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Increased expression of activation markers and adhesion molecules on lung T-cells compared with blood in the normal rat

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Increased expression of activation markers and adhesion molecules on lung T-cells compared with blood in the normal rat. T. Tschernig, F. Fliegert, J. Westermann, R. Pabst. ©ERS Journals Ltd 1999.

ABSTRACT: Lymphocytes play an important role in many lung diseases and are routinely accessible by bronchoalveolar lavage (BAL). Lymphocytes from the BAL (BAL pool) have a different subset composition to those from peripheral blood, consisting mainly of activated T-cells. The aim of this study was to examine whether preferential migration of activated T-cells to the bronchoalveolar space or factors of the specific microenvironment mediate this phenomenon.

The expression of adhesion molecules and cellular activation markers (intercellular adhesion molecule-1, leukocyte function-associated antigen-1, CD2, CD44, interleukin-2 receptor and L-selectin) was studied on T- and B-cells not only in the BAL and peripheral blood (blood pool), but also in the compartments in between, such as the lung vascular perfusate (marginal pool) and the lung interstitium (interstitial pool), with the experiments being performed simultaneously in the same animals.

Low levels of adhesion molecule expression were observed on T-cells in the blood and marginal pool, medium levels in the lung interstitium and the highest levels in the BAL. "Memory" ($CD45R^{low}$) and "naive" ($CD45R^{high}$) T-cells in the lung compartments showed a higher expression of adhesion molecules compared with blood. However, the predominating $CD45R^{low}$ T-cells showed a significantly higher expression than the $CD45R^{high}$ cells, indicating that CD4+ $CD45R^{high}$ T-cells had changed their phenotype to $CD45R^{low}$.

In conclusion, a high level of expression of leukocyte function associated antigen-1 and intracellular adhesion molecule-1 on the bronchoalveolar lavage and interstitial T-cells is more likely to be the result of local, lung-specific induction than a prerequisite for migration into the bronchoalveolar space.

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Lymphocytes play a significant role in lung disorders, *e.g.* sarcoidosis, asthma, and rejection after transplantation (for review see [1, 2]). However, even basic kinetic aspects of the bronchoalveolar lavage (BAL) pool of lymphocytes, such as their origin, lifespan, proliferation, apoptosis and recirculation, are largely undefined [3]. Lymphocytes in the bronchoalveolar space are routinely accessible by BAL, which is the most important tool for immunological examination in lung disease. In contrast to the peripheral blood, mainly activated (CD4+, $CD45R^{low}$ and L-$selectin^{low}$) T-cells were found in the BAL of humans [4] and mice [5]. In humans only blood and BAL have been studied [6]. Since the lungs have no afferent lymphatics the blood is the starting point for lymphocyte immigration. Lymphocytes marginate to the lung vascular endothelium (marginal pool) and enter the interstitial lung tissue (interstitial pool), where the lymphocyte composition was different from blood and BAL when examined in healthy rats [7]. The number and composition of lymphocytes might be regulated by adhesion molecules [8], even though a homing receptor for the lung has not been found. A few data are available on leukocyte–endothelial interactions on pulmonary endothelial cells in an *in vitro* assay [9]. Clinical implications of adhesion molecules in pneumology have been shown for eosinophils in a primate model of allergic asthma [10].

The aim of this study was to characterize the expression of adhesion molecules and cellular activation markers on lymphocytes, determined simultaneously in the same animals, not only in the BAL and the circulating blood, but also in the marginal vascular pool and the lung interstitium.

Materials and methods

Animals

Eight male Lewis rats (Central Animal Laboratory, Medical School of Hannover, Hannover, Germany), 12–14 weeks old, with a mean weight of 370 g, were used. All animals were kept under specified pathogen-free conditions. The experiments were approved by the local authorities according to German animal welfare law.

