Reduced expression of the αβ T-cell antigen receptor by alveolar T-cells

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ABSTRACT: A previous study revealed that reduced expression (modulation) of the CD3 antigen is a common characteristic of alveolar T-cells in health and disease. As CD3 molecules are noncovalently bound to T-cell antigen receptors (TCR), it was hypothesized that modulation of TCR was also a feature of alveolar T-cells.

To demonstrate this, lymphocytes from bronchoalveolar lavage fluid were stained with an anti-αβTCR antibody and analysed by flow cytometry. The expression of αβTCR by alveolar T-cells was evaluated by calculating mean fluorescence intensity (MFI) and was compared with αβTCR expression by autologous blood T-cells.

As anticipated from a previous study, modulation of TCR was observed not only in healthy volunteers but also in patients with pulmonary sarcoidosis, other pulmonary diseases, and nonpulmonary diseases. There were no significant differences in MFI of alveolar T-cells among the study groups. The degree of modulation assessed by the difference of MFI between blood and alveolar T-cells was greater for CD4+ cells than for CD8+ cells owing to the higher MFI of CD4+ blood T-cells. Coculture of alveolar macrophages with blood T-cells *in vitro* induced partial modulation of TCR.

These results demonstrate the ubiquity of modulation of T-cell receptors on alveolar T-cells and suggest, in contrast to a previous report by other investigators that it is caused by some nonantigenic mechanism possibly inherent in the alveolar milieu. The implications of this phenomenon in *in vivo* immune responses of the lung need to be examined.
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Pulmonary sarcoidosis is characterized by the accumulation of abundant CD4+ T-cells in the alveolar milieu [1]. Most investigators favour the notion that these T-cells are responding to a causative antigen, if any, of sarcoidosis [2, 3], because effector T-cells in the cell-mediated immune response are believed to belong to the CD4+ cells [4]. Antigens that fit within the unique pockets of major histocompatability complex (MHC) class II molecules are presented to T-cells by antigen-presenting cells such as alveolar macrophages and dendritic cells [5]. These molecular complexes are recognized by T-cell antigen receptors (TCR). The TCR is noncovalently associated on the cell surface with a group of five invariant polypeptides designated γ, δ, ε, and ζ–ζ, which collectively represent the CD3 complex responsible for signal transduction [6]. The T-cell coreceptor, CD4, is bound to the β_2 domain of MHC class II molecules [7] and transduces signals supplementary to those generated through TCR/CD3-MHC complexes [6]. One notable phenomenon at this stage of the immune response is downregulation of the expression of TCR/CD3 molecular complexes on the cell surface which results from internalization of the complexes [8–11]. This has customarily been called modulation and is associated with hyporesponsiveness of T-cells to antigens or mitogens [12–14].

A previous report has revealed the modulation of TCR composed of α and β subunits (αβTCR) on alveolar T-cells recovered by bronchoalveolar lavage (BAL) from patients with pulmonary sarcoidosis [15]. The study was conducted only on pulmonary sarcoidosis, and the authors considered modulation to be the result of local T-cell triggering in this disease. Modulation of CD3 on alveolar T-cells compared with autologous T-cells in pulmonary sarcoidosis has been demonstrated previously [16]. As CD3 and TCR are noncovalently bound, it was assumed that this observation represented indirect evidence of modulation of TCR. More importantly, modulation of CD3 was not accompanied by disease or pathological state specificity in that it was found not only in pulmonary sarcoidosis, but also in normal subjects and other pulmonary and nonpulmonary diseases. Therefore, it was concluded that modulation of CD3 in pulmonary sarcoidosis did not necessarily suggest local activation of T-cells through an antigen/MHC-TCR interaction. The present study was undertaken to further examine this notion by direct demonstration of the modulation of αβTCR.

