

Physiological parameters of the isolated perfused mouse lung and their dependency on age

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Abstract.

BACKGROUND: The “Isolated lung perfusion system” has been established to study lung physiological parameters of mice or other small animals, but little is known about the influence of age on the lung function in mice.

METHODS: We used a buffer-perfused lung system with negative-pressure ventilation to study the effect of age on physiological parameters in the isolated mouse lung. Our study included C57BL/6N mice when they were young (3 to 4 months), adult (5 to 9 months) or old (24 months).

RESULTS: The *ex vivo* data indicated that maximal function of the lung is achieved in the early adulthood (5 to 6 months of age). In this period, lung developed highest maximal expiratory flow rate which declined steadily with increasing age. After this age (>7 months) the dynamic lung compliance increased with significant changes in the 24-month-old mice. However, mouse age had no effect on airway resistance and pulmonary artery pressure. Moreover, we did not find an influence of age on the integrity of the endothelial-epithelial barrier.

CONCLUSION: Our findings indicate the importance of age, in particular on the respiratory mechanics in isolated mouse lungs, which should be considered in the pulmonary research using the “Isolated lung perfusion system”.

Keywords: Airway resistance, lung compliance, C57BL/6N mice, maximal expiratory flow rate

Abbreviations

ANOVA	analysis of variance
BAL	broncho-alveolar lavage
C _{dyn}	dynamic lung compliance
F	air flow
KHL	Krebs-Henseleit

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MIF	maximal inspiratory flow
MEF	maximal expiratory flow
R_{dyn}	dynamic airway resistance
SD	standard deviation
TNF- α	tumor necrosis factor- α
TRITC	tetramethyl-rhodamine-5-(and 6)-isothiocyanate
V_T	tidal volume

1. Introduction

Mice are important tools to investigate the effect of substances, pathogens and diseases on the respiratory function in combination with genetic variations or active genetic manipulations. Moreover, there is an increasing interest in studying the basal or additive effect of normal aging processes on the respiratory function of mice [1, 2]. Non-invasive and invasive methods are available, which can assess physiological parameters of the respiratory system in mice [3–5]. In this regard, the noninvasive whole body plethysmography and two invasive technologies using a pre-programmed ventilator each are currently applied [5].

The respiratory system includes upper airways, lung with lower airways (i.e. lung parenchyma) and thoracic cage with the respiratory muscles. In some experimental issues, such as the lung vascular effects of acute hypoxia [6], or the mechanical effect of lung-related genetic modifications [7], it is desirable to investigate specifically physiological parameters of the lung without simultaneous effect of the thoracic cage. In other approaches, it is also desirable to exclude the influence of other organs and/or the immune system. The “Isolated lung perfusion system” for mice and other small or larger animals which operates with either positive- or negative-pressure ventilation, is preferred to test new substances *ex vivo* for their action and toxicity prior to their application *in vivo*. Despite some limitations, such as the relatively short measurement time and replacement of blood by perfusion buffer, it allows continuous monitoring of several physiological lung parameters. Depending on the individual equipment of the perfusion system this can be lung compliance and airway resistance as main parameters of the respiratory mechanics as well as the pulmonary vascular resistance, edema formation and gas exchange.

Lung physiological parameters measured in mice are dependent on their genetic background [8–10]. Also, the age of mice might significantly influence respiratory mechanics, pulmonary circulation and other physiological parameters due to morphological changes of the lung during growth and aging. Morphological changes mainly include alterations in number, structure and size of the lower airways and alveoli, vessel density and elasticity of the extracellular matrix [11–15]. Although these age-related changes are mainly described for human and rat, they seem to be similar in mouse lung [16–20]. The altered ratio of lung weight to body weight with increasing somatic growth and eventually age-related cachexia is also to consider in experimental settings and data interpretation [16, 21].

Little is known about age-related physiological changes in the mouse lung under normal and/or hyperventilated conditions. However, this information might be useful for designing and interpreting physiological studies using isolated mouse lungs. Most laboratories working with the “Isolated lung perfusion system” apply low tidal volume conditions for up to three hours because long-term ventilations at higher tidal volume cause damage of the mouse lung tissue [22, 23]. This again impairs the validity of data as well as the quality of lung tissue for post-analyses. Therefore, our present study aimed at the analysis of basic physiological parameters of the lung from growing, adult and old mice by use of the “Isolated lung perfusion system” ventilated at normal breathing conditions. In additions, we aimed at

the identification of potential weaknesses of the “Isolated lung perfusion system” when used for mice of very different ages.

