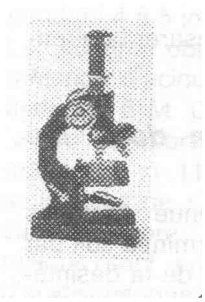

ANALYSE D'IMAGES DE MACROPHAGES ALVEOLAIRES (PAM) PAR CYTOMETRIE CHEZ DES PATIENTS SOUFFRANT DE PATHOLOGIES PULMONAIRES INTERSTITIELLES

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CHARACTERIZATION OF HUMAN PULMONARY ALVEOLAR MACROPHAGES IN INTERSTITIAL LUNG DISEASES, USING IMAGE ANALYSIS CYTOMETRY

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RESUME

Une cytométrie par analyse d'images des macrophages alvéolaires (PAM) a été réalisée sur des frottis de cellules recueillies par lavage broncho-alvéolaire (BAL) chez des volontaires (n = 6) et chez des patients souffrant de pathologies pulmonaires interstitielles (n = 24). La surface des cellules (SC), le facteur de forme (FF) et le rapport nucléo-cytoplasmique (RNP) ont été mesurés sur un échantillon moyen de 300 cellules choisies au hasard par patient. A partir de critères morphométriques multi variés utilisant ces paramètres, 6 sous-populations de PAM ont été définis et leur distribution a été étudiée dans l'échantillon de chaque patient.

Les résultats ont montré des différences dans le profil cytologique des PAM selon l'état physiopathologique du patient. En particulier, la répartition des monocytes-macrophages et celle des grands PAM ont été significativement différents ($p < .001$) entre les groupes physiopathologiques. En revanche, le profil des sous populations macrophagiques a été similaire dans la plupart des patients au sein du même groupe pathologique.

Ces résultats suggèrent l'existence d'une relation significative entre la répartition des sous-types de PAM et l'état de l'interstitium pulmonaire. Une fréquence relative des types de monocytes-macrophages 1 + 2) atteignant 0,36 de cellules récupérées par BAL semble être de grande valeur prédictive de la sarcoïdose (> 90%). Ainsi, l'automatisation de la procédure peut devenir un outil utile dans le diagnostic et le suivi dans de nombreuses maladies du poumon.

Mots clés : Sous-types de Macrophage Alvéolaires / Cytométrie par Analyse d'Images / Sarcoïdose / sclérodermie / SIDA

SUMMARY

Image analysis cytometry of Pulmonary Alveolar Macrophages (PAM) was performed on smears of cells recovered by bronchoalveolar lavage (BAL) from volunteers (n = 6) and patients suffering lung interstitial derangements (n = 24). Cell area, form factor and nuclear to cytoplasmic ratio were measured on an average sample of 300 randomly chosen cells per patient. From morphometric multivariate criteria using these parameters, 6 subpopulations of PAM were defined and their distribution was studied in the sample from each patient.

Results showed differences in PAM cytology profile according to patient physiopathologic state. Particularly, the distribution of monocyte-macrophages and large PAM were significantly different ($p < .001$) between physiopathological groups. By contrast, the PAM subsets profile was similar in most of patients within the same pathology group.

These findings suggest the existence of a valuable relation between the distribution of PAM subtypes and the state of the lung interstitium. A relative frequency of monocyte-macrophage types achieving 0.36 of cells recovered by BAL appeared to be of high predictive value of sarcoïdosis (> 90%). Thus, as automated, the procedure can become a useful tool in diagnosis and survey in lung diseases.

Key words : Pulmonary Alveolar Macrophage subtypes/Image Analysis Cytometry/ Sarcoïdosis/ Scleroderma/ AIDS

INTRODUCTION

Identification and quantitative study of cell subpopulations remain the basis of bronchoalveolar lavage fluid (BAL) cytological examination. Assessed by total and differential cell counts, these samples provide a valuable means of evaluating inflammatory and immune processes of the human lung [1, 3, 9, 30, 31, 43]. BAL constituents have been shown to reflect inflammatory and immune processes occurring in the alveolar structures [18, 24, 38]. Thus, BAL cell characterization is the current method for classification of alveolitis, whose evaluation is of major interest during interstitial and infectious lung disorders [12, 26, 31, 35]. In this purpose, lymphocytosis and polymorphonuclear levels are the most investigated criteria, and the existence of correlations were established between lymphocytosis, lymphocyte subsets, polymorphonuclear levels and lung diseases i. e. sarcoïdosis, idiopathic pulmonary fibrosis (IPF), tuberculosis or connective systemic disorders [8, 12, 14, 16, 22, 23, 28, 34, 42]. Pulmonary alveolar macrophages (PAM) represent an average of 90% of cells recovered by BAL [20, 28, 38, 40]. Furthermore, they have been found to play a central role during interstitial lung diseases [4, 25, 27, 37]. However, changes in PAM morphology in these circumstances remain poorly investigated.

