Recovery of Rat Alveolar Macrophages by Bronchoalveolar Lavage under Normal and Activated Conditions

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When rat (female Wistar) lungs were lavaged (bronchoalveolar lavage, BAL) six times with physiological saline, approximately the same number of alveolar macrophages (AM) were found in the first and second BAL, whereas in the third fourth, fifth, and sixth BAL, the number of AM decreased exponentially. Morphometric counting of the number of AM in histological sections of lung tissue showed that only 14% of the AM population had been recovered by BAL. Although additives to the BAL fluid such as lidocaine and/or fetal calf serum increased the AM count in the first washing considerably, the total number of AM washed out remained unaltered. Addition of the phagocytosis stimulant zymosan increased the AM count in BAL by a factor of more than 2. On stimulation of the lungs with an inert dust (silicon carbide), the AM count in the BAL and the lung was only slightly increased 8 weeks after intratracheal instillation. In contrast, after exposure to fibrogenic and cytotoxic quartz, the AM count in BAL and lung was significantly increased, and the recovery of AM had also increased by a factor of approximately 2. The experiments show that it is the micromilieu of the alveoli and the condition of the AM (certain physiological activation states, such as phagocytic activity) that essentially determine the degree of recovery.

Introduction

Bronchoalveolar lavage (BAL), i.e., washing terminal bronchioles and alveoli with physiological saline via a bronchoscope, is of increasing importance in diagnosing and monitoring the course of lung disease. Cellular and noncellular components are flushed out of the alveoli by BAL and are hence rendered accessible to diagnosis (*1,2*). Analysis of the lavage fluid can give an indication of the state of the alveolar micromilieu. With regard to dust-induced occupational lung diseases, analysis of the lavage fluid may give some insight into the nature and degree of dust exposure (*3-5*).

The technique of BAL also has increasing importance in clinical research, with the aim of gaining greater understanding of causes of disturbances to the micromilieu of the lung. BAL analysis can therefore extend existing research methods for investigating pathogenic reactions caused by inhaling toxic substances. In addition, results from clinical research show excellent comparability to those from animal experiments.

A fundamental question in such investigations is the extent to which the lavage fluid represents the micromilieu of the alveoli. The number of alveolar macrophages (AM) flushed out is of particular importance, as this cell type plays a key role in alveolar

pathology. However, the AM count in lavage fluid may also vary considerably with different lavage techniques. This applies particularly to animal experiments because no guidelines exist for conducting BAL. Individual laboratories use different techniques for BAL, e.g., different lavage fluids are employed and the number of individual washes performed can vary from 1 to 18. This second point appears to be of importance as, over several lavages of the lung, the number of AM in the lavage sequence does not follow a simple dilution series. For example, there is evidence that in rodents more AM may be present in the second lavage than in the first (*5,6*). It can also be demonstrated that the number of AM obtained can be affected by the lavage fluids used. Removing the divalent cations Mg^{2+} and Ca^{2+} increases recovery of AM significantly (*7*). Studies by Miller and Foster (*8*) showed that addition of the local anesthetic lidocaine to the lavage fluid increases the recovery of AM due to an effect on the macrophage cell membrane. Different adhesive properties of the AM (as determined by their particular functional characteristics) obviously affect their recovery. To estimate the total number of AM in the lung from the number recovered by BAL, it is important to know how many AM the lung itself contains and what factors determine their recovery.

The aim of this investigation was therefore to define quantitatively the recovery of AM in the rat lung model. The effect of changes in macrophage adhesion were to be induced by external factors, i.e., different lavage techniques, and internal factors such as pathogenic conditions in the alveoli.

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Materials and Methods

Female Wistar rats (190–260 g body weight) were used in this study. Groups of 20 rats were exposed by intratracheal instillation to either 50 mg silicon carbide or 5 mg quartz (DQ12) suspended in 0.5 mL physiological saline solution. The animals underwent BAL 2 and 8 weeks after dust administration.

Bronchoalveolar Lavage

Animals were sacrificed by IP injection of hexobarbital. After opening the abdominal cavity and exsanguination via the aorta abdominalis, the lungs were exposed and a cannula (1.2 mm internal diameter) fixed in the trachea. The lungs were lavaged gently, via this cannula, to avoid tissue rupture. Lavage fluid (5 mL) was slowly injected into the lung using a syringe and then sucked out again. The operation was repeated six times with fresh, cold (4°C) saline. The following lavage fluids were used: physiological saline solution (PSS; 0.9% NaCl) supplemented with 10% fetal calf serum (FCS), PSS with lidocaine (12 mM) and 10% FCS, and a zymosan suspension (2% zymosan in PSS).