Isolation of lymphocytes from the blood and three lung compartments

The procedure for cell preparation used for the blood, marginal vascular lymphocyte pool, lung tissue and BAL has been described recently [7]. In brief, the animals were anaesthetized and blood from the abdominal aorta was collected in heparinized tubes. Red blood cells were removed by incubating each 0.5 mL aliquot in 10 mL ammonium chloride solution (0.83% NH_4Cl; Merck, Darmstadt, Germany) for 10 min. After centrifugation the pellet was resuspended in phosphate-buffered saline (PBS; 1% bovine serum albumin (BSA) and 0.1% NaN_3). This cell preparation was denoted the blood pool. The lungs were lavaged by the instillation of 5 mL cold (4°C) 0.9% sodium chloride solution into the trachea followed by gentle aspiration, which was repeated 15 times. All fractions were pooled and >90% of the instilled volume was recovered. After centrifugation, cells were resuspended in PBS (containing 1% BSA and 0.1% NaN_3) (BAL pool). Control treatment with ammonium chloride solution was performed in separate experiments and did not alter the expression of the examined surface molecules. Lung perfusion was performed after dissecting the lungs from the chest. The pulmonary trunk was cannulated, the left ventricle was opened and the lung vascular bed was perfused 12 times with fractions of 10 mL cold (4°C) perfusate consisting of RPMI medium (Biochrom KG, Berlin, Germany) with 35 $g \cdot L^{-1}$ dextran (70 kDa). The first and second fractions were withdrawn and the following fractions were pooled (marginal pool) and handled as described for blood. After removing the trachea and major bronchi including hilar lymph nodes, cells of the lung tissue were extracted using mechanical disruption to avoid alteration of the adhesion molecules by enzymatic digestion [8]. To this end, the lung tissue was completely disaggregated by passing through a metal sieve. Then, cells were separated from debris by filtration through a 75-mm nylon mesh and processed in the same way as blood (interstitial pool).

Immunofluorescence of adhesion molecules on lymphocyte subsets

Adhesion molecules were measured on αβ T-cells, CD4+, CD8+ T-cells (double staining with R73 and OX8), CD4+ $CD45R^{low}$ (recently activated) and CD4+ $CD45R^{high}$ (resting) T-cells (double staining with W3/25 and OX22) as summarized previously [7]. In this technique the subset-defining antibodies are detected in fluorescence 1 (fluorescein isothiocyanate; FITC) or fluorescence 3 (Red 670). The subpopulation of interest was gated then in fluorescence 1 and 3 and the adhesion molecule was measured in fluorescnce 2 (phycoerythrin). The monoclonal antibody (mAb) OX8 was labelled with FITC and measured in fluorescence 1. R73 was biotinylated and traced with Red 670 for fluorescence 3. The double staining with R73 and OX8 also traced the CD4+ T-cells (R73+ OX8-). Intracellular adhesion molecule-1 (ICAM-1; 1A29, mouse immunoglobulin (Ig)G_1 [11]), L-selectin (HRL3, hamster IgG_1 [12]), interleukin-2 receptor (IL-2R; OX39, mouse

Table 1. – Subset composition of lymphocytes derived from different lung compartments and the peripheral blood, determined by flow cytometry

	T-cells	CD4+	CD8+	CD45-R^{low}	CD45-R^{high}	B-cells
Blood pool	71±2	54±1	16±1	25±1	75±1	21±1
Marginal pool	57±4*	44±4*	12±1*	36±3*	64±3*	20±2
Interstitial pool	51±4	42±4	10±1	70±6*	30±6*	17±2
BAL pool	75±2*	62±3*	8±2	99±1*	1±1*	8±2*

Percentages are given of T- and B-cells among all lymphocytes gated, $CD4^+$ and $CD8^+$ cells among the T-cells and $CD45R^{low}$ and $CD45R^{high}$ cells among the CD4+ cells. BAL: bronchoalveolar lavage. Data are mean±SEM. *: $p<0.05$ in comparison with the preceding compartment.

