

## Suppressive mechanisms of alveolar macrophages in interstitial lung diseases: role of soluble factors and cell-to-cell contact

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*Suppressive mechanisms of alveolar macrophages in interstitial lung disease: role of soluble factors and cell-to-cell contact. E. Fireman, S. Ben-Efraim, S. Spinrad, M. Topilsky, J. Greif. ©ERS Journals Ltd 1993.*

**ABSTRACT:** Alveolar macrophages (AMs) from patients with interstitial lung diseases, such as sarcoidosis and idiopathic pulmonary fibrosis, suppress the phytohaemagglutinin (PHA) stimulation of autologous peripheral lymphocytes. The aim of this study was to determine whether the suppressive effect of alveolar macrophages of patients with interstitial lung disease is due, not only to the secretion of soluble factors prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), interleukin-1 (IL-1) but is also correlated to a direct effect of AMs on the expression of IL-2 receptors (IL-2R: CD25) and on the induction of IL-2 activity.

We studied 26 subjects, 8 with sarcoidosis, 7 with idiopathic pulmonary fibrosis, and 11 controls.

Alveolar macrophages of sarcoid and idiopathic pulmonary fibrosis patients suppressed proliferation of autologous peripheral lymphocytes by 68±14% and 53±4.5%, respectively, compared to enhancement of 19±11% in three controls and suppression of 25±11% in the other six controls; the difference between subjects with interstitial lung disease and controls was significant. As already reported, the alveolar macrophages of sarcoid patients secreted large amounts of IL-1 (184±59 U·ml<sup>-1</sup>) whereas the alveolar macrophages from idiopathic pulmonary fibrosis patients secreted large amounts of PGE<sub>2</sub> (3.6±2 ng·ml<sup>-1</sup>·10<sup>5</sup> cells) compared with 23±19 U·ml<sup>-1</sup> IL-1 and 0.34±0.15 ng·ml<sup>-1</sup>·10<sup>5</sup> cells respectively, of controls. Suppression by supernatants recovered from lipopolysaccharide (LPS) stimulated alveolar macrophages can only partially explain the high suppressive effect of alveolar macrophages of interstitial lung diseases. Co-culture of autologous peripheral lymphocytes with alveolar macrophages of sarcoid and idiopathic pulmonary fibrosis patients markedly reduced the expression of CD25 (down to 66% of the initial value) and decreased induction of IL-2 activity (down to 47% of the initial value).

We conclude that alveolar macrophages of patients with sarcoidosis and idiopathic pulmonary fibrosis suppress expression of IL-2R and decrease induction of IL-2 activity mainly by cell-to-cell contact.

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Macrophages or cells of the monocyte-macrophage lineage play a critical role in the effector phase of a number of phenomena of cell-mediated immunity: they can either enhance or suppress immune reactions [1]. It has been postulated that the macrophages act both *via* soluble monokines [2], and by cell-to-cell contact [3]. The down-regulation activity of macrophages was reported to be due to soluble factors, such as prostaglandins [4, 5], interferons [6], reactive oxygen species [7], low molecular weight peptides [8], or excess interleukin-1 (IL-1) [9].

In previous studies [10-12], we demonstrated that alveolar macrophages (AMs) obtained from patients with interstitial lung disease (ILD) suppress phytohaemagglutinin (PHA) stimulation of autologous peripheral blood lymphocytes (APL). This down-regulation correlated well with the high prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production by AMs recovered from patients with idiopathic pulmonary fibrosis (IPF), and to an excess production of IL-1 by AMs

of sarcoidosis (SA) patients [11]. The role of other soluble factors, such as metabolites of oxygen, in the AM and peripheral monocyte-mediated suppression in ILD was also studied [12]. Surface receptors are essential for the suppression or proliferation of lymphocytes in response to foreign antigens, other immune cells, and cytokines [13]. Hydrophobic protein components [14], and increase of intracellular calcium concentration in lymphocytes [15], were suggested as mechanisms of cell-to-cell contact in the immunosuppressive activity of normal human alveolar macrophages, but little is known about the mechanism of AM-mediated suppression in pathogenesis of the lung.

The aim of the present study was to investigate the extent to which the immunosuppression by AMs in ILD is correlated to their effect on T-cells. We show here that ILD AMs suppress expression of IL-2 receptor (IL-2R: CD25) and decrease induction of IL-2 activity by cell-to-cell contact.

## Patients

Twenty seven patients were included, belonging to three groups:

1. Pulmonary sarcoidosis (SA). Diagnosis was made in eight untreated patients (five males and three females,  $37 \pm 7$  yrs of age), by clinical and roentgenological presentation, a positive Kveim test, or a positive biopsy of non-caseating granuloma. All patients were in Stage II of the disease and none was a smoker.

2. Idiopathic pulmonary fibrosis (IPF). Seven patients (five women and two men) were included. Diagnosis of IPF was made by roentgenological evidence of reticular infiltration, and different degrees of interstitial fibrosis were demonstrated by transbronchial biopsy. The mean age of the patients was  $61 \pm 10$  yrs and none was a smoker.

3. Controls (C). Eleven patients with a mean age of  $21 \pm 9$  yrs (nine males and two females) were admitted for investigation due to persistent cough. All of them presented chest roentgenograms within normal limits. Three of them were present smokers.

None of the patients received any drugs prior to the study. Written consent was obtained from each subject before bronchoscopy. Characterization of cell populations present in bronchoalveolar lavage (BAL) of all patients examined is summarized in table 1.