Subjects and methods

Study subjects

The study subjects consisted of four distinct groups: 12 healthy volunteers, 28 patients with pulmonary sarcoidosis, 10 patients with other pulmonary diseases, and 10 patients with nonpulmonary diseases. Their demographic

data and results of BAL are presented in table 1. No healthy volunteers had a history of lung disease or evidence of lung disease on physical examination, chest radiography, and pulmonary function tests. All had visibly normal airways within reach of the fibreoptic bronchoscope. Each patient with pulmonary sarcoidosis had a compatible clinical picture without evidence of mycobacterial, fungal, or parasitic infection and compatible chest radiographic findings including bilateral hilar and/or paratracheal lymph node enlargement with or without parenchymal infiltrates. By chest radiographic staging, two subjects were in stage 0, 13 in stage I, 11 in stage II, and two in stage III. Of the 26 sarcoid patients with pulmonary involvement, six had active eye lesions. Thus, 26 patients had one or more organs affected by active sarcoid lesions and were regarded as being in the active state. The group of other pulmonary diseases included three patients with idiopathic pulmonary fibrosis (IPF), three with interstitial pneumonia associated with rheumatoid arthritis, one with pneumoconiosis, one with bronchopneumonia, one with diffuse panbronchiolitis, and one with hypersensitivity pneumonitis. All patients with IPF fulfilled the clinical and radiographic criteria for IPF without evidence of other interstitial lung diseases that potentially cause pulmonary fibrosis. They had transbronchial or thoracoscopic lung biopsies showing varying degrees of interstitial fibrosis. All patients with rheumatoid arthritis fulfilled the established criteria of the disease [17]. Of the 10 patients with nonpulmonary diseases, four had uveitis, two had Crohn's disease, one had ulcerative colitis, two had viral hepatitis, and one had adult T-cell leukaemia without evidence of lung involvement. Informed written consent was obtained from all subjects, and the study was approved by the Ethical Committee of the School of Medicine, Hokkaido University, Japan.

Immunocytometry

Fluorescein isothiocyanate (FITC)-conjugated or unconjugated anti-$\alpha\beta$TCR antibody TCR-1 [18] was purchased from Becton Dickinson Immunocytometry Systems (San Jose, CA, USA), and phycoerythrin (PE)-conjugated T4

(anti-CD4 antibody) and T8 (anti-CD8 antibody) from Coulter Immunology (Hialea, FL, USA). The FITC-conjugated anti-CD45RO antibody, UCHL-1, was purchased from Nichirei (Tokyo, Japan) and PE-conjugated polyclonal antimouse immunoglobulin (Ig) was from Dakopatts (Glostrup, Denmark).

BAL cells were washed twice with Hank's balanced salt solution (HBSS; GIBCO, Grand Island, NY, USA) and resuspended in autologous serum at 5×10^6 cells·mL^{-1} to obtain conditions similar to those of whole blood. In single-colour flow cytometric analysis, 50 μL of heparinized whole blood or BAL cell suspension were placed in 16×100 mm plastic tubes. A 25 μL aliquot of FITC-conjugated TCR-1 diluted with phosphate-buffered saline (PBS) containing 0.1% sodium azide was then added to the appropriate tubes, and the samples were mixed by brief agitation using a vortex. After incubation for 30 min at 4°C in the dark, 2 mL of 1% lysing solution (Immuno-Lyze, Coulter Immunology, Hialea, FL, USA) were added to each tube. The samples were incubated for 5 min and washed twice with PBS containing azide. After removal of the supernatant, the stained cells were resuspended in 500 μL of PBS containing azide and immediately analysed by a FACScan (Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA). A live gate for lymphocytes was set using forward light scatter and side scatter. The percentage of positive cells was determined by comparing an experimental and a control histogram obtained by staining samples with control immunoglobulin (FITC-conjugated mouse IgG1, Becton Dickinson Immunocytometry Systems, San Jose). A <1% threshold was used to define $\alpha\beta$TCR positive cells. The fluorescence signal of each cell was plotted on a log scale, FL^{-1}, and converted to a linear channel number ranging 0–255. The mean fluorescence intensity (MFI) of fluorescence-positive cells was calculated by computer analysis of the number of cells fluorescing in each channel of the flow cytometer.

