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Flow cytometric characteristics of alveolar lymphocytes (AL) obtained from the control group – proposal of normal value range of AL subsets in nonsmokers

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Abstract

Background: *Immunological and cytological examination of bronchoalveolar lavage (BAL) fluid is frequently used for diagnostics and research of interstitial lung diseases (ILD). Flow cytometry (FC) is a common tool applied to investigations on biology of alveolar lymphocytes (AL). However, there is no consensus on the processing of immunophenotyping of BAL samples. As a consequence, the normal values of AL subsets are not definitely established.*

The aim of the study *was to determine the normal phenotype values of AL, using precise inclusion criteria in a relatively large cohort of healthy individuals. The data generated in this way could serve as a reference point for investigations and diagnostics of lower airways.*

Material and methods: *AL were obtained from BAL performed in 41 individuals free of any lung pathology, incl. 27 nonsmokers, NS, and 14 smokers, S. Direct double- and three-colour immunophenotyping was used for analysis of AL subsets, as well as for the markers of their activation and apoptosis susceptibility. Precise criteria of flow cytometry acquisition and analysis were employed. Mean fluorescence intensity (MFI) was determined for major AL subsets. The parallel typing of peripheral blood lymphocytes (PBL) was carried out.*

Results: *Both in NS and S, alveolar lymphocytes were almost exclusively T cells (90.1±1.4% and 89.3±1.6% respectively, median ± SEM). B and NK cells in BAL fluid occurred infrequently (2.7±0.4 and 4.2±0.8% in NS, 2.6±0.9 and 3.6±0.9% in S, respectively), as compared to peripheral lymphocytes (10.1±1.2% and 9.3±1.7% in NS, 17.9±3.4% and 14.0±1.9% in S, respectively, $p<0.0001$ for both). The AL were mostly CD45R0+ and CD95+ cells, regardless of analyzed subset (CD4 or CD8) and smoking status. Low BAL CD4/CD8 ratio in smokers, due to increased percentage of CD8+lymphocytes presenting T cytotoxic cell phenotype (CD8+CD11b–) was found (39.9±4.4% vs. 21.8±2.7% in NS, $p<0.01$). Only some AL CD8+ expressed suppressor cell phenotype (CD8+CD11b+), in opposite to the results obtained from peripheral blood. BAL fluid recovery (high value reflects increased content of alveolar cells vs. bronchial contamination) in NS (but not in S) was positively correlated with percentage of AL CD4+ (R Spearman +0.46, $p=0.04$) and CD4/CD8 ratio (R Spearman +0.45, $p=0.02$). MFI of CD3 and CD4 superficial antigens was significantly decreased on AL as compared to PBL ($p<0.001$ and $p<0.01$ resp.).*

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Conclusions: *The normal value range was proposed for BAL lymphocyte immunophenotyping. The results support the hypothesis that T cells as a major subset of AL, they are chronically stimulated in lungs by specific antigens and carry the phenotype of primed memory/effector cells. Helper T cells are the lymphocytes that accumulate preferentially in alveoli in physiological conditions.*

Key words: *alveolar lymphocytes, bronchoalveolar lavage, flow cytometry, lymphocyte subsets, mean fluorescence intensity*

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Introduction

In the past ten years, the authors used flow cytometry (FC) in order to test more than 500 BAL materials originating from patients suspected of interstitial lung diseases (ILD). BAL, including alveolar lymphocyte (AL) typing, is considered as an important diagnostic tool in ILD patients, as in sarcoidosis, hypersensitivity pneumonitis, transplant recepients and many others [1-6]. Moreover, the investigations on pathogenesis of ILD, as well as of bronchial asthma, lead to conclusions emphasizing the principal role of lower airways lymphocytes in lung pathology. In the studies concerning the subject, lymphocyte phenotype and function were largely discussed [7-10].