For lavage with zymosan, the initial suspension was left in the lung for 15 min and the next five lavages were performed with PSS in the customary manner. As a control, a second group was lavaged with PSS using the same method.

Cells from the individual lavages were centrifuged (150g; 4°C), resuspended in 5 mL phosphate-buffered saline (PBS), and centrifuged again (150 g; 4°C). After resuspension in medium (RPMI 1640), the cell count was made using a Coulter counter.

For histological examination, the exposed lungs were fixed via the cannula with 5 mL of a cold (4°C) solution of 8% paraformaldehyde in 0.1 M phosphate buffer. The trachea was clamped to prevent leakage of the fixing solution. The lungs were then postfixed at 4°C for 4 hr. At the end of this period, the fixing solution was removed from the lung and the number of AM in the solution determined with a Coulter counter.

For histological examination of the lung, two tissue samples were taken from the left lobe and processed by the method of Lojda (9) for displaying enzyme activity with paraffin embedding. The nonspecific esterase content was then determined on 9-μm-thick paraffin sections by the method of Davies and Ornstein (10). AM have a high content of nonspecific esterases (11) and are therefore preferentially displayed by histochemical staining. Hence they are easier to record during morphometric tissue analysis.

Histological preparations were evaluated under a light microscope at a magnification of 200×. Two sections of the left pulmonary lobe were counted per lung; 32 fields per section were selected randomly from the entire section area. The individual fields were 550 × 425 μm in size, so for each lung the macrophage count was recorded over an area of 15 mm². In a preliminary investigation we found no significant differences between the left and right lobes of the lung. On the basis of the section thickness (9 μm), and taking shrinkage from fixation into account, an alveolar volume of 0.1348 mm³ was calculated for the measured section area. Determination of the total inner volume per lung gave an average lung volume of 1.73 cm³. A factor (×2845) was calculated from the lung volume and the alveolar area of the section, so the total number of AM in the lung could be estimated. For more precise evaluation of AM numbers in the

lung, the number of AM found in the fixing solution was added to this figure.

Unless otherwise noted, the results are given as mean values of the absolute values. Statistical differences were calculated via the t-test (BMDP Statistical Software, Los Angeles, CA).

Results

After lavaging six times with PSS, summation of the cell counts from the individual lavages gave, on average, recovery of $9.12 \pm 0.76 \times 10^5$ AM. However, considerable individual variations were revealed, with values ranging from 4.67×10^5 to 14.3×10^5 cells per BAL.

It is evident that the decrease in cell count is not exponential over the lavage sequence, but takes the form of an S curve. Thus the first and second lavages contain virtually the same number of AM, although in some animals it is possible for the cells recovered in the second lavage to be greater than those in the first. A significant decrease in cell count, which can be confirmed statistically, occurs between the third and fourth lavages. Based on the total cell recovery, approximately 60–70% of recoverable AM are obtained from lavages 1–3 (Fig. 1).

Analysis of the recovered fixing solution for its AM content showed that it contained a significant number of AM (on average 4.86×10^5 cells). There was also considerable scatter of the individual values (between 7.8 and 1.65×10^5 cells).

Evaluation of histological preparations from normal non-lavaged lungs and calculation of the AM count gave an average value of 10×10^6 cells per lung, including AM in the fixing solution. In comparison, an average of 10.7×10^6 AM per lung was obtained for the lavaged lungs (Fig. 2). The numbers of AM remaining in the lungs showed a range of between 7.8×10^6 and 13.5×10^6. Comparison of the AM count of nonlavaged lungs with the recovery of AM in six lavages shows that only about 14% of the AM population is recovered by the lavage.

The extent to which the AM content of the lavage fluid can be modified by altering simple physiological parameters, e.g., altering AM adhesion by adding protein (FCS), or introducing substances that act on the AM membrane (lidocaine), was tested. Lidocaine (12 mM), FCS (10%), or lidocaine (12 mM) and FCS (10%) were added to the PSS used for BAL.