In two-colour analysis, 50 μL of heparinized blood or BAL cell suspension were placed into 16×100 mm plastic tubes. A 25 μL aliquot of FITC-conjugated TCR-1 and 25 μL of either PE-conjugated T4 or T8 diluted with PBS containing azide were simultaneously added to the appropriate tubes. The samples were mixed and treated in the

Table 1. – Demographic data and bronchoalveolar lavage (BAL) findings for study subjects

	Healthy volunteers	Sarcoidosis	Other pulmonary diseases	Nonpulmonary diseases
Subjects n	12	28	10	10
Male/female	7/5	10/18	5/5	7/3
Age yrs	33±16	41±16	57±17	32±12
Smoker/nonsmoker	3/9	11/17	5/5	2/8
BAL findings				
Total cells $\times 10^4$ cells·mL^{-1} BAL fluid	12±7	20±9	28±21	16±12
Alveolar macrophages %	90±8	65±20	62±28	72±20
Lymphocytes %	9±8	34±20	25±24	25±20
CD2 %[†]	83±14	95±7	90±6	94±5
$\alpha\beta$TCR %[†]	68±20	86±12	81±16	85±11
CD4/CD8	2.0±0.8	6.4±5.1	3.5±5.7	2.3±2.7
Neutrophils %	0.6±0.7	0.5±0.4	11.1±20.6	0.7±1.1
Eosinophils %	0.2±0.2	0.5±1.3	2.2±3.5	0.3±0.7
Mast cells %	0±0	0±0.1	0±0	0±0.1

Data are expressed as mean±SD. TCR: T-cell receptor. [†]: percentage of lymphocytes gated on a forward scatter and side scatter plot of a flow cytometer.

same way as for the single-colour analysis. For evaluating TCR expression on memory and naive blood T-cells separately, 5×10^5 blood mononuclear cells in a 50 µL aliquot of PBS containing azide and 0.1% bovine serum albumin (BSA) were first stained with 5 µL of TCR-1 for 30 min at 4°C, followed by staining with 5 µL of PE-conjugated anti-mouse Ig. After washing, the remaining anti-mouse Ig was adsorbed with 50 µL of mouse Ig (Cappel, Durham, NC, USA) suspended in PBS containing azide at 2 mg· mL^{-1}) for 20 min, and stained with 5 µL of FITC-conjugated UCHL-1 for 30 min. The expression of TCR was assessed by calculating the MFI of FL-2 after gating CD45RO+ cells or CD45RO- cells using fluorescence profiles of FL-1.

To evaluate the TCR expression by T-cells stained in conditions without alveolar macrophages (AMs), BAL cells or blood mononuclear cells were mixed with neuraminidase-treated sheep erythrocytes, and incubated for 1.5 h at 4°C, followed by Ficoll-Paque (Pharmacia, Uppsala, Sweden) centrifugation. The sedimented cell population was used as T-cells (E rosette purification) and stained with FITC-conjugated TCR-1.

To assess the effects of AMs on the TCR expression, the E rosette purification was performed on BAL cells obtained from two healthy volunteers, two patients with pulmonary sarcoidosis, two patients with IPF, and one patient with hypersensitivity pneumonitis, and the nonrosette fraction collected and used as AMs. Blood T-cells (1×10^6 cells) were mixed with the same number of AMs, suspended in a 1 mL aliquot of RPMI-1640 (GIBCO) containing 10 mM HEPES, 1×10^{-5} M 2-mercaptoethanol, 100 U·mL^{-1} penicillin-G, and 100 µg·mL^{-1} streptomycin and supplemented with 10% foetal calf serum (GIBCO; complete medium), and incubated for 48 h or 96 h using 24-well tissue culture plates (Falcon 3047, Becton Dickinson, Lincoln Park, NJ, USA). Cells recovered by washing the wells were stained with FITC-conjugated TCR-1.

To assess the effects of BAL fluid on TCR expression, BAL fluid was concentrated 100-fold using nitrocellulose filters (Diaflo PM-10, Amicon, Danvers, MA, USA) and added to blood T-cell suspensions (1×10^6 cells·mL^{-1} of complete medium) in various proportions. Aliquots of cell suspension (1 mL) were incubated for 48 h using 24-well culture plates and cells were recovered and stained with FITC-conjugated TCR-1.