However, in the papers published by other authors we encountered the problem of reference values for AL phenotypes. The difficulties could be summarized as follows:

1. The technical problems related to FC analysis of alveolar lymphocytes involve:
   a) AL are different in shape as compared to PBL, so special gating procedures are discussed [11, 12];
   b) AL flow cytometric gate is commonly contaminated by large red cells and monocytic forms of alveolar macrophages (AM) [13];
   c) antigen sharing between AL and AM occurs [11];
   d) poor lymphocytic materials (as for example in healthy smokers) are difficult to be analyzed; a double-colour FC analysis is often insufficient then [12, 14];
   e) results calculated from BAL material (in this number cytology and cell phenotype data) present nonparametric distribution of variables and a wide range of values [15].
2. The control groups collected for different research purposes are not numerous enough, if to take into account the technical complications listed in the point 1. For example, the nonparametric distribution of data could be avoided in part by collecting the numerous group of healthy individuals, but this condition is difficult to accomplish [2, 11, 14, 16, 17].
3. Any BAL used for ILD diagnostics needs a reference point, i.e. especially numerous and carefully yielded control group of AL collected in the respective center [2]. There is a remarkable necessity to elaborate basic principles of BAL material sampling and AL subset analysis, parallel to PBL processing. Additionally, the absence of uniformity in lymphocyte typing of BAL fluid has made comparisons between various reports difficult or even impossible [18].
4. Controls should be collected separately for nonsmoker and smoker group, due to the distinct cytoimmunological pattern observed in the latter. These fundamental conditions are not always exactly fulfilled [19-21]. It should be remember, that in healthy nonsmokers, lymphocytes constitute about 10% (or even more) of reactive cells obtained from lungs by BAL procedure. The respective value in smokers is about 5%; the majority of rest alveolar cells are macrophages [22]. This cytological pattern seems to be physiological.
5. Up to now, the role of alveolar lymphocytes in the local immunity has not yet been fully understood. Some investigators consider that AL simply reflect the inflammatory process in the surrounding interstitium [19, 23]. Some others, however, accentuate the biased (i.e. preferential) accumulation of T lymphocytes on the surface of lower airways [24]. These differences implicate the way, that the authors interpret their results.
6. There is no consensus on some other important phenotypic and functional features of alveolar lymphocytes, including so basic parameters as proportions of T, B and NK cells. It is also unclear, if apoptosis occurs frequently in AL population and if AL proliferate in the alveoli. The studies reporting the lymphocyte migration from lower airways back to the local lymphatic nodes also need confirmation [17, 25-28].

In the current study we presented the particular characteristics of AL phenotypes, examined with use of FC. The material, obtained from the relatively large group of healthy individuals, has been collected by our team for several years. The attention was focused on AL harvested on physiological conditions, i.e. from nonsmoking subjects free of any lung pathology. Additionally, the group of "healthy" smokers was established, as *sine qua non* for all diagnostic and research BAL procedures concerning the smoking patients. The results of nonsmokers and smokers were presented separately. The smoker control group was

not numerous enough to constitute the normal value range of AL phenotypes.

The careful, standardized BAL fluid collection and FC evaluation of AL was proposed as the optimal solution for troubles mentioned above. Furthermore, the BAL fluid recovery was used as an important indicator of pure alveolar space content versus bronchial contamination. The mean fluorescence intensity (MFI) assessment was included. The parameters tested in the study were selected in order to better understand the role of alveolar lymphocytes (and their subsets) in the local immunity.

The purposes of the study were:

1) to propose the normal value range of AL for further diagnostics and research investigations;
2) to characterize the phenotype of normal alveolar lymphocytes obtained by bronchoalveolar lavage (BAL);
3) to assess the relation of AL immunophenotyping results to fluid recovery.

Material and methods

Study population

The studied group consisted of 41 individuals who underwent bronchoscopy for diagnostic reasons and were retrospectively free of any infectious, inflammatory or malignant lung disease. Microbiological analysis of the BAL fluid was negative for bacteria, mycobacteria and fungi. Complete clinical investigation (incl. chest X-rays, lung function tests, DLCO and arterial blood gas analysis) definitely excluded presence of any lung pathology. No subjects were treated with corticosteroids, immunosuppresive therapy or any other drugs known as a potential cause of ILD (nitrofurantoin, amiodaron, PAS, etc.).

The characteristics of the control population are summarized in table 1.

The Bioethical Commission of Collegium Medicum, University of Nicolaus Copernicus, Bydgoszcz, gave the permission (no KB/294/2003) for the study.