In summary, it can be shown that the total number of AM recovered in BAL was not increased by any of the additives, but remained at or below values obtained by lavage with PSS. It is striking, however, that with addition of FCS, the cell count of the individual BAL sequences differed in comparison to the values obtained by lavage with PSS. Although there was no significant difference between cell counts of the individual BAL sequences with PSS and those of the lidocaine solution, the addition of FCS led to a significant increase in the cell count in the first lavage. Thus, with the addition of 10% FCS, approximately 40% of the recoverable AM were obtained in the first sequence of the BAL. Only 10–15% of the total AM recovered were found in each of the subsequent lavages. A similar exponential decrease in cell count, although not as pronounced, was observed during lavage with 12 mM lidocaine and 10% FCS. Although the recovery of AM here, approximately 27%, was below the value for lavage with added FCS, it was still significantly above the control values and the values for lavage with added lidocaine (Fig. 3).
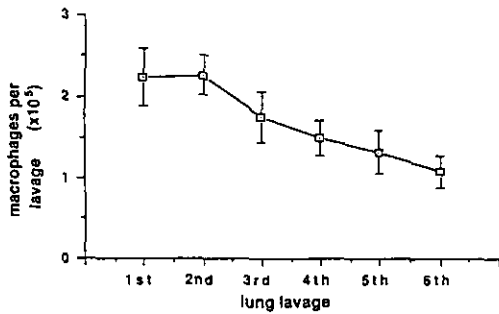
FIGURE 1. Number of alveolar macrophages per lavage of six subsequent lavages with physiological saline solution (PSS). Mean of 20 animals.
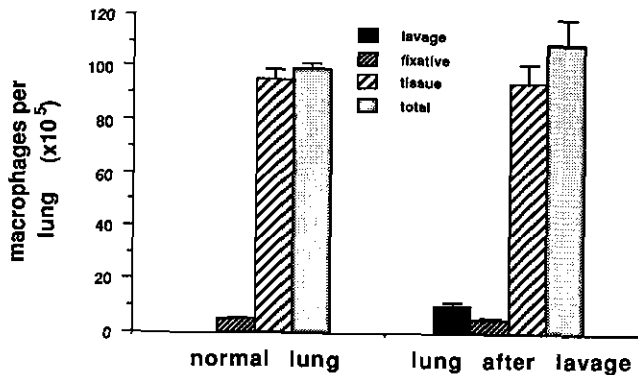

FIGURE 2. Number of alveolar macrophages in the lavage solution, fixing solution, in the lung tissue section, and the resulting total number of alveolar macrophages per lung (sum of the individual values) in nonlavaged lungs and lungs subsequently lavaged six times. Mean of six animals in each case.
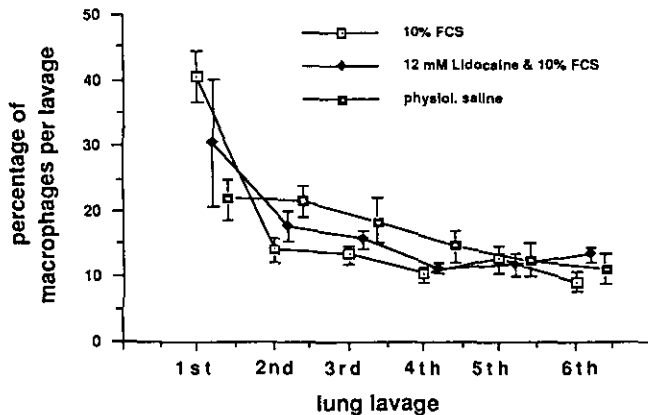

FIGURE 3. Percentage of alveolar macrophages recovered in each lavage based on total number of alveolar macrophages obtained by six washings. Comparison of lavage with physiological saline solution (PSS), PSS with fetal calf serum (10%), and PSS with lidocaine (12 mM) plus fetal calf serum (10%). Mean of six animals in each case.

Morphometric determination of the count in lungs lavaged with FCS gave an average AM count of 9.2×10^6 remaining in the lung, a value comparable to the PSS controls. Likewise, only a small proportion (13%) of the available AM were recovered by lavage.

The subsequent experiment was designed to investigate the extent to which phagocytic activity of AM influences their recoverability by BAL. To stimulate phagocytosis, the first lavage

of the sequence was performed with zymosan added to the PSS, and the lavage fluid was left in the lung for 15 min to prolong its action (for details see "Material and Methods").

