden between infected ( $3.03 \times 10^5 \pm 1.77 \times 10^5$  CFU) and uninfected lung ( $74.13 \pm 73.13$  CFU; paired *t* test, *P* = 0.0079; *n* = 5) were observed. Histological examination (dose, 200,000 CFU) revealed signs of pneumonia exclusively affecting lobes of the right lung (Figure 4G), whereas the left lung was unaffected (Figure 4F).

Finally, we used bronchoscopy for targeted allergen delivery in a murine model of allergic airway inflammation. We sensitized mice systemically with OVA, followed by local application of a single OVA dose into the right main bronchus.

At 48 hours after unilateral OVA challenge, markers of allergic airway inflammation were observed only in the right lung, including increased numbers of eosinophils and macrophages (Figure 5A). In addition, BAL fluid of OVA-challenged right lungs exhibited enhanced levels of cytokines known to be involved in allergic disorders, such as IL-5, KC, and regulated upon activation (Figure 5B). These findings were corroborated by lung histology (Figures 5C and 5D), showing a moderate multifocal peribronchial and perivascular interstitial pneumonia only in the affected right lung.

## Discussion

In summary, miniaturized bronchoscopy allows for almost simultaneous comparative investigation of both lungs in living mice, and is suitable for long-term monitoring. In addition, bronchoscopy enables researchers to selectively administer substances into one lung.

Thus, whereas one lung could serve for interventions, such as pathogen or drug application, the other might serve as intraindividual control. Introduction of the bronchoscope into the left and right lung is possible without restrictions; however, for consistency, we focused on applications into the right lung in this study.

Histological analysis after unilateral instillation of green dye revealed that liquids applied bronchoscopically into the right main bronchus were detectable in the right lung only. Distribution was irregular within right lung lobes, which was expected, as a fluid, and not an aerosol, was applied. Localized instillation of the synthetic TLR-2 agonist, MALP-2, induced leukocyte recruitment and cytokine release into the bronchoalveolar space of right lungs, corresponding to previously established whole-lung models in different species, including mice (13, 17, 18). Analogously, unilateral OVA application in previously sensitized mice induced the typical eosinophilic airway inflammation (19). As the respective left lungs remained uninfamed, MALP-2 and OVA are capable stimuli for murine models of specific unilateral lung inflammation. Murine infection models are commonly used in experimental research. First analyses addressing unilateral application of seasonal influenza A virus demonstrated typical macrophage recruitment and pathological alterations in the infected right lung compared with the noninfected contralateral side. Macrophage numbers were lower compared with previously described models (20, 21), probably due to the early time point of analysis (22). After unilateral infection with *S. pneumoniae*, histopathological signs of pneumonia were observed only in the infected lung. As current results were derived from early stages of viral and bacterial infection, further analysis considering progression of infection and pathological alterations are warranted, although bacteremia after pneumococcal lung infection may already be observed 24 hours after infection (13, 14) that would result in bilateral stimulation of the lungs.

Moreover, this study demonstrated feasibility and investigated side effects of repetitive intravital BALs. Frequent repetitive BAL, such as every third day with a volume of 300  $\mu$ l, induced