## Methods

### Bronchoalveolar lavage (BAL)

BAL was performed with a BF-B2 flexible fiberoptic bronchoscope (Olympus Optical Co., Ltd, Tokyo, Japan). Subjects were premedicated with diazepam 5 mg, pethidine 50 mg, atropine 0.5 mg, and the airways were anaesthetized by inhalation of 4% xylocaine. Boluses of 50 ml of 0.9% saline, previously warmed at  $37^\circ\text{C}$ , up to a total volume of 150–200 ml, were instilled, with the bronchoscope wedged into a subsegmental bronchus of the right or left lower lobe. The cells were recovered by gentle aspiration. The percentage of lavage fluid ( $\pm$ SD) recovered from each group of patients was as follows:  $67 \pm 10\%$  from C and  $58 \pm 8\%$  from ILD cases.

The average total cells recovered was:  $5 \pm 1.3 \times 10^6$  cells from C and  $17 \pm 7.2 \times 10^6$  cells from ILD patients.

### Preparation of alveolar macrophages (AMs)

The recovered fluid was collected into specimen traps, filtered through sterile gauze and centrifuged at  $400 \times g$  for 15 min at  $4^\circ\text{C}$ . Differential cell count was performed on a Giemsa-stained cytocentrifuge preparation (Cytospin; Shandon, Southern Products Ltd, Runcorn, Cheshire, UK), by counting a minimum of 500 cells. The pellet was washed three times with cold phosphate buffered saline (PBS) (Biological Industries, Beit Haemek, Israel) and cells were adjusted to a final concentration of  $10^6$  cells·ml<sup>-1</sup>, in RPMI 1640 medium, supplemented with 10% fetal calf serum (FCS), 1% L-glutamine, and 1% streptomycin, penicillin, and mycostatin (Biological Industries, Beit Haemek, Israel). The AMs were purified by adherence for 1 h, at  $37^\circ\text{C}$ , in a 5% CO<sub>2</sub> humidified atmosphere. Identification of macrophages was made by morphology and nonspecific esterase staining, and counted with an objective micrometer (Olympus Optical Co. Ltd, Tokyo, Japan). The adherent cell population contained more than 90% AM.

### Preparation of autologous peripheral lymphocytes and peripheral lymphocytes

Autologous peripheral lymphocytes (APLs) were obtained from heparinized venous blood from each subject on the same day as the BAL AMs. Peripheral lymphocytes (PLs) were collected from normal volunteers, on the day of culturing with AM supernatants. Peripheral mononuclear cells were isolated by centrifugation on Ficoll-Hypaque gradient (Pharmacia Fine Chemicals AB, Uppsala, Sweden) washed three times with PBS, and resuspended in RPMI 1640, supplemented with 10% FCS, 1% L-glutamine, streptomycin, penicillin, and mycostatin (Biological Industries, Beit Haemek, Israel). The cells were incubated for the same period of time, and tested before and after incubation for naphthyl esterase activity. They were found to be 25 and 7% positive, respectively. Non-adherent APLs and PLs were resuspended to a final concentration of  $7.5 \times 10^6$  cells·ml<sup>-1</sup>.

Table 1. – Differential counts of bronchoalveolar lavage (BAL) cells

		Macro %	Lymph %	Neut %	Bas %	Eos %
SA	n=8	$59 \pm 20$ (29.8–80.6)	$39 \pm 20^*$ (16–69)	$2 \pm 1$ (1–4)	$0.2 \pm 0.01$ (0.2–0.25)	$0.5 \pm 0.4$ (0.2–1.2)
IPF	n=7	$56 \pm 24$ (28.2–82.8)	$11 \pm 9$ (2.0–25)	$24 \pm 27^*$ (6.4–69.8)	$0.6 \pm 0.4$ (0.2–0.8)	$18 \pm 25^{**}$ (1.8–61.6)
C	n=11	$87 \pm 6$ (80–92)	$10 \pm 7$ (1.8–20)	$1 \pm 0.7$ (0.4–1.8)	-	-

Results are expressed as mean  $\pm$  SD of percentage of total cells counted, and range in parentheses. \*:  $p < 0.02$  compared to C; \*\*:  $p < 0.05$  compared to SA and C; Macro: macrophages; Lymph: lymphocytes; Neut: neutrophils; Bas: basophils; Eos: eosinophils; SA: sarcoidosis; IPF: idiopathic pulmonary fibrosis; C: control.

### Suppressive cell activity assay

The lymphocyte suspension was overlaid on the adherent cells ( $6.5-8.0 \times 10^5$  cells·ml<sup>-1</sup>) at a final concentration of  $7.5 \times 10^5$  cells·ml<sup>-1</sup> (1:1 ratio). Each co-culture was performed in quadruplicate, and stimulated with 1 µg·ml<sup>-1</sup> PHA (Wellcome Research Lab., Beckenham, Kent, UK). Control cultures were incubated at 37°C, in a 5% CO<sub>2</sub> humidified atmosphere, for 72 h. A quantity of 1 µCi of tritiated thymidine was added to each culture for the last 4 h of incubation. The cells were harvested through glass fibre filters and counted in a liquid scintillation counter. The results were expressed as mean counts·min<sup>-1</sup>, with the background counts·min<sup>-1</sup> in unstimulated cultures subtracted. The percentage suppression or percentage enhancement was calculated by rapport between lymphocytes with and without AM.