Recent data suggest that morphological characteristics of PAM might reflect their functional state. Indeed the existence of correlations between PAM morphological characteristics and lung state was recently proposed in human. First studies were performed mainly on histological specimens [10, 19, 20]. Then, authors described morphological aspects of PAM recovered in BAL during some interstitial lung derangements [6, 22, 39, 41]. In our experience of evaluating alveolitis, including assessment of PAM morphology in addition to conventional cell count and differential count, we observed differences in PAM types present in samples obtained from patients with different lung disorders and similarities in samples from patients suffering from the same disorders. Since there may be questions of objectivity and reliability of such observations based on direct eye assessment on microscopical preparation [2, 5, 32, 33, 36], we undertook quantitative evaluation of morphometrical characteristics of PAM in BAL from patients with different lung pathologies by using image analysis cytometry. Our aim was first to test the existence of differences in distribution of PAM types in these circumstances. In addition, we attempted to determine if morphometric multivariate criteria applied to PAM subsets recovered by BAL might be of high predictive value in diagnosis and/or survey in lung diseases.

MATERIALS AND METHODS

Study population

Thirty (30) bronchoalveolar lavage fluids (BAL), rated satisfactory according to criteria previously described [1, 11, 30, 43], were examined by image analysis cytometry. The samples were taken from patients of the same hospital, mainly the department of pneumology unit. The study population was divided into 4 groups: volunteers (A = 6) including two smokers; patients with sarcoïdosis (B = 9) with lung involvement in different stages of the disease course; patients with scleroderma (C = 8), with or without clinical or radiological evidence of lung involvement; immunocompromised patients (D = 7) investigated for infectious pneumonitis research.

These patients consisted of 15 males and 15 females, with a mean age of 38.8 (23-75). More detailed information concerning the subjects and the physiopathologic groups is summarized in table 1.

Table 1: Distribution of patients according to their physiopathological state.

State	Size (Male-Female)	Mean Age (range)	Clinical informations
Volunteers (A)	**6** (5-1)	**29.6** (24-38)	-1 of them had an abnormal cell differential count and might not represent a healthy volunteer.*
Sarcoidosis ** (B)	**9** (4-5)	**40** (26-50)	-5 in stage I or not specified, 3 in stage II and 1 in an outbreak evolutive state.
Scleroderma (C)	**8** (1-7)	**45.6** (23-62)	-3 of them had clinical and radiological evidence of lung involvement.
Immuno-compromised (D)	**7** (5-2)	**37.6** (27-75)	-5 of them were positive for VIH; 1 was a Hodgkin lymphoma patient being treated by chemotherapy; the last one was at the beginning of investigations for probable AIDS. -All the patients presented clinical symptoms of pneumonitis.
Total	**30** (15-15)	**38.8** (23-75)	

* This patient's BAL cell differential count was: **Lymphocyte** 12%, **Polymorphonuclear** 7%, **Macrophage** 81%
** In the sarcoidosis group, all differential counts except 1 (12%) revealed a lymphocyte level > 25%.
The last one presented lung interstitial abnormality revealed by X-ray at the time of BAL recovery.

Bronchoalveolar and cell processing

The BAL procedure was similar for all cases, as recommended elsewhere [7, 21, 29]. The lavaged area was the lingual. The mean fluid recovered was 85 ml. pooled fluid was transported in siliconized sterile glass flask to the laboratory within 30 minutes of recovery.

Before any filtration or centrifugation, a total cell count was performed in a hemocytometer, coupled with a viability test (trypan blue 1%). Lavage fluid was then divided in two conic tubes and centrifuged at 250g for 10mn. Monolayer smears were prepared manually from the cell pellets. The smears were air dried and stained by the May-Grunwald Giemsa method (MGG) and then submitted to microscopical image analysis.

