Limited data exist on the cytokine and cellular changes in the alveolar environment in immunocompromised patients during *Pneumocystis jirovecii* infection. A cellular and a cytokine analysis were performed on bronchoalveolar lavage (BAL) samples from three groups of patients, i.e., an initial study group of 64 immunocompromised *P. jirovecii*-positive individuals and two control groups of *P. jirovecii*-negative patients who had been or not immunosuppressed (65 patients). The results were related to alveolar dilution as determined by urea measurement. Compared with non-infected groups, *P. jirovecii*-infected patients had a lower level of alveolar macrophages (AM), particularly those with high burdens of *P. jirovecii*. Alveolar macrophages over-expressed the Dectin-1 receptor, which was largely implicated in *P. jirovecii* clearance. The alveolar CD8+T and CD4+T lymphocyte counts were increased and an inverse correlation was observed between the alveolar CD4+ cell count and the *P. jirovecii* burden. Although the alveolar IL-6 level was considerably increased, alveolar IL-17, IL-10, TNF-α, TGF-β concentrations of *P. jirovecii* patients were not different from the control groups. Changes in the pulmonary environment were also highlighted during *P. jirovecii* colonization. Our study suggests that there is a correlation between the *P. jirovecii* burden in the alveolus (from colonization to a high *P. jirovecii* burden), and the degree of impairment of the alveolar immune response.

**Keywords** *Pneumocystis jirovecii*, immunosuppression, bronchoalveolar lavage, alveolar leukocytes, cytokines, colonization

**Introduction**

*Pneumocystis jirovecii* is an opportunistic fungus localized in the alveoli of the lung that causes pneumonia. *Pneumocystis* pneumonia (PjP) remains a frequent cause of morbidity and mortality in immunocompromised patients including those with congenital immunodeficiencies or acquired immune deficiency syndrome, as well as those receiving immunosuppressive medication, patients with haematological or solid malignancies, and transplant recipients [1,2]. The host immune response during PjP involves complex interactions between CD4+T cells, CD8+T cells, neutrophils, alveolar macrophages (AM) and soluble mediators that facilitate the clearance of the pathogen [3–6]. The increase in the incidence of PjP among HIV-infected patients and results from animal model studies of PjP [7] clearly confirm CD4+T lymphocytes critical role in the pathogenesis of this infection in that there is an inverse
relationship between the number of blood CD4+ lymphocytes and the risk of subsequent PjP [8]. CD8+ T cells, which are recruited to the lung in large numbers in response to *P. jirovecii* infection, have been associated with lung injury in murine models [9–11]. Several observations support an important role for AM in the successful host response to *P. jirovecii* [12]. Rats selectively depleted of AM were found to have impaired clearance of *P. jirovecii* [5]. AMs require cytokine activation to maximize their activity [13,14] and therefore a cytokine and chemokine network is necessary for *P. jirovecii* clearance by activation of these cellular effectors. Different studies carried out principally in murine models, have shown an important role for TNF-α or IL-10, two opposing cytokines in the T helper response [15,16]. The characterization of another novel T helper subtype, Th17 cells, has complicated the basic Th1/Th2 model of *P. jirovecii* infection [17].

Even if the alveolar environment seems to be essential in *P. jirovecii* infection pathophysiology, much information needs to be clarified and there is a need for an overall view of the evolution of the immune response during this disease in man. The aim of our study was to examine the changes in the alveolar cytokinetic and cellular environment in *P. jirovecii*-infected patients according to the *P. jirovecii* alveolar burden.

**Materials and methods**

**Patients’ specimens**

Studies of fresh bronchoalveolar lavage (BAL) samples that had been submitted to the Parasitology-Mycology unit of the Toulouse University Hospitals (France) were conducted from November 2007 to April 2008. The clinical unit systematically transmitted these samples to the different laboratories of the Toulouse Hospitals for investigation or pneumopathies tests. BAL fluid was recovered and treated according to the usual routine protocols of the Toulouse Hospitals. Investigations were performed in the Parasitology-Mycology unit for *Toxoplasma gondii* (direct examination and PCR) and different fungi (direct examination, culture and identification) including *P. jirovecii* (direct examination and PCR). In the Virology unit, real-time PCR assays were used to detect the most frequent viral agents of infections of the lungs (1 and 2 Herpes simplex virus, Varicella zona virus, Influenza virus, Cytomegalovirus, Rhinovirus and Adenovirus), whereas direct examination, culture and identification were conducted for potential pathogenic bacteria in the Bacteriology unit. In the Cytology unit, a total and differential leukocyte count was done on the BAL.