2. Methods

2.1. Animals

Mice with C57BL/6N background (Charles River, Sulzfeld, Germany) were investigated at different ages in this study (Table 1). All mice were housed in polycarbonate cages (550 cm² area; Ehret, Emmendingen, Germany) at standard housing conditions (21 °C, 45–60% humidity, 12:12 light:dark cycle, and they were regularly controlled for state of health. Out of the mice originally kept for lung investigations at advanced age, only 60% reached the age of 24 months. This animal study was performed in accordance with the guidelines approved by the local Animal Care Usage Committee, Halle (Saale).

2.2. Lung function

The “Isolated lung perfusion system” for mouse (Hugo Sachs Elektronik-Harvard Apparatus) was used to study the *ex vivo* respiratory function. The mice were anesthetized intraperitoneally with ketamine and xylazine (20 µg each. kg⁻¹ body weight) and placed in the artificial thoracic chamber. Then, the trachea was cannulated to provide constant positive-pressure ventilation (90 breaths · min⁻¹) with room air. After abdominal incision and transdiaphragmatic injection of sodium heparin (25 units) into the heart, the mouse was exsanguinated by transection of the vena cava. The subdiaphragmatic part and the anterior chest plate were excised and discarded. The pulmonary artery was cannulated to provide a constant perfusion flow of the lung vascular bed. Krebs-Henseleit (KHL) buffer (Sigma-Aldrich, Steinheim, Germany) supplemented with 0.1% glucose, 0.3% HEPES and 2% bovine serum albumin (BSA) fraction V (AppliChem, Darmstadt, Germany) was used as perfusion buffer. This buffer was oxygenated with carbogen (5% CO₂, 95% O₂) to yield a pH of 7.4 and passed by means of a roller pump at constant flow (1 ml · min⁻¹). After starting the perfusion via the pulmonary artery, the left ventricle was immediately opened with a small incision at the apex of the heart to allow the efflux of the perfusate. The left atrium was cannulated by passing the cannula through apex and mitral valve. The respiratory mechanics and perfusate characteristics were continuously acquired by the PULMODYN[®] Pulmonary Mechanics Data Acquisition software (Hugo Sachs Elektronik-Harvard Apparatus). The lid of the artificial thoracic chamber was closed, and the ventilation was switched to negative pressure ventilation (90 breaths · min⁻¹) with regular induction of a deep inspiration (one augmented breath after four minutes). KHL perfusion buffer and isolated lung were maintained at 37 °C throughout the experiment by use of a circulatory water bath. In all cases, we applied a constant end-expiratory pressure of -2 cm H₂O and a variable inspiratory pressure (-8 to -11 cm H₂O) in the artificial thoracic chamber (corresponds to the minimal and maximal negative pleural pressure (P_{pleu}), respectively) to reach a tidal volume (V_T) of 7 µl · g⁻¹ body weight. After an equilibration period of 20 min, the lung physiological data were recorded for 90 min. Figure 1 shows the raw data of five breathing cycles of a selected experiment for the airflow (F), V_T, P_{pleu} and pulmonary artery pressure (PAP). Dynamic airway resistance at 70% V_T (R_{dyn}) and dynamic lung compliance at zero air flow (C_{dyn}) were calculated simultaneously according to the given equations (Fig. 1).

At the end of each experiment, lung was perfused with KHL buffer containing 1 mg · ml⁻¹ tetramethylrhodamine-5-(and 6)-isothiocyanate (TRITC)-labeled dextran (65–85 kDa; Sigma-Aldrich,

