

**Additional file 1 – Additional information on the model (PDF)**

A more detailed description of the method, including the infection model, the behavioral score system and the spirometric measurements

Description of the spirometric parameters from a raw flow curve (1 figure)

Additional spirometric parameters omitted in the main text (1 figure)

Complete table of spirometric values prior to infection for the investigated study group

Histological sample slides at various time points post infection (1 figure)

**Additional file 2 – Short movie of the spirometric procedure (.wmv)**

A short movie showing the procedure of placing the mice in the inserts and starting the spirometric measurement.

**Head-out spirometry accurately monitors the course of *Pseudomonas aeruginosa* lung infection in mice**

Florian Wölbeling, Antje Munder, Frauke Stanke, Burkhard Tümmler, Ulrich Baumann

**Supplementary material**

**Bacterial Infection.** Prior to the start of the experiments animals were acclimatized for at least seven days. *Pseudomonas aeruginosa* strain TBCF10839 was grown in Luria broth (LB) overnight at 37°C (230 rpm), pelleted by centrifugation (5000 x g, 10min), washed twice with sterile phosphate buffered saline (PBS) and the optical density of the bacterial suspension was determined by spectrophotometry at 578 nm. The intended number of colony forming units (CFU) was extrapolated from a standard growth curve, and appropriate dilutions with sterile PBS were made to prepare the inoculums for the mice ( $6 \times 10^5$  CFU/30 $\mu$ l). To verify the correct dilution, an aliquot was serially diluted using PBS and plated on LB agar plates. Anesthetized mice were inoculated via view-controlled intratracheal instillation.

**Score.** The behavioral score utilizes the visual inspection of the parameters vocalization, piloerection, attitude, locomotion, breathing, curiosity, nasal secretion, grooming and dehydration for an estimation of the health status of the mouse. Each parameter is assigned a rank number from 0 to 2. The combined score gives an impression on the overall health status of the animal. The analysis scale ranges from 0 (undisturbed) to 11 (moribund). Values higher than 11 most definitely result in the death of the animal. The behavioral score reaches values of 11 around 6 to 8 hours post infection for females and 8 to 10 hours post infection for males and returns to normal values at 24 to 48 hours post infection for both genders. For higher doses the moribund condition would be irreversible and fatal, for the utilized dose a recovery and return to normal values is possible. For a detailed description of the method and an example of its utilization please refer to [E1].

**Spirometry.** Prior to the start of the infection experiment mice were familiarized to the spirometric procedure for five days. The first two days are not used for analysis due to high variability caused by movement artifacts. Of the remaining three days, the median was

calculated for each mouse and parameter and defined as the starting value.

Post infection first spirometric measurements were taken after four hours and then in the acute phase of infection at time points 6, 8, 10, 12, 18 hours post infection. From time point 24 hours post infection until the end of a measurement series after eight days (192 hours post infection) measurements were taken every 24 hours at the same time of day. Previous experiments showed that 192 hours is sufficient for a longitudinal investigation of bacterial lung infections at the applied dosage. By that time all changes in the lungs were reversed – a further extension of the measurement period did not yield more information (data not shown). Spirometric measurements using the Notocord HEM (Version 4.2.0.241) software were performed in a custom made head-out spirometry apparatus and took about four minutes. Four mice can be measured in parallel. For this, mice are placed in glass inserts with their heads sticking out through a double layer of membranes. These membranes ensure an airtight fit between the outside air and the air volume in the insert, as well as restraintment of the mice. Three different sizes of inserts were engineered to hold mice of varying sizes ranging up to 36 grams.

The smallest inserts are 85 mm in length with an outer diameter of 30 mm and an opening at one end of 25 mm in diameter. They can accommodate young mice with a body weight up to 24 grams. For mice weighing 24 to 32 grams inserts with a longer tube are available. They measure 110 mm in length and the same diameter and opening size as mice in this weight range increase only in length rather than diameter. For mice above 32 grams a new set of inserts was developed which have a slightly larger diameter and a larger opening for easier access of the mice. These are 115 mm in length with an outer diameter of 35 mm and an opening of 35 mm. This makes the system very versatile and underlines the usability for different mouse groups/ages.

Respiration causes air to flow through a pneumotachometer positioned above the thorax of the

mouse. The airflow is converted into an electrical signal by a pressure transducer and amplified and digitalized before it reaches the computer for analysis. Data evaluation is done with the help of the Notocord HEM software. In brief, two markers are set within the four minutes measurement period for every mouse individually thus compensating for inter-individual duration of acclimatization periods. A time period of about one minute is chosen for analysis. Therefore, together with a sampling rate of 500 Hz about 30,000 data points only for the analysis period were acquired for each mouse (1 data point every 2 ms). The resulting raw data were imported into Excel 2003. Mean values for 14 spirometric parameters for the chosen time of evaluation were gathered after quality control (filtering and smoothing, Filtered mean (5 sec, Exclusion % for filtered mean: 10%)).