All sequential BAL samples found to be positive for *P. jirovecii* DNA were selected during the six month period for this investigation. To choose the negative controls, we decided to include in our analysis all the first *P. jirovecii* DNA negative samples after each positive patient.

Immunosuppression and its causes was analysed for all *P. jirovecii*-positive or negative patients selected for this study. Patients were considered as immunocompromised when an immunosuppressive therapy was carried out (chemotherapy for solid or hematologic cancer, treatment for systemic disease, immunosuppressive therapy post transplantation) or when they were positive for HIV with blood lymphocyte CD4+ T cells < 500/mm³.

To determine the influence of immunosuppression or *P. jirovecii* infection on the different parameters, patients were classified according to two criteria, i.e., the immunosuppression status (IC) and the presence of *P. jirovecii* DNA (Pnj) in the BAL fluid. The patients positive for *P. jirovecii* but without immunosuppression were excluded from this study. Among the remaining patients, three groups of patients were incorporated into the study consisting of two control groups and one study group. The first control group was composed of patients without *P. jirovecii* DNA in their BAL and without immunosuppression [IC(−)/Pnj(−)], the second control group contained patients without *P. jirovecii* DNA but who were immunosuppressed [IC(+)Pnj(−)] and the third (study group) contained only immunocompromised patients with *P. jirovecii* DNA [IC(+)Pnj(+)]. The patients in the latter group [IC(+)Pnj(+)] were ranked according to their PCR cycle threshold (Ct) which was reflective of the alveolar fungal burden of *P. jirovecii*, as described in a previous study [18]. Thus, these patients were further sub-divided into three groups, i.e., PCR with Ct ≥ 28 (low fungal burden: improbable PjP), PCR with 22 ≤ Ct < 28 (moderate fungal burden: possible PjP) and PCR with Ct < 22 (high fungal burden: authentic PjP) [18]. Patient identifiers were eliminated from all clinical and biological data.

**BAL**

Briefly, after topical 2% lidocaine anesthesia of the oropharynx, a fiberoptic bronchoscope was passed into the airways and wedged in a segment of the right middle lobe. BAL was performed by instilling 150 ml of warm saline solution (0.9%), followed by gentle suction. The BAL obtained was sent to the different laboratories for routine evaluation of pathogens (virus, bacteria, mycobacteria, fungus, and parasites).

**Urea determination**

BAL fluids were collected from patients who had varying clinical and pulmonary presentations. Because of the presence of pulmonary oedema, the alveolar lining fluid (ALF) levels were not identical among all the patients. To
Changes in the alveolar environment during \textit{P. jirovecii} infection

The information in the image is a page from a scientific publication discussing the methodology and results of a study on \textit{P. jirovecii} infection in the alveolar environment. The page contains detailed experimental procedures, including the use of \textit{P. jirovecii} DNA, urea quantification, and flow cytometry for characterizing leukocytes and cytokines.

**Total and differential leukocyte counts and cell characterization by flow cytometry in blood and BAL**

In blood, total and differential leukocytes counts were performed in the haematology laboratory with automated routine haematology analyzers: Beckman Coulter LH 750 (Beckman Coulter, Miami, USA) and Sysmex XE-2100 (TOA Medical Electronics Co., Kobe, Japan).

In the BAL, a total cell count was done in the cytology unit using a Malassez hemocytometer. Then each BAL fluid specimen was cytopspun (95 g for 8 min) and the smear obtained stained by the May Grünwald Giemsa (MGG) method to numerate the patient’s alveolar cells.

Lymphocytes were characterized in BAL and blood by the immunology laboratory using standard flow cytometry following routine methods. CD3-FITC, CD4-APC and CD8-PE monoclonal antibodies (Multiset BD, BD Biosciences, San Jose, USA) were used with BD TruCOUNT tubes (BD Biosciences, San Jose, USA) according to the manufacturer’s instructions. Analyses were performed on a FACScalibur flow cytometer (BD Biosciences, San Jose, USA).

AMs in ALF were characterized using Dectin-1, Mannose Receptor, CD14 and CD36 -monoclonal antibodies. After centrifugation of the BAL (438 g for 5min), monoclonal antibodies directed against Dectin-1(IgG2b mice; R&D Systems, Minneapolis, USA), the mannose receptor (IgG1 mice-APC; Serotec, Oxford, UK), CD36 (IgM mouse-FITC) and CD14 (IgG2a mouse-PerCPCy5.5; BD Biosciences, San Jose, USA) were added to each pellet and incubated in the dark for 30 min. After one further wash (1 ml PBS: 438 g for 5min), an appropriate amount of secondary antibody (goat IgG PE anti-mouse IgG2b) was added and incubated for 30 min, followed by another wash. All analyses were performed on a FACSscan flow cytometer (BD Biosciences, San Jose, USA) using CellQuest software (BD Biosciences, San Jose, USA). The AM population was gated using CD11b labelling. Fluorescence in the macrophage gate was assessed and quantified in arbitrary units as mean fluorescence intensity (MFI). For every patient, the MFI was assessed for each antibody-labelled cell suspension and divided by the MFI of the same isotype.

