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IL-12 and IL-18 Are Increased and Stimulate IFN-γ Production in Sarcoid Lungs

Katsunori Shigehara,*‡ Noriharu Shijubo,† Mitsuhide Ohmichi,§ Ryuji Takahashi,‡ Shin-ichiro Kon,† Haruki Okamura,‖ Masashi Kurimoto,|| Yohmei Hiraga,§ Tachio Tatsuno,* Shosaku Abe,† and Noriyuki Sato‡

Sarcoidosis is a systemic chronic granulomatous disease of unknown cause. Recent investigations revealed that the cytokine profile in inflamed lesions of sarcoidosis is Th1 dominant. To obtain better immunopathologic understanding of sarcoidosis, we examined the expression of IL-12 and IL-18 and their roles in IFN-γ production in pulmonary sarcoidosis. Sarcoid cases had significantly elevated levels of IL-12 (p40 and p70) and IL-18 in bronchoalveolar lavage (BAL) fluids compared with healthy subjects. IL-12 p70 and IL-18 were immunohistochemically expressed in the epithelioid cells and giant cells of sarcoid granulomas. Significant induction of IFN-γ, IL-12 p70, and IL-18 was observed from sarcoid BAL fluid cells with LPS stimulation, whereas LPS tended to induce only IL-12 p70 in BAL fluid cells from healthy subjects. Sarcoid cases had significantly greater IFN-γ induction with LPS stimulation than healthy subjects did. IL-18 mRNA expression was observed in freshly isolated sarcoid BAL fluid cells as well as in LPS-stimulated sarcoid BAL fluid cells, but IFN-γ and IL-12 mRNA expression was observed only in LPS-stimulated BAL fluid cells. Treatment with anti-IL-12- and anti-IL-18-neutralizing Abs significantly inhibited IFN-γ production from LPS-stimulated BAL fluid cells of sarcoid cases. Coadministration of rIL-12 or rIL-18 induced greater IFN-γ production in sarcoid BAL fluid cells than in normal BAL fluid cells. We concluded that bioactive IL-12 and IL-18 were produced in sarcoid BAL fluid cells and synergistically induced IFN-γ production, indicating important cytokines in the Th1 response of sarcoidosis. The Journal of Immunology, 2001, 166: 642–649.

Interleukin-18 is a recently described member of the IL-1 cytokine family and was initially defined as an IFN-γ-inducing factor (1). Subsequent studies have elucidated a broad array of effector functions beyond lymphocyte activation that implicates IL-18 as an important regulator of both innate and acquired immune responses (2, 3). Several recent reports have indicated that IL-18 is expressed at sites of chronic Th1-mediated inflammatory diseases such as Crohn’s disease (4, 5) and rheumatoid arthritis (6). IL-18 promotes type 1 cytokine production from NK cells and T cells in leprosy (7). Increased levels of circulating IL-18 correlate with increased levels of circulating IFN-γ in human tuberculosis (8).

In the type 1 immune response (Th1 responses), IL-12 and IL-18 are important cytokines that synergistically stimulate IFN-γ production (9–11) and enhance NK and T cell-mediated cytototoxicity (12, 13). Furthermore, IL-12 induces the differentiation of T cell precursors (Th0 cells) into Th1 cells (14). IL-12 and IL-18 play critical roles in resistance to infection with intracellular parasites and bacteria and in some kinds of autoimmune diseases and tumor toxicity (2, 3, 15).

Sarcoidosis is a systemic disease of unknown origin that is characterized by noncaseating epithelioid cell granuloma with dominant infiltration of CD4+ Th cells and macrophages (16). Many cytokines and chemokines play an important role during the processes of sarcoid granuloma (17). On the basis of recent Th1 and Th2 paradigms, the cytokine profile of sarcoidosis is Th1 dominant when disease activity is high (17–19). Recent studies have demonstrated enhanced expression of IL-12 (20–22) and IL-12R (23, 24) in sarcoid lungs. IL-18 expression was reported to be increased in the airway epithelial cells of sarcoid cases (25). However, the cooperative roles of IL-12 and IL-18 in sarcoidosis have not been fully resolved in Th1 response.

In this study we investigated local production of IL-12 and IL-18 in patients with sarcoidosis and their influences on the Th1 response of sarcoidosis concerned with IFN-γ production in pulmonary sarcoidosis.