Microscopical image analysis of PAM

Analysis was performed by using a microscope-linked, PC-based image analyser SAMBA TM 2005 (Alcatel, TITN Meylan, France). Each smear was analysed with a 40 X objective (X 400) by a stereotyped scanning method sufficient for accurate sampling in all slide areas. An average sample of 300 randomly chosen PAM was analysed for each smear. The image of each microscopical field was taken by a CCD camera. The video image was digitalized with 256 grey-value resolution in an image format of 512 X 512 pixel, and sent to a PC monitor. From a preliminary study, from each microscopical field, an automated selection of PAM, based on criteria of cell size was performed. Nevertheless, the decision to validate a cell as PAM was made by the operator (cytologist) who could observe the cell in the selected microscopical field before making the decision. For each selected cell, different parameters were measured: cell area (CA); cell perimeter (P); cell form factor (FF = $P^2/4\pi CA$); and the nuclear area (NA). The nuclear to cytoplasmic ration (NCR) was computed secondarily (as NA/CA).

From combinations of the measured parameters and by referring to PAM morphology as described by cytologists, different classes of PAM were defined, according to multivariate criteria (figure 1).

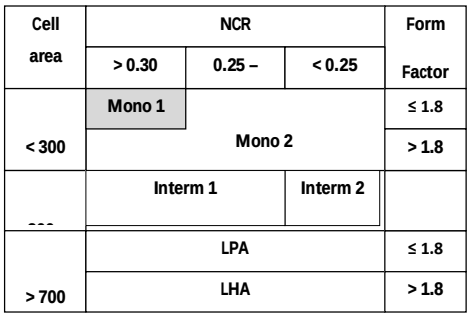

Figure 1: Criteria used for determination PAM subpopulations *
Double stroke indicates that corresponding parameter was not used in the definition of the macrophage subtype.
*** Mono 1** = true monocyte-macrophage; **Mono 2** = monocyte-like macrophage
Interm 1= Intermediate macrophage; **Interm 2** = Intermediate-like macrophage
LPA = Large poorly active macrophage; **LHA** = Large highly active macrophage.

One BAL was used for validation of the procedure. For' this purpose, the BAL fluid was divided in 3 aliquots which underwent successive centrifugations in the same conditions. During management of one aliquot, the others are placed at + 4°C to prevent cell dying. From the cell pellet of each aliquot, two smears were prepared, air dried, and then stained by MGG. The resulting 6 smears were subjected to microscopical image analysis. Furthermore, to assess the repeatability of the measurements, three cells (a small PAM, a medium sized PAM and a large PAM were analysed 30 times. The procedure of data acquisition was newly initiated for each of the 30 measurements.

Data analysis
For the statistical analysis the data in all groups were tested for normality.
In descriptive statistics, data are expressed as mean $\pm$ SEM, or frequency distributions of classes of PAM within the groups.
Comparative analyses of the means of parameters within and between groups were carried out by variance analysis (ANOVA), using PLSD of Fischer and Dunn's tests.
Comparative analysis of the distribution of cell types within a group and between groups was carried out by using a contingency table (Chi^2 test).

RESULTS

Validation of morphometric analysis of PAM
To assess the reproducibility of measurements, 6 smears obtained from the same BAL were compared. Cell area, form factor and nuclear to cytoplasmic ratio were estimated for samples of PAM present in these smears (Table 2).

Table 2: Validation of the procedure of PAM image analysis cytometry: between smears variation.*
6 smears were performed from the same BAL. Morphological parameters were assessed by image analysis on PAM in the different smears.

Slide Number	Cell sample size	Cell area (μm^2) (mean ± SEM)	Form Factor (mean ± SEM)	NCR (mean ± SEM)
1	325	**266** (± 7)	**1.67** (± 0.04)	**0.368** (± 0.007)
2	223	**257** (± 7)	**1.58** (± 0.04)	**0.368** (± 0.007)
3	274	**272** (± 9)	**1.67** (± 0.04)	**0.369** (± 0.006)
4	262	**262** (± 8)	**1.65** (± 0.04)	**0.370** (± 0.006)
5	227	**254** (± 8)	**1.59** (± 0.04)	**0.364** (± 0.007)
6	221	**253** (± 8)	**1.61** (± 0.04)	**0.366** (± 0.007)

* Between smears variation compared by variance analysis revealed not significant ($p > 0.05$) whatever the parameter and/or the compared smears.

