Altered accessory cell function of alveolar macrophages: a possible mechanism for induction of Th2 secretory profile in idiopathic pulmonary fibrosis

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ABSTRACT: Alveolar macrophages (AMs) are considered to play a central role in the pathogenesis of idiopathic pulmonary fibrosis (IPF). Recent studies have revealed a predominance of the type-2 T-helper (Th2) cytokine pattern of inflammatory response in the pulmonary interstitium in IPF. The aim of the present study was to determine whether or not the altered accessory cell function of AMs could account for the Th2 pattern of chronic inflammation in IPF.

The levels of various cytokines were measured in the supernatants of soluble anti-CD3 monoclonal antibody (MoAb)-stimulated T-cells, co-cultured with autologous AMs, by enzyme-linked immunosorbent assay (ELISA). Cells from six patients with IPF and from nine normal volunteers (five nonsmokers and four smokers) were examined. The inhibitory effect of interleukin (IL)-10 on the accessory cell function of AMs and the expression of CD80 and CD86 on AMs were also investigated.

IL-4 and IL-5 levels were significantly higher in the co-cultures from patients with IPF than in those from normal volunteers. IL-2, interferon-γ (IFN-γ), and IL-10 production in these co-cultures did not differ. IL-10 suppressed T-cell proliferation in co-cultures with AMs from healthy volunteers (smokers and nonsmokers), but not with AMs from patients with IPF. Expression of CD80 and CD86 on AMs from these groups did not differ.

Thus, the altered accessory cell function of alveolar macrophages from patients with idiopathic pulmonary fibrosis may possibly relate to the pattern of type-2 T-helper cytokine production in response to inflammation.

Idiopathic pulmonary fibrosis (IPF), a chronic inflammatory disorder limited to the lung, is characterized by the presence of alveolitis and interstitial pneumonitis. Whereas the aetiology of the disease is unknown, the pathogenesis is considered to involve a persistent inflammatory reaction to an unidentified agent in the lung, resulting in tissue damage. Pathologic findings are identical to usual interstitial pneumonia (UIP). IPF is diagnosed by elimination of neoplasm, collagen vascular disease, work exposure, toxic treatments, and the presence of sarcoidosis or other granulomatous diseases. Examination of affected pulmonary tissue reveals inflammatory infiltrates, composed principally of T-lymphocytes and macrophages, with variable numbers of mast cells, neutrophils and eosinophils, and distinct B-lymphocyte aggregates [1, 2]. Bronchoalveolar lavage (BAL) is useful in the diagnosis and management of patients with IPF. There are no specific BAL features that are diagnostic of IPF.

However, lavage lymphocytes often identify a predominantly cellular histopathology with a greater likelihood of response to corticosteroid therapy; and, in contrast, lavage neutrophilia, eosinophilia or both, appear to identify a more advanced, fibrotic stage of the disease and lack of response to corticosteroid therapy [3, 4].

Recent advances in our understanding of inflammatory reactions have shown that different patterns of cytokine production by inflammatory cells are associated with different patterns of inflammatory response, and, on this basis, have been described as type-1 T-helper (Th1)-like and type-2 T-helper (Th2)-like [5]. WALLACE et al. [6] immunohistochemically examined diffusely infiltrating cells within the interstitium from patients with IPF, and showed that most of the diffusely infiltrating mononuclear cells stained for interleukin (IL)-4 and IL-5 (>50%) in all 10 cases of IPF, and a small minority (<7%) of the cells stained for interferon-γ (IFN-γ). Their results suggest that a Th2-like pattern of cytokine production predominated in the interstitium of patients with IPF.