Figure E1 shows an overview of the investigated parameters in an actual flow curve and its integral.

Please refer to the video file in the online supplemental material for an example of how the spirometric process is performed in our laboratory.

**Additional spirometric parameters.** Three parameters (Inspiratory Time, Relaxation Time and Time of Brake) showed less overall changes in the chosen setting and no clear tendency in their course. These parameters were therefore not presented in the section bacterial infection and spirometry. However, these parameters showed usability in different settings (higher infectious dose and/or different mouse/pseudomonas strain, data not shown).

The parameters Peak Inspiratory Flow (PIF) and Peak Expiratory Flow (PEF) were very similar to their IF50 and EF50 values, indicating that at 50% of inspiration (or expiration, respectively) the maximal/peak flow values were almost reached. Due to space constraints these two parameters were allocated to the supplement (Figure E2).

**Histological sample slides.** Mice from the same mouse strain and of comparable age (20 weeks) as well as the same infectious dose as in the main experiment were chosen. To investigate the signs of inflammation the right lungs were lavaged in situ, with approximately 0.7 ml sterile PBS, which flushed the organ twice. Afterwards the lungs were formalin (4%) fixed and embedded in paraffin. The paraffin blocks were cut into 4 µm slices, stained with haematoxylin/eosin and twenty fields of view per specimen slide were assessed regarding the inflammation in the lung parenchyma and in the bronchial areas using a Zeiss Axiophot photomicroscope and a semi-quantitative score system. Please refer to figure E3 for histological sample slides.

**Complete table of spirometric and physiological standard values.** Please refer to table E1 for a complete list of spirometric and physiological standard values for the investigated study group. P-values that test for intrinsic differences between male and female mice show that only three of these 14 spirometric parameters were initially significantly different at the 0.05 significance level. However, only body weight was significant after Bonferroni correction. For all parameters median and interquartile range data from three independent measurements prior to infection are shown.

## Supplemental Material References

- E1. Munder A, Zelmer A, Schmiedl A, Dittmar KE, Rohde M, Dorsch M, Otto K, Hedrich HJ, Tümmler B, Weiss S, Tschernig T. Murine pulmonary infection with *Listeria monocytogenes*: differential susceptibility of BALB/c, C57BL/6 and DBA/2 mice. *Microbes Infect* 2005;7:600-611. )