**Real-time PCR assay**

A real-time PCR assay to detect \textit{P. jirovecii} DNA was performed with probes labelled with fluorescein and Red 640 (FRET), as described previously [18] as an adaptation of the Larsen et al. method [20]. All acquired fluorescence data were analyzed and the quantification of the \textit{P. jirovecii} DNA was represented by the cycle threshold (Ct) measured by the LC software. A Ct value was reported for each positive sample.

**Cytokine measurement in BAL**

The lavage fluid was centrifuged for 5 min at 2594 g to remove cells and \textit{P. jirovecii}. Supernatants were stored at \(-80^\circ\text{C}\) until used. IL-6, IL-10, IL-17, TGF-\(\beta\) and TNF-\(\alpha\) were measured by using commercially available ELISA assays (ELISA BD OptEIA, BD Biosciences, San Jose, USA for IL-6, IL-10 and TNF-\(\alpha\); ELISA eBIOSCIENCE, San Diego, USA for IL-17 and TGF-\(\beta\)) according to the manufacturer’s instructions.

**Statistical methods**

Bivariate analysis was performed using the \(\chi^2\) test or Fisher’s exact test to compare patient characteristics in each group. The odds ratio and 95% confidence intervals (OR \([95\% \text{ CI}]\)) were used to describe associated factors. For continuous parameters, the data distribution was found to be non-Gaussian. The distributions were displayed as median along with interquartile ranges (med [IQR]). These data were analyzed using the non-parametric Mann-Whitney rank sum test for two group comparisons. The relationships between two variables were analyzed by the Pearson product-moment correlation. A comparison was considered statistically significant if the \(P\) value was \(\leq 0.05\).

All tests were performed using the SigmaStat (2.03) statistical program (SigmaStat, Heame Scientific Software, Chicago, USA).
Results

Patient characteristics (Table 1)

After analysis for the presence of \( P. jirovecii \) DNA, 135 BAL samples collected from November 2008 to April 2009 were initially selected for inclusion in this study. Seventy of the samples were positive for \( P. jirovecii \) DNA, while specimens of 65 \( P. jirovecii \)-negative patients were included to serve as controls. The median age of all these patients was 56.7 years (IQR 41.8; 65.7) and the sex ratio (M:F) was 1.75. According to the criteria of inclusion described in the methods section, six patients with a positive PCR for \( P. jirovecii \), but without identifiable immunosuppression factors were excluded from the investigation. All the latter patients had very low \( P. jirovecii \) burden (Ct >28) clinically compatible with colonization. The remaining 129 patients were classified in one of the three groups, i.e., a control group of non-immunocompromised patients without \( P. jirovecii \) DNA \( [IC(−)/Pnj(−)] \) (group 1 with 32 patients), a second control group of immunocompromised patients without \( P. jirovecii \) DNA \( [IC(+)/Pnj(−)] \) (group 2 of 33 patients) or a third study group of only \( P. jirovecii \)-infected patients \( [IC(+)/Pnj(+) \) (group 3 composed of 64 patients).

In the two immunocompromised groups (group 2 and 3), the four main sources of immunosuppression were (a) cancerous malignancies and associated chemotherapy [lymphoma, myeloma, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myelogenous leukemia (AML), solid tumor (oesophagus, cerebral, epidermoid carcinoma)], (b) HIV-infection with blood lymphocyte CD4+T cells < 500 mm\(^3\), (c) transplantation and associated therapy (bone marrow, kidney, liver, heart, lung), and (d) systemic or autoimmune disease and associated treatment (rheumatoid arthritis, temporal arteritis, Wegener’s granulomatosis, myasthenia, idiopathic thrombocytopenic purpura, sarcoidosis, mixed connective tissue disease, Sweet’s syndrome). There were no significant differences among the patients in the two groups in terms of age, sex, presence of treatment or prophylaxis anti-\( P. jirovecii \) or cause of immunosuppression (HIV, immunosuppressant treatment post-graft or transplant, immunosuppressant treatment for autoimmune or systemic disease, cancer/chemotherapy). The ratio among patients with or without viral, fungal and bacterial co-infection was not significantly different between the two groups.