Macrophages are considered to play a central role in the pathogenesis of IPF. Stimulated macrophages produce a variety of enzymes, complement components, cytokines, and other mediators of inflammatory and fibroblast cell function, that could initiate or maintain the inflammatory processes that proceed and result in...
the fibrotic stage of IPF. Macrophages are not only antigen-presenting cells but are also a source of cytokines that can manipulate the efficient activation of T-cell response. In addition, macrophages are an important source of co-stimulatory molecules, which play a crucial role in the efficient activation of T-cells. Various combinations of cytokines and co-stimulators are capable of producing a wide range of outcomes. Macrophages and T-cells are, thus, indisputably linked in activation of inflammatory reactions. Recent work has described many of the individual components of this system [7]. CD80 on AMs acts preferentially as a co-stimulator for the generation of Th1 cells, whilst CD86 on AMs co-stimulates and induces Th2 cells in the murine system [8, 9]. IL-12 and prostaglandin E2 (PGE2) are secreted by macrophages and other accessory cells. IL-12 is an effective inducer of IFN-γ and generation of Th1. PGE2 inhibits the production of IFN-γ, whereas the production of IL-4 and IL-5 is not affected [10].

Gant et al. [11] reported a correlation between AM accessory cell function and the number of BAL lymphocytes in bronchial asthma. They discussed the possibility that the presence of an AM population with abnormally enhanced accessory cell function may be responsible for the infiltration of increased numbers of activated T-lymphocytes in asthmatic airways.

We hypothesized that an alteration in accessory cell function in AMs from patients with IPF may induce a Th2-like response, and would explain the mechanism behind the predominance of a Th2-like pattern of response in IPF. To test this hypothesis, the levels of various cytokines were measured in the supernatants of soluble anti-CD3 monoclonal antibody (MoAb)-stimulated T-cells, co-cultured with autologous AMs from patients with IFP and normal volunteers (nonsmokers and smokers). IL-10 acts on macrophages, and macrophages suppress IFN-γ production by Th1 clones. In contrast, IL-10 does not impair the potential of macrophages to stimulate cytokine production by Th2 cells [12-14]. The inhibitory effect of IL-10 on the accessory cell function of AMs was also investigated. If altered accessory cell function of AMs from patients with IPF induces a Th2-like response, IL-10 may not suppress the accessory cell function of AMs from patients with IPF.

Methods

Subjects

This study included six IPF patients (1 current smoker, 3 ex-smokers and 2 nonsmokers) and nine normal volunteers (4 smokers and 5 nonsmokers). The current smoker had smoked for 40 yrs, with a mean consumption of 1.5 packs·day⁻¹. Ex-smokers among the IPF patients had smoked for an average of 18.8±11.2 yrs, with a mean consumption of 1.0±0.5 packs·day⁻¹. All the IPF patients had clinical and radiographic evidences of interstitial lung disease, with pathologic confirmation of UIP by thoracoscopic lung biopsy, and by elimination of other lung diseases. The IPF patients had received no steroid therapy for 1 month before the study. Normal volunteers had taken no medication for 1 week before the study. No volunteer had a past history of lung disease or viral illness in the preceding 2 weeks. All patients with IPF and volunteers gave informed consent for this study.

Bronchoalveolar lavage

BAL was performed using a fiberoptic bronchoscope, as described previously [15, 16]. Premedication with 0.8 mg atropine was given intramuscularly. For topical anaesthesia, 0.5% xylocaine was applied locally in the nose and posterior pharynx. The fiberoptic bronchoscope was wedged into a subsegmental bronchus of the right middle lobe, and 50 mL sterile saline at 37°C was injected and aspirated by hand suction into the syringe three times. The aspirate was placed in 50 mL centrifugation tubes and stored at 4°C until processed. Only the second and third aliquots of fluid were used in this experiment, because the first aliquot involves much contamination of the lavage fluid by mucus and large airway cells.

Antibodies

Murine monoclonal antibodies (MoAbs) UCHT-1 (CD3), LT14 (CD14), 3G8 (CD16) and SJ25-C1 (CD19) were purchased from Biodesign (Kennebunk, MA, USA). UCHT-1 recognizes a T-cell surface glycoprotein; its isotype is mouse immunoglobulin G1 (IgG1) [17]. LT14 recognizes a cell surface glycoprotein expressed on most peripheral blood monocytes [18]. 3G8 reacts with CD16 antigen, which is found on all granulocytes and natural killer (NK) cells [19]. SJ25-C1 recognizes B-cell surface glycoprotein, that is present at all stages of maturation. BB1 (CD80) and IT2.2 (CD86) were purchased from PharMingen (San Diego, CA, USA). Fluorescein isothiocyanate (FITC)-conjugated sheep anti-mouse immunoglobulin G (IgG) was purchased from Biodesign, and used as the secondary antibody. Rat MoAb JES3-9D7 (anti-human IL-10) was a kind gift of PharMingen. This antibody can neutralize human IL-10 bioactivity in functional assays [20].