Materials and Methods

Study population

The diagnosis of sarcoidosis was established in 58 individuals (23 men (mean age, 26.0 years) and 35 women (mean age, 47.2 years); 36 nonsmokers and 22 smokers). They had histologic evidence consistent with sarcoidosis in the lung (showing noncaseating epithelioid cell granuloma) without any evidence of mycobacterial, fungal, or parasitic infection. None had a history of exposure to organic or inorganic materials known to cause granulomatous lung diseases. No patient had received corticosteroid therapy at the time of the study. Forty-eight patients had abnormal chest x-ray findings; 36 patients demonstrated hilar lymphenadenopathy alone, 11 patients had hilar lymphadenopathy and interstitial infiltrates of the lung field, and 1 patient had interstitial infiltrates of the lung field alone. Ten patients had no abnormal chest x-ray findings, but had positive biopsy findings of the lung and uveitis and thus were strongly suspected to have sarcoidosis. The assessment of disease activity included clinical features, chest x-ray,
high resolution computed tomography, lung function tests, bronchoalveolar lavage (BAL) assay, 68Ga scan, and routine blood studies. The examinations revealed 35 extrapulmonary sarcoid lesions (27 uveitis, and 6 skin, 1 bone, and 1 parotid gland) and 28 sarcoid patients; five patients had two extrapulmonary lesions, and one patient had three extrapulmonary lesions. For comparison, 23 normal individuals were studied (13 men and 10 women: mean age, 34.8 years). Thirteen individuals were nonsmokers, and 10 were smokers. No healthy individuals had a history of cardiopulmonary or other illness. There were no abnormal findings on physical examination or chest x-ray, and all results showed lung function tests within normal limits. Informed consent was obtained from the patients with sarcoidosis and normal volunteers.

**BAL analysis**

BAL analysis was performed on 58 sarcoid cases and 23 healthy subjects as previously described (26). Three 50-ml aliquots of 0.9% sterile saline were instilled into a bronchus in the right middle lobe through a fiberoptic bronchoscope in routine BAL analysis. To obtain more BAL fluid cells for further examinations, six 50-ml aliquots were instilled in some sarcoid cases and healthy subjects. BAL fluids were recovered by gentle suction immediately after the infusion of each aliquot. BAL fluids were filtered through a single layer of sterile gauze. After centrifugation (4000 g, 10 min), supernatants were removed. BAL fluids were stored at −80°C. To synthesize cDNA, 1 μg of total RNA was incubated in the presence of Moloney murine leukemia virus reverse transcriptase (Life Technologies, Gaithersburg, MD) and 5 ng/ml oligo(dT)12–18 primers (Pharmacia Biotech, Uppsala, Sweden) for 30 min at 42°C using reaction conditions described by the manufacturer (Life Technologies), and samples were stored at −80°C.

**Analysis of cytokine gene expression**

For cytokine gene expression analysis in BAL fluid cells, nine sarcoid cases and six healthy nonsmokers were selected from among increased total BAL fluid cell count study subpopulations (sarcoid cases, >3 × 10^6 cells; healthy subjects, >2 × 10^6 cells of six 50-ml aliquots). Total RNA of 5 × 10^6 cultured BAL fluid cells was isolated using Isogen (Nippon Gene, Toyama, Japan) and quantified by measurement of absorbance at 260 nm. To synthesize cDNA, 1 μg of total RNA was incubated with reverse transcriptase (Life Technologies, Gaithersburg, MD) and 5 ng/ml oligo(dT)12–18 primers (Pharmacia Biotech, Uppsala, Sweden) for 30 min at 42°C using reaction conditions described by the manufacturer (Life Technologies), and samples were stored at −80°C.

**Immunooassay of BAL fluids and cultured supernatants of BAL fluid cells**

BAL fluids were concentrated 10-fold by membrane dialysis using Vivaspin (Vivascience, Stonehouse, Glostrup, Denmark) for cytokine immunoassays. We measured IFN-γ, IL-12 p40 and p70, and IL-18 and GAPDH. Cycle numbers corresponding to exponential phase were individually determined for each primer set. The cycles numbers were 45 for IFN-γ, IL-12 p40, and IL-12 p35 and 30 for IL-18 and GAPDH. BC products were stained with ethidium bromide after electrophoresis, and the intensities of the bands on photographs of the agarose gels were quantified using a CCD image sensor (Molecular Dynamics, Tokyo, Japan) using optical software (ImageQuant, version 3.3, Molecular Dynamics).