Cell perimeter was used only for computing the cell form factor. Variance analysis revealed no differences ($P > 0.05$) among data obtained from the 6 stained smears from the same BAL. repeatability was also assessed on 3 selected cells. Thirty analysis of each cell yielded coefficient of variation of less than 5% for all parameters.

Morphological characteristics of PAM

In table 3 are summarized descriptive results of data from the study population as mean $\pm$ SEM. PAM area ranged between 87 and 2040μm^2. More than 92% of the total PAM sample (9735 cells) were less than 700μm^2 i.e. 30µm diameter. The higher means of cell area were observed in scleroderma and AIDS, while lower mean was observed in sarcoïd patients (45% of PAM from sarcoïd patients measured less than 300μm^2 i.e. 20µm diameter vs only 22% of such cells in scleroderma patients). The mean PAM area was significantly different between all physiopathological groups (ANOVA: $P < 0.0001$).

Cell for factor (FF) ranged between 1.11 (in sarcoïd group) and 16.42 (observed in an immunocompromised patient). Form factor mean was lower in sarcoïd sample and higher in volunteers sample (72% vs 55% with FF $\leq$ 2, sarcoïd vs volunteers respectively). The mean FF of PAM was not significantly different between sarcoïd and immunocompromised patients (ANOVA; $P = 0.24$).

Nuclear to cytoplasmic ratio ranged between 0.011 and 0.87 (both in sarcoïd group). 90% of the total PAM sample had a NCR > 0.20, equally distributed between those $\geq$ 0.33 and those with NCR > 0.20 < 0.33. NCR mean was higher in sarcoïd group and lower in immunocompromised sample. The mean NCR of PAM was different between all physiopathological groups (ANOVA; $P < 0.0001$).

Table 3: Data from PAM measurements in physiopathological groups; as mean ± SEM*

Group	Patients sample size	Cell sample size	Cell area (µm^2) (mean ± SEM)	Form Factor (mean ± SEM)	NCR (mean ± SEM)
Volunteers	6	2011	359 (± 4) **(a)**	2.36 (± 0.03) **(a)**	0.337 (± 0.002) **(a)**
Sarcoidosis	9	2875	348 (± 3) **(b)**	1.98 (± 0.02) **(b)**	0.354 (± 0.002) **(b)**
Scleroderma	8	2639	448 (± 4) **(c)**	2.10 (± 0.02) **(c)**	0.321 (± 0.002) **(c)**
Immuno-compromised	7	2210	464 (± 5) **(d)**	2.02 (± 0.03) **(d)**	0.308 (± 0.002) **(d)**
Total	30	9735	404 (± 2)	2.10 (± 0.01)	0.331 (± 0.001)

*** (a, b, c, d)** indicate significantly different values of the parameter between the four groups, revealed by variance analysis. The use of the same letter for the same parameter in two or more groups means that there is not statistical difference for the parameter values in the corresponding groups, at least by one of the comparison tests of the variance analysis (PLSD test of Fischer and Duncan test).

Subpopulations of PAM from different physiopathological groups

Figure 2 summarizes frequency distributions of the PAM subtypes defined as described previously (Figure 1) in the physiopathological groups. The frequency of the Mono 1 cell type was significantly higher in the sarcoïdosis group than in other groups, including volunteers (Chi2; $P < 0.0001$). All the patients of the sarcoïdosis group exceeded 15% Mono 1 while less than 50% of the samples in the other groups achieved this proportion.