Preparation of AMs

BAL cells were passed through gauze to remove any particulate matter and excess mucus, and then centrifuged at 900×g [18]. The sedimented cells were washed twice in Iscove’s modified Dulbecco’s medium (Life Technologies, Grand Island, NY, USA) with 10% heat-inactivated foetal calf serum, 5×10⁻⁵ M 2-mercaptoethanol, and antibiotics (IMDM with 10% FCS). Cells were counted in a haemocytometer and a cytocentrifuge preparation was made to determine the cellular composition. The BAL cells were placed in plastic culture dishes and incubated in a humidified CO₂ incubator (5% CO₂ in 95% air) for 30 min at 37°C. The nonadherent cells were discarded and the dish was rinsed gently to remove residual nonadherent cells. IMDM with 10% FCS at 4°C was then added in plastic culture dishes. The adherent cell populations were removed by thorough washing and used as AMs; over 95% of the cells proved to be AMs on differential staining with Diff-Quik.
Preparation of peripheral blood T-lymphocytes

Heparinized peripheral blood was obtained from every bronchoscopy subject prior to the procedure [19]. The mononuclear cells were isolated on a Ficoll-Hypaque (sp. gr. 1.077) (Pharmacia, Uppsala, Sweden) gradient, washed, and allowed to adhere to plastic dishes in a humidified CO₂ incubator (5% CO₂ in 95% air) for 30 min at 37°C. Nonadherent cells were then removed and applied to a nylon wool column. The filtered cells were incubated with MoAb against cell type-specific markers on human monocytes (CD14), B-cells (CD19), and NK cells (CD16) for 30 min at 4°C in a rotary mixer. Labelled cells were washed three times (300 × g for 7 min at 4°C), and then resuspended in phosphate buffered saline (PBS) with 1% FCS in a 3 mL conical-bottomed plastic tube. Washed immunomagnetic beads coated with sheep antimouse IgG (M450 Dyna-beads; Dynal AS, Oslo, Norway) were incubated with the MoAbs-coated beads with sheep antimouse IgG (M450 Dyna-beads; Dynal AS, Oslo, Norway) were incubated with the MoAbs-coated beads were removed by magnetic extraction for 5 min. Supernatant containing the purified T-cells was aspirated and centrifuged (300 × g for 7 min at 4°C) and resuspended in IMDM with 10% FCS. The purity of CD3+ T-cells from each individual exceeded 95%, as determined by fluorescence-activated cell (FAC)-scan analysis. IMDM with 10% FCS at 4°C was then added to the plastic culture dishes. The adherent cell populations were removed by thorough washing and used as monocytes; over 95% of the cells proved to be monocytes on differential staining with Diff-Quik.

Flow cytometry

Purified T-cells, monocytes and AMs were washed in PBS with 1% heat inactivated FCS and 0.05% sodium azide, and seeded in a 96-well round-bottomed plate. Fc receptors were blocked by incubation with human IgG for 10 min at 4°C. Cells were then incubated with specific MoAbs (anti-human CD3, CD14, CD80 and CD86) at saturating concentrations for 30 min at 4°C, washed twice in washing solution, and stained with FITC-conjugated goat antimouse IgG (Biodesign) for another 30 min at 4°C. The cells were then washed twice in washing solution and fixed with 1% paraformaldehyde in PBS. Flow cytometric analysis of surface marker expression was performed using a FACscan cytometer (Becton Dickinson & Co., Mountain View, CA, USA) equipped with Cell Quest program (Becton Dickinson & Co.). Nonviable cells were excluded from analysis on the basis of forward and side scatter, and in some cases, propidium iodide was used (Sigma Chemical Co., St. Louis, MO, USA). In every case, isotype-matched mouse immunoglobulin was used as the background control.