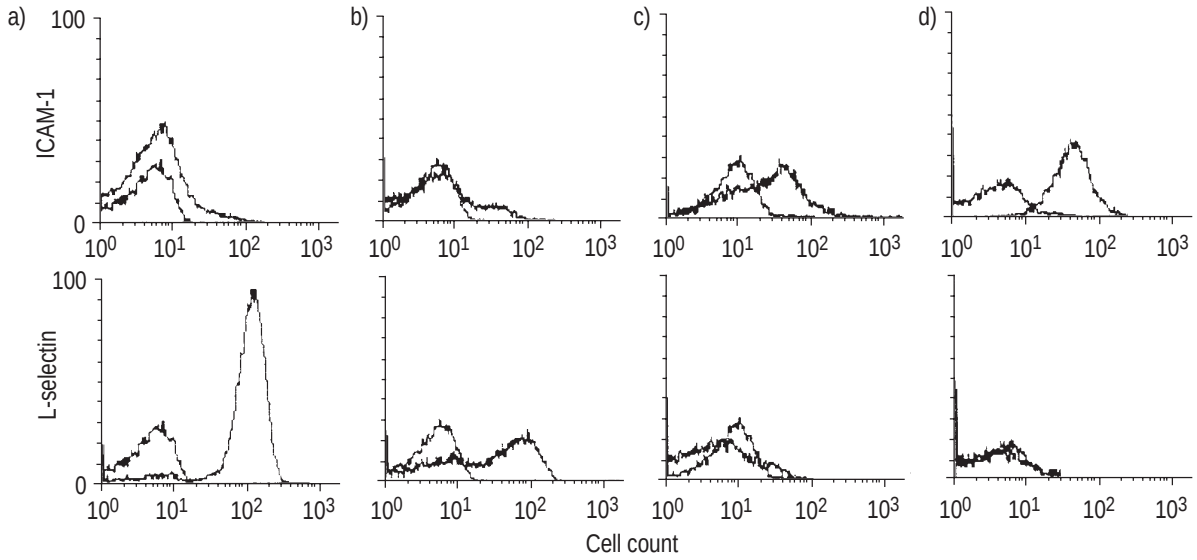

Fig. 1. – Representative fluorescence-activated cell sorter (FACS) profiles of intercellular adhesion molecule-1 (ICAM-1) and L-selectin on CD4+ T-cells demonstrating increasing activation from a) blood to b) the marginal pool (perfusate), c) lung interstitium and d) the bronchoalveolar lavage.

IgG_1 [13]), leukocyte function-associated antigen-1 (LFA-1; WT1, mouse IgG_{2a} [14]), CD2 (OX34, mouse IgG_{2a} [15]) and CD44 (OX49, mouse IgG_{2a} [16]) were traced with a phycoerythrin (PE)-conjugated second-step antibody and measured in fluorescence 2 (for details see [17]). Isotype-matched, irrelevant antibodies served as controls. In separate experiments it was shown that the data from double and three-colour staining experiments were similar to those obtained by single staining. Within the lymphocyte cluster 5×10^4 cells were analysed. In the BAL only one or two activation markers could be determined, depending on the small number of lymphocytes. Therefore, in BAL the number of animals studied was three or a single value was given, as indicated in the figure legends, in contrast to the blood, perfusate and lung interstitium (n=8). The viability of the lymphocytes determined by propidium iodide staining was always >90%. Results were given as the percentage of each T-cell subset positive for the adhesion molecules and the mean fluorescence intensity for comparing the grade of expression.

Analysis of data

SPSS-Windows 6.0.1 (SPSS, Chicago, IL, USA) was used for calculations. Results are presented as mean±SEM. Differences between group means were analysed using the Wilcoxon test. Differences with $p<0.05$ were taken as significant.

Results

Lymphocyte subsets

The composition of lymphocyte subsets in the various lung compartments and the blood differed, as shown in table 1. The number of B-cells was lower in the BAL than in the other compartments. The opposite was true for T-cells, most of which showed a CD4+ "recently activated" phenotype in the BAL.