Table 1. Nonsmokers and smokers – BAL cytology

Parameter		Nonsmokers n=25	Smokers n=14	p
age*		32.9±12.7 (16-65)	37.1±13.4 (19-63)	ns
sex	M/F	10/15	11/3	ns
smoking history*	no of pack-years**	-	16.1±13.3 (2-42)	
BAL fluid recovery	%	60±3 (36-75)	54±3 (34-70)	ns
total BAL cell no	cells x 10^3/ ml	197±30 (55-310)	495±55 (187-780)	<0.01
alveolar macrophages (AM)	%	87.0±1.3 (60.6-96)	92.9±0.9 (86.1-97.1)	<0.01
alveolar lymphocytes (AL)	%	11.8±1.3 (3.0-37.8)	5.6±0.6 (2.5-10.1)	<0.001
neutrophils	%	1.3±0.2 (0-4)	1.4±0.3 (0.3-3.3)	ns
eosinophils	%	0.3±0.06 (0-1.3)	0.3±0.08 (0-0.8)	ns
mast cells	%	0.5±0.1 (0-0.8)	0.7±0.2 (0-1.0)	ns

** mean ± SD; all other data were presented as mean ± SEM (range)*
*** number of packs smoked per day times number of years smoked (data collected from 12 individuals)*
ns – non significant (U Mann-Whitney test)

Bronchoalveolar lavage performance

BAL was carried out, as previously described [10, 16]. In brief, midazolam 2.5-5.0 mg IV, followed by local anesthesia with 2% lidocaine solution was used for premedication. The Olympus Bf 20 bronchofiberoscope was introduced to the middle lobe or to the left lung lingula, alternatively. Lavage with 200 ml of 0.9% NaCl sterile solution (37°C) was carried out by sequential instillation of four 50 ml aliquots of saline. The BAL fluid fractions were retrieved carefully by gentle suction, then pooled and filtered. The fluid recovery was calculated as the percentage of instilled volume [29]. The material was send to the laboratory and cytoimmunological examination was undertaken without delay (as for flow cytometry, average time from BAL collection to the beginning of staining was 45 minutes).

BAL fluid cytology

BAL cell fluid viability determined by trypan blue exclusion test was >90% in all samples. Total cell number was calculated and aliquots of 100-300 ul fluid were cytocentrifuged (Cytospin Shandon 3, 1500 r.p.m./min, 5 min.). Cytospin preparations were stained simultaneously with hematoxylin-eosin (HE) and May-Grünwald-Giemsa (MGG). The differential count of BAL fluid reactive cells was calculated in each case as the average value of both preparations (at least 500 cells were counted) [12]. For mast cells, toluidine blue staining was applied, as previously described [30].

AL subsets phenotyping

BAL material was centrifuged (300 g, 10 min.) and a cell pellet was resuspended with PBS to 2-10 x 10^6 cells per ml. The samples containing 50 ul of cell suspension were incubated with mixture of saturating amounts (5-10 ul usually) of monoclonal antibodies for 30 minutes in dark. Double- or three-colour typing was performed, according to the percentage of AL (more or less than 5% of BAL fluid reactive cells, respectively). Mouse anti-human monoclonal antibodies (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) used in the study, were listed in the table 2. For three-colour immunostaining, anti-CD45 PECy5 (DAKO A/S, Glostrup, Denmark, 5 ul per sample) was given additionally. Negative isotype control was used for each sample series. After incubation, cells were washed with PBS (300 g, 10 min.) and resuspended in 300 uL of PBS containing 1% formaldehyde [12, 21].

Parallel PBL typing was performed due to BD Simultest IMK protocol. In brief, heparinized peripheral blood was

Table 2. Al (alveolar lymphocyte) phenotype staining. Monoclonal antibodies (MoAb) used for direct immunofluorescence

Sample no	FL 1 MoAb FITC Conjugated	FL 2 MoAb PE Conjugated	Notes
1	**CD45***	**/**	BAL lymphocyte gate defining
2	**CD15***	**CD14***	granulocyte/macrophage contamination in AL gate
3	**CD3**	**CD19**	T&B cells
4	**CD4***	**CD8**	CD4/CD8 ratio
5	**CD3**	**HLA-DR***	activated T cells ("late" activation marker)
6	**CD3**	**CD16* + CD56**	T&NK cells
7	**CD25***	**CD4***	activated Th cells ("early" activation marker)
8**	**CD95***	**CD4* (CD8)**	marker of cells (Th/Tc) susceptible to apoptosis (Fas)
9**	**CD178***	**CD4* (CD8)**	marker of cells (Th/Tc) inducing apoptosis (Fas Ligand)
10	**CD45RO***	**CD4* (CD8)**	primed memory cells (Th/Tc)
11	**CD19**	**CD5***	B cell subsets
12	**CD8**	**CD11b***	Tc&Ts cells
13	control antibody (IgG 1)	control antibody (IgG 2a)	negative isotype control

** antigen sharing with alveolar macrophages*
*** n=13*
Mouse anti-human CD45 PECy5 (for three-colour AL analysis) was applied to samples 2-7, 10-13.

incubated with monoclonal antibodies; hemolysis with BD Lysing Solution was performed subsequently. Next steps were carried out according to AL staining procedure.