Evaluation of accessory activity of AMs and monocytes

Purified AMs or monocytes were used as accessory cells and were co-cultured with purified T-cells and soluble anti-CD3 MoAb (10 ng·mL⁻¹) in a 96-well flat-bottomed plate (Becton Dickinson & Co.). Each well contained 1×10⁵ T-lymphocytes and various ratios of accessory cells in 200 mL of IMDM with 10% heat-inactivated FCS. The plates were incubated for 72 h at 37°C in a humidified atmosphere containing 5% CO₂. The cultures were pulsed with 1 μCi [³H]-thymidine for the last 14 h. The cells were then harvested onto glass-fibre filters and the radioactivity was counted in a scintillation counter. The data were obtained as mean counts per minute (cpm) of triplicate determinations, after subtracting the background from unstimulated cultures.

In vitro assays for immunosuppressive activity of IL-10

The potential of IL-10 to inhibit the accessory cell function of AMs and monocytes from patients with IPF and normal volunteers was also investigated. Human recombinant IL-10 (11F1645) was obtained from Biodesign, the source being Escherichia coli. For most in vitro applications, IL-10 exerts its biological activity in the concentration range 0.2–20.0 ng·mL⁻¹. Small numbers of AMs enhance and larger numbers inhibit T-cell proliferation; therefore, AMs or monocytes with autologous T-lymphocytes at various ratios (0.003:1, 0.01:1, 0.03:1, 0.1:1, 0.3:1, 1:1) were stimulated by soluble anti-CD3 MoAb and co-cultured for 72 h in the presence or absence of IL-10 (10 ng·mL⁻¹) in 96-well microtitre plates [21]. Because the baseline soluble anti-CD3 MoAb-induced T-cell proliferation differed between individuals, the results are expressed as percentage maximum cpm as follows:

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\frac{\text{(cpm in the present)-(cpm background)}}{\text{maximum cpm)-(cpm background)}} \times 100\%
\]

Soluble anti-CD3 MoAb-induced T-cell proliferation in co-culture with AMs was maximum when 30% AMs was added. At the beginning of the present study, T-cells were cultured for various times in the presence of various doses of IL-10. Culture with 10 ng·mL⁻¹ of IL-10 for 4 days yielded a sufficient inhibition. The dose-dependency of the IL-10 inhibitory effect on the accessory function of AMs was also examined.

Detection of IFN-γ, IL-2, IL-4, IL-5 and IL-10 levels in culture supernatants

After 24 h incubation, protein levels of IFN-γ, IL-2, IL-4, IL-5 and IL-10 in the supernatants of T-cell co-cultured with AMs were measured by enzyme-linked immunosorbent assay (ELISA), using a system purchased from R & D systems (Minneapolis, MN, USA). The objective was to determine whether AMs from patients with IPF induced the Th2-like pattern of cytokine production. In addition, protein levels of these cytokines were measured in unconcentrated BAL fluid from patients with IPF and normal volunteers. Sandwich ELISA was performed according to the manufacturer’s instructions. The sensitivity of ELISA for each cytokine was as follows: IL-2 6.0 pg·mL⁻¹; IL-4 4.1 pg·mL⁻¹; IL-5 3.0 pg·mL⁻¹; IL-10 15 pg·mL⁻¹; and IFN-γ 3.0 pg·mL⁻¹.
Statistical analysis

Results are expressed as the mean±SD. Differences between the groups were assessed by the Mann-Whitney test. A p-value less than 0.05 was considered to have statistical significance.

Results

The BAL cells of patients with IPF and of normal volunteers (nonsmokers and smokers) included, on average 86, 90, and 96% AMs, respectively. The remaining cells consisted of lymphocytes, neutrophils and eosinophils. The number of BAL cells and the absolute number of AMs were increased in patients with IPF and smokers compared with the nonsmokers (table 1).