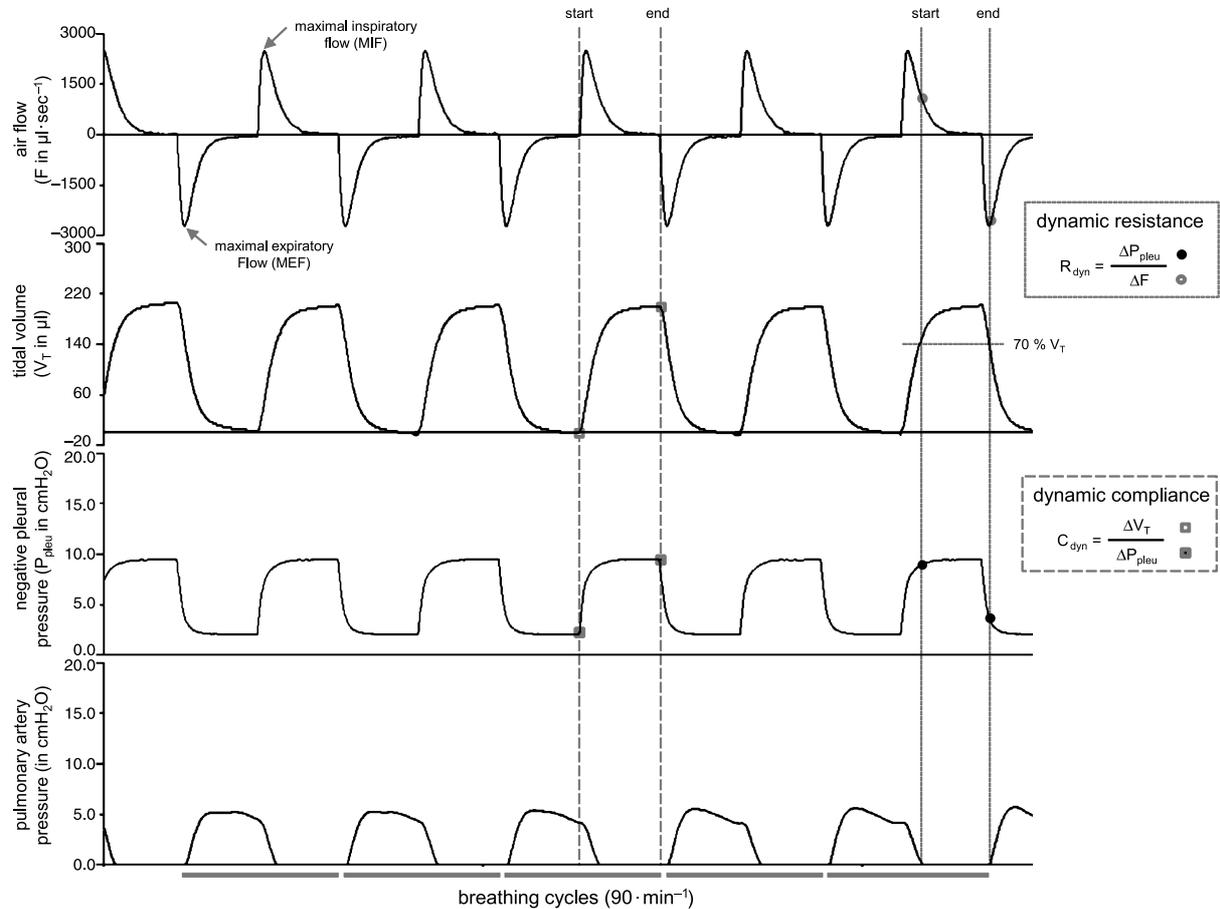


Fig. 1. Selected raw data for respiratory mechanics and perfusate pressure of the isolated mouse lung as acquired and computed by the “Isolated lung perfusion system” with Data Acquisition software. R_{dyn} and C_{dyn} were calculated according to the given equations [26].

Steinheim, Germany) to determine the vascular permeability. TRITC-labeled dextran-containing buffer was replaced after 7 min by standard KHL buffer and the perfusion was continued for further 3 min. Thereafter, the lung was lavaged three-times with 0.5 ml saline via the cannulated trachea. The bronchoalveolar lavage (BAL) fluids were pooled, centrifuged for cell removal and stored at -80°C for further analysis.

2.3. BAL analysis

Lung vascular permeability was determined by quantifying TRITC-labeled dextran in the BAL fluid per TRITC-dextran standard series (550 nm excitation, 590 nm emission) using the infinite[®] M1000 fluorescence reader (Tecan, Grödig, Austria). Total protein was determined with the BCA Protein Assay Reagent kit (Pierce, Rockford, IL) and tumor necrosis factor- α (TNF- α) with the mouse TNF- α ELISA Ready-SET-Go kit (eBioscience, Frankfurt, Germany) according to manufactures’ instructions.