Statistical analysis

All data were expressed as mean±SD. The Wilcoxon matched-pairs signed-rank test was used for comparison of the TCR-MFI between alveolar and blood T-cells, between alveolar CD4+ and CD8+ cells, and between cultured blood T-cells with and without AMs at individual time points. The Mann–Whitney U-test was used to assess the differences in MFI among study groups. Multiple testing was corrected by Bonferroni′s method. Repeated-measures analysis under a general linear model was used to assess the effect of AMs on the cultured blood T-cells. Statistical analyses were performed using the SPSS statistical package (SPSS, Chicago, IL, USA). Differences with a p-value <0.05 were considered statistically significant.

Results

BAL cells and blood mononuclear cells were stained with the FITC-conjugated anti-αβTCR antibody, TCR-1. Alveolar lymphocytes and blood lymphocytes were gated, and their flow cytometric profiles in a patient with pulmonary sarcoidosis are shown in figure 1. The histograms clearly demonstrated reduced staining of αβTCR+ alveolar lymphocytes compared with blood counterparts. This finding was similar to that for anti-CD3 stained lymphocytes, as previously reported [16]. All subjects had bimodal distribution of αβTCR expression for both blood and alveolar lymphocytes as did the sarcoid patient shown in figure 1. Hence, αβTCR+ cells formed a single population in terms of αβTCR expression. Interestingly, this pattern of TCR expression was observed not only in patients with pulmonary sarcoidosis, in whom antigen-driven immune responses are likely to be present in the local milieu of the lung, but also in healthy volunteers and patients with various other pulmonary or nonpulmonary diseases. Thus, the MFI of αβTCR+ alveolar lymphocytes was significantly reduced compared with that of αβTCR+ blood lymphocytes in all study groups (fig. 2). In addition, there were no significant differences in the MFI of alveolar T-cells and blood T-cells among all study groups

Differences in TCR MFI (ΔTCR) between blood and alveolar T-cells were calculated and compared among the study groups (fig. 3). There was no significant difference between the control group and individual disease groups. A significant difference was observed only between sarcoidosis and other pulmonary diseases (p=0.04); however, after correction for multiple testing by Bonferroni′s method, it was no longer significant (p=0.24).

As the reduced expression of αβTCR by alveolar T-cells was so common, it seemed possible that the result was influenced by the method employed. One possibility was that an intact anti-TCR antibody with an Fc domain was bound to the Fc-receptors of the abundant AMs leading to a reduced antibody concentration during staining. To exclude this possibility, alveolar and blood T-cells were separated by E-rosette formation, stained, and analysed in
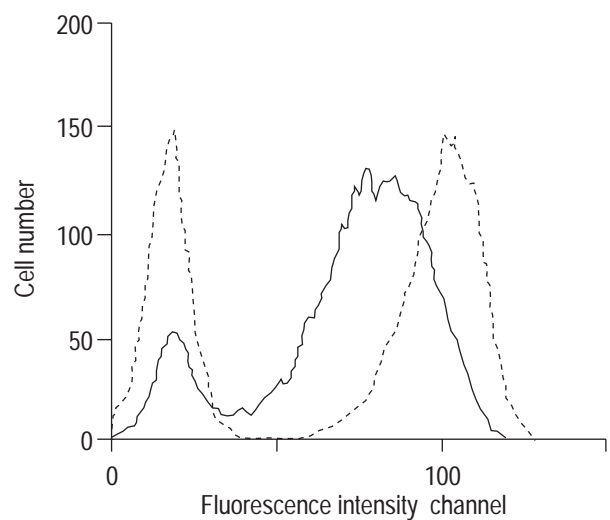

