Eur Respir J, 1996, 9, 1456–1462
DOI: 10.1183/09031936.96.09071456
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European Respiratory Journal
ISSN 0903 - 1936

Altered immunosuppressive activity of alveolar macrophages in farmer's lung disease

A. Dakhama, E. Israël-Assayag, Y. Cormier

Altered immunosuppressive activity of alveolar macrophages in farmer's lung disease. A. Dakhama, E. Israël-Assayag, Y. Cormier. ©ERS Journals Ltd 1996.
ABSTRACT: Since normal alveolar macrophages (AMs) can suppress T-cell proliferation to mitogenic and antigenic stimuli both *in vitro* and *in vivo*, we questioned whether an altered AM immunosuppressive activity could account for the alveolar lymphocytosis observed in farmer's lung (FL) and whether granulocyte/macrophage colony-stimulating factor (GM-CSF), a cytokine able to abrogate AM-induced immunosuppression, is involved in the process.

The ability of different concentrations of AMs to inhibit lymphocyte proliferation in response to the T-cell-specific mitogen phytohaemagglutin (PHA) after *in vitro* culture was tested in three groups of subjects: 12 patients with FL; four asymptomatic farmers (AS); and six normal volunteers (N). Release of GM-CSF by AMs was also measured.

At all ratios tested, AMs from patients with FL did not suppress the lymphoproliferation but instead had an enhancing effect. In AS, AMs enhanced the proliferation at a lower ratio but inhibited it at high ratios. In N subjects, as described previously, AMs increasingly inhibited the blastogenesis of lymphocytes (L) at increasing ratios of AM:L. In some patients with FL, AMs spontaneously released more GM-CSF than in normal volunteers (206±84 *versus* 29±14 pg·mL^{-1}, respectively). In AS, GM-CSF release was intermediate (74±36 pg·mL^{-1}).

In conclusion, a defect in the ability of alveolar macrophages to suppress the proliferation of lymphocytes in the lung of patients with farmer's lung is a major factor accounting for the development of the observed lymphocytic alveolitis. Granulocyte/macrophage colony-stimulating factor could be one factor which may contribute to this alteration.
Eur Respir J., 1996, 9, 1456–1462.

Unité de Recherche, Centre de Pneumologie de l'Hôpital Laval, Université Laval, Sainte-Foy (Québec), Canada.

Correspondence: Y. Cormier
Centre de Pneumologie
Hôpital Laval
2725 Chemin Sainte-Foy
Sainte-Foy
Québec
Canada G1V 4G5

Keywords: Alveolar macrophages
hypersensitivity pneumonitis
interstitial lung disease

Received: October 30 1995
Accepted after revision March 29 1996

Supported by the Respiratory Health Network of Centres of Excellence, Canada.

Farmer's lung (FL) is a type of hypersensitivity pneumonitis characterized by a massive proliferation of lymphocytes, mostly of the cytotoxic/suppressor subtype (CD8+), which accumulate within the lung parenchyma and alveolar space of sensitized individuals, and may mediate tissue damage [1–3]. The resulting alveolar lymphocytosis can persist after an acute episode of the disease in subjects who are still exposed to the offending antigens that occur in the farm environment [4, 5]. A milder lymphocytosis can be observed in asymptomatic but exposed dairy farmers [6]. A recent investigation suggested that both acute and persisting alveolar lymphocytosis are sustained by an *in vivo* production of interleukin (IL)-2 [7]. Different hypotheses to explain why only a proportion of exposed individuals develop the symptomatic disease include: a massive exposure to the antigens [8]; a viral infection [9]; or a co-exposure [10]. However, direct data in humans supporting such concepts are not yet available. Individual susceptibility may be an important predisposing factor to the disease; however, some studies have failed to detect genetic markers for FL [11]. We postulated that changes in host cell functions could be important in the development of symptomatic FL.