AL flow cytometry analysis

FC data were acquired within 24 hours after staining, using CellFit software and FACSCalibure cytometer (BD Immunocytometry Systems). An argon ion laser was used for 488 nm excitation. Emitted light was detected by logarithmic amplification through barrier filters specific for the emission range of the different fluorophores: 530/22 nm for FITC (fluorescence channel FL1), 585/42 nm (FL2) for PE and >650 nm (FL3) for PE Cy5. From each sample 8000-12000 events (cells) were acquired.

AL gate was determined due to the cell granularity (SSC, side scatter) and intensity of staining with CD45 FITC ("back gating"). The gated cells were assessed by quadrant analysis of FL1 vs FL2 channel dot plot. Results were presented as the percentage of all gated lymphocytes [31]. Mean fluorescence intensity (MFI) was calculated for CD3, CD4, CD5, CD8 and CD45. Arbitrary units were used, according to the reference [32]. We resigned to assess MFI for B and NK cell markers, because these subsets of AL were sparse or absent.

Phenotype and MFI data were calculated with PC-Lysys software. Criteria for correct FC analysis of AL were listed in the table 2 (they were commented on elsewhere) [12, 14]. Materials, which failed to fulfill these criteria, were not included in the study.

Statistics

All BAL cytology and phenotype results were presented as median ± SEM (due to nonparametric distribution of values, see introduction). The Mann-Whitney U test to compare the obtained data (NS vs S) and paired Student's test to analyze pairs of values (AL vs PBL) were used. The Spearman's rank correlation coefficient r_s was applied to test for correlation between two random variables. P-values less than 0.05 were considered statistically significant.

Results

Results of BAL fluid cytology were shown in the table 1. Significant increase in total cell number and AM percentage, as well as significant decrease in AL percentage was found in smokers, as compared with nonsmoker group.

AL phenotype results were compared to PBL and presented in the figures 1-2 and the table 4. In both, nonsmokers and smokers, AL were dominated by T cells with only few B and NK cells. In each individual case, BAL fluid CD3+ and CD5+ percentage was higher than respective PBL data; percentage of alveolar B cells (CD19+) and NK cells (CD3–CD[16+56]+) was lower (fig. 1).

AL and PBL CD4/CD8 results were presented in the fig. 2: the CD4/CD8 ratio decreased significantly in smokers, as compared to nonsmokers (0.94±0.37 vs. 1.87±0.21, p<0.001). On the contrary, PBL CD4/CD8 ratio was higher in smokers (2.04±0.18 vs. 1.32±0.1, p<0.05).

Table 3. Criteria of FC analysis – alveolar lymphocyte phenotypes

Criterion	Accepted value
epithelial cells in BAL material	<3%*
lymphocytes in BAL material	>10000
lymphocytes in a single sample	>500
total AL percent (CD45/SSC) in lymphocytic gate	>95%
AM contamination in AL gate	<4%
granulocyte contamination in AL gate	<4%
all contamination together (AM, granulocytes, red cells, debris)	<40%
T+B+NK cells	>90%
CD3+ percentages in different samples of the same patient - difference of separate results	<5%

Criteria based on the references for PBL immunotyping, modified for AL subset [12, 14, 31]

** In all BAL materials involved in the study we obtained <1% of epithelial cells (calculated as percentage of all cells)*

BAL fluid obtained from smokers was characterized by significant decrease in CD4+ and CD5+CD19+ cell percentage, as well as elevated proportion of CD8+ and CD8+CD11b– (phenotype of Tc) cells.

The majority of both CD4 and CD8 cells in BAL fluid, in opposite to PBL parallel results, were CD45R0+ (phenotype of primed memory cells) and CD95+, i.e. Fas+ (up to 100% alveolar T cells seem to be susceptible to membrane pathway of apoptosis induction). These data were presented in brief in the table 4, without further distinction to CD4 and CD8 cells. Fas Ligand (CD178) was expressed more frequently in AL than in PBL, and the percentage of CD8+CD178+ cells was increased in BAL fluid of S, as compared with NS (p<0.05).

No differences in expression of activation markers between tested groups were found.