Moreover, there were no significant differences among the patients in the two groups without detectable \( P. jirovecii \) \([IC(−)/Pnj(−)] \) and \([IC(+)/Pnj(−)] \) groups relative to age, sex, or viral, fungal and bacterial co-infection. Nevertheless, treatment or anti-\( P. jirovecii \) prophylaxis before BAL sample analysis, were statistically lower in the \([IC(−)/Pnj(−)] \) than in the \([IC(+)/Pnj(−)] \) group \([IC(−)/Pnj(−): 3.5% vs IC(+/Pnj(−): 48.5%].

Alveolar leukocytes changes among \( P. jirovecii \)-infected patients

The number of alveolar leukocytes was not statistically different in the three groups of \([IC(−)/Pnj(−)] \), \([IC(+)/Pnj(−)] \) and \([IC(+)/Pnj(+) \) patients (data not shown). However, there was an inverse correlation between the \( P. jirovecii \) burden and alveolar leukocyte number (Fig. 1A).

Differential leukocyte counts did not show significant differences among the samples from the three groups with respect to alveolar neutrophil and eosinophils polynuclear cell numbers (data not shown).

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The number of alveolar macrophages (AM) was significantly lower in the IC(+/Pnj(+)) group compared with the IC(−)/Pnj(−) group (15,384 IQR [7962; 33684] vs 26,985 IQR [17,743; 61,058]) but no significant difference was observed between the IC(−)/Pnj(−) and the IC(+/Pnj(+)) groups (Fig. 1B). The decrease in macrophages affected more specifically the patients with high (13,334 IQR [5,101; 23,710]) or middle P. jirovecii burden (10,803 IQR [8,261; 26,992]) (Fig. 1B) but there was no correlation between the AM number and fungal burden (data not shown). Peripheral blood leukocytes were analysed to determine whether the macrophage count was decreased only in lung alveoli. The peripheral blood monocyte count was not significantly different in the three groups (data not shown), suggesting that the decline in monocytes/macrophages was localized to the lung.

The alveolar lymphocyte count was statistically higher in the IC(+)/Pnj(+) than in the IC(−)/Pnj(−) group (4,858 IQR [1,057; 14,562] vs 1,469 IQR [789; 2,896]) (Fig. 1C). There was no significant difference between IC(−)/Pnj(−) and IC(+)/Pnj(+) groups. In the IC(+)/Pnj(+) group, the increase was specific to the patients with a low (7,017 IQR [1,385; 21,649]) or middle P. jirovecii burden (10,091 IQR [4,340; 33,079]) (Fig. 1C). This increase was specifically observed in the lungs as the lymphocyte count in the blood was not significantly different between the two
Changes in AM receptor expression in \textit{P. jirovecii}-infected patients

CD14 and CD36 expression were very low on the AM of all the patients (data not shown). The mannose receptor was expressed but there was no significant difference among the three groups for this marker (Fig. 3A). However, Dectin-1 had a significantly higher expression on the AM of the IC(+)/Pnj(+) group than on the IC(+)/Pnj(−) group (1.87 IQR [1.48; 2.57] vs 1.40 IQR [1.18; 1.65]) (Fig. 3B). There was no significant difference between the IC(−)/Pnj(−) and IC(+)/Pnj(−) groups suggesting that this increase was related to \textit{P. jirovecii}.

Aveolar lymphocyte profiles among \textit{P. jirovecii}-infected patients

The blood and alveolar CD8+T lymphocyte counts were determined. The number of alveolar CD8+T lymphocytes was similar in the two groups without detectable \textit{P. jirovecii} DNA (Fig. 2A). Conversely, CD8+T lymphocytes were significantly increased in the IC(+)/Pnj(+) group compared with the IC(+)/Pnj(−) group (4,233 IQR [2,330; 5,790] vs 606 IQR [345; 971]) (Fig. 2A). Thus, the presence of \textit{P. jirovecii} in the lung led to an increase in alveolar CD8+T lymphocytes. Nevertheless, the \textit{P. jirovecii} burden was not correlated with alveolar CD8+T lymphocyte counts (data not shown) as the latter were increased whatever the burden. This result was not linked to HIV status since a high level of CD8+ was often found (data not shown) with \textit{P. jirovecii} (+)/HIV(−) patients. The blood CD8+ lymphocyte count was not statistically different in the three groups and not correlated with fungal burden (data not shown).