In normal lung, where they constitute the majority of the alveolar cell population, alveolar macrophages (AMs) have the ability to suppress antigenic and mitogenic [12–14], as well as IL-2-induced lymphocyte proliferation [15]. AMs can also suppress T-cell cytotoxicity [16], and immunoglobulin production [17, 18]. Recent studies demonstrated that AMs selectively inhibit the proliferation of T-cells without affecting their initial activation and expression of effector functions [19, 20]. Thus, by limiting the overall expansion of activated T-cells *in vivo*, this immunosuppressive activity of AMs would be necessary to protect against T-cell-mediated lung tissue damage that may occur in response to repeated inhalations of nonpathogenic antigens [21]. A previous study [22] showed that AM-induced T-cell suppression is reversible by granulocyte/macrophage colony-stimulating factor (GM-CSF). GM-CSF is secreted by airway epithelial cells and macrophages upon stimulation by antigen or cytokines, such as IL-1 and tumour necrosis factor-α (TNF-α) [23]. Moreover, GM-CSF may contribute to the proliferation of AMs and the recruitment of lymphocytes [24].

In FL, AMs are actively involved in the disease and release proinflammatory mediators and cytokines [25, 26]. Although their absolute number is increased in the bronchoalveolar lavage (BAL) of patients with acute FL,

their relative proportion is considerably diminished whilst lymphocytes become the predominant alveolar cell population [27]. It thus appears feasible that an alteration (downregulation) of the immunosuppressive activity of AMs is likely to occur in FL, therefore allowing for the development of the lymphocytic alveolitis. To our knowledge, no study has reported the lymphosuppressive function of AMs from patients with FL or any other form of hypersensitivity pneumonitis.

To test this hypothesis, we evaluated the ability of AMs to suppress autologous lymphocyte proliferation *in vitro* in three groups of subjects (patients with FL, asymptomatic farmers, and normal nonfarming volunteers). The results show that the immunosuppressive activity of AMs is markedly altered in patients with acute FL, and to a lesser degree in asymptomatic farmers with a lymphocytic alveolitis. We also measured the amounts of GM-CSF produced spontaneously by AMs cultured *in vitro* for 24 h. A positive correlation was found between amounts of GM-CSF secreted by macrophages and the percentage of proliferation of AM-peripheral blood mononuclear cell (PBMC) co-cultures. This may be one of the mechanisms involved in the alteration of the normal suppressive AM activity in patients with acute FL.

Materials and methods

Subjects

The study included 12 patients with FL, four asymptomatic farmers with a lymphocytic alveolitis (AS), and six normal nonfarming controls (N). The diagnosis of FL was based on the criteria of Chemlik *et al.* [28]. All patients were dairy farmers and had a clinical history suggestive of FL, including inspiratory crackles on physical examination, interstitial infiltration on chest radiographs, and altered lung functions. All subjects were nonsmokers, and none was receiving immunosuppressive therapy at the time of this study. BAL and venous blood were obtained from all patients as a part of their clinical evaluation and from other subjects on written consent.

Bronchoalveolar lavage

BAL was performed using a fibreoptic bronchoscope as described previously [4]. In brief, a total volume of 300 mL of sterile nonpyrogenic saline was instilled in 60 mL aliquots into a wedged segment, usually of the right middle lobe. After each instillation, the fluid was gently aspirated, placed in 50 mL centrifugation tubes and kept on ice until processed.

Preparation of alveolar macrophages (AM)

Cells were separated from BAL fluids by centrifugation at 400×g for 10 min at 4°C. After two successive washes with Hank's balanced salt solution (HBSS), the cells were counted on a haemacytometer and resuspended at 10^7 cells·mL^{-1} in RPMI-1640 (Roswell Park Memorial Institute-1640) medium (Gibco-Canada) supplemented with 10% heat-inactivated foetal bovine serum and 1% penicillin-streptomycin (complete medium). Differential cell counts were performed on Diff-Quik® and esterase-stained cytocentrifuge preparations.

AMs were purified by adherence on sterile plastic dishes for 1 h at 37°C in a 5% CO_2-enriched humidified atmosphere. After removal of nonadherent cells by several washes with HBSS, the adherent cells were incubated at 4°C in ice-cold HBSS for 30 min, and finally detached by gently scraping with a sterile rubber policeman. Typically, this method yielded 90–95% AM-enriched cell preparation as indicated by esterase staining. Cell viability, as estimated by trypan blue exclusion, was above 97%.