### Preparation of AM supernatants

AMs were cultured in complete medium (RPMI 1640, supplemented with 1% FCS, glutamine and antibiotic-antimycotic mixture) at  $1 \times 10^6$  cells·ml<sup>-1</sup>, in 3 cm diameter plastic Petri dishes (Sterilin, Hounslow, Middlesex, UK), either for 24 h or 72 h. The 24 h period was found to be optimal for testing production of IL-1 in the presence of lipopolysaccharide (LPS) *E. coli* 055:B5 (Difco Lab., Detroit, USA, 10 µg·ml<sup>-1</sup>). The 72 h period was chosen as the optimal time for release of PGE<sub>2</sub> in the absence of LPS. Supernatants tested for their effect on CD25 expression and IL-2 production were collected from AM cultures incubated for 24 h with or without LPS. Supernatants were recovered, filtered (Acrodisc 0.2 µ, Gelman Sciences) and stored at -70°C until determination for IL-1, and not longer than two weeks for PGE<sub>2</sub>.

### Assay of prostaglandin and IL-1 production

Aliquots of AM supernatants were assayed for PGE<sub>2</sub> and IL-1 production. PGE<sub>2</sub> measurement was done by an enzyme-linked immunosorbent assay (ELISA) kit (Advanced Magnetic Inc., Massachusetts, USA), and IL-1 by a C3H/HeJ thymocyte comitogenic assay, as described previously [12]. Specific IL-1 α and β were measured by radio-immunoassay (RIA) kits (Amersham M<sup>MT</sup> magnetic separation, Amersham International PLC Amersham, UK).

### Indirect immunofluorescence method for monoclonal antibodies staining

PLs and APLs at a final concentration of  $7.5 \times 10^5$ ·ml<sup>-1</sup> were incubated with AM supernatants (1:1 dilution), and with adherent AMs ( $6.5-8.0 \times 10^5$  cells·ml<sup>-1</sup>) respectively, and pulsed with 1 µg·ml<sup>-1</sup> PHA for 72 h. At the end of the incubation period, the non-adherent cells were washed

with cold PBS, supplemented with 2% FCS and 0.1% sodium azide, and resuspended to a final concentration of  $1 \times 10^6$  cells·ml<sup>-1</sup>. PLs and APLs were stained by the indirect immunofluorescence method. The cells were incubated with 0.02 ml of first antibody: CD25 (anti IL-2R Becton Dickinson, Mountain View, CA, USA), for 30 min, washed with cold PBS, and incubated with 0.005 ml of second antibody (Goat fluorescein isothiocyanate (FITC) conjugated immunoglobulin G (IgG) antimouse, Becton Dickinson) for 30 min. After washing, cells were fixed with paraformaldehyde and analysed using a Becton Dickinson fluorescence-activated cell sorter (FACS) with 300 W 488 nm laser excitation, and with 525 nm (±20 nm) band filter. Control cultures were performed with unstimulated cultures, with or without AMs or AM supernatants. The high side scatter population (macrophages) were excluded from the final analysis. Exogenous IL-1α and IL-1β (Glaxo-IMB, Geneva, Switzerland) and PGE<sub>2</sub> (Sigma Chemical Co., St. Louis, USA) were added and PLs tested for CD25 expression.

### IL-2 Determination

The IL-2 dependent cytotoxic murine T-cell line, CTLL-2, was used for assay. CTLL-2 cells were extensively washed and resuspended in complete RPMI 1640 medium, (supplemented with 1% sodium pyruvate, 1% non-essential amino acids, 1% glutamine, and 1% antibiotic-antimycotic mixture). Flat-bottomed 96-microwell plates were filled with complete RPMI 1640 medium, containing 10% FCS, 1% sodium pyruvate, 1% non-essential amino acids, 1% glutamine, 1% antibiotic-antimycotic mixture and  $5 \times 10^{-5}$  M mercaptoethanol. Serial dilutions (1:2-1:1,024) of undiluted standard IL-2 and supernatant samples were performed in triplicate horizontal rows. Five  $\times 10^4$ ·ml<sup>-1</sup> CTLL-2 cells·well<sup>-1</sup> were added and incubated for 48 h. Control cultures were performed by incubating cells with medium alone.

Standard IL-2 prepared from Wistar splenocytes, with assigned activity of 100 U·ml<sup>-1</sup>, were tested with each set of test samples. The resultant dose response data were used to plot the regression lines for the standard and the test samples. The sample dilution yielding 30% of the maximum counts per minute (cpm) obtained with the standard IL-2 preparation was determined from the regression lines. The activity in the test samples was transformed into units according to the formula:

$$\frac{\text{Reciprocal titre of test sample at 30\% of maximal cpm of standard}}{\text{Reciprocal titre of standard at 30\% of maximal cpm of standard}} \times 100 = \text{U}\cdot\text{ml}^{-1} \text{ sample}$$

The <sup>3</sup>H-thymidine incorporation in CTLL-2 cells, in the presence of supernatants from the incubation of PL+AM supernatants and APL+AM cultures, was compared with that of PL and APL alone in the same experiment.

Specific determination of IL-2 was also measured by an ELISA kit (Advanced Magnetic Inc., Massachusetts, USA).

## Statistics

The data were expressed as mean $\pm$ SD. The analysis of variance (ANOVA) test was used for statistical evaluations, using the Epistat software, Copyright (c) 1984TL Gustafson.