MFI of CD3+ and CD4+ cells was significantly lower in BAL fluid, as compared with PBL, both in nonsmokers (101±2.2 vs 120±1.9, p<0.001, and 91±10.7 vs 111±7.4, p<0.01 mean ± SEM, resp.) and in smokers (96±11 vs 116±12.4, p<0.001, and 94±17.9 vs 109±21, p<0.01). Remarkable decline in MFI of CD3 and CD4 antigens assessed on AL surface was observed in all examined subjects, as compared to PBL. No changes in MFI of other lymphocyte markers were found.

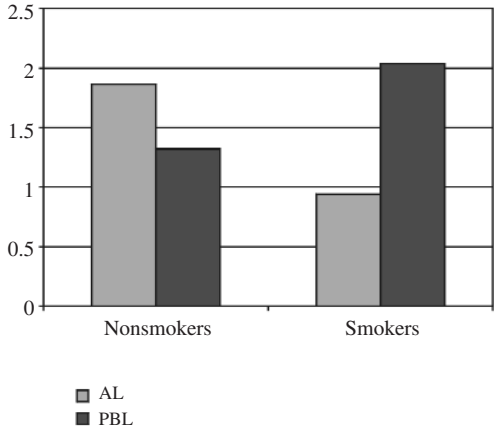

Fig. 1. CD4/CD8 ratio. Alveolar versus peripheral blood lympocytes

In nonsmokers BAL fluid recovery was positively correlated with AL CD4+ percentage (r_s +0.46, p=0.04), and inversely with AL CD8+ (r_s –0.36, p=0.09; fig. 3). It finally resulted in BAL fluid recovery significant positive correlation with AL CD4/CD8 ratio (r_s +0.45, p=0.02). Additionally, CD4+CD25+ subset percentage and BAL eosinophil percentage was correlated negatively with fluid recovery (r_s –0.67, p=0.03 and r_s –0.77, p=0.01, respectively) in smokers.

The example of FC analysis of AL subsets was presented in the fig. 4.

In the table 5 we proposed the normal value range of major alveolar lymphocyte subsets in healthy nonsmokers. It could serve as the baseline for future studies and diagnostics, which will include AL typing.

Discussion

The first studies describing AL as a separate lymphocyte population were published in the late 80's and early 90's [11, 16, 33]. Initially, many authors did not find important differences between AL and PBL in distribution of main lymphocyte subsets: they reported relatively low percentage of T cells in BAL in these studies, and the proportions of BAL B or NK cells were similar to those found in peripheral blood [14, 16, 19, 22]. Additionally, there was no consensus on the flow cytometric criteria of AL gating and subset analysis, in contrast to the precise guidelines established for PBL [18].

In the current study, the standardized method of AL immunophenotyping resulted in the observations described above. The attention should be paid to the fact that in each evaluated subject, the sum of T+B+NK cell percentage (indicator of correct FC analysis, see table 3) was over 90% (and usually more than 95%) [31].

It should be regarded as a rule that BAL fluid of every healthy individual contains higher proportion of T cells with corresponding decrease in percentage of B and NK cells, as compared to peripheral blood. It did not matter, which particular CD antigen (CD3 or CD5) was used for T cell detection. Data presented here were in general consistent with findings of other researchers. However, some authors reported relatively high values of NK and/or B cells in BAL fluid [16, 34]. In the current study we defined NK cells with