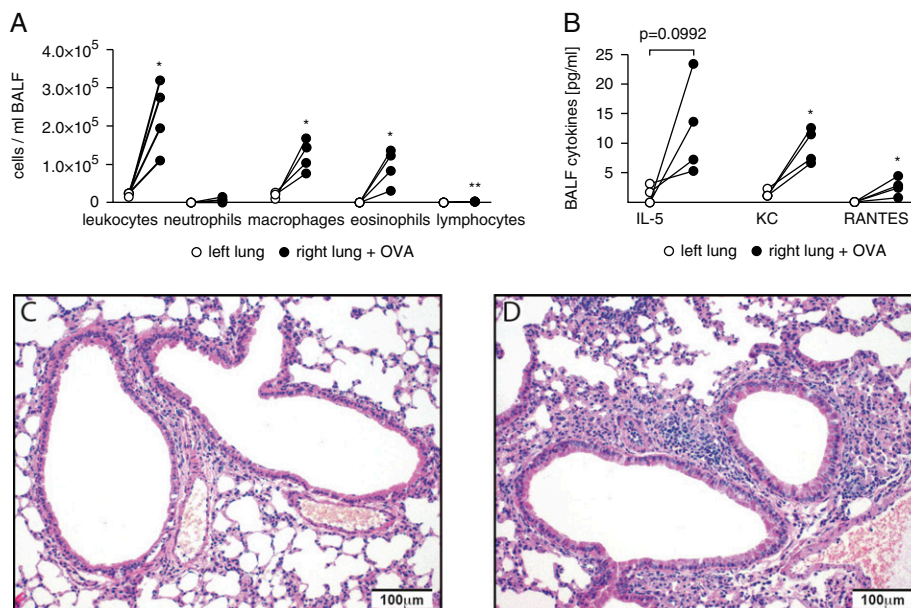


**Figure 5.** Unilateral ovalbumin (OVA) treatment as murine model of local allergic airway inflammation. Mice were systemically OVA sensitized on Days 0 and 14, challenged with a single dose of OVA applied to the right lung on Day 28, and killed after a further 48 hours. (A) OVA treatment resulted in a strong ipsilateral recruitment of eosinophils and macrophages into the alveolar space and (B) elevated BALF cytokine levels as compared with the untreated left lung. The unilateral cell influx could be confirmed by histological analysis of (C) the untreated left and (D) the OVA-treated right lung, showing moderate multifocal peribronchial and perivascular interstitial pneumonia associated with leukocyte infiltration in the treated right lung (representative details of two unilaterally OVA-challenged murine lungs are shown). Values are given as scatter plots and lines connecting paired values of the same animal (*n* = 4). \**P* < 0.05, \*\**P* < 0.01 versus untreated left lung using paired *t* test (A and B).

recruitment of immune cells 24 hours after the last BAL, limiting the frequency and time frames of procedures. However, single BAL did not induce leukocyte recruitment. Thus, adequate frequencies, recovery times, and BAL volumes have to be determined for every longitudinal study. From our data, it may be inferred that unilateral BAL could also be feasible, as we could demonstrate a strictly unilateral application as well as bilateral BAL.

The procedure of bronchoscopy and application or lavage is relatively easy to learn (23), and, with some experience, does not exceed 2 minutes. Although the newly developed bronchoscopy has proven to be useful for different experimental mouse studies, a few limitations need to be considered. Reduced oxygen saturation during examinations could provoke systemic effects. For example, with increasing examination times, bronchoscopy-induced hypoxia might promote bacterial translocation (24). Short examination times or oxygen supply could overcome this problem. Apart from this, the risk of

complications is negligible. The structure of mouse lung tissue is more delicate compared with that of larger animals; however, the diameter of the bronchoscope is far less than the size of mouse bronchi. Therefore, we never observed glottic edema, perforation, or other complications. BAL might increase lung granulocytes, according to reports on BAL in intubated mice (4). Finally, even unilateral stimulation of the lung might affect the contralateral lung via systemic effects. Thus, possible systemic side effects of bronchoscopic examinations should be considered.

Bronchoscopy as a minimally invasive approach promotes the concept of reduction, replacement, and refinement, which is the widely accepted cornerstone in laboratory animal welfare, and is becoming part of animal welfare legislation (25). In addition, several life science technologies, such as knockout breeds, are much more advanced for mice than for larger animals. Thus, the use of mice for a particular research question becomes more and more relevant.

Therefore, many experimental mouse models will benefit from the use of a bronchoscopic approach. Endoscopic optical imaging has made great progress in recent years (26, 27), fostering an interest in applying endoscopic techniques in laboratory rodents.

In conclusion, this report provides a description of a novel advancement in experimental lung research. Miniaturized bronchoscopy may provide an important tool for examination and treatment of specific lung segments in mice, and may thereby improve the quality of experimental *in vivo* studies and reduce the number of mice needed. ■

**Author disclosures** are available with the text of this article at [www.atsjournals.org](http://www.atsjournals.org).

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