## Results

*Effect of AMs on PHA-induced proliferation of APLs*

The effect of AMs on PHA-induced proliferation of APLs was tested in 21 patients (fig. 1). Twelve patients suffering from interstitial lung disease (ILD) showed a high rate of suppression of 68 $\pm$ 14% in the SA group, and 53 $\pm$ 4.5% in the IPF-group ( $p < 0.001$ , between ILD and the C group). In the C group, there was an enhancement, with a mean value of 19 $\pm$ 11% in three of the patients, but a suppression of 25 $\pm$ 11% was seen in six of the others.

*PGE<sub>2</sub> and IL-1 production by AMs*

In confirmation of previous results [11] analysis of PGE<sub>2</sub> secretion revealed a marked, significant increase in IPF patients, whereas release of PGE<sub>2</sub> from AMs of SA patients was within normal limits. LPS-induced production of IL-1 by AMs of SA patients was markedly increased, whereas IL-1 production was normal in IPF (table 2). IL-1 secretion without LPS was between 0.05–0.07 ng·ml<sup>-1</sup> for IL-1 $\alpha$  and IL-1 $\beta$ , in all groups. IL-1 secretion without LPS measured by thymocyte assay showed measurable levels in only two patients with SA (2–14 U·ml<sup>-1</sup>) and one patient with IPF (9 U·ml<sup>-1</sup>).

*Effect of AM supernatants on PHA-induced PL proliferation*

Supernatants from stimulated and non-stimulated AMs were tested (fig. 2). Supernatants from non-stimulated AM supernatants recovered from IPF and SA patients produced only a slight suppression (11 $\pm$ 7% and 16 $\pm$ 11%, respectively). In LPS-stimulated AMs, the suppressive effect was moderately stronger (21 $\pm$ 8% and 29 $\pm$ 10%, respectively). In the C group, the non-stimulated AM supernatants induced proliferation of PL by 4 $\pm$ 2.2% in three cases, and was suppressed by 23% in the fourth.

Table 2. – PGE<sub>2</sub> secretion and IL-1 release by alveolar macrophages (AMs)

	PGE <sub>2</sub> secretion*		IL-1 production**	
	ng·ml <sup>-1</sup> ·10 <sup>-5</sup>	U·ml <sup>-1</sup>	IL-1 $\alpha$ ng·ml <sup>-1</sup>	IL-1 $\beta$ ng·ml <sup>-1</sup>
SA	0.40 $\pm$ 0.2	184.4 $\pm$ 59.3*	0.8 $\pm$ 0.7*	2.1 $\pm$ 2.4*
IPF	3.56 $\pm$ 2.0**	33.7 $\pm$ 28.7	0.15 $\pm$ 0.02	0.16 $\pm$ 0.11
C	0.34 $\pm$ 0.15	23.1 $\pm$ 19	0.08 $\pm$ 0.02	0.17 $\pm$ 0.08

The results are expressed as mean $\pm$ SD. \*: PGE<sub>2</sub> cells were tested in supernatants recovered from unstimulated AMs after 72 h of culture; \*\*: IL-1 was tested in supernatants recovered from LPS stimulated AMs after 24 h of culture; \*:  $p < 0.001$  compared to C and IPF; \*\*:  $p < 0.001$  compared to C and SA; #:  $p < 0.05$  compared to C; PGE<sub>2</sub>: prostaglandin E<sub>2</sub>; IL-1: interleukin-1; LPS: lipopolysaccharide. For further abbreviations see legend to table 1.