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**Table I. Characteristics of BAL analysis**

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>Sarcoidosis</th>
<th>Healthy Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Recovery (%)</td>
<td>65.2 ± 2.9</td>
<td>66.3 ± 1.8</td>
</tr>
<tr>
<td>Total cell</td>
<td>18.8 × 10^6</td>
<td>11.9 × 10^6</td>
</tr>
<tr>
<td>Macrophage (%)</td>
<td>63.9 ± 4.3</td>
<td>86.3 ± 2.1</td>
</tr>
<tr>
<td>Lymphocyte (%)</td>
<td>35.9 ± 4.3</td>
<td>12.6 ± 0.3</td>
</tr>
<tr>
<td>Neutrophil (%)</td>
<td>0.7 ± 0.2</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td>Eosinophil (%)</td>
<td>0.3 ± 0.1</td>
<td>0.8 ± 0.4</td>
</tr>
<tr>
<td>CD4/CD8</td>
<td>5.1 ± 0.7</td>
<td>1.7 ± 0.7</td>
</tr>
</tbody>
</table>

* Abbreviation used in this paper: BAL, bronchoalveolar lavage.

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3 Data are expressed as mean ± SEM. * p < 0.01 compared with healthy subjects.
Results

IL-12 and IL-18 protein levels in BAL fluids

We measured protein levels of IL-12 p70, IL-12 (p40 and p70), and IL-18 in BAL fluids using a specific ELISA system. IL-12 p70 was not detected in any BAL fluids, nor was it observed in 10-fold-concentrated BAL fluids of the examined cases. When IL-12 (p40 and p70) levels were compared between nonsmokers and smokers, sarcoid and healthy smokers had significantly lower IL-12 (p40 and p70) levels in BAL fluids than sarcoid (p < 0.0001) and healthy nonsmokers (p = 0.0008), respectively. Sarcoid nonsmokers and smokers had significantly increased IL-12 (p40 and p70) levels in BAL fluids compared with healthy nonsmokers (p < 0.0001) and smokers (p = 0.0012), respectively (Fig. 1).

There were no significant differences in BAL fluid IL-18 levels between nonsmokers and smokers in healthy subjects or sarcoid cases. Sarcoid nonsmokers and smokers had significantly increased IL-18 levels in BAL fluids compared with healthy nonsmokers (p < 0.0001) and smokers (p = 0.0131), respectively (Fig. 1). Sarcoid cases had significantly increased levels of IL-12 (p40 and p70) and IL-18 proteins in BAL fluids compared with healthy subjects.

Immunohistochemistry of IL-12 and IL-18 in sarcoid lungs

In normal lungs, very little positive staining of IL-12 and IL-18 was found. Only some alveolar macrophages showed weak staining for IL-12 and IL-18 (data not shown). In contrast, positive staining of IL-12 p70 and IL-18 was found in epithelioid cells of granulomas and macrophages in alveolar lumens and in the interstitium of the sarcoid lungs, especially in the cytoplasm (Fig. 2, A and C). Giant cells were also positive for IL-12 p70 and IL-18. Lymphocytes and fibroblasts around granulomas were negative.

IFN-γ, IL-12, and IL-18 mRNA expression and their production in cultured BAL fluid cells

Freshly isolated sarcoid BAL fluid cells expressed IL-18 mRNA, but not IFN-γ, IL-12 p40, or IL-12 p35 mRNA. IFN-γ and IL-12 p40 mRNA expression was observed at 4 and 48 h after initial culture with LPS stimulation, and IL-12 p35 mRNA expression was observed at 4 h after LPS stimulation. IL-18 mRNA expression was also observed at 4 and 48 h after initial culture with LPS stimulation (Fig. 3A). IL-18 mRNA expression was observed in BAL fluid cells freshly isolated from healthy subjects, and it was observed at 4 and 48 h after initial culture with LPS stimulation (data not shown).

Spontaneous release of IFN-γ and IL-18 was observed in sarcoid cases and healthy subjects, whereas spontaneous release of IL-12 p70 was only found in five sarcoid cases and one healthy subject (Fig. 3B). There was a significant positive correlation between spontaneous releases of IFN-γ and IL-18 from unstimulated BAL fluid cells in sarcoid cases (Fig. 3C), but not in healthy subjects.