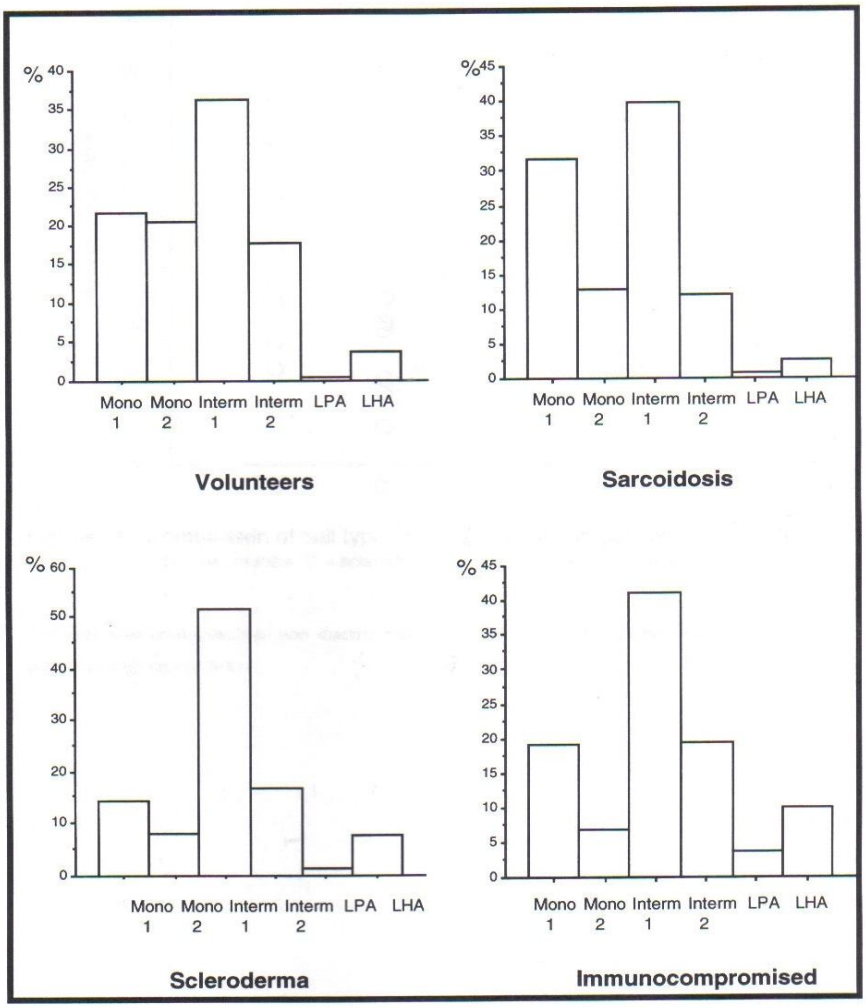

Figure 2: Distribution of multivariate PAM subtypes in physiopathological groups

The subtypes of PAM were defined according to the criteria described Figure 1.

The predictive value of sarcoïdosis in pathological groups achieved 91% when the proportion of monocyte-macrophages (Mono 1 + Mono 2) achieved 36% (Figure 3).

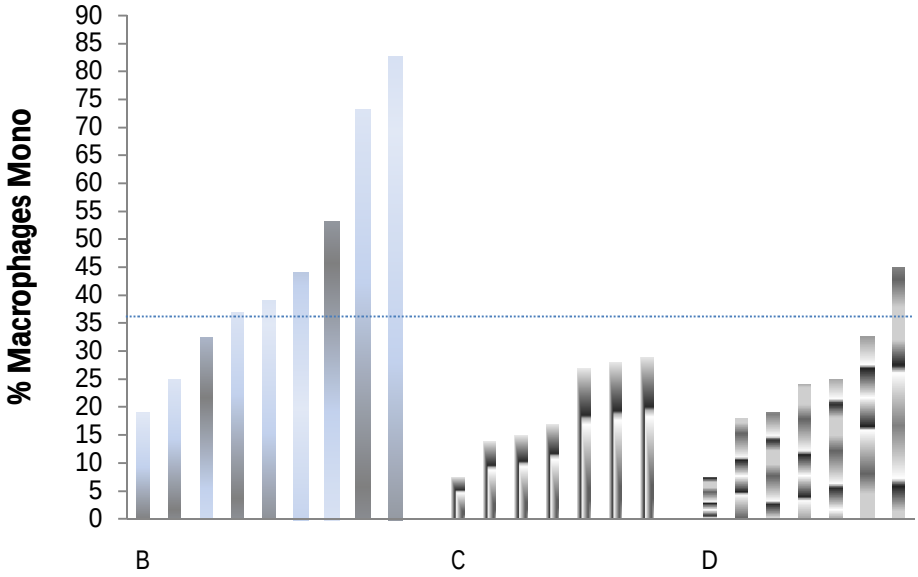

Figure 3: Comparison of cell type Mono distribution in patients:
B = sarcoidosis, **C** = scleroderma, **D** = immunocompromised

The area above the line (36%) corresponds to non discriminant values of Mono subtypes between pathological groups