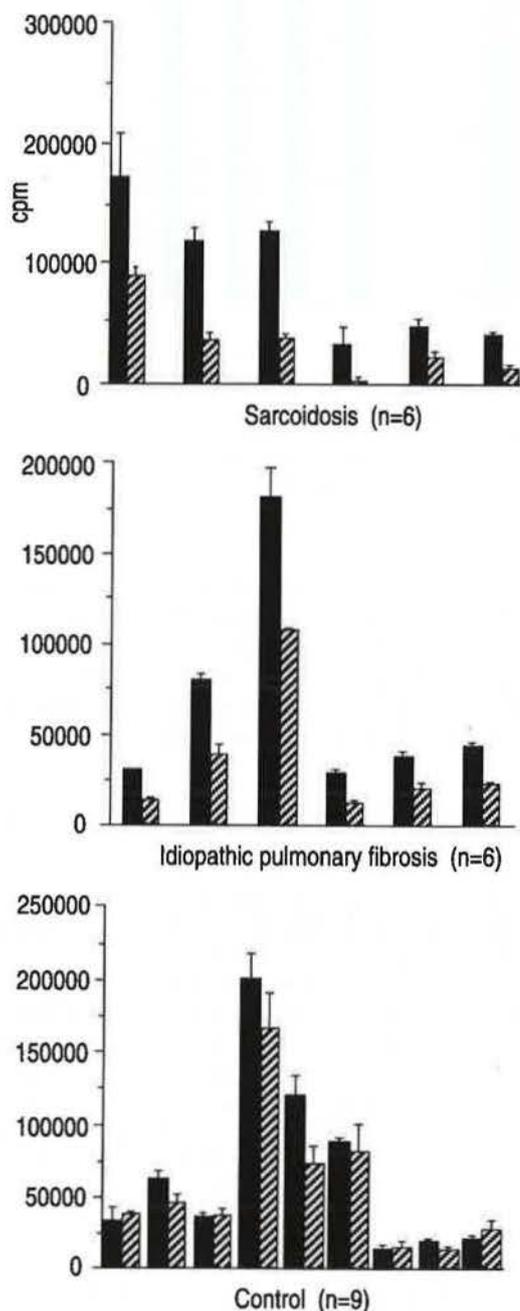


Fig. 1. – Effect of AMs on PHA-induced blastic proliferation of APLs. Cells were co-cultured for 72 h; 1  $\mu$ g·ml<sup>-1</sup> <sup>3</sup>H-thymidine was added for the last 4 h of incubation. Results are expressed as mean $\pm$ SD of counts per minute (cpm) of triplicate well cultures for each subject, with the background of unstimulated response subtracted. ■: APLs stimulated by PHA. ▨: APLs incubated with AMs and stimulated by PHA. AM: alveolar macrophage; PHA: phytohaemagglutinin; APL: autologous peripheral blood lymphocyte.

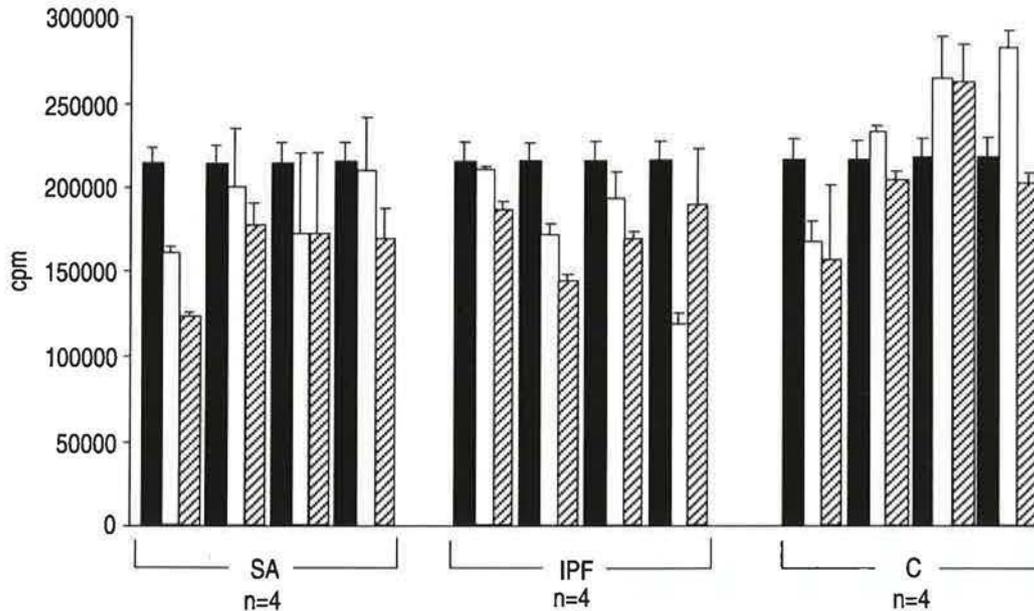


Fig 2. - Effect of AM supernatants on PHA-induced blastic proliferation of PLs. Cells were co-cultured for 72 h;  $1 \mu\text{g}\cdot\text{ml}^{-1}$  3H-thymidine was added for the last 4 h of incubation. ■: PL stimulated by PHA; □: PL incubated with AM supernatants (-LPS) and stimulated by PHA; ▨: PL incubated with AM (+LPS) and stimulated by PHA. SA: sarcoidosis; IPF: idiopathic pulmonary fibrosis; C: controls; PLs: peripheral blood lymphocytes. For further abbreviations see legend to figure 1.

In the AM stimulated supernatants the PL proliferation was suppressed by  $13\pm 11\%$  by three supernatants, and enhanced by 20% in the fourth. ( $p < 0.02$ , between SA or IPF versus C in the effect of supernatants from AM cultures stimulated with LPS).

#### Effect of AMs on the induction of CD25 expression and IL-2 secretion of T-lymphocytes

In view of the wide variation in the percentage expression of CD25 positive cells, and in the induction of IL-2 activity, in control samples of various experiments, the effect of AM co-culturing with APLs was compared with that of APLs in each of the individual experiments. Co-culturing with AMs in SA and IPF cases led to suppression of  $38\pm 17\%$  of CD25 positive cells, as compared with corresponding APL cultures alone. The inhibitory effect of AM from ILD cases was observed in all 10 cases: five SA and five IPF cases. AMs from controls reduced CD25 expression by a mean of 8% in five cases (table 3).

Supernatants collected from AM cultures stimulated or not stimulated with LPS had no significant effect on expression of CD25 positive cells, as compared with the percentage of CD25 positive cells in a PL population cultured in the absence of supernatants (table 4).

Supernatants from APL cells cultured alone or together with AMs were added to CTLL-2 IL-2 dependent line. Addition of supernatants from APLs co-cultured with AMs of SA and IPF led to reduction in thymidine incorporation by  $53\pm 5\%$ , in comparison with supernatants from corresponding APLs cultured without AMs. On the other hand, addition of supernatants from C APLs

cultured with AMs resulted in only a slight reduction ( $19\pm 4\%$ ), as compared with supernatants from C APLs cultured alone. Thus, induction of IL-2 secretion was reduced in the presence of AMs from SA and IPF patients. Similar results were shown when supernatants were tested by an ELISA immunoassay (table 5).