Activation markers

The percentage of T-cells positive for ICAM-1 and IL-2R was low in the blood and perfusate, clearly higher in the lung interstitium and reached nearly 100% in the BAL (figs. 1 and 2). No difference was observed between T-cells and the CD4+ and CD8+ subsets. The opposite was true for L-selectin; however, this also demonstrates increasing activation of T-cells from the blood to BAL because L-selectin is typically lost during activation [18]. The "memory" ($CD45R^{low}$) T-cells were significantly more activated than the "naive" $CD45R^{high}$ T-cells. In contrast, the B-cells showed a homogeneous pattern.

Adhesion molecules

The vast majority of T-lymphocytes were positive for LFA-1, CD2 and CD44, with no differences between the compartments (data not shown). However, the mean fluorescence intensity showed a differential amount of molecules expressed on the cell surface (fig. 3). The mean fluorescence intensity of LFA-1 and CD2 of the cells from the lung tissue and BAL was higher than that in the blood and marginal pool, whereas the mean fluorescence intensity of CD44 was lower in the blood than in any of the lung compartments. The cells in the marginal pool showed a higher mean fluorescence intensity of CD44 than those in the blood or lung interstitium. Data on very late antigen-4 (VLA-4) were similar to the LFA-1 data, showing increasing mean fluorescence intensity from the blood pool to the BAL pool (data not shown). $CD45R^{low}$ T-cells showed a higher mean fluorescence intensity for LFA-1, CD2 and CD44 than for the $CD45R^{high}$ T cells.

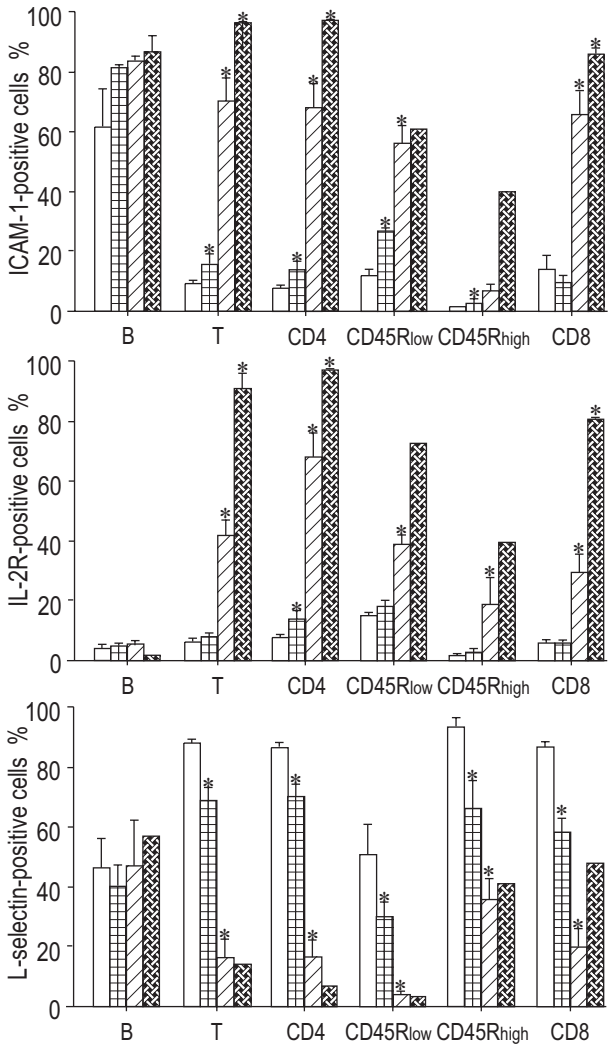

Fig. 2. – Percentages of lymphocyte subsets derived from the blood and three lung compartments expressing different activation molecules. The mean±SEM of flow-cytometric data from eight animals are presented for the blood (□), perfusate (▦) and lung interstitium (◰), whereas data from three animals or single values are shown for the bronchoalveolar lavage (▩) because of the low number of lymphocytes in healthy rats. *: $p<0.05$ in comparison with the preceding compartment. Comparison of $CD45R^{low}$ and $CD45R^{high}$ CD4+ T-cells revealed significant differences in the blood, perfusate and lung interstitium for interleukin-2-receptor (IL-2R), L-selectin and intercellular adhesion molecule-1 (ICAM-1) (not indicated).

