The Journal of Immunology

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J Immunol 1998; 161:6413-6420; ;
http://www.jimmunol.org/content/161/11/6413

This information is current as of October 22, 2021.

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.

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Involvement of the IP-10 Chemokine in Sarcoid Granulomatous Reactions[1]

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The accumulation of T cells and monocytes at sites of ongoing inflammation represents the earliest step in the series of events that lead to granuloma formation in sarcoidosis. In this study, we evaluated the pulmonary production of IFN-inducible protein 10 (IP-10), a CXC chemokine that stimulates the directional migration of activated T cells. Striking levels of IP-10 were demonstrated in the bronchoalveolar lavage (BAL) fluid of 24 patients with pulmonary sarcoidosis and lymphocytic alveolitis, as compared with patients with inactive disease or control subjects. A positive correlation was demonstrated between IP-10 levels and the number of sarcoid CD45R0$^+$/CD4$^+$ cells in the BAL. Immunochemistry, performed with an anti-human IP-10 polyclonal Ab in lymph nodes displaying prominent sarcoid granulomas, showed that cells bearing IP-10 were mainly epithelioid cells and CD68$^+$ macrophages located inside granulomatous areas. Macrophages recovered from the BAL of sarcoid patients stained positive for IP-10 protein. Furthermore, alveolar macrophages isolated from sarcoid patients with T cell alveolitis and cultured for 24 h in presence of IFN-γ secreted definite levels of IP-10 capable of inducing T cell chemiotaxis. Interestingly, alveolar lymphocytes recovered from patients with active sarcoidosis were CD4$^+$ T cells expressing Th1 cytokines (IL-2 and IFN-γ) and high levels of CXCR3. Taken together, these data suggest the potential role of IP-10 in regulating the migration and activation of T cells toward sites of sarcoid inflammatory process and the consequent granuloma formation. *The Journal of Immunology,* **1998, 161: 6413–6420.**

Sarcoidosis is a chronic, multisystem disorder of unknown cause(s) characterized in involved organs by an accumulation of activated CD4$^+$ T cells and macrophages, noncaseating epithelioid cell granuloma, and tissue injury. Although sarcoidosis can affect any organ in the body, the pulmonary tract is most frequently involved. Morphologically, sarcoid granuloma represents a typical delayed-type hypersensitivity granuloma. The central core is made up of a number of monocytes/macrophages and epithelioid cells, which are derived from mononuclear phagocytes; it also contains giant, multinucleated cells that derive from the aggregation of macrophage cells. Epithelioid cell foci are surrounded by a mantle of CD4$^+$ T lymphocytes and plasma cells (1, 2).

Although the etiology of sarcoidosis is still unknown, the characteristic immunologic and immunohistologic patterns of the granulomatous lesions suggest that they are the result of an Ag-driven response. Studies of sarcoid macrophages have shown that these cells behave as versatile secretory cells that, acting as APCs, release a great variety of cytokines, including TNF-α, IL-12, IL-15, and growth factors (3–6). There is also evidence that sarcoid T cells, which are biased in expression of genes for the β-chain V region of the TCR (7–9), spontaneously release IFN-γ and IL-2 (10–11). Taken together, these data have suggested the concepts that 1) sarcoid granulomas are formed in response to a persistent and poorly degradable antigenic stimulus that locally induces an oligoclonal Th1-type T cell-mediated immune response; 2) as a consequence of their chronic stimulation, macrophages locally release mediators of inflammation, leading to accumulation of Th1 cells at sites of ongoing inflammation and contributing to the development of the granuloma structure.

The superfamily of chemokines consists of an array of chemoattractant proteins that has been divided into four branches (C, CC, CXC, and CXXXC) on the basis of the relative position of the cysteine residues in the mature protein (12, 13). Structural variations of chemokines have been demonstrated to be associated with differences in their ability to regulate the trafficking of immune cells during inflammatory responses (13). For instance, IFN-inducible protein 10 (IP-10)[3], a CXC chemokine that is induced in monocytes/macrophages by IFN-γ (14), has been shown to be chemotactic for IL-2-activated human T cells, but not for neutrophils (15). The fact that IP-10 favors the migration of T cells to sites of inflammation makes it an attractive candidate to investigate the mechanisms promoting the development of sarcoid granuloma. Interestingly, IP-10 expression has been seen in the epidermis of patients with another delayed-type hypersensitivity granulomatous disorder, i.e., tuberculoid leprosy (16).