Table 1
Mice of study

Group	Age		Number <i>n</i>	Gender <i>n</i>	Body weight Mean \pm SD
	Median	Total range			
Young	3 months	89–96 d	10	4 ♀, 3 ♂	23.3 \pm 3.6
	4 months	124–133 d	5	5 ♀, – ♂	24.3 \pm 3.8
Adult	5 months	141–169 d	6	5 ♀, 1 ♂	25.8 \pm 1.9*
	6 months	179–186 d	7	7 ♀, – ♂	27.2 \pm 1.4*
	7 months	206–215 d	5	3 ♀, 2 ♂	31.1 \pm 3.1*
	8 months	231–245 d	6	3 ♀, 3 ♂	30.4 \pm 4.2*
	9 months	364–376 d	5	4 ♀, 1 ♂	31.7 \pm 2.8*
Old	24 months	700–738 d	14	6 ♀, 8 ♂	28.9 \pm 3.7*

* $P < 0.01$ versus 3-month-old as determined by ANOVA test (further significances are not indicated).

2.4. Statistical analysis

Data are reported as mean \pm standard deviation (SD) or as median with range or individual data. Data presentations and statistical calculations were performed by use of the SigmaPlot 10 and SigmaStat 3.5 softwares (Systat Software Inc., San Jose, CA). The one-way analysis of variance (ANOVA) procedure followed by Student-Newman-Keuls method or, in the case of non-parametric data, the one-way ANOVA procedure on Ranks followed by Dunn's method was applied as statistical test. P values < 0.05 indicate a significant difference between the groups.

3. Results

Lung physiological parameters of C57BL/6N mice were studied *ex vivo* by use of a buffer-perfused lung system which operates with negative-pressure ventilation similar to *in vivo* conditions. As listed in Table 1, we studied mice when they were young (3 to 4 months), adult (5 to 9 months) or old (24 months). The terms “young”, “adult” and “old” have been assigned according to both biological age and weight development of the mice (Table 1). Our age groups comprised female and male mice in different numbers (Table 1), however, in contrast to the whole respiratory system [10], no sex-dependent differences in the biomechanics of the isolated lung could be identified [7]. In order to avoid inhomogeneity in ventilation due to differences in the lung size, all lungs were ventilated constantly at a body weight-adapted V_T . Although it does not absolutely ensure the same air volume in the lung in relation to the total lung capacity, the V_T adjustment according to the body weight is still the best method to reach similar ventilations and, therefore, it is applied by most groups [6, 22, 24, 25].

Due to age-related differences in the body weight (Table 1), the experimental V_T was lower in young mice than in adult and old mice (Fig. 2A). Moreover, dynamic compliance (C_{dyn}) of isolated mouse lungs was dependent on age. As shown in Fig. 2B, C_{dyn} remained constant for mice between 3 and 6 month of age but increased at higher ages with significant changes in 24-month-old mice. In comparison to static lung compliance (C_{stat}), C_{dyn} is also a function of the resistive force ($R \cdot F$), which needs to be overcome. Since C_{dyn} is computed when the air flow is zero (Fig. 1), the calculation of C_{dyn} corresponds with the