## Figures

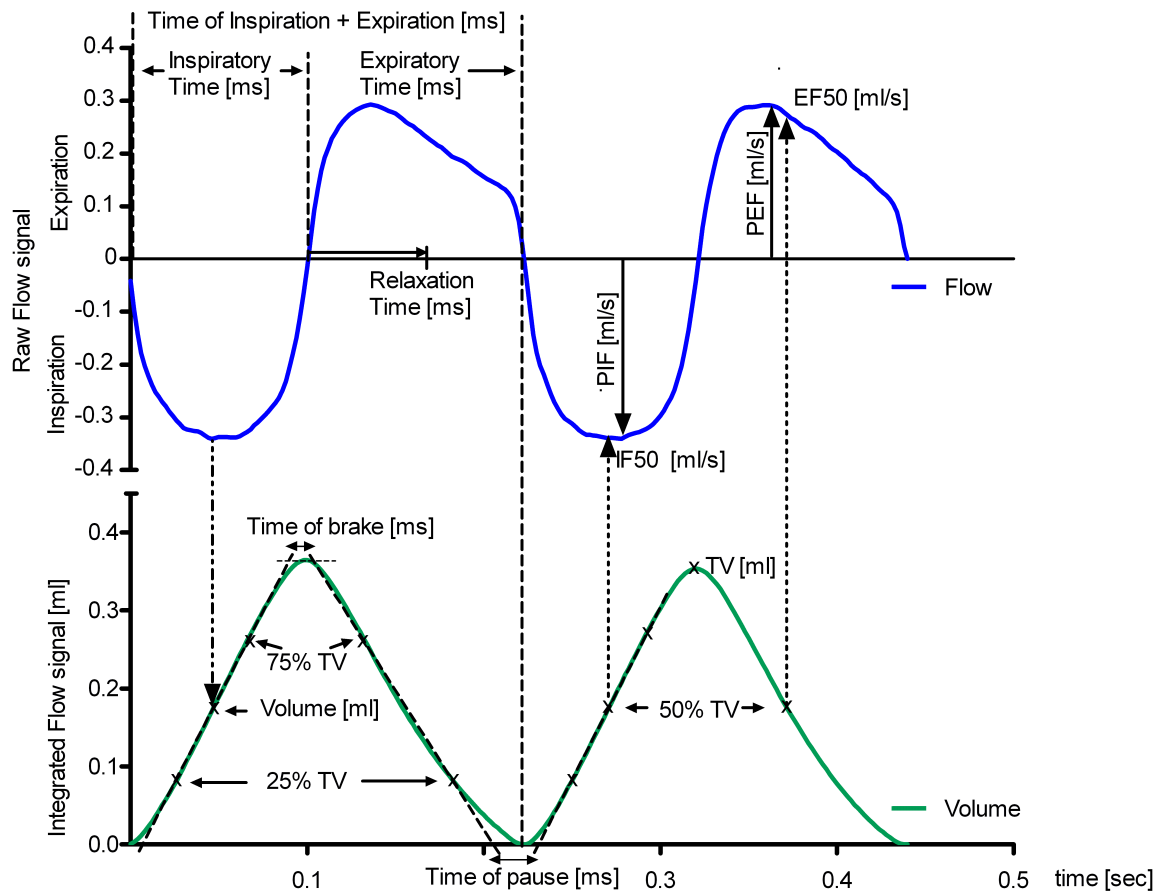


Figure E1 - Raw flow curve of two breath cycles and integrals thereof. Parameters with further explanation: Relaxation time - Time required to exhale  $1-e^{-1}$  (64 %) of tidal volume. Tidal volume - maximum point of integral curve. Volume - projection from the maximum value of inspiration of the flow curve to the integral curve; Time of brake and time of pause - intersection of the tangents through the 25% and 75% of tidal volume with the maximum/minimum point of the integral curve. EF50 and IF50 - projection from the 50% Tidal volume of expiration and inspiration to the point on the flow curve.