In this study, we investigated whether IP-10 is expressed by inflammatory cells at sites of delayed-type hypersensitivity cellular immune response characterizing sarcoidosis. Specifically, we evaluated the in situ expression of IP-10 in tissues presenting granulomatous lesions related to sarcoidosis, the relationship between

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Received for publication October 6, 1997. Accepted for publication July 27, 1998.

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[1] This work was supported by a grant from Regione Veneto (Venezia, Italy) and Italian Ministry for Education and Research (M.U.R.S.T.) and a National Institutes of Health grant (CA69212). A.D.L. is the recipient of a Cancer Research Institute/Benjamin Jackson Family Investigator Award and a Culpeper Medical Scholars Award.

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[3] Abbreviations used in this paper: AM, alveolar macrophage; BAL, bronchoalveolar lavage; IP-10, interferon-inducible protein 10.

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the levels of IP-10 in the bronchoalveolar lavage (BAL) fluid and the degree of sarcoid $CD4^+$ T cell alveolitis, and the in vitro production of IP-10 by sarcoid macrophages. Furthermore, we evaluated whether sarcoid T cells express CXCR3, i.e., the receptor for IP-10 and other related lymphotactic chemokines (Mig, I-TAC) (13, 17, 18). Data reported herein suggest the direct involvement of macrophage-derived IP-10 in the local accumulation of $CD4^+$ T cells and formation of sarcoid granuloma.

Materials and Methods

Study populations

Twenty-four patients with sarcoidosis were analyzed (6 male and 18 female; mean age 37.3 ± 7.4 yr; 4 smokers and 20 nonsmokers). In all cases, the diagnosis was made by biopsy obtained either from the lungs or from lymph nodes and showing noncaseating epithelioid granulomas, with no evidence of inorganic material known to cause granulomatous diseases. According to our staging system for sarcoidosis (19), each patient underwent BAL analysis.

Seventeen sequentially enrolled sarcoid patients presenting with an episode of pulmonary involvement were evaluated at the onset of the disease. They were defined as having an active disease on the basis of the following characteristics: 1) lymphocytic alveolitis ($>30 \times 10^3$ lymphocytes/ml); 2) positivity to ^{67}Ga scan; and 3) lung CD4/CD8 ratio more than 5. Apart from the BAL analysis, the assessment of disease activity included clinical features, chest radiograph, lung function tests, high resolution computed tomography, and routine blood studies.

Other seven BAL samples were obtained by as many patients with previously diagnosed pulmonary sarcoidosis who repeated BAL analysis during their follow-up period. All of these patients were in the inactive phase of the disease because sequential BAL data performed during their follow-up period showed a regression of the CD4 alveolitis either spontaneously or with therapy. In particular, at the time of our analysis, all patients had normal lung function, normal BAL cell numbers, negative ^{67}Ga scan, and no clinical signs of acute disease. Four of these seven patients were previously given steroid therapy (prednisone 1 mg/kg/day), but no patients received immunosuppressive therapy for 6 mo before the BAL analysis. The average period of follow-up for this group of patients was 35 ± 17.1 mo (range, 18–46 mo).

Six normal subjects were accepted as controls for the BAL studies (3 male and 3 female; mean age 29.8 ± 5.8 yr; 2 nonsmoking healthy persons and 4 subjects evaluated for cough complaints without lung disease). All had normal physical examination, chest x-ray, lung function tests, and BAL cell numbers.

Preparation of cell suspensions

Following administration of local anesthesia, the BAL was performed as previously described (20). Briefly, a total of 150–200 ml of saline solution was injected via fiberoptic bronchoscopy in 25-ml aliquots, with immediate vacuum aspiration after each aliquot. The fluid was filtered through gauze, and its volume was measured: 55.1 ± 4.9% of the injected fluid was recovered. Cells recovered from the BAL were washed three times with PBS, resuspended in endotoxin-tested RPMI 1640 (Sigma Chemical, St. Louis, MO) supplemented with 20 mM HEPES and L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (ICN Flow, Costa Mesa, CA), and then counted. Alveolar macrophages (AMs), lymphocytes, neutrophils, and eosinophils were differentially counted in a total count of 300 cells, according to morphologic criteria, in cytocentrifuged smears stained with Wright-Giemsa.