By culture with LPS stimulation, IFN-γ, IL-12 p70, and IL-18 production was significantly induced from sarcoid BAL fluid cells, whereas culture with LPS tended to induce only IL-12 p70 production (p = 0.06; Fig. 4) in BAL fluid cells from healthy subjects, but not IFN-γ or IL-18 production. When compared cytokine induction by LPS stimulation between sarcoid cases and healthy subjects, sarcoid cases had significantly higher IFN-γ production than healthy subjects (p < 0.0001). There were no significant differences in IL-12 p70 or IL-18 induction with LPS stimulation from BAL fluid cells between sarcoid cases and healthy subjects. There was a significant positive correlation between IFN-γ and IL-12 p70 production from LPS-stimulated BAL fluid cells in sarcoid cases (Fig. 3C), but not in healthy subjects.

FIGURE 1. The concentrations of IL-12 (p40 and p70) and IL-18 in BAL fluids of patients with sarcoidosis and in healthy volunteers. BAL fluids were concentrated 10-fold. IL-12 (p40 and p70) and IL-18 were measured by specific ELISA. Each circle represents a single individual. ○, Nonsmokers; ●, smokers. The dashed lines indicate the levels of sensitivity of the IL-12 (p40 and p70) assay (5 pg/ml) and the IL-18 assay (10 pg/ml). Tobacco smoking affected BAL fluid IL-12 (p40 and p70) levels, but it did not affect BAL fluid IL-18 levels. Sarcoid patients had significantly elevated levels of IL-12 (p40 and p70) and IL-18 in BALF compared with healthy subjects. All comparisons were made using the Mann-Whitney U test.
Effects of IL-12- and IL-18-neutralizing Abs on IFN-γ production in BAL fluid cells

When using isotype-matched mouse IgG and/or nonimmune goat IgG as a negative control, LPS-stimulated sarcoid BAL fluid cells produced significantly higher IFN-γ (578 ± 219 pg/ml) than healthy subjects (67.3 ± 30.1 pg/ml). Anti-IL-12-neutralizing Ab or anti-IL-18-neutralizing mAb inhibited IFN-γ production from LPS-stimulated BAL fluid cells in 13 sarcoid cases (percent inhibition: anti-IL-12 Ab, 30.0 ± 5.1%; anti-IL-18 mAb, 39.8 ± 6.4%) and five healthy subjects (percent inhibition: anti-IL-12 Ab, 37.2 ± 7.8%; anti-IL-18 mAb, 44.4 ± 11.2%). With coadministration of anti-IL-12-neutralizing Ab and anti-IL-18-neutralizing mAb, IFN-γ production from BAL fluid cells was extremely decreased in sarcoid cases (percent inhibition, 58.5 ± 5.6%) and healthy subjects (percent inhibition, 78.0 ± 4.7%). Inhibition by treatment with anti-IL-12- and/or anti-IL-18-neutralizing Ab was significant in sarcoid cases (all p < 0.05). In addition, IFN-γ mRNA expression was obviously decreased in LPS-stimulated sarcoid BAL fluid cells when concomitantly treated with anti-IL-12- and anti-IL-18-neutralizing Abs. These results indicated that IL-12 and IL-18 produced from sarcoid BAL fluid cells were bioactive and were synergistic potent stimulators of IFN-γ in sarcoid lungs.

Effects of rIL-12 and/or rIL-18 on IFN-γ production in BAL fluid cells

We investigated IFN-γ production with stimulation of rIL-12 and/or rIL-18 in three sarcoid cases and three healthy subjects (Fig. 5). Under stimulation with rIL-12 alone, inducible IFN-γ levels were very low in both sarcoid cases and healthy subjects, whereas under stimulation with rIL-18 alone, IFN-γ was inducible in a dose-dependent manner in two sarcoid cases (cases 2 and 3) and one healthy subject (case 2). With the concurrent application of both recombinants, IFN-γ was inducible in a dose-dependent manner. With stimulation of rIL-12 and rIL-18, sarcoid BAL fluid cells exhibited greater IFN-γ production compared with normal BAL fluid cells. However, high dose stimulation reached a plateau of IFN-γ production in all sarcoid cases. Recombinant IL-12 and IL-18 synergistically stimulated IFN-γ production in BAL fluid cells of sarcoid cases and healthy subjects.