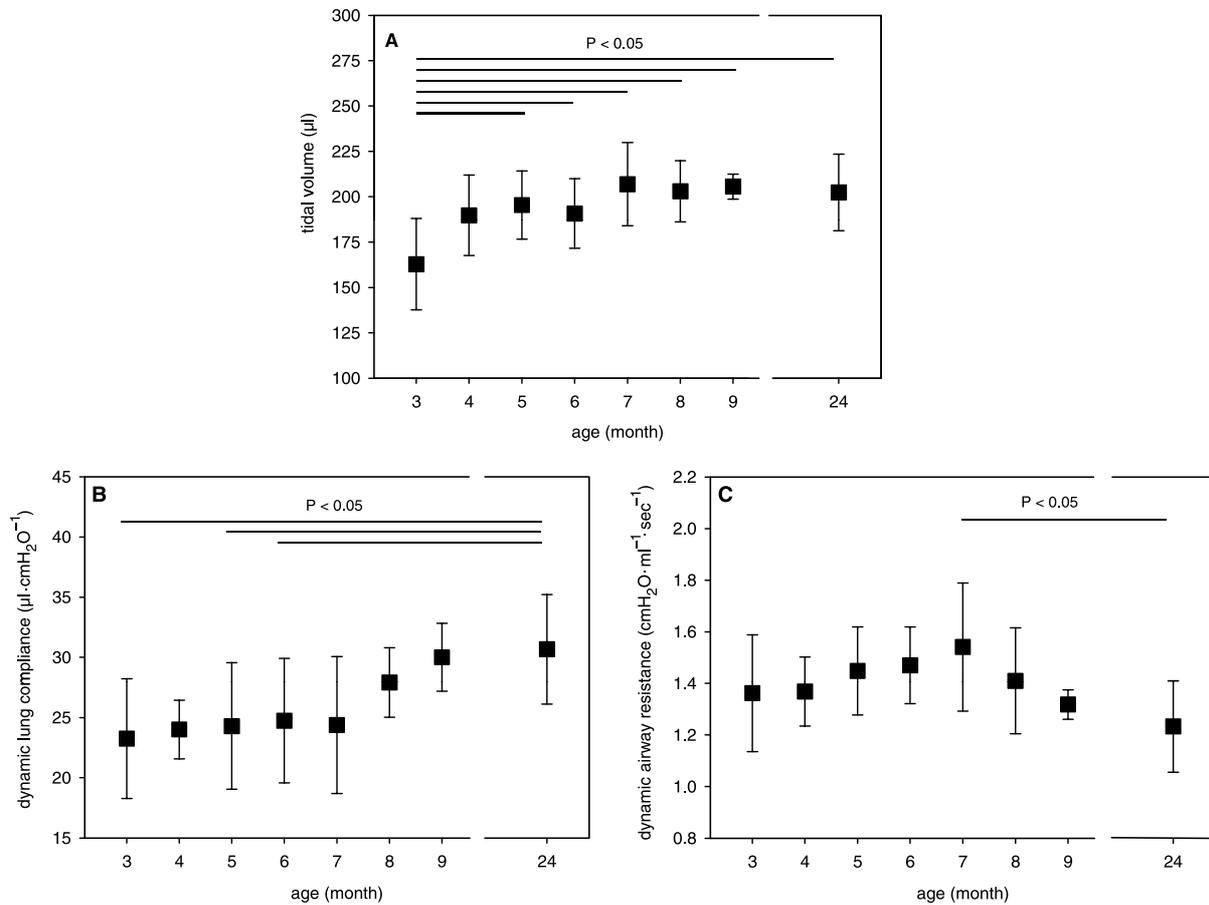


Fig. 2. Age-dependency of A) experimental V_T , B) C_{dyn} at zero air flow and C) R_{dyn} at 70% V_T in isolated mouse lungs. Mean data \pm SD are given with $P < 0.05$ as determined by ANOVA test.

C_{stat} calculation [26]. Moreover, the dynamic airway resistance (R_{dyn}) was only moderately influenced by age with slight reduction in lungs from old mice as compared to adult mice (Fig. 2C).

Peak flow analyses showed that the maximal expiratory flow (MEF) at weight-adapted V_T was lower in the youngest age group than in the other age groups (Fig. 3A). Since youngest mice were also ventilated with the lowest V_T , our data indicate a relationship between MEF and lung volume. In growing humans, this relationship was described by the power law equation: $MEF = a \text{ lung volume } (V)^b$ [27–29]. Therefore, we analysed the relation between MEF and weight-adapted V_T applied for ventilation in our isolated mouse lungs. Regression analyses calculated the exponent b (slope of the regression) with 0.534 for all mice (Fig. 3B). Subsequent correction of the MEF data by the individual $V_T^{0.534}$ revealed no significant differences between the age groups but still indicated highest MEF in 4- to 6-month-old mice (Fig. 3C). However, age-dependent subgroup analyses calculated different exponents for the relation between MEF and V_T with highest values for the young mice (Fig. 3B). Since the airway resistance was less influenced (Fig. 2C), we then normalized the MEF per maximal inspiratory flow (MIF), which was independent of age (data not shown). This MIF-corrected MEF was highest in isolated lungs from 5-month-old mice as compared to lungs from younger mice and decreased steadily with increasing age (Fig. 3D).