Levels of IFN-γ, IL-2, IL-4, IL-5 and IL-10 were measured in the supernatants from soluble anti-CD3 MoAb-stimulated T-cell co-cultures with AMs or monocytes at an accessory cell:T-cell ratio of 0.1:1 (10% AMs) and 1:1 (100% AMs). IL-4 and IL-5 levels were significantly higher in co-cultures with AMs from patients with IPF than in co-cultures with AMs from normal volunteers (nonsmokers and smokers) at both ratios (fig. 1a and b). IFN-γ and IL-10 levels did not significantly differ in the supernatants between the three groups (fig. 1c and d). IFN-γ, IL-4, IL-5 and IL-10 levels in the supernatants from co-culture with monocytes were also not significantly different between the three groups. IL-2 was below detection level (5 pg·mL⁻¹) in the supernatants from any co-culture. This absence of detectable levels of IL-2 in the T-cell co-culture stimulated by soluble anti-CD3 MoAb has been noted by other authors [22]. IFN-γ, IL-2, IL-4, IL-5 and IL-10 were also measured in unconcentrated BAL fluid from patients with IPF, and normal volunteers (nonsmokers and smokers), however, none of these cytokines were detected.

No inhibition by IL-10 (10 ng·mL⁻¹) was observed for soluble anti-CD3 MoAb (10 ng·mL⁻¹)-induced autologous T-cell proliferation in co-culture with AMs from patients with IPF (fig. 2a). However, marked inhibition by IL-10 was observed with AMs from normal volunteers (nonsmokers and smokers) at AM:T-cell ratios of 0.003:1, 0.01:1, 0.03:1, 0.1:1, 0.3:1 and 1:1 (fig. 2b and c). IL-10 inhibited soluble anti-CD3 MoAb-induced autologous T-cell proliferation in co-culture with peripheral blood mononuclear cells from patients with IPF and normal volunteers (nonsmokers and smokers) (fig. 2d–f). The AMs and monocytes added were not irradiated. In preliminary work, we had compared AMs irradiated by 3,500 rad X-ray and nonirradiated AMs, and similar results were obtained (data not shown).

The dose-dependency of IL-10 inhibitory effects on T-cell proliferation was observed. IL-10 dose-dependently inhibited T-cell proliferation in co-culture with AMs from normal volunteers (nonsmokers and smokers), but not from patients with IPF (fig. 3). The AM:T-cell ratio was 0.1:1 (10% AMs). These inhibitory effects were specific for IL-10, since they were neutralized completely by anti-IL-10 MoAb (fig. 4).

The expression of CD80 and CD86 on AMs and monocytes from patients with IPF and normal volunteers (nonsmokers and smokers) was then examined (table 2) and no significant difference was found between groups. The average proportions of CD86 antigen-positive AMs from patients with IPF, nonsmokers, and smokers were 30, 22, and 27%, respectively.

Discussion

We have obtained evidence that IL-4 and IL-5 production in co-culture of T-cells with AMs from patients with IPF is significantly higher than that from normal volunteers, and that IL-10 inhibits accessory cell function of AMs from normal volunteers but not of AMs from patients with IPF. The absence both of Th1 secretory profile and the inhibitory effect of IL-10 is not sufficient to demonstrate the presence of Th2 profile. These results suggest that Th1-like cell proliferation occurs in co-culture with AMs from normal volunteers, and non-Th1-like cell proliferation occurs in co-cultures with AMs from patients with IPF. Thus, the abnormal function of AMs from patients with IPF may be related to the Th2-like response in the pulmonary interstitium in IPF.

Experiments were performed without separating CD4+ and CD8+ T-cells. CD4/CD8 ratios of the purified peripheral blood T-cells ranged 1.1–2.2, and were not significantly different between patients with IPF and controls. The majority of CD8+ T-lymphocytes show a Th1-like secretory profile, even when CD4+ T-lymphocytes showed the Th0 as well as the Th2 profile [5, 23, 24]. The IFN-γ released by CD8+ T-lymphocytes may prevent development of the Th2-like profile [25].