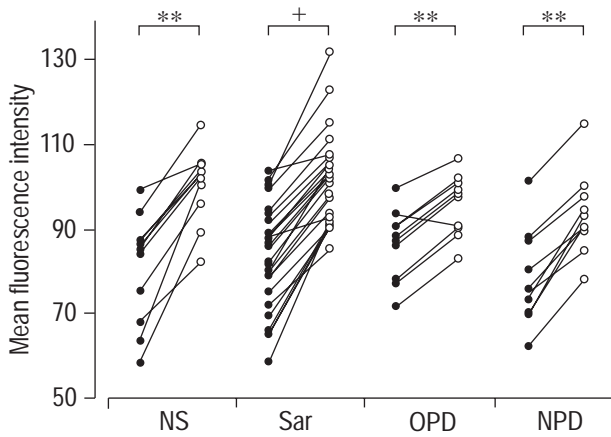Fig. 1. – Flow cytometric profiles of alveolar (——) and blood (- - -) T-cells stained with anti-αβ T-cell receptors (TCR) antibody.

Fig. 2. – Expression of αβ T-cell receptors (TCR) by alveolar (●) and blood (○) T-cells in the different study groups. NS: normal subjects (n=12); Sar: sarcoidosis (n=28); OPD: other pulmonary diseases (n=10); NPD: nonpulmonary diseases (n=10). **: p<0.01; +: p<0.0001.

the same manner as the BAL cells and whole blood. As shown in table 2, the reduced expression of αβTCR by alveolar T-cells was observed again.

To investigate which of the two main subsets of T-cells was amenable to modulation, BAL cells and blood mononuclear cells were simultaneously stained with FITC-conjugated TCR-1 and PE-conjugated T4 or T8 in eight patients with pulmonary sarcoidosis. After gating T4+ or T8+ cells, the MFI of αβTCR+ cells was measured and the difference (ΔMFI) between blood and alveolar T-cells of the same subset was calculated. Both alveolar CD4+ and CD8+ cells expressed significantly less TCR antigen than their blood counterparts (table 3). In addition, CD4+ cells exhibited more intense reduction of TCR than CD8+ cells (table 3). These observations were consistent with previous findings regarding CD3 [16]. The ΔMFI of CD4+ cells did not correlate with a CD4/CD8 ratio of BAL fluid lymphocytes.

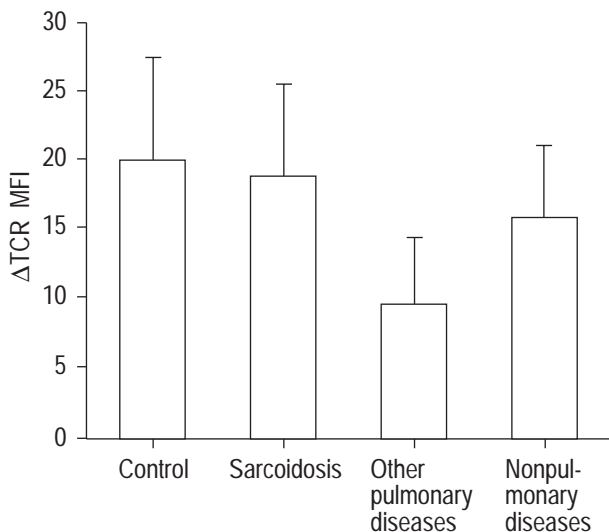As pulmonary surfactant is the main component of the epithelial lining fluid of the lung and is known to be

Fig. 3. – Differences in the expression of αβ T-cell receptors (TCR) between blood and alveolar T-cells (ΔTCR) in the different study groups. Data are presented as mean±SD. MFI: mean fluorescent intensity.

Table 2. – Expression of T-cell receptors by E-rosette purified T-cells

| Case No. | Disease | MFI | |
		Alveolar	Blood
1	Sar	96	108
2	Sar	60	79
3	FLD	71	86

MFI: mean fluorescence intensity; Sar: sarcoidosis; FLD: farmer's lung disease.

immunosuppressive to lymphocytes [19], various proportions of 100-fold concentrated BAL fluid obtained from a patient with pulmonary sarcoidosis were added to cultures of blood T-cells purified from healthy volunteers. Cells were incubated for 48 h using 24-well culture plates. They were then recovered by washing the wells and stained. As shown in table 4, BAL fluid did not affect the expression of TCR in the selected experimental conditions.