Discussion

In this study, the probable route used by lymphocytes to move from the blood to the bronchoalveolar space was

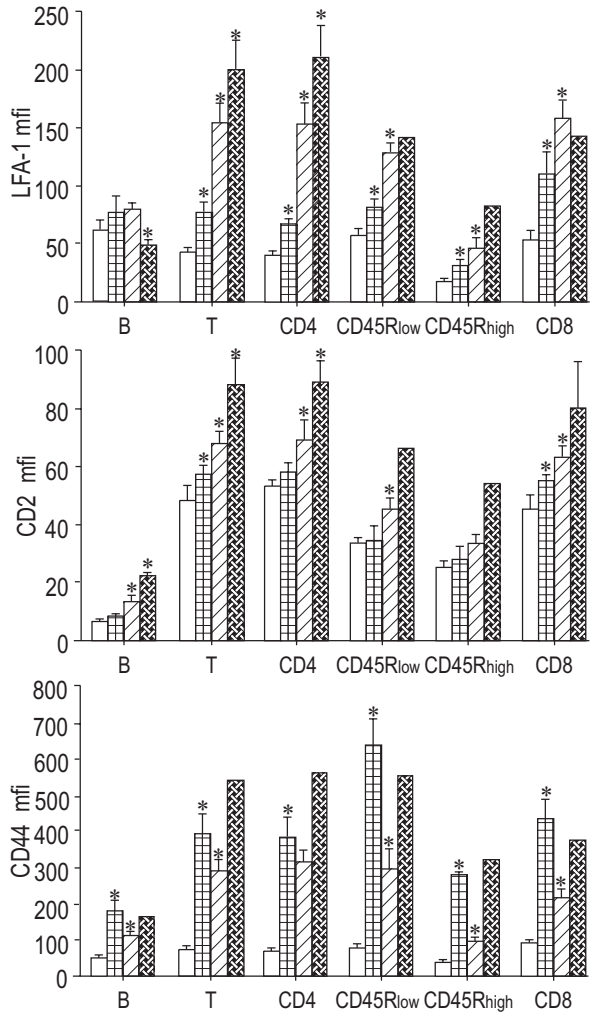

Fig. 3. – The mean fluorescence intensity (mfi) of leukocyte function-associated antigen-1 (LFA-1), CD2 and CD44 on lymphocyte subsets. The percentage of T-cells positive for these surface molecules was 80–100% without significant differences between cell preparations (not shown). The meanSEM of flow-cytometric data from eight animals are presented for the blood (□), perfusate (▦), and lung interstitium (◪), whereas data from three animals or single values are shown for the bronchoalveolar lavage (▩). *: $p<0.05$ in comparison with the preceding compartment. Comparison of $CD45R^{low}$ and $CD45R^{high}$ CD4+ T-cells revealed significant differences in the blood, perfusate and lung inter-stitium for LFA-1 and CD44 (not indicated).

examined in normal rats, and a differential expression of adhesion and activation molecules on lymphocytes in different lung compartments was found.

The composition of lymphocytes in the blood and three lung compartments was distinct and comparable to published data [7]. Increasing activation of T-cells and T-cell subsets was observed from the blood to the bronchoalveolar space (IL-$2R^{high}$, L-$selectin^{low}$, ICAM-1^{high}, LFA-1^{high}, $CD2^{high}$). In this regard, only minor differences were found between CD4+ and CD8+ T-cells. Therefore, the dominance of CD4+ T-cells in the lung interstitium and the BAL does not depend on activation as a prerequisite for entry. Different levels of activation were found between the $CD45R^{low}$ and $CD45R^{high}$ CD4+ T-cells but this was not restricted to a certain lung compartment, indicating constant differences between these cell types. The predominance of recently activated $CD45R^{low}$ T-cells in the lung interstitium and the BAL might be related to a phenotype change as a process of activation or stimulation [19]. B-cells, representing <20% of lymphocytes in the lung, showed no differences in the expression of the surface molecules examined in these compartments [7]. The high expression of CD44 on lymphocytes in the marginal lung vascular pool was in accordance with the suggested role of this molecule, *i.e.* rolling, which is the first step in the leukocyte–endothelial interaction [20].