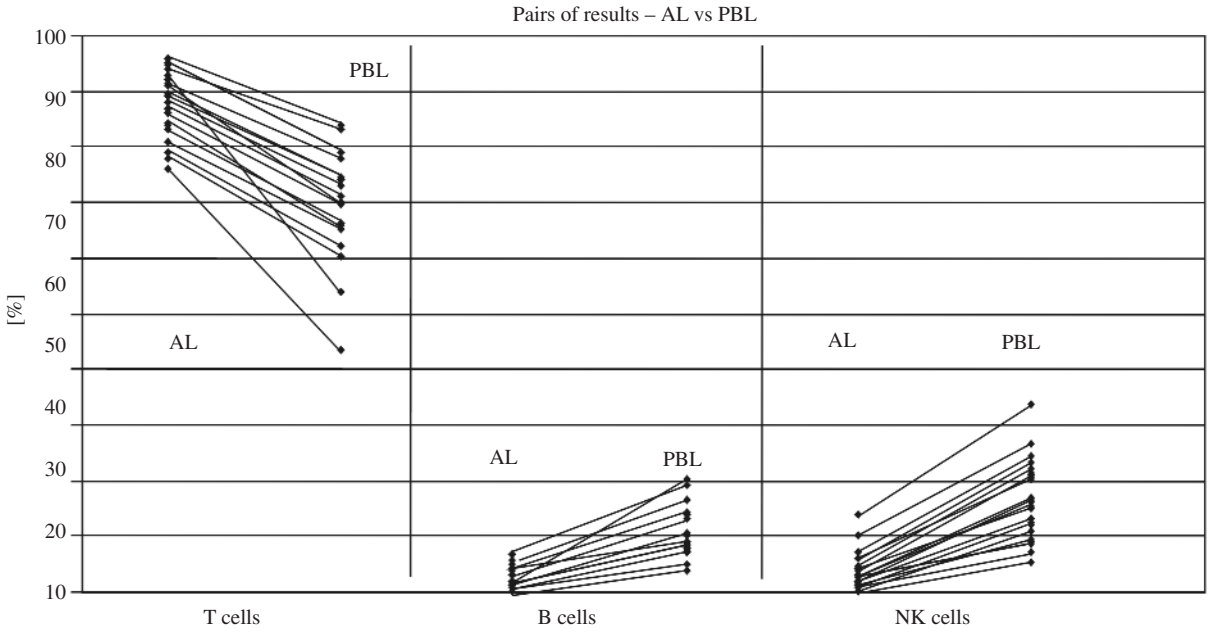

Fig. 2. Main subsets of BAL (AL) and blood (PBL) lymphocytes: T, B and NK cells. Data from nonsmokers and smokers were pooled

Table 4. AL & PBL subsets – comparison

Parameter		Nonsmokers [%]	Smokers [%]	P
AL	CD4+	**51.4**±2.8 (34-78)	**43.1**±3.2 (23-56.8)	<0.01
	CD4+CD25+/CD4+ *	**9.7**±1.5 (0-22.2)	**12.8**±1.3 (0.6-16.7)	ns
	CD8+	**30.5**±2.1 (19.1-58)	**46.5**±2.6 (33.9-60.0)	<0.001
	CD8+CD11b-/CD8+ *	**88.5**±1.8 (85-93)	**94.4**±1.4 (92-97)	<0.05
	CD5+CD19+	**1.5**±0.3 (0-3.8)	**0.5**±0.2 (0-1.4)	<0.001
	CD45R0 + **	**85.1**±5.3 (68-99)	**81.6**±7.2 (70-95)	ns
	CD95+ **	**89.4**±2.9 (73-96)	**77.4**±5.2 (56-100)	ns
	CD4+CD178+	**5.0**±2.4 (1.9-17)	**5.3**±2.2 (2.1-10)	ns
	CD8+CD178+	**2.3**±0.7 (1.5 -6)	**8.3**±3.2 (1.9-13)	<0.05
PBL	CD4+	**40.0**±1.4 (24.4-51)	**49.0**±1.9 (40.7-59.3)	<0.001
	CD4+CD25+/CD4+ *	**5.1**±0.9 (1.3-15.9)	**7.1**±1.3 (0.7-16.3)	ns
	CD8+	**32.0**±1.2 (23.9-44)	**24.9**±1.4 (17-33.1)	<0.01
	CD8+CD11b-/CD8+ *	**47.2**±1.6 (12.3-55.9)	**33.8**±1.3 (11.3-48.6)	ns
	CD5+CD19+	**1.9**±0.3 (0.5-4.3)	**1.9**±0.4 (0.5-5.0)	ns
	CD45R0 + **	**47.0**±3.2 (33-55)	**42.4**±5.1 (36-59)	ns
	CD95+ **	**32**±7.2 (3-42)	**31.5**±13 (13-44)	ns
	CD4+CD178+	**1.4**±0.4 (0.5-3.5)	**0.6**±0.2 (0-3.5)	ns
	CD8+CD178+	**3**±0.7 (0.3-5.4)	**0.6**±0.3 (0.2-3.3)	ns

All results were presented as median ± SEM (range)
** results presented as percentage of CD4+ (CD8+) cells (respectively); all other results presented as proportion of total AL*
*** data shown in brief, for total AL only;*
ns – non significant
All P values concern comparison between nonsmokers and smokers (Mann-Whitney U test)