AMs are also known to suppress proliferative responses of lymphocytes, especially when cultured in excess proportions [20, 21]. AMs were purified from one healthy volunteer and six patients with various pulmonary diseases, and mixed with autologous blood T-cells at a ratio of 1:1. The effect of AMs on the expression of TCR was significant overall by repeated-measures analysis under the general regression model (fig. 4). With respect to the differences at individual time points, a slight but significant difference of TCR expression was observed at 48 h between blood T-cells cultured with and without AMs; however, this was due to a slight increase in TCR expression in the absence of AMs. At 96 h, the difference between the cultures with and without AMs was significant. However, the average difference of MFI between pre- and post-culture was smaller than that observed between alveolar and blood T-cells of individual subjects (fig. 2). Longer periods of culture resulted in the generation of substantial cell debris in the presence of AMs, which made it impossible to clearly determine the lymphocyte area on dot plots of the flow cytometer. The presence of cell debris mainly derived from AMs had a negligible effect on MFI in the staining process (data not shown). The coculture of blood monocytes with blood T-cells was not associated with similar downregulation of TCR (data not shown).

Discussion

The current study revealed that the expression of αβTCR by alveolar T-cells was decreased as compared with autologous blood T-cells, not solely in sarcoidosis but in a wide variety of pathological and physiological conditions of the lung. It has previously been shown that the expression of CD3 antigen by alveolar T-cells is likewise reduced in health and disease [16]. Since TCR and CD3 molecules are noncovalently bound, it was anticipated that modulation of CD3 would also involve the extracellular domain of TCR. The present study offered direct evidence for this theoretical deduction.

Several stimuli, including antigens, mitogens, and anti-CD3 antibodies, induce modulation of the TCR/CD3 complex [8–13]. As demonstrated by Du Bois et al. [15],

Table 3. – Difference in modulation between CD4+ and CD8+ cells in patients with pulmonary sarcoidosis (n=8)

BALF CD4/CD8	CD4+			CD8+		
	Blood	Alveolar	$\Delta MFI^{\dagger}$	Blood	Alveolar	$\Delta MFI^{\dagger}$
8.7±7.1	108±14*	87±11+	21±8#	101±13	89±12+	12±5

Data are expressed as mean fluorescence intensity (MFI)±SD. BALF: bronchoalveolar lavage fluid. †: difference in MFI between blood and alveolar αβ T-cell receptor (TCR)+ cells; *: $p < 0.05$, compared with blood CD8+ cells; +: $p < 0.05$, compared with blood counterparts; #: $p < 0.05$, compared with ΔMFI of CD8+ cells.

obvious modulation of alveolar T-cells in pulmonary sarcoidosis could be shown. This was considered to be a sign of local triggering of alveolar T-cells in this disease and it was suggested that those T-cells accumulated at sites of disease through an immune response in which antigens or self-antigens stimulate T-cells to proliferate in the local milieu. However, the results of the current study cast doubt upon this notion. Firstly, modulation was present for T-cells from healthy subjects and patients with various pulmonary and nonpulmonary diseases. Secondly, the extent of modulation as expressed by the MFI of TCR did not differ among all study groups. Thus, modulation of αβ-TCR of alveolar T-cells was not a specific phenomenon seen only in pulmonary sarcoidosis. Accordingly, modulation *per se* may not necessarily suggest recent activation by antigens directly responsible for the disease process. Rather, it is possible that modulation is induced by some unique physiological conditions common to the alveolar milieu.