#### Effect of exogenous $\text{PGE}_2$ and IL- $1\alpha$ and $\beta$ on CD25 expression

Exogenous  $\text{PGE}_2$  was added at increasing concentrations to normal peripheral lymphocytes stimulated by PHA, and its effect on CD25 expression was determined. Low concentrations of  $\text{PGE}_2$  produced a mild suppression of CD25 expression ( $15\pm 3\%$ ). Only very high concentrations of  $\text{PGE}_2$  ( $1,000\text{--}10,000 \text{ ng}\cdot\text{ml}^{-1}$ ) reduced the number of IL-2R on lymphocytes by  $36\pm 2\%$ . Increasing concentrations of IL- $1\alpha$  and  $\beta$  were added to normal peripheral lymphocytes stimulated with PHA. A maximal suppression of CD25 expression was achieved by  $100 \text{ ng}\cdot\text{ml}^{-1}$  IL- $1\alpha$  ( $25\pm 0.7\%$ ) and IL- $1\beta$  ( $18\pm 7\%$ ) (table 6).

#### Effect of anti-human leucocyte antigen-DR (HLA-DR) and anti-IL- $1\alpha$ on CD25 expression

The inhibitory effect of AMs from ILD cases on CD25 expression was compared with that of anti HLA-DR and anti IL- $1\alpha$  antibodies, in order to test the possible role of HLA-DR antigens and the membranal form of interleukin 1 (IL- $1\alpha$ ) on the AM-mediated suppression of peripheral lymphocytes. AMs were preincubated, with and without monoclonal antibodies (MoAbs) to HLA-DR

(1:50 dilution, Becton Dickinson, Mountain View, CA, USA) and to IL-1 $\alpha$  (2.8  $\mu\text{g}\cdot\text{ml}^{-1}$ , Genzyme Co., Boston, MA, USA). The results showed that these membrane antigens had only helper function, as CD25 expression was reduced by 29 and 30% when lymphocytes were cultured

with AMs preincubated with MoAbs to HLA-DR and IL-1 $\alpha$  receptors, respectively. Maximal suppressive effect (40%) was seen when AMs were preincubated with both MoAbs (fig. 3). Controls were performed by stimulating APLs and AMs separately.

Table 3. - Effect of alveolar macrophages (AMs) on the % of IL-2R (CD25) positive APL\*

Pt no.	SA**					IPF*					C**				
	1	2	3	4	5	1	2	3	4	5	1	2	3	4	5
% APL*	54	37	49	36	52	39	50	65	64	30	76	23	82	57	11
% APL+AM*	32	31	31	19	24	22	34	50	56	11	62	19	79	50	11
% Suppression*	40	15	37	50	60	44	31	22	13	64	19	17	3	1	0

\*: percentage positive APL cells (incubated with and without AM) out of 10,000 cells counted; AMs were co-cultured for 72 h with autologous peripheral lymphocytes (APL) in the presence of 1  $\mu\text{g}\cdot\text{ml}^{-1}$  PHA; \*\*: mean expression of CD25 APL and APL+AM in SA group=46 $\pm$ 8.5 and 27 $\pm$ 5.7%, respectively, (p<0.008); \*: mean expression of CD25 APL and APL+AM in IPF group=47 $\pm$ 13.2 and 28 $\pm$ 14.8, respectively, (p<0.001); \*\*: mean expression of CD25 APL and APL+AM in C group=44 $\pm$ 32 and 36 $\pm$ 32, respectively, (ns); #: percentage suppression from APL positive cells; PHA: phytohaemagglutinin; IL-2R: interleukin-2 receptor; APL: autologous peripheral blood lymphocytes. For further abbreviations see legend to table 1.

Table 4. - Effect of alveolar macrophage (AM) supernatant\* on the IL-2R (CD25) positive PL\*\*

Pt no.	SA			IPF			C	
	1	2	3	1	2	3	1	2
% PL	52	30	47	17	56	39	56	59
% PL+Sup	50	34	46	15	62	27	49	52
% Suppression or enhancement*	4	-4	-3	7	-1	30	-6	-5

\*: supernatants were recovered from AMs after 24 h of incubation with LPS; peripheral lymphocytes were incubated for 72 h with the recovered supernatants and pulsed with 1  $\mu\text{g}\cdot\text{ml}^{-1}$  PHA; \*\*: percentage positive PL cells (incubated with and without supernatant) out of 10,000 cells counted; AM were co-cultured for 72 h with autologous peripheral lymphocytes (APL) in the presence of 1  $\mu\text{g}\cdot\text{ml}^{-1}$  PHA. No significant differences were found between PL and PL+Sup corresponding groups; #: percentage suppression or enhancement from PL positive values. Positive values mean suppression; negative values mean enhancement. PL: peripheral lymphocyte; Sup: supernatant. For further abbreviations

Table 5. - Effect of alveolar macrophages (AMs) on the induction of IL-2 activity

AM origin	APL*	IL-2 secretion			pg·ml <sup>-1</sup> †		
		U·ml <sup>-1</sup> **	APL+AM*	% supp **	APL	APL+AM	% supp**
SA	165782 $\pm$ 13800	71694 $\pm$ 5527	57*	48	20	59	
SA	12062 $\pm$ 2054	6138 $\pm$ 1330	48	40	2	95	
IPF	10682 $\pm$ 1526	4318 $\pm$ 1336	60*	30	2	94**	
IPF	20519 $\pm$ 1819	10771 $\pm$ 2318	48	300	25	92	
C	81362 $\pm$ 4828	63371 $\pm$ 16256	22	72	50	31	
C	61949 $\pm$ 1010	52203 $\pm$ 1029	16	40	30	25	

\*: the cells were cultured for 72 h in the presence of supernatants originating either from APL cultures only, or from same APL co-cultured with AMs; \*\*: <sup>3</sup>H-thymidine incorporation by the CTLL-2 IL-2-dependent cell line; †: measured by ELISA method; \*\*: percentage suppression from values of IL-2 secretion of APL alone; #: p<0.05, IPF and SA compared to C; \*\*: p<0.05, IPF compared to C. CTLL-2: cytotoxic murine T-cell line; ELISA: enzyme-linked immunosorbent assay; supp.: suppression. For further abbreviations see legends to tables 1-3.