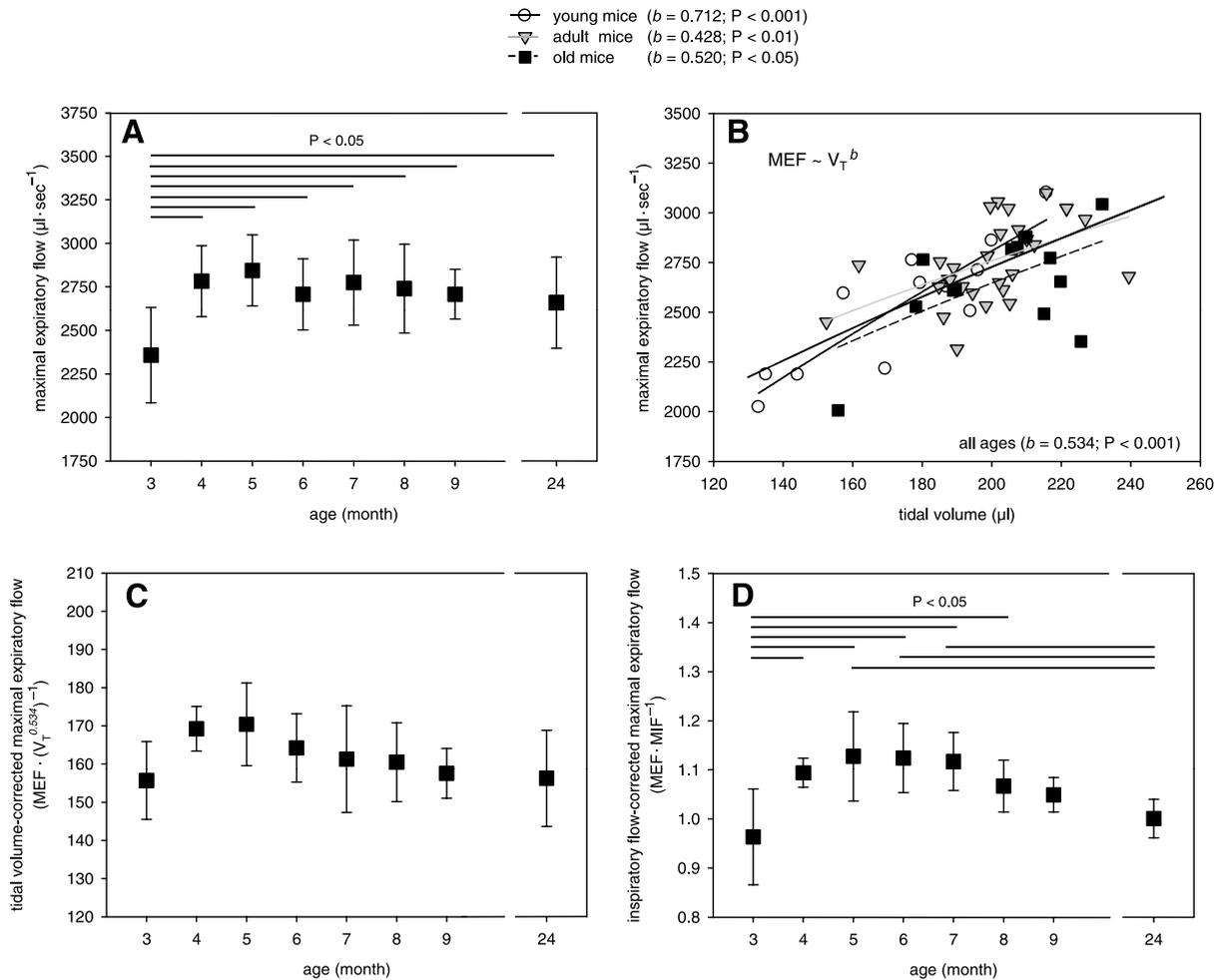


Fig. 3. Effect of growth and aging on the airflow in isolated mouse lungs. A) MEF, B) MEF in relation to V_T , C) V_T -corrected MEF, and D) MIF-corrected MEF. Mean data \pm SD are given with $P < 0.05$ as determined by ANOVA test.

Our buffer-perfused lung system also allowed the detection of the mean perfusion pressure in pulmonary arteries, but these measurements did not reveal a significant effect of age (Fig. 4). The integrity of the endothelial-epithelial barrier was assessed by detection of TRITC-labelled dextran in the BAL, which was added to the perfusate shortly before. The quantification of diffused TRITC-labelled dextran did not indicate an influence of age on the lung vascular permeability and, therefore, edema formation in the isolated mouse lungs (Table 2). This observation was also confirmed by an unchanged protein concentration in the BAL fluid (Table 2). Moreover, we did not find an influence of mouse age on the BAL concentration of TNF- α released from the lung cells before and/or during the experiment (Table 2).