In this regard, AMs could be the candidate to induce modulation, since they have been shown to suppress antigen- or mitogen-induced proliferative responses of lymphocytes depending on the *in vitro* conditions, such as their ratio to lymphocytes and the concentrations of stimuli [20–23] and to attenuate intracytoplasmic free calcium ion responses of T-cells [24]. In the present study, it was found that coculture of AMs with autologous blood T-cells for 4 days reduced the expression of αβTCR. Thus, AMs seemed to be, at least in part, responsible for the modulation. However, whether this process actually involves antigen presentation or is a result of some nonspecific mechanism is not clear. Airborne particles and micro-organisms that may exist in the alveolar milieu, irrespective of the presence or absence of pulmonary diseases, are candidate antigens presented to alveolar T-cells. Alternatively, modulation may be an antigen-independent phenomenon. The observation that coculture of T-cells with AMs could induce partial modulation in the absence of antigens *in vitro* supports this notion. Another alternative explanation is the selective recruitment of recently activated T-cells from the

Table 4. – Effect of concentrated bronchoalveolar lavage fluid (BALF) expression of T-cell receptor by blood T-cells

Experiment 1	Pre-culture		100
	BALF+	0%	90
		5%	89
		10%	93
		20%	94
Experiment 2	Pre-culture		97
	BALF+	0%	94
		2%	94
		20%	93

Data are presented as mean fluorescence intensity. +: volume percentage of concentrated BALF.

bloodstream because T-cells with expression of αβTCR comparable to that by alveolar T-cells are present in blood as demonstrated in figure 1.

Pulmonary surfactants are important components in epithelial lining fluid of alveolar spaces and have long been known to exert suppressive effects on the proliferative responses of lymphocytes [19]. In this study, they were added to the cultures of blood T-cells as concentrated BAL fluid. The results did not support the notion that surfactants or other components in BAL fluid are responsible for modulation of TCR. However, as the concentration of surfactants in epithelial lining fluid *in vivo* cannot be accurately estimated, the results may only be valid for the selected experimental conditions.

Modulation of TCR is in general associated with lowered responsiveness to antigens and mitogens [12–14]. In this regard, the results are superficially inconsistent with previous reports that demonstrated expected heightened or equivalent responses of alveolar T-cells to specific or recall antigens compared with blood T-cells [25, 26]. One possible explanation is that the augmented responsiveness inherent in memory T-cells counterbalances the lowered responsiveness due to modulation of TCR. If this is the case, modulation may serve to prevent exaggerated immune phenomena in the alveolar milieu.

In conclusion, the expression of a T-cell receptor is nonspecifically reduced for alveolar T-cells and therefore, does not represent a sign of activated states induced by specific antigens of a lung disease. The results from *in vitro*
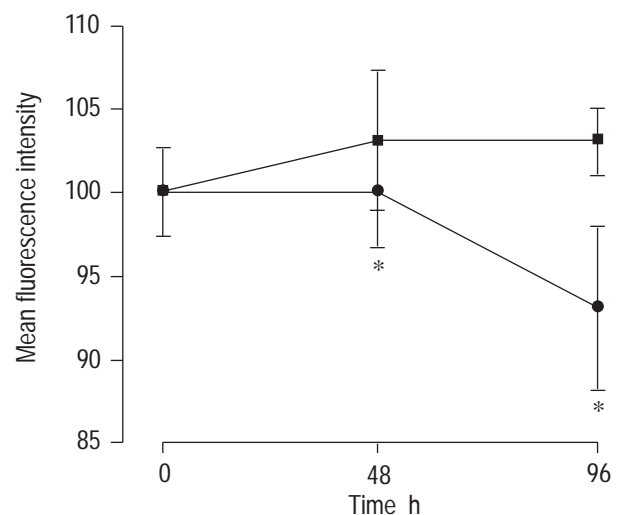
Fig. 4. – Expression of αβ T-cell receptors (TCR) by cultured blood T-cells in the presence (●) or absence (■) of alveolar macrophages. The effect of alveolar macrophages on the expression of TCR was significant overall by repeated-measures analysis under the general regression model ($p < 0.05$). *: $p < 0.05$, compared with blood T-cells cultured in the absence of alveolar macrophages at individual time points.

experiments suggest that alveolar macrophages may be responsible for this phenomenon. Further studies are needed to elucidate the role of modulation in the *in vivo* immune responses of the lung. In addition, its potential influences on *in vitro* proliferative responses of alveolar T-cells should always be borne in mind.

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