Preparation of PBMC

Heparinized blood was obtained from all patients and controls. PBMC were separated from whole blood by Ficoll-paque density gradient centrifugation, washed three times in HBSS and resuspended in complete RPMI at 0.5×10^6 cells·mL^{-1}. These cells were more than 97% viable. Whole PBMC were used in co-cultures in order for blood monocytes to provide the accessory function for the lymphocyte response to phytohaemagglutin (PHA).

Preparation of blood monocytes

Monocytes were prepared according to a method described previously [29]. Briefly, PBMC were allowed to adhere to serum precoated plastic dishes for 1 h at 37°C. Nonadherent cells were removed by washing. Adherent cells were covered with ice-cold HBSS and incubated at 4°C for 30 min. The cells were removed by gentle scraping with a rubber policeman and resuspended at various concentrations in RPMI medium. These cells were found to be 90–95% positive for esterase.

In vitro *assays for immunosuppressive activity of AMs*

Purified AMs, or in some cases monocytes, were co-cultured for 72 h with autologous PBMC at various ratios (0.5:1, 1:1, 2:1) in the presence or absence of PHA (1 µg·mL^{-1}) in 96-well microtitre plates, each well containing 200 µL of complete medium and 5×10^4 PBMC. Similar co-cultures were also obtained at a 2:1 ratio of monocytes:PBMC, *i.e.* a triple concentration of monocytes. The cultures were pulsed with 1 µCi tritiated-thymidine for the last 12 h. The cells were then harvested onto fibreglass filters and the radioactivity counted in a Packard matrix β counter. The data were obtained as mean counts per minute (cpm) from quadruplicate values after subtracting the background from unstimulated cultures, and the results were expressed as a percentage of PHA-stimulated PBMC without AMs.

Detection of spontaneous release of GM-CSF

GM-CSF was measured by enzyme-linked immunosorbent assay (ELISA) (PerSeptive Diagnosis, Cambridge, MA, USA) in BAL fluid and in the supernatants from AMs after 24 h of culture at 5×105 cells·mL^{-1} in complete RPMI medium.

Statistical analysis

For graphical presentation of data, results of representative measures were expressed as mean±SEM. For comparisons between group means, a one-way analysis of variance (ANOVA) was performed. Normality and homogeneity of variances was verified to validate tests. When a significant overall difference was observed, pairwise differences between group means were evaluated using Scheffe's comparison. Pearson's correlation coefficient was used for the relationship between GM-CSF and lymphoproliferation. The statistical software package Statistical Analysis System (SAS) was used for all analyses.

Results

Although the percentage of macrophages was considerably decreased in the BAL from patients with FL (fig. 1a), there was a significant increase in their absolute number with respect to total cells when compared to normal control values: $201\pm18\times10^3$ *versus* $59\pm12\times10^3$ macrophages·mL or recovered fluid, respectively ($p<0.05$) (fig. 1b). BAL from asymptomatics showed intermediate values for macrophage counts. A significant increase in neutrophil numbers was observed only in the FL group.