Table 6. - Effect of increasing concentrations of IL-1- $\beta$  and  $\alpha$  and PGE<sub>2</sub> on the % of CD25 positive cells

Reagent added	Concentration of reagent							
	0	0.01	0.1	1	10	100	1000	10,000ng·ml <sup>-1</sup>
IL-1 $\beta$	55±1.2	51±6.8	50±3.8	48±4.6	48±1.0	45±1.0		
IL-1 $\alpha$	55±1.2	48±1.2	46±1.0	47±5.0	44±2.0	42±0.3		
PGE <sub>2</sub>	54±0.9	48±0.4	44±0.5	44±2	45±1	44±3	37±1	34±2

Results are expressed as % CD25 (IL-2R) positive cell for each concentration of reagent added. Normal peripheral lymphocytes were incubated for 72 h with PGE<sub>2</sub> and IL-1 stimulated with 1  $\mu$ g·ml<sup>-1</sup> PHA and expression of CD25 was determined. The mean±SD values represents the results of two consecutive experiments. For abbreviations see legends to tables 2 and 3.

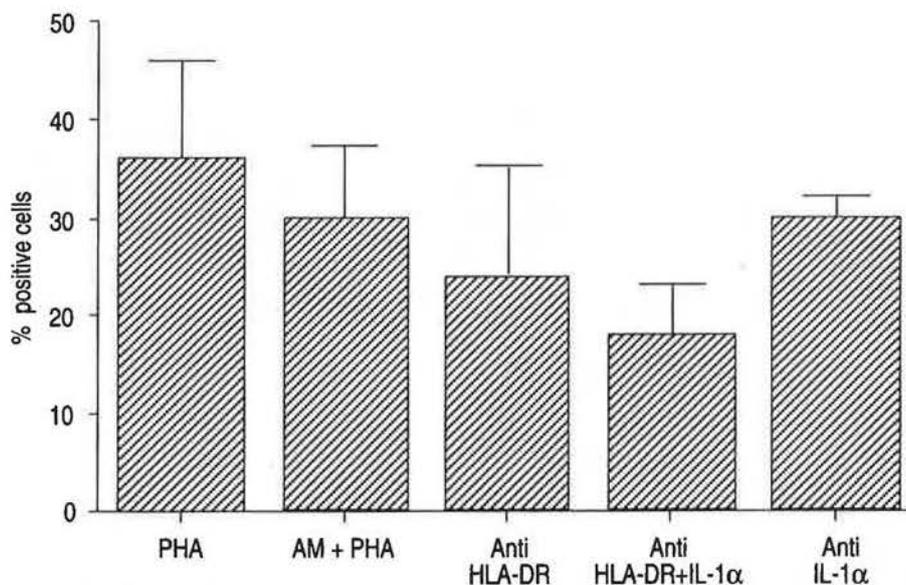


Fig. 3. - Effect of MoAbs to HLA-DR and IL-1 $\alpha$  on the CD25 expression of lymphocytes. AMs were incubated for 1 h with respective MoAbs (anti-HLA-DR, anti-IL-1 $\alpha$ ), washed and then overlaid by APLs of ILD patients (1 SA and 2 IPF). Both types of cells were stimulated for 72 h with 1  $\mu$ g·ml<sup>-1</sup> PHA. APLs were tested for the expression of (CD25) IL-2R. MoAb: monoclonal antibody; HLA-DR: human leucocyte antigen-DR; IL-1 $\alpha$ : interleukin-1 $\alpha$ ; ILD: interstitial lung disease. For further abbreviations see legends to figures 1 and 2.

### Discussion

Detailed studies on the mechanism of human blood monocyte and alveolar macrophage-mediated suppression of T-cell activation, suggest that more than one pathway is operative. Both soluble factors [16-19], and macrophage interaction [20-22] with T-lymphocytes have been shown to participate in macrophage-mediated suppression of lymphocytic proliferation [23-25]. We have previously shown that the immunomodulating effect of AMs was related to the number of AMs added to APLs: enhancing at low ratios and suppressing at high ratios [10]. These results confirm previously published studies, which show enhanced stimulatory activity of AMs on lymphocyte proliferation when small quantities of AMs are added to culture [26, 27], but not when a high ratio of AM:APL is used [28]. Since AMs greatly outnumber lymphocytes in normal bronchoalveolar lavage, it would seem that the highest suppression seen with 50% AMs resembles the *in vivo* situation.