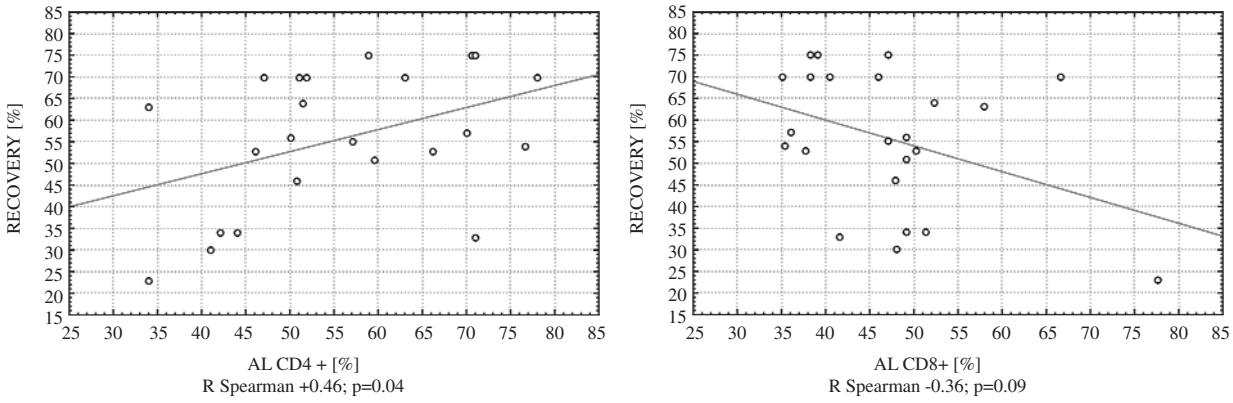

Fig. 3. Nonsmokers. Correlation between AL subsets (CD4, CD8) and BAL fluid recovery