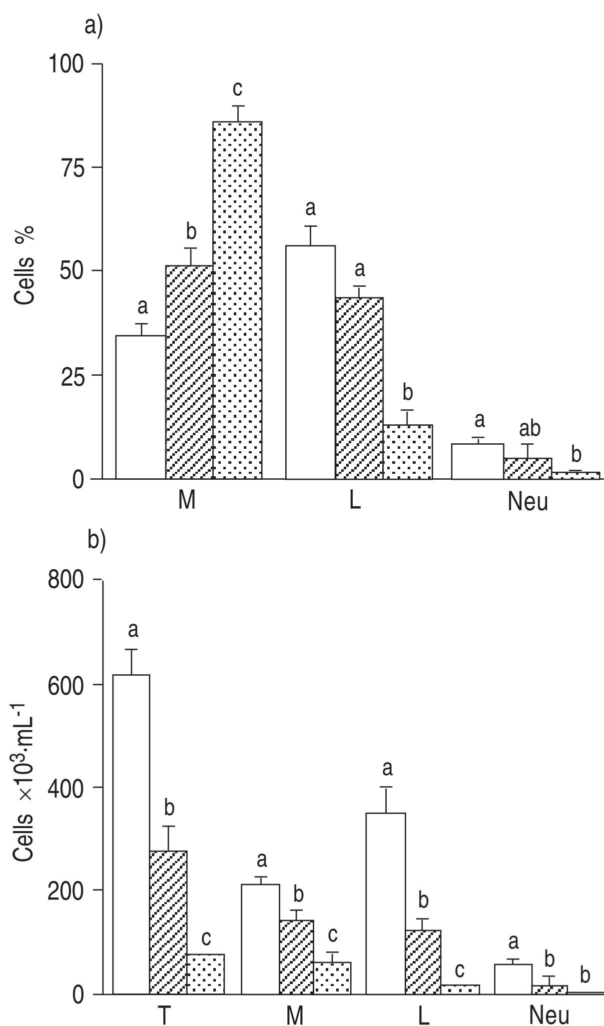

Fig. 1. – a) Bronchoalveolar lavage (BAL) differential cell counts, in percentage of total cells; b) BAL total cell counts in number of cells·mL^{-1}. Values are presented as mean±SEM. For total cells and each cell type, bars with different letters are significantly different ($p<0.05$) between groups of subjects. T: total cells; M: macrophages; L: lymphocytes; Neu: neutrophils; ▭ : patients with farmer's lung (n=12); ▨ : asy-mptomatic farmers (n=4); ▤ : normal non-farmer volunteers (n=6).

The proliferation responses of PBMC to PHA after *in vitro* culture in the presence of increasing proportions of autologous AM are presented in figure 2. Results are given in percent proliferation of PHA-stimulated PBMC without AM (100%). As expected, AM from normal subjects suppressed PHA-induced proliferation of autologous PBMC. This suppression increased with increasing AM:PBMC ratios. P values for ratios 0.5:1, 1:1, and 2 were respectively: 0.101, 0.023 and 0.002. In asymptomatic farmers, AM at low ratio (0.5:1) did not suppress but stimulated lymphoproliferation to PHA ($p=0.001$). However, when higher concentrations of AM were added, a normal suppressive activity was restored (ratio 2:1, $p=0.023$). AM from patients with FL failed to suppress PBMC proliferation at any ratio of AM:PBMC tested. A significant enhancing effect was seen at ratios 0.5:1 and 1:1 ($p=0.002$ and 0.023). At the 2:1 ratio, proliferation was not different than control cultures ($p=0.55$). Comparison between groups shows that AM from normal subjects had significantly higher suppressive activity at all ratios tested than AM from subjects with acute FL ($p<0.01$). AM from asymptomatic farmers had intermediate effects. At low ratios they failed to suppress PBMC proliferation, but at higher ratios their suppressive function was significantly greater than that of AM from the subjects with acute FL ($p<0.01$). AS had more suppression at higher AM:PBMC ratios. Figure 3 shows the results of an experiment where additional purified blood monocytes were added in the cultures. When AM were substituted by monocytes, no suppression was observed, whereas

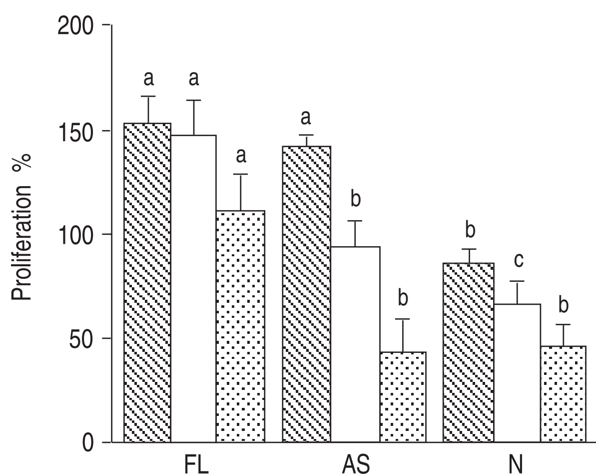

Fig. 2. – Phytohaemagglutinin-induced proliferation of lymphocytes of peripheral blood mononuclear cells (PBMC) in the presence of increasing concentrations of autologous alveolar macrophages. Values are presented as mean±SEM. The basal counts for unstimulated PBMC with or without AMs at each ratio were negligible and not different within or between the groups. Results are expressed as percentage of PHA-induced PBMC response (without AM=100%). Values with different letters are significantly different between groups ($p<0.01$). AMs from FL patients significantly stimulated PBMC proliferation, while those from normal subjects suppressed this activity. AMs from asymptomatics farmers were able to suppress PBMC proliferation only at high AM:PBMC ratios. PHA: phytohaemagglutinin; AMs: alveolar macrophages; FL: farmer's lung. For further definitions see legend to figure 1. ▨ : 0.5:1 (AM:PBMC); ▭ : 1:1 (AM:PBMC); ▤ : 2:1 (AM:PBMC).