Alveolar and blood CD4+T lymphocytes were evaluated. These cells evolve in the same way as alveolar lymphocytes. There was no significant difference between the two groups IC(−)/Pnj(−), IC(+)/Pnj(−) but the alveolar CD4+ lymphocyte count was statistically higher in the IC(+)/Pnj(+) than in IC(+)/Pnj(−) group (2,544 IQR [758; 10,761] vs 435 IQR [242; 1,331]) (Fig. 2B). This increase was specific to the patients with a low (3,973 IQR [880; 22,049]) or middle \textit{P. jirovecii} burden (6,073 IQR [4,178; 29,921]) (Fig. 2B). Moreover, the number of CD4+T lymphocytes was inversely correlated with the fungal burden in immunocompromised \textit{P. jirovecii} infected patients (Fig. 2C). This inverse correlation was also obtained with the percentage of alveolar CD4+T lymphocytes (Fig. 2D) and the CD4+/CD8+ ratio (Fig. 2E). In blood, no increase in CD4+ cells was observed, i.e., in the two groups of immunocompromised patients [IC(+)/Pnj(+) and IC(+)/Pnj(−)], the CD4+T lymphocyte count was not different whatever the \textit{P. jirovecii} burden, but was logically higher in the IC(−)/Pnj(−) patients than in the IC(+)/Pnj(−) group (data not shown).

Discussion

The results of the present study show that human \textit{P. jirovecii} infection was linked to changes in the local alveolar microenvironment, particularly in terms of cell numbers and in cytokine levels. These data were obtained by comparing different groups of patients with or without \textit{P. jirovecii} infections. In this study, one of the difficulties was to separate changes in the alveolar environment induced by the pathogen from those caused by immunosuppression. To solve this problem, three groups were defined among the patients recruited. We compared a group of non-immunocompromised patients with or without \textit{P. jirovecii} infections to study the specific influence of \textit{Pneumocystis} on the alveolar environment. It would be interesting to subgroup patients with \textit{P. jirovecii} according to the cause of the immunosuppression (HIV, transplantation, etc.) but the resultant patient numbers would probably be too small for accurate analysis. However, statistical analyses showed that the three groups consisted of the same proportion of HIV-positive patients, and those treated...
Changes in the alveolar environment during P. jirovecii infection

There was homogeneity within each group as to the etiology of immunosuppression that minimized the impact of this factor on the parameters studied. Another difficulty was that patients infected by P. jirovecii were quite often co-infected with a second pathogen (bacteria, fungus or virus).

However, the data show that the three groups were homogeneous relative to pulmonary infections other than P. jirovecii. In each group, the proportions of bacterial, viral and fungal infections were the same and so that reduced the influence of the other pathogen on the markers analyzed for P. jirovecii.

**Fig. 2 Alveolar lymphocyte profiles among Pneumocystis jirovecii-infected patients.** Comparison of (A) alveolar CD8+ T cell counts and (B) alveolar CD4+ T cell counts in IC(-)/Pnj(-), IC(+)/Pnj(-) and IC(+)/Pnj(+) groups (Log10). Among the IC(+)/Pnj(+) group, low, moderate and high P. jirovecii burden patients were individualized (Log10). Correlation between (C) alveolar CD4+ T cell counts (Log10) or (D) alveolar CD4+ T cell percentage (%) or (E) CD4+/CD8+ ratio in BAL (Log 10) and P. jirovecii burden (Ct).

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*Calculated by Mann-Whitney rank sum test.
*Calculated by Pearson product moment correlation test.
__ median of the population.

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Iriart et al. The direct comparison of the concentrations of cellular or soluble material in the BAL among different patients is not ideal because of their different pulmonary status (oedema). In order to more accurately standardize the quantification of the elements within the BAL, we decided to correct for the dilution of the alveolar component to take into account any eventual oedema. According to Dargaville et al. [21], urea is the most appropriate choice of a marker since the lavage/plasma urea ratio appears to provide a meaningful estimate of ALF recovery even in the diseased lung. The volume of ALF (including the volume from an eventual oedema) and the BAL dilution was established for each patient to enable the determination of the absolute quantity of ALF components. Nevertheless, it is noteworthy to point out that directly using BAL concentrations without corrections (expressed as ‘per ml of BAL’), would have provided the same results in the inter-group comparisons, as well as for the correlations.