Discussion

In the present study we demonstrated that IL-12 (p40 and p70) and IL-18 were increased in BAL fluids of patients with sarcoidosis. However, we failed to detect IL-12 p70 in 10-fold concentrated BAL fluids of the sarcoid cases examined, although IL-12 p70 was immunohistochemically observed in epithelioid cells and macrophages in sarcoid lungs. Sample concentration and subsequent freezing and thawing may make the exact measurement of IL-12 p70 uncertain (20). BAL fluid levels of IL-12 (p40 and p70) were significantly lower in smokers than in nonsmokers, while tobacco smoking did not affect BAL fluid levels of IL-18. Tobacco smoking affects cytokine production by alveolar macrophages (27, 28). Therefore, we studied only the nonsmoker population for various analyses using BAL fluid cells. In addition, the present study had a study design limitation of the use of BAL fluid cells, but it is well known that BAL fluid cells indirectly reflect granulomatous events in active sarcoidosis (16–18, 21, 22). This study demonstrated that intensive expression of IL-12 p70 and IL-18 was immunohistochemically observed in sarcoid lungs.
IL-18 mRNA expression was observed in BAL fluid cells freshly isolated from sarcoid cases as well as healthy subjects, although IFN-γ and IL-12 mRNA expression was observed only after LPS stimulation in sarcoid BAL fluid cells. Constitutive IL-18 mRNA has been found in various organs and macrophage lineage cells and cell lines (2, 13, 30). The lungs are constantly stimulated through various exogenous stimuli. IL-18 may constantly produce and demonstrate an innate immunity even if virulent pathogens do not attack in the respiratory tract. This study demonstrated that IL-18 was significantly induced with LPS stimulation from sarcoid BAL fluid cells, while very weak induction was found in BAL fluid cells from healthy subjects.
was markedly enhanced with LPS stimulation in a murine *Propionibacterium acnes*-conditioned liver injury model. Alveolar macrophages of sarcoidosis are thought be activated by unknown stimuli. Activation of alveolar macrophages may be a clue to elucidate marked induction of IL-18 with LPS stimulation from BAL fluids of patients with sarcoidosis.

**FIGURE 4.** Effects of anti-IL-12 and anti-IL-18 neutralization on IFN-γ production of BAL fluid cells. A, BAL fluid cells from 13 sarcoid cases and five healthy subjects were incubated in the presence of anti-IL-12-neutralizing polyclonal Ab (10 µg/ml) and/or anti-IL-18-neutralizing mAb (125-2H, 1 µg/ml) with LPS stimulation for 48 h. Normal goat IgG and/or isotype-matched mouse IgG was used as a negative control. Culture supernatants were collected, and the IFN-γ concentration was measured using a specific ELISA. Data are expressed as the percent inhibition. *p < 0.05, IFN-γ levels in cells treated with neutralizing Ab(s) vs IFN-γ levels in cells treated with negative control IgG. Treatment with anti-IL-12 and anti-IL-18 Abs markedly inhibited IFN-γ production by LPS-stimulated BAL fluid cells (sarcoid cases, 58.5 ± 5.6%; healthy subjects, 78.0 ± 4.7%). B, mRNA expression of IFN-γ in sarcoid BAL fluid cells with concomitant use of anti-IL-12- and anti-IL-18-neutralizing Abs. IFN-γ mRNA expression was obviously down-regulated with concomitant use of anti-IL-12- and anti-IL-18-neutralizing Abs.

**FIGURE 5.** Effects of human rIL-12 and/or rIL-18 on IFN-γ production by BAL fluid cells. BAL fluid cells (three sarcoid cases and three healthy subjects) were incubated with rIL-12 and/or rIL-18 for 48 h. Culture supernatants were collected, and IFN-γ concentrations were measured by specific ELISA. Remarkable synergistic enhancement of IFN-γ production was observed with coadministration of rIL-12 and rIL-18. Enhancement of IFN-γ production was dose dependent; at high concentrations of rIL-12 and rIL-18, IFN-γ production reached a plateau. Sarcoid cases had greater IFN-γ induction with coadministration of rIL-12 and rIL-18 than did healthy subjects.
IL-12 and IL-18 stimulate IFN-γ production in sarcoid lungs

References


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