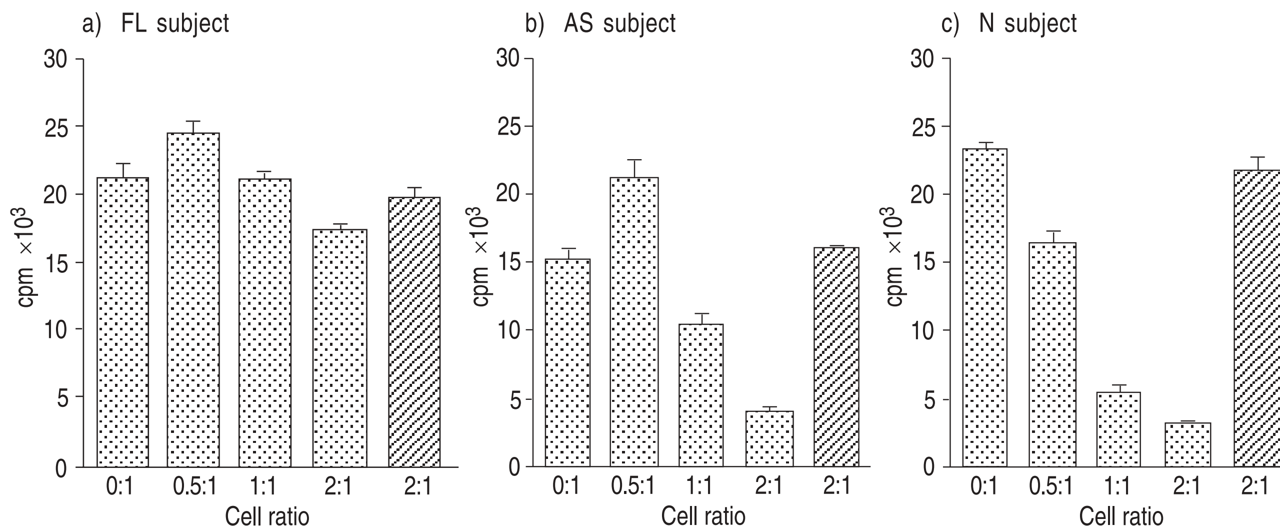

Fig. 3. – Phytohaemagglutinin-induced proliferation responses of triated-thymidine labelled lymphocytes of peripheral blood mononuclear cells (PBMC) in the presence of increasing concentrations of autologous alveolar macrophages (AMs) or purified blood monocytes (Mono), for one typical subject from each group: a) patient with farmer's lung (FL); b) asymptomatic farmer (AS); c) normal nonfarming volunteer (N). Values are presented as mean±SEM. The persisting proliferative response when AMs were substituted by monocytes confirms that the suppression observed was not due to cell crowding. ▭ : AM:PBMC; ▨ : Mono:PBMC; cpm: counts per minute.

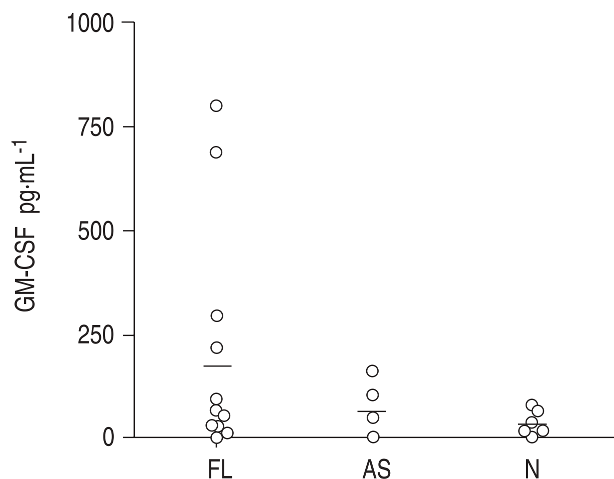

Fig. 4. – Spontaneous *in vitro* release of granulocyte/macrophage colony-stimulating factor (GM-CSF) by alveolar macrophages from the three groups of subjects. Although four subjects in the FL group had higher levels of GM-CSF, the difference between groups did not reach statistical significance. Horizontal bar indictes mean value. For further definitions see legend to figure 1.