It was noted that the prolonged duration of the first lavage sequence in the lung had a significant effect on the number of AM recovered by BAL. In comparison to the "normal" controls, the number of AM found in the first lavage was increased by a factor of four. This number was again doubled by the addition of zymosan (Fig. 4). In the second to sixth lavages of the sequence (PSS; short duration of action), the values of the two groups did not differ. However, they were significantly greater than the values of the normal control group (Fig. 4). The AM count was, on average, approximately 32×10^5 in the zymosan group and 22×10^5 in the control group, and this difference was attributable to the value of the first lavage. Both values were considerably greater than that obtained with normal controls (about 9×10^5 AM). With regard to the recovery pattern over the six lavages, it can be seen that, in contrast to lavage supplemented with FCS, the AM count decreased exponentially over the sequence. In the zymosan group, the jump in the AM count from the first to the second lavage was distinctly more pronounced than in the control group (Fig. 4).

Morphometric evaluation of the histological lung preparations gave values for the number of AM remaining in the tissue of approximately 8.1×10^6 for the zymosan group and 8.4×10^6 in the zymosan control group. These values were significantly below those of the normal controls (9.4×10^6 AM in the lavaged lung). A significantly higher percentage of AM was obtained by lavage in both the zymosan control group (26%) and the zymosan group(35%) than in the normal controls (14%) (Fig. 5). The slight differences in the total number of AM between the zymosan-exposed rats (11.3×10^6), the zymosan control group (10.2×10^6), and the normal controls (10.3×10^6) were statistically not significant.

To investigate the influence of internal factors, i.e., pathogenic processes in the alveoli on the recoverability of AM, rats were lavaged 2 and 8 weeks after intratracheal instillation of 5 mg quartz (DQ12) and 8 weeks after intratracheal instillation of 50 mg silicon carbide. In contrast to fibrogenic quartz, we assumed
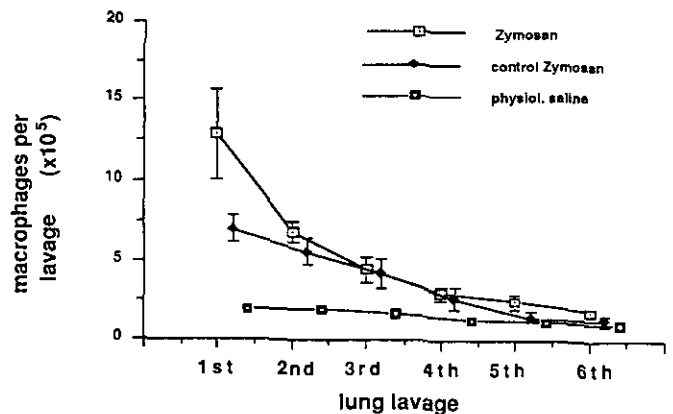

FIGURE 4. Number of alveolar macrophages per lavage by six washings with physiological saline solution (PSS) (control zymosan) or with the addition of zymosan. The lavage liquid in each case was left in the lung for 15 min during the first lavage. For comparison, macrophage count for lavage with PSS without prolonged duration is shown. Mean of six animals in each case.
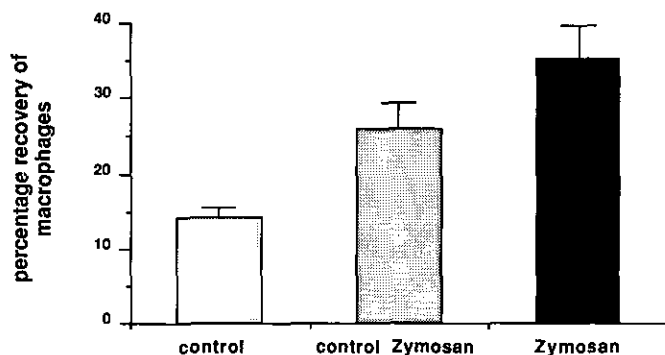
FIGURE 5. Percentage recovery of alveolar macrophages based on the total cell count per lung. Comparison of values obtained by lavage with physiological saline solution (PSS) (control), PSS with 15 min duration of first lavage (zymosan control) and addition of 2% zymosan (zymosan). Mean of six animals in each case.

that silicon carbide, an inert dust, induces no substantial pathogenic changes in the lung.