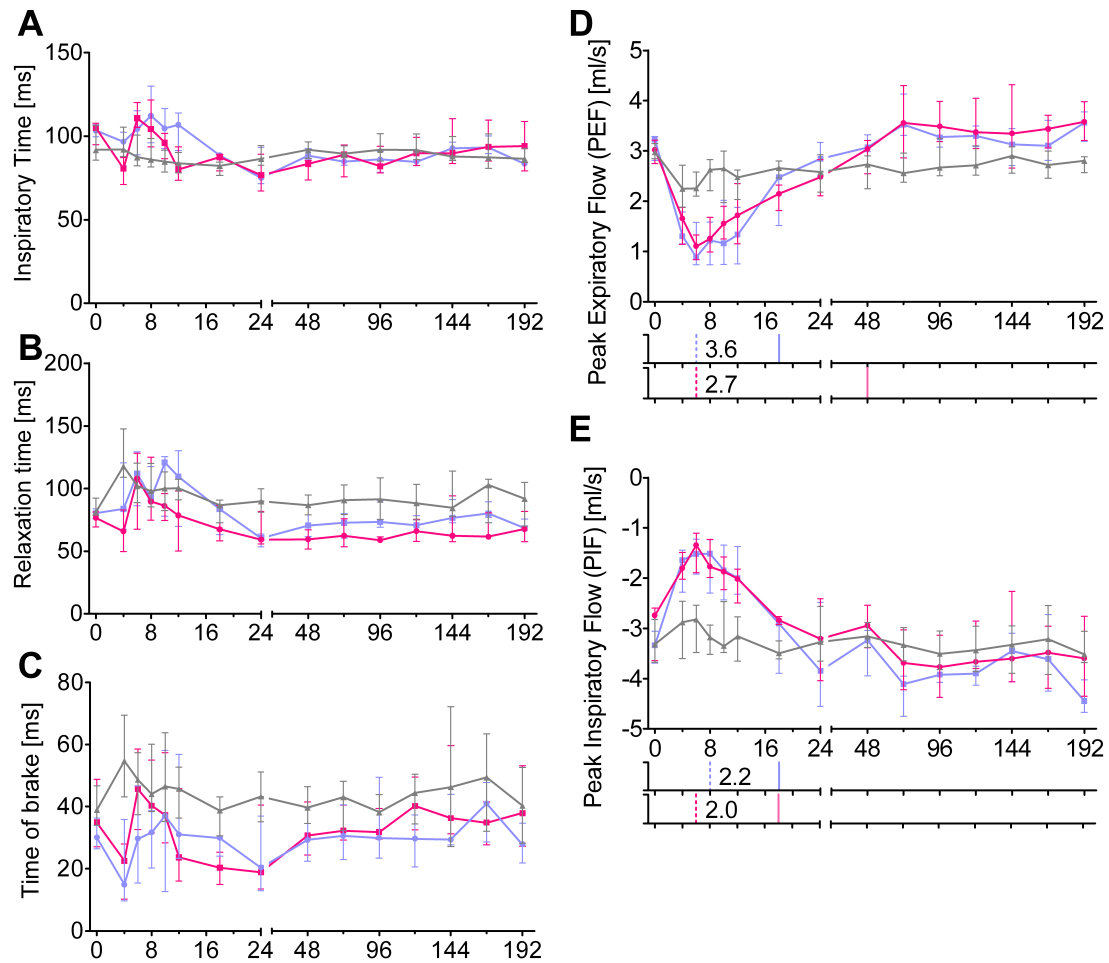


Figure E2 - Respiratory parameters Inspiratory time (A), Relaxation time (B) and Time of Brake (C), which showed no clear tendency at the utilized infectious dose and mouse strain. Parameters Peak Inspiratory Flow (PIF;(D)) and Peak Expiratory Flow (PEF;(E)), which were moved to online supplemental material due to space constraints and high similarity to their related parameters IF50 and EF50.