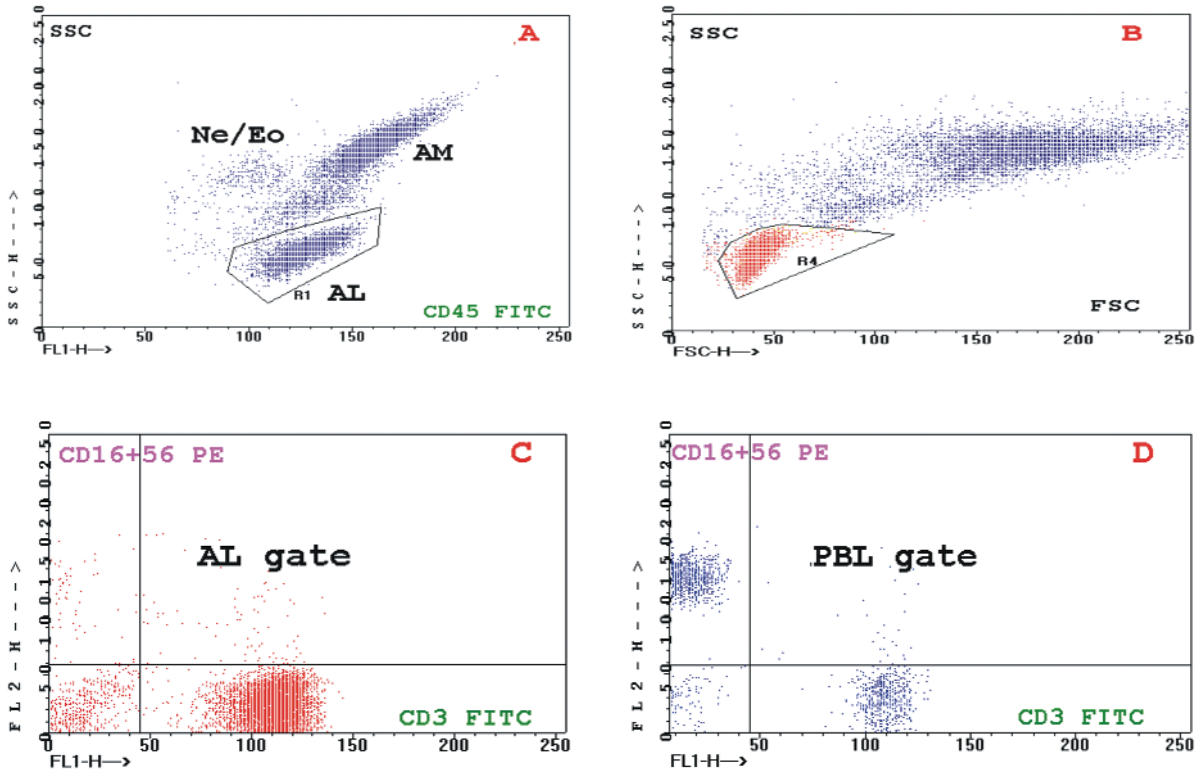

Fig. 4. AL analysis in FC – example

Table 5. AL subsets of nonsmokers (NS) – proposal of normal value range

Subset	Median ± SEM %	Critical Range* %	Comments
T cells **CD3+**	90.1±1.4	**76-95**	more than in parallel peripheral blood staining
B cells **CD19+**	2.7±0.4	**0-6**	less than in parallel peripheral blood staining
NK cells **CD3-(CD16+56)+**	4.2±0.8	**0-10**	less than in parallel peripheral blood staining
T helper cells **CD4+**	51.4±2.8	**38-71**	
T cytotoxic cells **CD8+**	30.5±2.1	**19-48**	
Th/Tc ratio			
CD4/ CD8	1.87±0.21	**0.9-3.5**	
activated T cells **CD3+HLA-DR+**	15.6±3.6	**6-38**	
T cells **CD5+CD19-**	90.0±2.3	**74-96**	more than in parallel peripheral blood staining

** the column contains the values between 5 and 95 percentyl;*
higher or lower results indicate pathology or inapropriate BAL material processing

use of monoclonal antibodies (CD3 FITC/CD[16+56] PE) excluding any antigen sharing with T cells. Results obtained in this way confirmed view on the minimal role of NK cells in lung local immunity [25], both in nonsmokers and smokers.

Low MFI value of CD3 and CD4 on alveolar lymphocytes, observed in ILD by other authors [7, 8, 17], seems to reflect local chronic antigen stimulation of AL. In this study, similar results (low CD3 and CD4 MFI) were also observed in healthy individuals.

In practice, low MFI of CD3+CD4+ cells in BAL fluid results in underestimation of proportion of CD3+ cells, sum of T+B+NK cells and CD4/CD8 ratio. For this reason, flow cytometry should be used preferably in processing of BAL material, including AL typing; the immunoenzymatic techniques (as immunoperoxidase staining) should be avoided [12].

Summing up, the flow cytometric characteristics of AL subsets established in the current study supports the hypothesis on biased T cell accumulation on the surface of pulmonary alveoli [24, 25]. Moreover, our results suggest that in nonsmokers the accumulation concerns mostly CD4+ T cells (T helpers). The crucial role of lung Th cells in the local immune defense is largely discussed in the literature [8, 17, 26, 34]. For instance, they could be stimulated by contact with dendritic and other antigen presenting cells [35]. They can be also active as cytotoxic cells [12]. In our material, the CD4/CD8 ratio in BAL fluid of nonsmokers was significantly higher than respective PBL value.

The decreased CD4/CD8 ratio in BAL fluid of smokers, referred by many authors [6, 21, 36], was completed by our observation of local predominance of T cells which express cytotoxic (but not suppressor) phenotype. We did not find any increase in number of neutrophils in smokers, reported by some authors [21, 22]. Relatively increased proportion of BAL eosinophils in smoking subjects constituted the contamination of upper airways, since this parameter was significantly and negatively correlated with BAL fluid recovery.

Interleukin-2 receptor (CD25), commonly applied as a marker of AL activation, should not be recommended. The number of BAL CD4+CD25+ lymphocytes was negatively correlated with fluid recovery in nonsmokers. It means that CD25+ cells originate partially from the upper airways.

The majority of AL, both Th and Tc, were positive for CD95. This finding reflected high AL susceptibility to membrane proapoptotic stimuli [27]. Paradoxically, lymphocyte apoptosis is not a common event in BAL material [10]. Fas Ligand (CD178) positive lymphocytes are relatively rare in BAL material; the remarkable increase in proportion of CD8+CD178+ cells in healthy smokers needs further investigation.

Conclusions

1. The normal value range of major subsets of alveolar lymphocytes was proposed for healthy nonsmokers. It was based on standardized approach to flow cytometric analysis of BAL material collected in a relatively large group of people.
2. Alveolar lymphocytes in the control group, both in nonsmokers and smokers, are dominated by T cell subset. B and NK cells occur infrequently in BAL fluid, as compared to PBL.
3. BAL fluid recovery in nonsmokers is positively correlated with BAL CD4/CD8 ratio.
4. Mean fluorescence intensity of CD3 and CD4 superficial antigens is significantly decreased on AL as compared to parallel PBL staining.
5. The results support the hypothesis that T cells as a major subset of AL, they are chronically stimulated in lungs by specific antigens and carry the phenotype of primed memory/effector cells. Helper T cells are the lymphocytes that accumulate preferentially in alveoli in physiological conditions.

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