Thus, there is increasing adhesion molecule expression and activation of T-lymphocytes in lung compartments. This immunological status is appropriate for such a vulnerable organ. The expression of adhesion molecules depends on the compartment from which the cells were extracted, leading to evidence that local activation and expression of adhesion molecules is induced by the microenvironment. Possible candidates for such activators are dendritic cells and macrophages or components of the extracellular matrix [21, 22].

The key mechanisms of lymphocyte migration into the lung tissue and bronchoalveolar space are still not known. In addition, differences between the normal situation and lung disease are likely. However, as shown in blocking studies in experimental immune responses, the interaction between VLA-4 and vascular cell adhesion molecule-1 plays a predominant role in the recruitment of lymphocytes to the bronchoalveolar space [23]. In a model of the isolated buffer-perfused lung the release of injected leukocytes was enhanced after blocking LFA-1 on the injected cells [24]. Intravital microscopy is used for studying adhesion and transmigration mechanisms in the peritoneum or lymph nodes but is difficult to perform in lung tissue. Recent data obtained using this method showed a suppression of leukocyte rolling in arterioles by L-selectin inhibition, whereas this was not true for venules or capillaries [25]. The characteristics of the pulmonary microvasculature seem to be quite different to those of other organs.

The analysis of different leukocyte compartments of the healthy lung is a useful basis for studying the effects of functionally relevant markers in inflammation. Moreover, the follow-up of labelled cells into lung compartments may clarify the mechanisms of the subset-specific distribution of lymphocytes in different lung compartments [26].

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References

1. Richeldi L, Franchi A, Rovatti E, Cossarizza A, duBois RM, Saltini C. Lymphocytes. *In:* Crystal RG, West JB, eds. The Lung. New York, Raven, 1996; pp. 803–820.
2. Berman JS, Beer DJ, Theodore AC, Kornfeld H, Bernardo J, Center DM. Lymphocyte recruitment to the lung. *Am Rev Respir Dis* 1990; 142: 238–257.
3. Pabst R, Tschernig T. Lymphocyte dynamics: caution in interpreting BAL numbers. *Thorax* 1997; 52: 1078–1080.
4. Saltini C, Kirby M, Trapnell BC, Tamura N, Crystal RG. Biased accumulation of T lymphocytes with "memory"-type CD45 leukocyte common antigen gene expression