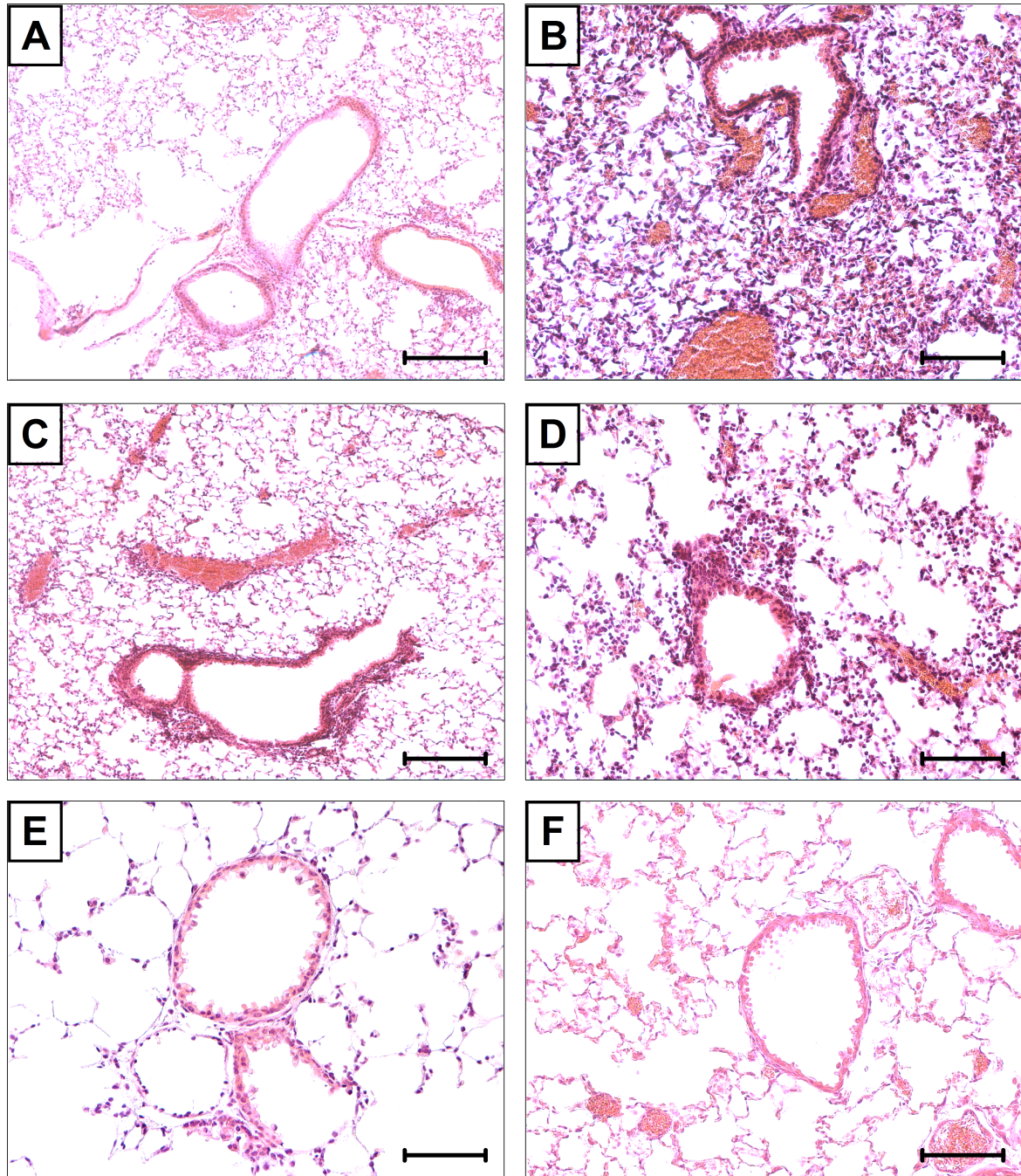


Figure E3 - Pathohistological findings in C57BL/6J murine lungs at different time points after intratracheal infection with  $6.0 \times 10^5$  CFU of *P. aeruginosa* TBCF10839 (A-E); vehicle control (F, 30  $\mu$ l PBS). Hematoxylin-eosin staining; original magnification x 100, scale bar: 100  $\mu$ m. A) 4 hours post inoculation: Very weak parenchymal and almost no peribronchial

inflammation. B) 8 hours post inoculation: Moderate purulent alveolar pneumonia with numerous parenchymal and peribronchial inflammatory infiltrates. C) 12 hours post inoculation: Slight leucocyte accumulation around a bronchus and only faint parenchymal inflammation. D) 24 hours post inoculation: Minor amount of inflammatory cells in the alveolar region, but clear peribronchial conglomerates. E) 48 hours post inoculation: Very weak parenchymal and peribronchial inflammation. F) Vehicle Control: normal lung parenchyma after instillation of 30  $\mu$ l sterile PBS i.t..

Table E1. Complete list of spirometric and physiological values for the investigated study group prior to infection

Parameter	Unit	Sex	Median (and interquartiles)*	P-value <sup>†</sup>
Tidal Volume (Total lung volume)	ml	male female	0.265 (0.240 - 0.281) 0.225 (0.210 - 0.252)	<b>0.018</b>
Volume (Inspiration volume)	ml	male female	0.134 (0.117 - 0.147) 0.115 (0.107 - 0.125)	<b>0.012</b>
Minute Volume (Total volume breathed in one minute)	ml/min	male female	68.859 (62.439 - 75.677) 60.763 (51.813 - 69.978)	0.113
Expiratory time (Time required for expiration)	ms	male female	128.92 (124.28 - 132.80) 119.91 (111.39 - 127.11)	0.227
Inspiratory time (Time required for inspiration)	ms	male female	103.42 (99.65 - 106.96) 105.02 (94.85 - 107.73)	0.603
Time of Inspiration plus Expiration (Time required for one breath)	ms	male female	232.30 (221.75 - 237.04) 222.19 (202.81 - 234.06)	0.227
IF50 (Flow at 0.5 VTI) (Midtidal inspiratory flow at 50% inspiration)	ml/s	male female	-3.226 (-3.624 - -2.959) -2.666 (-3.553 - -2.500)	0.372
EF50 (Flow at 0.5 VTE) (Midtidal expiratory flow at 50% expiration)	ml/s	male female	2.936 (2.633 - 3.118) 2.861 (2.620 - 3.028)	0.086
PIF (Peak Inspiratory Flow)	ml/s	male female	-3.333 (-3.692 - -3.056) -2.740 (-3.636 - -2.593)	0.164
PEF (Peak Expiratory Flow)	ml/s	male female	3.224 (2.814 - 3.291) 3.026 (2.756 - 3.220)	0.976
Respiratory Rate (Breaths per minute)	bpm	male female	262 (256 - 272) 278 (260 - 298)	0.407
Relaxation Time (Time required to expire (1-e <sup>-1</sup> ) of Tidal Volume)	ms	male female	80.28 (75.89 - 83.83) 76.53 (69.28 - 81.39)	<b>0.048</b>
Time of Pause (End expiratory pause (EEP))	ms	male female	23.02 (22.23 - 23.73) 22.30 (21.95 - 23.88)	0.976
Time of Brake (End inspiratory pause (EIP))	ms	male female	30.02 (26.37 - 36.26) 34.93 (26.99 - 48.73)	0.129
Body temperature	°C	male female	35.6 (35.3 - 36.0) 36.7 (35.9 - 37.3)	<b>0.004</b>
Body weight	g	male female	32.3 (30.8 - 33.3) 23.1 (22.5 - 24.7)	<b>0.000</b>

\* Data calculated from three independent measurements prior to infection

<sup>†</sup> 2-sided p-values were calculated utilizing Mann-Whitney U test. Significant values (p<0.05) are marked in bold. After correction for multiple testing only p-values below 0.0033 are maintained.