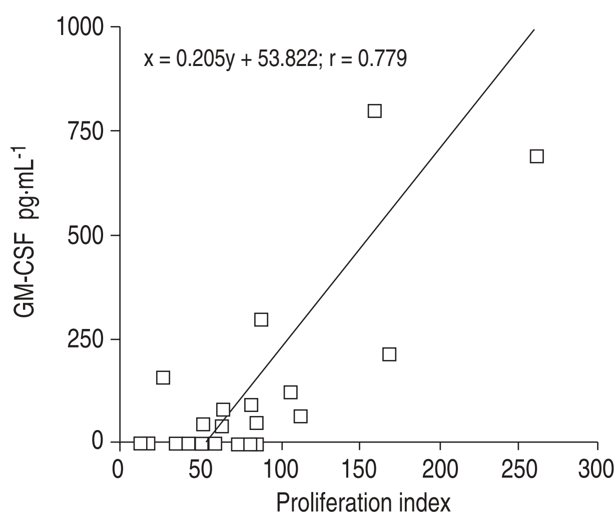

Fig. 5. – Correlation between amounts of granulocyte/macrophage colony-stimulating factor (GM-CSF) produced by *in vitro* cultures of alveolar macrophages and percentage of lymphoproliferation in AM:PBMC 2:1 ratio co-cultures for all subjects. AM: alveolar macrophage; PBMC: peripheral blood mononuclear cell.

in N and AS, increasing concentration of AM induced an increased suppression.

No GM-CSF was found in the BAL fluids. Figure 4 illustrates the values of spontaneous release of GM-CSF from AMs in the three groups of subjects. AMs from patients with acute FL released more GM-CSF (206±84 pg·mL^{-1}) than AMs from asymptomatic farmers (74±36 pg·mL^{-1}) or normal volunteers (29±14 pg·mL^{-1}). Although the differences between the groups did not reach statistical significance, AMs from some patients, but not all, spontaneously released substantial amounts of GM-CSF after *in vitro* culture. At all ratios tested, a positive correlation was found between the proliferation index and amounts of GM-CSF secreted by AMs (at ratio 0.5:1, r=0.666 and p=0.0013; at ratio 1:1, r=0.727 and p=0.0001; at ratio 2:1, r=0.779 and p=0.0001, for all subjects). Figure 5 illustrates this correlation at ratio 2:1.

Discussion

The present investigation focused on the immunosuppressive activity of AMs as a potential candidate which may be involved in the development of the lymphocytic alveolitis observed in FL disease. The results clearly demonstrate a defect in the ability of AMs from patients with FL to suppress *in vitro* mitogenic proliferation of autologous lymphocytes. The development of this downregulation of the immunosuppressive activity is probably progressive, as shown by a lesser degree of alteration in asymptomatic farmers. Since this immunosuppressive activity may play a role in protecting the lung against excessive lymphocyte accumulation and subsequent tissue damage in response to continuous antigenic challenges [21], such an altered activity may be of significance

in the pathogenesis of FL. Such an alteration is even more important when one considers that the *in vivo* ratio of AM:lymphocyte (as estimated by BAL) is significantly decreased in the lung of patients with FL and in asymptomatic farmers with a lymphocytic alveolitis (0.6 and 1.3, respectively) compared to normal subjects (6.8).

Although IL-2 may be one mechanism which contributes to lymphocyte accumulation in the lung of patients with FL [7, 30], since this cytokine does not inhibit the suppressive effect of AMs [31] it cannot be held responsible for the altered AM immunosuppressive activity observed in this study.