To evaluate the evolution of the number of cells among immunocompromised *P. jirovecii*-infected patients, we determined the alveolar lymphocyte, macrophage and total leukocyte counts. Despite a constant alveolar total leukocyte number, we showed that during *P. jirovecii* infection, lymphocytes increased whereas at the same time, the AM decreased. In line with a previous study, our results confirm the importance of these two cell types in the pathophysiology of *P. jirovecii* infection [12]. Moreover, although Vestbo et al. [22] observed a correlation in the

### Table 2 Alveolar cytokine profiles from *Pneumocystis jirovecii*-infected patients.

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n = 32)</th>
<th>Group 2 (n = 33)</th>
<th>Group 3 (n = 64)</th>
<th>P value between group 1 and 2</th>
<th>P value between group 2 and 3</th>
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<tbody>
<tr>
<td></td>
<td>IC(–)/Pnj(–) group</td>
<td>IC(+)/Pnj(–) group</td>
<td>IC(+)/Pnj(+) group</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TNF-α (pg)</td>
<td>187 [165; 435]</td>
<td>417 [251; 764]</td>
<td>384 [180; 826]</td>
<td>0.270 a</td>
<td></td>
</tr>
<tr>
<td>IL-10 (pg)</td>
<td>296 [215; 495]</td>
<td>266 [235; 728]</td>
<td>421 [259; 938]</td>
<td>0.992 a</td>
<td>0.162 a</td>
</tr>
<tr>
<td>IL-6 (pg)</td>
<td>1022 [481; 5221]</td>
<td>1185 [764; 2487]</td>
<td>9431 [4002; 39273]</td>
<td>0.771 a</td>
<td>0.001 a</td>
</tr>
<tr>
<td>TGF-β (pg)</td>
<td>36476 [17864; 48982]</td>
<td>40297 [16984; 52592]</td>
<td>36573 [24155; 56138]</td>
<td>0.973 a</td>
<td>0.488 a</td>
</tr>
<tr>
<td>IL-17 (pg)</td>
<td>179 [97; 253]</td>
<td>96 [86; 120]</td>
<td>105 [83; 198]</td>
<td>0.189 a</td>
<td>0.964 a</td>
</tr>
</tbody>
</table>

IC(–), patients without immunosuppression; IC(+), patients with immunosuppression; Pnj(–), patients without *Pneumocystis jirovecii* DNA; Pnj(+), patients with *Pneumocystis jirovecii* DNA.

1Data are presented as median IQR [25%;75%].

*Calculated by Mann-Whitney rank sum test.
lung interstitium between the amount of *P. jirovecii* and the accumulation of leukocytes, there was no data on this relationship in the alveolar environment where *P. jirovecii* was present and interacting directly with the immune cells. Our study demonstrated that the number of alveolar leukocytes (particularly AM and lymphocytes) was inversely associated with the *P. jirovecii* burden.

Binding and phagocytosis of *P. jirovecii* by AM is a critical first step in the initiation of the immune response and the eventual clearance of the organism [23]. Several studies have shown that the percentage of AM in BAL fluid of AIDS patients with PJP was reduced [24–26]. It is unknown whether this change was due to a decrease in the number of AM or an increase in other cell types. In the Dex-Pc rat model, Lasbury et al. determined that the absolute number of AMs was decreased in infected rats [27]. Using urea determinations, our study confirms that during PJP, the absolute AM number was significantly lower in patients with *P. jirovecii* infection whatever the cause of the immunosuppression (HIV or not). This is important data as some authors have shown that an increase in the numbers of alveolar macrophages was associated with a decreased mortality in animals [28] and man [29]. Moreover, the low AM count is linked to the presence of *P. jirovecii* and not to immunosuppression since there is no difference between the IC(−)/Pnj(−) and IC(+)/Pnj(−) groups of patients. The reduced AM count is a localized pulmonary phenomenon as the blood monocyte count was similar between controls reduced AM count is a localized pulmonary phenomenon and not to immunosuppression [30]. In our investigation, alveolar CD8+ T cells increased locally and specifically in lung, whatever the fungal burden. Studies of HIV *P. jirovecii*-infected patients [38,39,42] or of SIV-*Pneumocystis carinii*-co-infected Rhesus Macaques [43] have provided similar results but its origin was not explained (HIV or *Pneumocystis* infection). Studying only HIV-negative patients, we have demonstrated that the lymphocyte count increased during *P. jirovecii* infection whatever the fungal burden [25,27,38–41]. Nevertheless, even if our study showed an overall increase in alveolar lymphocytes among *P. jirovecii*-infected patients, we found that this lymphocyte count varied according to the *P. jirovecii* burden.

In our investigation, alveolar CD8+ T cells increased locally and specifically in lung, whatever the fungal burden. Studies of HIV *P. jirovecii*-infected patients [38,39,42] or of SIV-*Pneumocystis carinii*-co-infected Rhesus Macaques [43] have provided similar results but its origin was not explained (HIV or *Pneumocystis* infection). Studying only HIV-negative patients, we have demonstrated that *P. jirovecii* infection is specifically associated with a CD8+ T cell increase. In murine models, CD8+ T cells that are recruited to the lung in large numbers in response to a *P. jirovecii* infection do not seem to be directly implicated in *P. jirovecii* clearance but rather have been associated with lung injury [9–11].