On lavaging lungs exposed to silicon carbide at 8 weeks after administration, no significant differences were found in the number of lavaged AM in comparison to untreated animals. After exposure to quartz, however, there was a marked increase in the AM count. At 2 weeks after exposure, the number of AM was on average significantly higher (14×10^5) than in untreated animals (9×10^5). At 8 weeks after administration, the AM count rose further, and on average reached a value of approximately 48×10^5 cells, which was five times greater than the control value (Fig. 6). The course of values over the six individual lavages did not differ in either the animals exposed to silicon carbide or those exposed to quartz, as compared to the untreated controls, i.e., the decrease in cell count followed an S curve.

In the silicon carbide group, the number of AM in the tissue was slightly increased in comparison to the controls, although the difference was not statistically significant. In contrast, the lavaged lungs of the quartz group contained significantly more AM. Interestingly, the value 2 weeks after quartz exposure was significantly greater than the value at 8 weeks. Calculation of the AM population remaining in the lung for the silicon carbide group revealed that it was not significantly altered compared to untreated animals. In contrast, stimulation with quartz increased the AM population by approximately 50% at 2 weeks after exposure and approximately 80% at 8 weeks after exposure. It is striking that the AM count in the lavage did not reflect the increase in the AM count in the lungs (Fig. 6).

This observation is explained by comparing the relevant percentages of the recoverable macrophages to one another. As can be seen from Figure 7, a value of approximately 14% was obtained for the untreated animals. A similar value was found for the animals exposed to silicon carbide. In contrast, the value for animals exposed to quartz is low at 2 weeks after exposure (approximately 12%), whereas at 8 weeks after exposure the value is more than doubled (approximately 28%).

Discussion

An average AM count of 10×10^6 rats per lung was determined for female Wistar rats (190–260 g) by morphometric evaluation
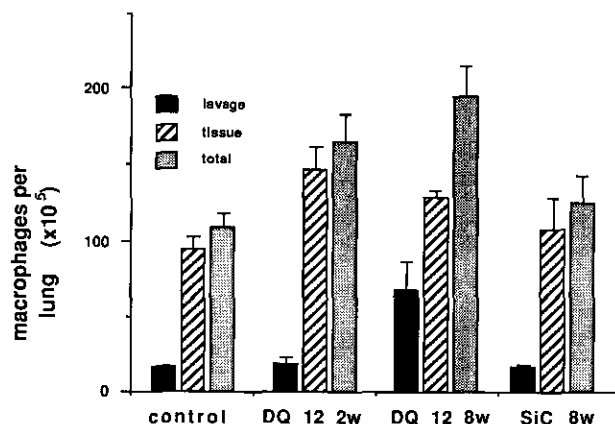

FIGURE 6. Number of alveolar macrophages in the lavage and tissue and the total number of alveolar macrophages calculated per lung (sum of individual values plus the value from the fixing solution) in lungs lavaged six times with physiological saline solution (PSS). Comparison between untreated rats and rats after exposure to silicon carbide (8 weeks after intratracheal injection) or quartz (2 and 8 weeks after intratracheal injection). Mean of six animals in each case.
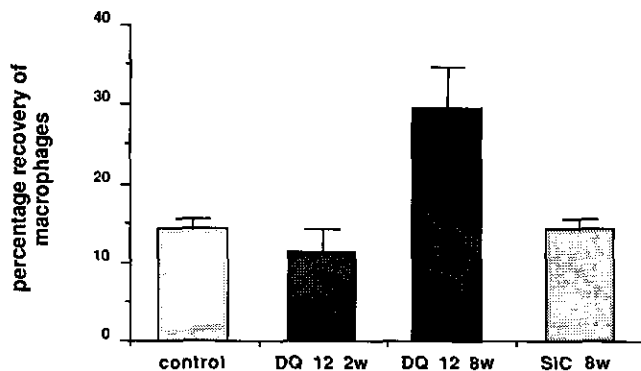

FIGURE 7. Percentage recovery of alveolar macrophages, based on the total cell count per lung. Comparison between untreated rats and rats after exposure to silicon carbide (8 weeks after intratracheal injection) or quartz (2 and 8 weeks after intratracheal injection).

of histological lung preparations. The total number of AM was calculated for nonlavaged lungs and also for lavaged lungs. As the values obtained were in relatively good agreement, it can be assumed that the method for quantitative evaluation of the histological preparations was valid and can by used for future investigations. However, it is essential for the calculations that the number of AM detached by the fixing solution be taken into account.