4. Discussion

Using the “Isolated lung perfusion system” we demonstrated that growth and aging is associated with biomechanical changes of the mouse lung under normal breathing conditions. Despite some disadvantages

Table 2
BAL after *ex vivo* lung perfusion

Age group	Permeable dextran (ng · ml ⁻¹) Mean ± SD	Protein (μg · ml ⁻¹) Mean ± SD	TNF-α (pg · ml ⁻¹) Mean ± SD
3 month	707 ± 396	357 ± 104	51 ± 13
4 month	639 ± 382	382 ± 139	98 ± 69
5 month	620 ± 181	296 ± 107	96 ± 45
6 month	1096 ± 628	358 ± 181	81 ± 53
7 month	1067 ± 803	390 ± 165	78 ± 15
8 month	657 ± 143	556 ± 155	61 ± 17
9 month	643 ± 132	275 ± 99	111 ± 69
24 month	648 ± 318	488 ± 115	88 ± 23

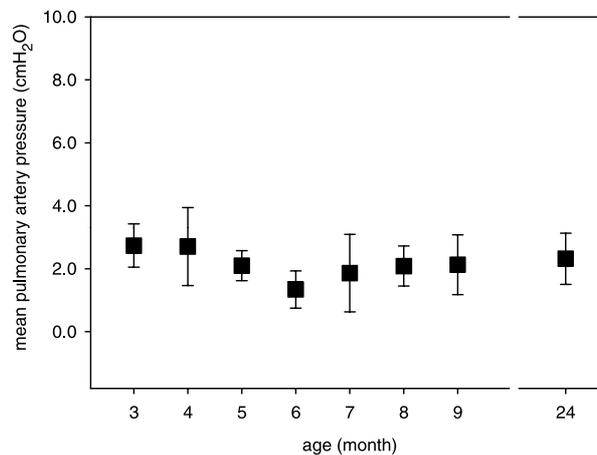


Fig. 4. Age-dependency of the mean pulmonary artery pressure in the isolated mouse lungs. Mean data ± SD are given with $P < 0.05$ as determined by ANOVA test.

of this *ex vivo* system, age-related changes have been particularly observed for the relative maximal expiratory flow rate and lung compliance, whereas perfusion flow and vascular permeability were not influenced by age. Changes in the biomechanical parameters indicated that maximal function of the lung is achieved in the early adulthood (5 to 6 months of age).

Developmental alveolarization (i.e. septal anlage) of the mouse lung is completed after about one month of age but growth of the lung parenchyma still continuous for more than two months [16, 20]. Due to concurrent changes in the somatic growth, the ratio of lung weight to body weight is gradually lowered with increasing age [16]. Since previous studies only analysed the development of mouse lungs in the first weeks after birth, it is difficult to assess when the ratio of lung weight to body weight reaches constant levels [16, 20]. According to plethysmographic investigations of growing mice, the inspiratory V_T is constant at the age of 3 months [10, 30], but at this age the body weight has not yet reached its maximum. Our own measurements in another study indicate a constant ratio of lung weight and body weight when C57BL/6N mice are between 4 to 4.5 months old (unpublished data). Since the buffer-perfused system for mouse lung, in which the lung remains in the opened thoracic cage, does not

allow the measurement of the lung weight, a lung weight-adapted V_T could also not be used. This is a disadvantage when investigating mice of very different ages with the negative pressure-based “Isolated lung perfusion system” or with other technologies, which are based on the weight-adapted respiratory volume. Consequently, the experimental V_T values we applied for lungs from young mice were probably lower in relation to their total lung capacity *in vivo* than in lungs from adult mice. Similar inaccuracy may have occurred for the V_T values in old mice due to the weight loss with advanced age. This fact might influence some of our findings, in particular the MEF data, which depend on the respiratory volume used for ventilation of mice [31]. Despite this disadvantage, it is important to note that studies at the weight-adapted V_T might still be preferred by most researchers because they ensure at least similar lung ventilations per total lung capacity.

Analysis of growing humans suggested that the MEF positively correlates with the lung volume^b where the exponent b is between 0.67 and 1.0 [29]. Although our study based on the weight-adapted V_T , we also calculated an exponent $b > 0.67$ for isolated lungs of young mice but lower values for lungs of adult and old mice. Since the relationship of MEF and weight-adapted V_T is influenced by age, a general correction of the MEF per weight-adapted V_T is not absolutely exact. Nevertheless, the V_T -corrected MEF indicated maximal expiratory lung function in the early adulthood (5 to 6 months of age). The maximum expiratory function of the isolated lung is also indicated by the highest MEF in relation to the MIF in this age. Our observations are partially in contrast to findings of whole-body plethysmographic investigations of growing mice showing either no changes or a parallel increase of MEF and MIF between 2.5-week-old and 6-month-old mice [10, 30]; however, this discrepancy may result from different airflows between the isolated lung and the total respiratory system.