A previous study has shown that cigarette smoke does not alter the immunosuppressive activity of AMs in normal subjects [32]. This observation is of great interest, since farmers who smoke are less likely to develop precipitating antibodies to the antigens responsible for farmer's lung and have a lower incidence of FL than nonsmokers [33]. In sarcoidosis, another disease where large numbers of lymphocytes accumulate in the lung parenchyma, there are conflicting data on the maintenance or alteration of this AM activity [34, 35].

The mechanisms by which AMs achieve this lymphosuppression are still incompletely characterized. It is generally admitted that this suppression primarily requires cell-to-cell contact [29, 31, 34], although some mediators of inflammation, such as reactive oxygen species and prostaglandins [15], and the transforming growth factor-β_1 (TGF-β_1) may also contribute to this effect [36, 37]. A recent study suggested that suppression of CD3-induced T-cell signal transduction by normal AMs may be mediated by surfactant protein-A (SP-A) [38]. In this study, in contrast to normal AMs, the amount of membrane-associated SP-A was substantially decreased in AMs from patients with interstitial lung disease where the suppressive activity was altered. Whether a normal immunosuppressive activity may be restored by addition of surfactant components needs further study. Other workers [22] have demonstrated that pretreatment of mouse AMs with GM-CSF (either recombinant form or natural form generated in lung-conditioned medium following exposure to lipopolysaccharide) abrogated mitogen-induced T-cell proliferation. In our study, we attempted to measure GM-CSF *in vivo* in concentrated BAL fluids from our subjects but failed to detect this cytokine, whilst substantial amounts were spontaneously released from AMs *in vitro*. This failure could be explained by the possible instability of this cytokine in the presence of a variety of proteolytic enzymes that might be recovered in the BAL fluids under such disease conditions. Apart from lymphocytes and macrophages, bronchial epithelial cells [39] and mast cells [40] may constitute other potential sources for GM-CSF *in vivo*. There is evidence that mast cells are increased in numbers in the BAL of patients with FL [41], however their exact role in the disease remains undefined. A more recent investigation successfully demonstrated GM-CSF by immunocytochemistry on mast cells and macrophages in the lung parenchyma of patients with FL [42].

Of particular interest in our study is the finding that AMs from patients with FL enhance, rather than suppress, the mitogenic response of autologous lymphocytes. This duality in AM function is likely to be reflected by the heterogeneity of this cell population. In FL, AMs are significantly increased in total numbers not only during the acute phase of the disease but also in asymptomatics. Compared to normal control values, in our study there were approximately threefold and twofold increases in this cell population in patients with FL and in asymptomatics, respectively. It is possible that such an increase might compensate for the normal immunosuppressive activity by diluting the local macrophage population. However, the stimulatory effect of these new macrophages adds another dimension to the alteration of the suppressive activity observed in this study. Whether this increase in AM population results from an *in situ* proliferation of a specific "subpopulation" of resident AMs or represents a pool of newly recruited monocyte-like macrophages need to be determined. Some recent observations may suggest the former possibility [43]; however, the lower proliferation indices reported may not account for the total increase observed in the present study. It is known that AMs can be induced to proliferate when stimulated by GM-CSF [44]. In addition, actively proliferating macrophages could be demonstrated in the peritoneum of transgenic mice for GM-CSF (45). In the present study, increased production of GM-CSF by AMs from patients with the active disease was detected and correlated with an increase in lymphoproliferation. Since this induction of AM proliferation might be important in the pathogenesis of FL, a possible involvement of IL-2 could not be totally excluded, since IL-2 receptor is more predominantly expressed on AMs in patients with FL [46]. Further studies are required to better characterize the stimulatory mechanism of these AMs and to determine whether an induction of AM proliferation would interfere with the immunosuppressive function. Other cytokines, with anti-inflammatory properties, such as TGF-β and IL-10, may also be involved in the disease. It would be interesting to look at the participation of these cytokines in different phases of FL in subsequent studies.

Based on the present observations, with the assumption that a heterogeneity in the alveolar macrophage population may also reflect a heterogeneity in alveolar macrophage function, we speculate that the prevalence of a specific "subpopulation" of alveolar macrophages (suppressive or stimulatory) might determine the normal immunological homeostasis or the disease condition. Better understanding of these regulatory mechanisms will help us determine why only some exposed individuals develop farmer's lung disease. Promoting the natural immunosuppression could be a promising avenue for the treatment of this disease.

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