The relative role of this cell-to-cell dependent suppressive pathway *versus* secretion of suppressive factors by AMs remains to be established. In the present work, two experimental systems were set up: cell-to-cell contact between AMs and APLs, and addition of supernatants of

AMs or soluble products of AMs to lymphocytes. The aim was to determine whether immunosuppression by AMs from ILD patients is due not only to high production of PGE<sub>2</sub> or IL-1, but is also correlated to the effect of AMs on IL-2R (CD25) expression and on the induction potency of IL-2 activity in peripheral lymphocytes.

The main finding arising from the present experiments is that co-culture with AMs from ILD patients, but not AM supernatants, markedly diminished the expression of CD25 in an APL population. Moreover, supernatants from cultures of APL+AM from ILD patients showed decreased IL-2 activity.

Similar work by KIERSZEMBAUM *et al.* [29] showed that in Chagas' disease, the *Trypanosoma cruzi* and their supernatant, cultured with peripheral blood mononuclear cells, produced a striking reduction of IL-2R after activation with either PHA or CD3 MoAbs. Inhibition of lymphoid cell growth during cell-to-cell contact was also found to be induced by a lipid-like component of macrophage hybridoma cells [30-32]. Moreover, IL-2 exerts a monocyte-dependent, prostaglandin (PG)-independent suppressive influence on human natural killer (NK) cells, which is associated with contact with the p55 ( $\beta$ ) moiety of the IL-2R [33].

AMs added directly to peripheral blood mononuclear

cells inhibited blastogenesis when present in co-culture with responder cells, but not when the cell populations were separated by 0.45  $\mu\text{m}$  filter, that permitted diffusion of soluble factors but not cells [14]. In our case, we showed partial suppression of blastogenesis by AM supernatants recovered from ILD patients. The differences between our findings and those of RICH *et al.* [14] may be due to the fact that supernatants recovered from AMs of ILD contain a high amount of soluble factors, which are partially suppressive.

It seemed of interest to us to determine whether addition of large amounts of PGE<sub>2</sub> or IL-1 would affect expression of IL-2R, in view of the release of high amounts of PGE<sub>2</sub> by AMs from IPF patients [11, 12], and of IL-1 by AMs from SA patients [12]. We found that exogenous PGE<sub>2</sub>, at concentrations of 10<sup>3</sup>–10<sup>4</sup> ng·ml<sup>-1</sup>, diminished CD25 expression by 26%. These PGE<sub>2</sub> concentrations are detected in AM supernatants from IPF cases (3.56±2.0 ng·ml<sup>-1</sup>·10<sup>5</sup> cells). The suppressive activity of PGEs and their precursors on IL-2 production and CD25 expression has already been reported [34]. Moreover, both exogenous administration and endogenous production of PGE<sub>2</sub> have been shown to inhibit several important lymphocyte and macrophage functions. PGE<sub>2</sub> inhibits NK cell activity [5], monokine release [35], and lung fibroblast growth [36]. Exogenous IL- $\alpha$  and  $\beta$  inhibited CD25 expression at concentrations of 1–100 ng·ml<sup>-1</sup> which are within the range of IL-1 present in the supernatants recovered from SA patients (0–4 ng·ml<sup>-1</sup>). Inhibitory effects of IL-1 have been reported in monocytes of tuberculous patients [37], and, together with tumour necrosis factor  $\alpha$  (TNF $\alpha$ ), on proliferating fibroblasts [38]. On the other hand, a stimulatory effect of IL-1 $\alpha$  has been reported [39, 40]. This effect is due to small amounts of IL-1 $\alpha$  (1 U·ml<sup>-1</sup>), which affect only the high affinity IL-2 binding sites and not the CD25 receptor. Moreover, the membranal form of IL-1 (IL-1 $\alpha$ ) [41], whether correlated or not with immune region associated (Ia) antigens, has no effect on the AM-mediated suppressive cell activity.

In sarcoidosis, the persistent recruitment of peripheral monocytes may explain the high suppressive activity of AMs, as immature macrophages were found to be highly suppressive [42]. Moreover, alveolar macrophages in their different stages of maturation/differentiation were found to be activated in sarcoidosis, releasing increased IL-1 [43]. This high secretion of IL-1, which was correlated with intensity of alveolitis, was found to down-regulate IL-2 secretion by T-cells in the lung in sarcoidosis [44]. On the other hand, in IPF, the high secretion of PGE<sub>2</sub> may be a suppressive factor for IL-2R and IL-2 secretion [34], down-regulating the IL-2-dependent CD4 cell proliferation. In fact, in IPF, a relative predominance of CD8<sup>+</sup> subsets was reported in lung lavage [45]. Although neutrophils seem to participate in the pathogenesis of this disease [46], T-cells appear to play an important role, since a collagen-induced production of migration inhibitory factor, and cytotoxicity by circulating T-cells, was reported in patients with IPF [47].

Our findings, showing a possible suppressive effect of soluble factors, may be important in the field of thera-

peutic inhibition or control of tissue fibrotic response, but the role of cell-to-cell contact interactions in the development of ILD is still unclear. BRODY *et al.* [48] showed that such interactions between epithelial and mesenchymal cells take place in certain cases of spontaneous pulmonary fibrosis. Moreover, when lung injury is induced in mice by a combination of butylated hydroxytoluene and hyperoxia, interaction between various alveolar cells was described [49]. Whether the cell-to-cell contact observed in these instances is accompanied by specific alterations in phenotypic expression of alveolar T-cells remains to be established.

In conclusion, identification of the potent immunosuppressant factor on AM membranes could advance research for a naturally occurring regulatory mechanism that controls fibrosis in patients with interstitial lung diseases.

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