on the epithelial surface of the human lung. *J Exp Med* 1990; 171: 1123–1140.
5. Curtis JL, Kim S, Scott PJ, Buechner-Maxwell VA. Adhesion receptor phenotypes on murine lung CD4+ T cells during the pulmonary immune response to sheep erythrocytes. *Am J Respir Cell Mol Biol* 1995; 12: 520–530.
6. Pabst R, Tschernig T. Lymphocytes in the lung: an often neglected cell. *Anat Embryol* 1995; 192: 293–299.
7. Fliegert F, Tschernig T, Pabst R. Comparison of lymphocyte subsets, monocytes, and NK cells in three different lung compartments and peripheral blood in the rat. *Exp Lung Res* 1996; 22: 677–690.
8. Mulder WMC, Koenen H, van de Muysenberg AJC, Bloemena E, Wagstaff J, Scheper RJ. Reduced expression of distinct T cell CD molecules by collagenase/DNase treatment. *Cancer Immunol Immunother* 1994; 38: 253–258.
9. Li X, Abdi K, Rawn J, Mackay CR, Mentzer SJ. LFA-1 and L-selectin regulation of recirculating lymphocyte tethering and rolling on lung microvascular endothelium. *Am J Respir Cell Mol Biol* 1996; 14: 398–406.
10. Letts LG, Wegner CD, Gundel RH. Adhesion molecules in acute and chronic lung inflammation. *Adv Exp Med Biol* 1993; 351: 39–45.
11. Tamatani T, Miyasaka M. Identification of monoclonal antibodies reactive with the rat homolog of ICAM-1, and evidence for a differential involvement of ICAM-1 in the adherence of resting *versus* activated lymphocytes to endothelial cells. *Int Immunol* 1990; 2: 165–171.
12. Tamatani T, Kuida K, Watanabe T, Koike S, Miyasaka M. Characterization of the LECAM-1 (L-selectin)-dependent adhesion pathway in rats. *J Immunol* 1993; 150: 1735–1745.
13. Westermann J, Smith T, Peters U, *et al*. Both activated and nonactivated leukocytes from the periphery continuously enter the thymic medulla of adult rats: phenotypes, sources and magnitude of traffic. *Eur J Immunol* 1996; 26: 1866–1874.
14. Tamatani T, Kotani M, Miyasaka M. Characterization of the rat leukocyte integrin, CD11/CD18, by the use of LFA-1 subunit-specific monoclonal antibodies. *Eur J Immunol* 1991; 21: 627–633.
15. Williams AF, Barclay AN, Clark SJ, Paterson DJ, Willis AC. Similarities in sequences and cellular expression between rat CD2 and CD4 antigens. *J Exp Med* 1987; 165: 368–380.
16. Arch R, Wirth K, Hofmann M, *et al*. Participation in normal immune responses of a metastasis inducing splice variant of CD44. *Science* 1992; 257: 682–685.
17. Klonz A, Wonigeit K, Pabst R, Westermann J. The marginal blood pool of the rat contains not only granulocytes, but also lymphocytes, NK-cells and monocytes: a second intravascular compartment, its cellular composition, adhesion molecule expression and interaction with the peripheral blood pool. *Scand J Immunol* 1996; 44: 461–469.
18. Richerson HB, Coon JD, Lubaroff D. Selective early increases of bronchoalveolar CD8+ lymphocytes in a LEW rat model of hypersensitivity pneumonitis. *J Allergy Clin Immunol* 1995; 96: 113–121.
19. Hargreaves M, Bell EB. Identical expression of CD45R isoforms by CD45RC+ 'revertant' memory and CD45RC+ naive CD4 T cells. *Immunology* 1997; 91: 323–330.
20. Dunon D, Piali L, Imhof BA. To stick or not to stick: the new leukocyte homing paradigm. *Curr Opin Cell Biol* 1996; 8: 714–723.
21. Holt PG. Regulation of antigen-presenting cell functions in lung and airway tissues. *Eur Respir J* 1993; 6: 120–129.
22. van Haarst JMW, Hoogsteden HC, de Wit HJ, Verhoeven GT, Havenith CEG, Drexhage HA. Dendritic cells and their precursors isolated from human bronchoalveolar lavage: immunocytologic and functional properties. *Am J Respir Cell Mol Biol* 1994; 11: 344–350.
23. Krug N, Tschernig T, Holgate S, Pabst R. How do lymphocytes get into the asthmatic airways? Lymphocyte traffic into and within the lung in asthma. *Clin Exp Allergy* 1998; 28: 10–18.
24. Klemm A, Tschernig T, Ermert L, *et al*. Anti leukocyte function-associated molecule-1 (αLFA-1) results in decreased adhesion of lymphocytes to the endothelium of the isolated buffer-perfused rat lung. *Am J Respir Crit Care Med* 1998; 157: A208.
25. Yamaguchi K, Nishio K, Sato N, *et al*. Leukocyte kinetics in the pulmonary microcirculation: observations using real-time confocal luminescence microscopy coupled with high-speed video analysis. *Lab Invest* 1998; 76: 809–822.
26. Tschernig T, Boeke K, Steinhoff G, Wonigeit K, Pabst R, Westermann J. The lung as a source and a target organ for T and B lymphocytes. *Am J Respir Cell Mol Biol* 1997; 17: 414–421.