CD4+T cells play crucial roles in the host defence against *Pneumocystis*, both in humans [44,45] and in animal models [7,46] of infection, by stimulating and maximizing the immune system. PJP development in HIV-infected patients or after intratracheal instillation in mice depleted apoptosis. These two explanations (lack of immune stimulation and apoptosis) may be complementary to explain the pathophysiology of this phenomenon.

The uptake of *P. jirovecii* by macrophages occurs through multiple receptor systems, including mannose receptors that interact with the major surface glycoprotein (gp-A) existing on the surface of *Pneumocystis* [34] cells, and the interaction between *P. jirovecii* β-glucans and the dectin-1 receptor [35]. Koziel et al. [36] observed that AM isolated from HIV-infected patients exhibited down regulation of mannose receptor expression and reduced binding and phagocytosis of *P. jirovecii*. However, we did not detect a decrease in AM mannose receptor expression in *P. jirovecii*-infected patients, whatever their HIV serological status. Interestingly, we showed that dectin-1 expression on AM is increased when patients are infected with *P. jirovecii*. Dectin-1 plays a part in *P. jirovecii* nonopsonic phagocytosis, in the production of AM cytokine and reactive oxygen species [35], and is particularly important in protection against *P. jirovecii* [37]. In a context of pulmonary deficiency of the innate immunity (dramatic decrease in AM), this over-expression of AM dectin-1 may enhance the host defence against *P. jirovecii*.

Lymphocyte responses seem to be essential for fungal elimination during *P. jirovecii* infections [3]. Our study demonstrated that the alveolar lymphocyte count increased during this disease. This change occurs principally among CD3+ T lymphocytes (CD8+ and CD4+T lymphocytes). Previous studies of BAL lymphocytes, principally of HIV patients, have already shown an increase in the alveolar percentage of lymphocytes during *P. jirovecii* infections [25,27,38–41]. Nevertheless, even if our study showed an overall increase in alveolar lymphocytes among *P. jirovecii*-infected patients, we found that this lymphocyte count varied according to the *P. jirovecii* burden.

for CD4+ T cells, proves the fundamental role of these cells in *P. jirovecii* infection pathophysiology. Recent studies in animals and humans, have begun to elucidate the mechanisms of the CD4+ T-cell activation during *P. jirovecii* infection. In healthy individuals, the release of TNF-α and IL-1 by AM is required for the initiation of the CD4+ T-cell response [47]. Moreover, CD4+ T cells proliferate in response to *Pneumocystis* antigens and generate cytokines, including IFN-γ and lymphotactin [3,48] required for additional lymphocyte recruitment [49]. Our results of the alveolar CD4+ T cell count and the existence of an inverse correlation between the *P. jirovecii* burden and CD4+ T cells were consistent with those of previous studies. The significant increase in alveolar CD4+ among patients with a low *P. jirovecii* burden reflects a sufficient proliferation and recruitment of these cells to maintain the fungal burden at a low level and to control the infection. Conversely, patients in the high burden group presented the same level of CD4+ as the controls. No recruitment or proliferation of the CD4+, which were necessary for an appropriate anti-*P. jirovecii* immune response, occurred among these patients. This alveolar immunosuppression explains the high *P. jirovecii* burden observed in these patients. On this assumption, there could be an equilibrium in the aevoli of the lungs between the fungal concentration and CD4+ T cell levels, underlined in this study by the inverse correlation between these two parameters. This strong and inverse correlation could be indicative of a more direct link between *P. jirovecii* and CD4+ T cells than between *P. jirovecii* and AM.