Like V_T -mediated correction of the MEF, its correction per MIF might also not be useful in all regards, especially when the airway resistance is increased [32, 33]. However, the resistance of conducting airways was even moderately reduced at advanced age in our and other mouse studies [1, 2] thereby suggesting no significant obstruction of the airflow with increasing age in mice. Since animal studies investigating rat lungs identified an age-dependent increase of the airway resistance first after inhalation of methacholine, which provokes bronchoconstriction [34], it is well conceivable that the methacholine challenge can only show an adverse effect of age on the resistance of conducting airways. Nevertheless, human studies have also indicated a rather small or even no dependency of the airway resistance on age as well [35–39].

Ageing of the respiratory system causes more problems with the expiration than inspiration [40]. This is reported in many human studies [37, 39, 41–50] and in few mouse studies as well [1, 2, 51]. If the lung volume is nearly constant the expiratory flow is mainly determined by the airway resistance and elastic properties of the lung. Since our findings did not indicate an age-related airway obstruction in mice, changes in the elastic recoil pressure seem mainly to be responsible for the reduced MEF in relation to MIF [52]. In this regard, the MIF-corrected MEF values showed a steady decline after reaching their maximum in the early adulthood of mice. This coincides with many studies exhibiting reduced elastic recoil of the lung (i.e. transpulmonary pressure) in humans [37, 42, 46, 49]. Although the respiratory musculature does not play a role when studying isolated lungs, the age-dependency of the MIF-corrected MEF shown for our isolated mouse lungs coincides with many human studies exhibiting highest forced expiratory flow rates in the early adulthood and subsequent decline with increasing age [39, 41, 44, 45, 47].

Changes in the lung elastic recoil are attributed to quantitative and qualitative changes in the extracellular matrix of the lung parenchyma, in particular elastin [53]. While in growing mice the high expression rate of elastin might contribute to the high elasticity, in aged mice qualitative changes of elastin significantly might impair the elastic recoil of the lung [53]. Moreover, quantitative and qualitative changes of col-

lagen are suggested to alter the elastic property of the lung parenchyma in an age-dependent manner [54, 55]. Age-dependent changes in the lung elasticity are also demonstrated in our *ex vivo* study by the higher compliance of isolated mouse lungs with increasing age. The age-dependent increase in either static or dynamic lung compliance has also been shown in other mouse studies [1, 2, 51] as well as in human studies [39, 48]. Although increased lung compliance can also be caused by greater lung volumes, for instance due to alveolar dilation [15], parallel age-related changes in the MIF-corrected MEF again indicate the pivotal role of the reduced lung elasticity in old mice. Surprisingly, lung compliance was nearly as high in 9-month-old mice as in 24-month-old mice, and also the MIF-corrected MEF was not significantly different between 9- and 24-month-old mice. Since only a part of the mice reaches the age of 24 months, this discrepancy could result from the positive selection of old mice. Nevertheless, relatively early changes in the lung elasticity normalized per lung volume have also been shown by static and dynamic compliance measurements in active humans [48]. Also surprisingly, expiratory flow and lung compliance were not that significantly influenced by higher mouse age as expected. One critical reason for this observation might be the application of a moderate ventilation mimicking normal breathing conditions in our *ex vivo* study instead of stressing the lungs with high ventilation to detect the impairment in the respiratory mechanics. In contrast to changes in the biomechanical parameters, i.e., relative maximal expiratory flow and lung compliance, we did not identify an influence of age on the arterial perfusion pressure in isolated lungs. This observation coincides with other studies demonstrating either no or a very moderate influence of increasing age on the pulmonary artery pressure in humans and dogs during rest [56, 57].

The respiratory system becomes more susceptible to bacterial infections with increasing age due to a functional decline of the immune system [58]. Nevertheless, the old lung is not characterized by chronic inflammation as demonstrated in many histological investigations [18, 59, 60]. This has also been indirectly confirmed by our study showing an unchanged level of pro-inflammatory TNF- α in the BAL fluid as well as an unchanged endothelial permeability in the isolated lungs of old mice.

Our findings indicate the importance of growth and ageing on the respiratory mechanics in isolated mouse lungs. This has to be considered in experimental pulmonary research when studying lung physiological parameters in the "Isolated lung perfusion system" for mice and most likely other species. For example, investigations of age-related changes in lung would only be meaningful when comparing aged mice with mice at the age of minimum 5 months but not younger.

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Conflict of interest

The authors have no financial, consulting or personal relationships with other persons or organizations which could have influenced the present work.

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