Other studies have suggested that IPF may be associated with a Th2-like response. Wallace et al., [6] using
Fig. 1. – Cytokine levels in the supernatants of soluble anti-CD3 MoAb-stimulated T-cells co-cultured with AMs or monocytes. a) IL-4; b) IL-5; c) IL-10; and d) IFN-γ levels in supernatants from soluble anti-CD3 MoAb-stimulated T-cell co-cultures with AMs; and e) IL-4; f) IL-5; g) IL-10; and h) IFN-γ levels in supernatant co-cultured with monocytes. Co-culture supernatants at accessory cell: T-cell ratios of 0.1:1 (10% AMs) and 1:1 (100% AMs) were measured by enzyme-linked immunosorbent assay (ELISA). Data are presented as mean± SD. *: p<0.01; **: p<0.05, compared to normal volunteers (smokers and nonsmokers). •: nonsmokers (n=5); □: smokers (n=4); △: patients with idiopathic pulmonary fibrosis (n=6). IL: interleukin; IFN-γ: interferon-γ; MoAb: monoclonal antibody; AMs: alveolar macrophages.
immunohistochemical identification of cytokines, stated that, while both Th1-like and Th2-like responses are present in IPF, the Th2-like pattern of cytokines appears to predominate. PRIOR and HASLAM [26] reported that IPF patients with the lowest plasma levels of IFN-γ showed the most marked deterioration in lung function on follow-up, and that in patients with sarcoidosis a high plasma level of IFN-γ was associated with resolution of interstitial shadowing on chest radiographs following immunosuppressive therapy.

The significance of the Th2-like response in IPF is uncertain. Several groups have noted that IFN-γ inhibits fibroblast proliferation and collagen production [27, 28]. SEMPOWSKI et al. [29] identified a subset of murine fibroblasts for which IL-4 promotes proliferation and production both of collagenous and noncollagenous extracellular matrix proteins. Thus, the Th2-like response may result in a relative imbalance of factors which regulate fibroblast function, and may contribute to the subsequent progressive scarring which characterizes IPF. The presence of a Th2-like cytokine pattern is also known to favour the development of a humoral response with persistent antibody production [30]. It was reported that synthesis of BAL fluid immunoglobulins (IgG, IgA, IgM) was...

Fig. 2. – Immunosuppressive effect of IL-10 on the accessory cell function of AMs or monocytes from patients with idiopathic pulmonary fibrosis (IPF; n=6) and normal volunteers (nonsmokers n=5, and smokers n=4). AMs or monocytes with autologous T-lymphocytes at various ratios were stimulated by soluble anti-CD3 MoAb and co-cultured in the presence or absence of IL-10. The cultures were pulsed with 1 µCi [3H]-thymidine, and the results are expressed as percentage maximum counts per minute (cpm). a) No inhibition by IL-10 (10 ng·mL⁻¹) was observed for soluble anti-CD3 MoAb (10 ng·mL⁻¹)-induced autologous T-cell proliferation in co-culture with AMs from IPF patients. However, marked inhibition by IL-10 was observed at AM:T-cell ratios of 0.003:1 (0.3%), 0.01:1 (1%), 0.03:1 (3%), 0.1:1 (10%), 0.3:1 (30%) and 1:1 (100%), with AMs from: b) normal volunteers who were nonsmokers; and c) normal volunteers who were smokers. : AMs; AMs + IL-10. IL-10 inhibited soluble anti-CD3 MoAb-induced autologous T-cell proliferation in co-culture with peripheral blood monocytes from d) patients with IPF; e) normal volunteers who were nonsmokers; and f) normal volunteers who were smokers. : monocytes; monocytes + IL-10. Data are presented as mean±SD. *: p<0.05; **: p<0.01, compared to co-cultures without IL-10. For definitions see legend to figure 1.
Macrophages are considered to play a central role in the pathogenesis of IPF and normal volunteers were not significantly different. Therefore, the abnormal function of AMs from patients with IPF may be related to the Th2-like response.

In summary, this study has shown that alveolar macrophages from patients with idiopathic pulmonary fibrosis induce a non-type-1 T-helper-like response and that this altered accessory cell function may contribute to the predominance of a type-2 T-helper-like inflammatory response in the pulmonary interstitium of patients with idiopathic pulmonary fibrosis.

References