The intermediate cell types (Interm 1 + Interm 2) were the most predominant PAM in BAL, with an average of 58.8% in the total study sample. The frequency of Interm PAM fluctuated from 56 ± 4% in groups A, B, and D, vs 68% in scleroderma group. The frequency of cell types LPA and LHA was too few in some groups to allow accurate statistical analysis. Consequently, we pooled them as larger macrophages (LM). Even though, their proportion was less than 5% in volunteers and sarcoïd groups, their frequencies reached respectively 9% and 13.6% in scleroderma and immunocompromised groups. The cell type LM was inequally distributed within groups. Individual sample analysis revealed a significant predominance of large macrophages (LM) in four patients. The four patients with a significant predominance of LM correspond also to those in which lung interstitial abnormality detected by X-rays was the reason for BAL. one patient of the volunteers group, with a similar level of LM presented an abnormal cell differential count, evocative of a pulmonary fibrosis (Table 1). The finding suggests that the increase of LM might be related to pulmonary inflammation.

DISCUSSION

Characterization of changes in alveolar macrophage during lung diseases remains the major problem of BAL cytological examination. Despite the numerical predominance and the central role played by these cell in lung physiopathology [4, 25, 27, 37]. PAM cytology remains poorly informative in diagnosis. Reasons for this situation arise mainly from unsuitability of new technology methods i.e. immunology or flow cytometry to investigate PAM [11, 13, 15, 30, 43]. Some useful patterns provided by PAM cytology, result from visual morphology assessment. The subjectivity and failure in reproducibility of the procedure prompted us to investigate PAM characteristics in various lung disorders by using image analysis cytometry.

The validation sample analysis suggests a reliability of the cytometrical method we used, from preparation of the slides to acquisition of data.

PAM morphological characteristics displayed by our series agree with those previously observed or assessed by authors in comparable staining conditions [17, 36, 39]. Studied parameters are those classically used for cell morphological classification by cytologists. By estimating the size, cytologist classifies cells as small, “normal” or large macrophages. Form factor is assumed to

reflect cell activity (i.e., production of pseudopods), traducing margin to the round form where FF = 1. We did not observe a difference between the sarcoïd and the immunocompromised groups as regards the form factor. However, reasons for this similarity are different. In sarcoïdosis, the phenomenon arose from a high number of small PAM (e.g. monocyte-macrophages), in which the FF is low. In the immunocompromised group, levels of this cell type were low (Figure 2); but the group contained the greatest percentage of large, presumably poorly active cells in which the form factor is low. Nuclear to cytoplasmic ratio (NCR) is recognized as a criterion of cell maturation. In the case of PAM, NCR values may decrease from the monocyte state to the mature macrophage stages [22, 36, 38, 41].

Visual assessment of morphology encountered three major problems: 1) values of cell parameter are continuous from the monocyte state to the macrophage stages. 2) Cell morphology assessment and classification require the evaluation of a combination of characteristics. 3) Assessment by eye is inherently subjective. In this model identification of a monocyte-macrophage was based on the following characteristics: 13-20µm diameter, NCR *around 1/3* and a round shape. Multivariate criteria used to determine the cell subtypes followed the same approach. The main difference from other approaches is that the threshold determination for each parameter is not linked to subjective opinion. In this purpose Mono 1 cell type may correspond to a true monocyte-macrophage. Mono 2 which has some of these characteristics, but not all may be recognized or not as this cell type, depending on the cytologist. The observation of higher rates of monocyte-macrophage in sarcoïdosis is in accordance with results from morphology assessment by other authors [6, 12, 15, 22, 41]. The high predictive value provided by monocyte-macrophages level (Figure 3) can be interesting in diagnosis and survey during lung sarcoïdosis. It is remarkable that intermediate cell types are homogeneously distributed in BAL whatever the physiopathological condition (50-70% in 26 of the 30 BAL).

The constancy of intermediate cell types and the differences observed in frequency of larger and smaller cells depending on the physiopathologic state suggest that the morphometric features on PAM may express the dynamic of subpopulations related to lung state.

Most prior studies in BAL utilizing computerized methods, mainly flow cytometry, focused on lymphocytes, as these methods appear unsuitable for PAM analysis [36, 43]. The image analysis method developed in this study allowed the identification of PAM subsets, determined by multivariate criteria from continuous data reproducibly measured. The analysis of the distribution of these subsets yielded valuable information about PAM changes during different lung diseases. As objective, reproducible, informative and automatable, image analysis cytometry of PAM may offer a new perspective in BAL cytological examination.

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