Morgan et al. (12) determined the size of the AM population in the rat lung via radioactive labeling and arrived at a figure of 20×10^6. However, rats weighing about 400 g were used in these studies, which possibly accounts for the difference. In better agreement are values obtained by Lehnert et al. (13) for Fischer rats (250–300 g). Using a dispersion model of the lung, they calculated that the AM population contained approximately 13×10^6 cells.

Dethloff et al. (14) also attempted to calculate the AM count via a flushing model and arrived at a value of 13×10^6 macrophages for rats weighing approximately 250 g. This estimated value is also in agreement with our value. In the model used by Dethloff et al. (14), an exponential decrease in the AM count over

the sequence of individual washes was assumed. With a comparable lavage modality, no exponential decrease in the AM count was found in the lavages performed here, but in contrast there was a tendency for the AM count to increase in the second or third washing. This finding agrees with studies by Brain and Frank (7), who argue that endogenous Ca^{2+} and Mg^{2+} cations must first be washed out to reduce the AM adhesion. In the results presented here, approximately 14% of the AM could be flushed out with lavages of PSS, and a maximum of approximately 35% of AM could be flushed out with the addition of zymosan to the lavage fluid.

In the lavages performed here, the cationic anesthetic lidocaine, fetal calf serum, or both substances were added to the lavage solution (PSS) as in previous studies by Rabinovitch and Destefano (15), Holt (16), and Miller and Foster (8). Lidocaine reduces the adhesion of the AM in the alveoli via membrane-related effects (8). This can be modulated further by adding fetal calf serum. Such an occurrence was confirmed in the present studies. The addition of lidocaine or fetal calf serum altered the AM recovery significantly, so approximately 40% of the recoverable AM were obtained in the first lavage. Nevertheless, no increase in the total number of AM in the lavage could be achieved. In regard to this finding, however, it should be noted that in our investigations the lavage fluid was cooled to 4°C, whereas in comparable studies, lavage was performed at 37°C. Clearly, there is a temperature dependence of the detachment processes of AM. Similar effects have also been described by Brain and Frank (7), who argued that at lavage temperatures in the physiological range the motility of AM is increased. It is probable therefore that AM are motile in a temperature range where few divalent cations are present, which would reduce binding of the AM to surfactant via its own negatively charged surface.

The addition of zymosan (powdered yeast cells) to the lavage medium showed that the functional states of AM must affect their recovery. In these lavages, the cell count in the total lavage and the cell count in the first sequence were increased significantly. Zymosan is used in cell biology to stimulate phagocytosis. In these studies, it must be assumed that the AM were stimulated to phagocytize by the additon of zymosan and were thereby activated in terms of their motility. Thus, adhesion in the alveoli was reduced considereably, and AM recovery significantly increased. These findings clearly suggest an influence of the functional status of AM on their recovery.

Confirmation of this assumption is found from an investigation of animals exposed to dust. Whereas in the above findings, changes in AM activity were induced externally via the lavage fluid, in this case an altered state of activation of the AM was due to the exposure and the resulting pathogenic reaction in the lung. It was demonstrated that the AM population was not significantly altered by the inert dust silicon carbide with respect to either its size or its recovery. In contrast, cytotoxic and fibrogenic quartz leads to stimulation of AM, changes in the alveolar micromilieu, and, in particular, changes to the surfactant-regulating system (4,11,14,17-22). In agreement with the findings of other workers, our results also showed a significant increase in the AM count in the lavage. This suggests that the number of AM in the lung was increased by the pathogenic processes induced by quartz and therefore more AM could be recovered by BAL. Calculation of the AM population via quantitative morphometric tissue analysis of the lung demonstrated, however, that not only was the number of AM increased, but that their recovery was also significantly

increased in comparison with normal AM. This finding supports the above claim that certain activation states lead to a reduced adhesion of AM to the alveolar surface, thereby increasing their accessibility to lavage.

In addition, these results demonstrate that a direct correlation between the number of lavaged AM and the total AM population in the lung cannot be assumed, as has often been done in the existing literature. It is essential to take into account the instantaneous state of the cells with respect to the effect on their adhesion to obtain valid results.

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