The alveolar environment also showed changes in the cytokine profile during *P. jirovecii* infection. Data have often been contradictory according to the study models (human or animal) or methodology [50–53]. Moreover, many of the previous studies did not take into consideration the different BAL dilutions, which is why we used the urea method to avoid this bias. In rodent models of PJP, an increase in IL-10 and TNF-α has been observed [54–56] but opposite results were obtained in rabbit models [48,57,58]. In humans, two *ex vivo* studies on HIV-positive or -negative patients with or without *P. jirovecii* infections did not demonstrate an increase in TNF-α or IL-10 release from BAL cells [52,59]. Our study indicated that there were no differences in the alveolar IL-10 or TNF-α levels between patients with or without *P. jirovecii* infection. These results relative to the IL-10 and TNF-α concentrations are in agreement with the findings reported by Israël-Biet *et al.* and Zhang *et al.* on humans [52,59] or with rabbit models of *P. jirovecii* [48,57,58]. Knowing that macrophages are the main cells producing these two cytokines and which are also the majority of cells in BAL, we can postulate that the low level of TNF-α and IL10 is attributable to the diminution of macrophages in the alveolar compartment. This low level of cytokines may cause a disruption in the cytokine network and lead to a deficiency in the immune response against *P. jirovecii*. The level of IL-6 was statistically higher in the *P. jirovecii* group. Although, this increase has been shown in many animal models of PJP [53,54,56,60], data on alveolar IL-6 concentrations in humans are extremely limited. Using the cell pellet or AM obtained from BAL fluid, two studies showed that IL-6 production was not increased *in vitro* [52,59]. The increase in alveolar IL-6 observed *in vivo* during *P. jirovecii* infection may originate from the production by *P. jirovecii* attachment to alveolar epithelial cells [61,62], rather than from the cells directly present in the BAL. A few data suggest that respiratory impairment and death are more closely correlated to the extent of lung inflammation than to the burden of the etiologic agent during PJP [63]. Agusti *et al.* [64] have shown that IL-6 levels in BAL fluid may help in the evaluation of the pulmonary inflammatory response and may be an independent predictor of mortality. Therefore, IL-6 levels may play an important role in the pathophysiology of serious PJP.

IL-6 is also implicated in the recently described Th17 immune response which is reported to be involved in clearing fungal pathogens [17]. IL-6 and TGF-β stimulate naive CD4+ T cells to differentiate into a novel subset called Th17 cells [65–67] which are distinct from the classical Th1 and Th2 cells. Moreover, recent experimental and clinical studies have shown an important role of the Dectin-1 pathway in the differentiation of Th17 and Th1 cells, particularly during fungal infections *in vivo* and in humans [68]. Only two studies considered the Th17 response in animal models of *P. jirovecii*. The first study indicated that in immunocompetent mice (non-depleted for CD4+ T cells), lower levels of IL-17 in the lung resulted in a transient decrease in the clearance of *Pneumocystis* cells [69]. The second study suggested that IFN-γ was needed to suppress Th17 differentiation and that the Th17 response seemed to be ineffective against *Pneumocystis* infection in immunocompromised hosts [70]. To evaluate the alveolar Th17 response in humans, we measured TGF-β and IL-17 in ALF. Levels of TGF-β and IL-17 were not statistically different between the group of *P. jirovecii*-infected patients and the control groups. These data may mean that the Th17 response is not predominant in the pathophysiology of PJP or that this response is not directly observable by measuring IL-17 or TGF-β in the BAL (binding of IL-17 or TGF-β). Moreover, these results were in apparent disagreement with the higher Dectin-1 expression on AM. The first explanation could be that the Th17 response is independent of Dectin-1 during *Pneumocystis* infection. The second hypothesis is that the Th17 response depended on Dectin-1 but was disrupted in its development. Even if Dectin-1 was...
overexpressed during \textit{P. jirovecii} infection, there was also a considerable decrease in AM numbers with an impairment of the function of these cells. As described in some studies for TGF-\(\beta\) [71,72], AM cytokine secretion and activation might be decreased, causing alterations in the interleukin collaboration and the ability to interact with lymphocytes to induce the Th17 response. Further studies will be necessary to better understand the role of this response in \textit{P. jirovecii} infection both in the immunocompetent and immunocompromised host.

In summary, this study showed that human \textit{P. jirovecii} infection was linked to changes in the local alveolar microenvironment. AM and lymphocytes play a major role in the pathophysiology of this disease. Even if there was an overall decrease in the AM with an increase in the alveolar lymphocytes, the count of these cells was very dependent on the \textit{P. jirovecii} burden. There was an equilibrium between the alveolar immune impairment and the development of \textit{P. jirovecii}. A severe pulmonary immune suppression, associated with a very low level of AM and a lack of recruitment and proliferation of CD4+, led to a very high burden of \textit{P. jirovecii}. The high level of IL-6 (probably secreted by alveolar epithelial cells) and of the CD8+T lymphocytes may also explain the pathophysiology of this infection by contributing to the inflammation and to the development of the lung injury observed in this disease. Moreover, our study shows changes in the pulmonary environment, even for low \textit{P. jirovecii} burdens. These low counts, clinically considered as colonization, involve an increase in the number of alveolar lymphocytes (CD8+ and CD4+T cells), IL-6 level and Dectin-1 expression on AM. These pulmonary colonizations may stimulate inflammation and may play a role in the development of lung diseases such as chronic obstructive pulmonary disease [73].

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Changes in the alveolar environment during P. jirovecii infection

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