AMs and BAL T cells were enriched from the entire mononuclear cell suspensions by rosetting with neuraminidase-treated SRBC, followed by Ficoll-Hypaque gradient separations, as previously described (20). Following this multistep selection procedure, more than 95% of the above cells were viable, as judged by trypan blue exclusion. Staining with CD3 (Immunotech, Marseille, France) mAb showed a percentage of CD3 T cells ranging from 98–100% in purified BAL T lymphocytes, while more than 98% of purified AMs expressed the macrophage-associated PAM-1 Ag (kindly provided by Dr. A. Mantovani, Milan, Italy).

IP-10 protein levels in BAL fluid

IP-10 protein levels were measured in the fluid component of BAL using a specific double-determinant RIA, as previously reported (21). Briefly, flat-bottom 96-well plates (MaxiSorp, Nunc, Naperville, IL) were coated with 100 μl/well of goat anti-IP-10 Ab (R&D Systems, Minneapolis, MN) (5 μg/ml in 0.1 M carbonate buffer, pH 9.5) for 24 h at 4°C and then extensively washed with PBS, pH 7.5, 0.05% Tween 20 (washing buffer). A total of 100 μl/well of either IP-10 standards (Peprotech, Rocky Hill, NJ) or BAL fluid was then added, followed by an overnight incubation at 4°C. Plates were rinsed with washing buffer before addition of 100 μl ^{125}I-labeled affinity-purified rabbit anti-human IP-10 polyclonal Ab (0.6 μg/ml in PBS-Tween with 50% FCS) and incubated overnight at 4°C (22, 23). After washings of the plates, 100 μl of 1 N NaOH was added into each well; samples were harvested after 30 min, and read in a gamma counter. This RIA had a detection limit of 10 pg/ml.

Immunohistochemical analysis of IP-10-producing cells

Fresh frozen tissue samples were obtained from lung and lymph nodes of five patients. Fragments of the tissue samples were covered with OCT medium (Ames, Elkhart, IN), snap frozen in liquid nitrogen, and stored at −80°C until analyzed. Five-micron cryostat sections were dried onto glass covered with polylysine adhesive (Sigma) and fixed in cold chloroform/acetone mixed in 1:1 ratio.

The alkaline phosphatase/anti-alkaline phosphatase or avidin-biotin peroxidase techniques were used for revealing IP-10-producing cells together with differentiation Ag (CD4 and CD68) on cell membranes on several sections, as previously reported (24). Specifically, to evaluate IP-10 immunohistochemistry, we covered cryostat sections with diluted swine serum to avoid nonspecific binding of Abs and then incubated them for 60 min in a moist chamber with a 1/750 dilution of unconjugated affinity-purified rabbit anti-human IP-10 polyclonal Ab.

Flow cytometry analysis of BAL cells

The commercially available conjugated or unconjugated mAbs used belonged to the Becton Dickinson (Sunnyvale, CA) and Immunotech series and included: CD3, CD4, CD8, CD29, and CD45R0. The unconjugated affinity-purified rabbit anti-human IP-10 polyclonal Ab, anti-IL-2 mAb (purchased from PharMingen, San Diego, CA), anti-IFN-γ mAb (PharMingen), anti-IL-4 mAb (PharMingen), anti-IL-15 mAb (M112; Genzyme, Boston, MA), and anti-hCXCR3 mAb (1C6; Leukosite, Cambridge, MA) were also used.

The frequency of BAL cells positive for the above reagents was determined by flow cytometry, as previously described (5). Briefly, 10×10^3 cells were acquired, and the analysis was determined by overlaying the histograms of the samples stained with the different reagents. Both BAL lymphocytes and AMs were gated in flow cytometry analysis using these two different approaches: 1) physical characteristics of cells, and 2) expression of the T-associated CD3 and AM-associated PAM-1 Ags on the area of lymphocytes and AMs, respectively. For direct fluorescence analysis, FITC- or phycoerythrin-conjugated control, isotype-matched mouse mAbs were used to set the negative control (IgG1, IgG2a, IgG2b; Becton Dickinson). In the indirect fluorescence analysis, cells were incubated with control purified isotype-matched mAb (Becton Dickinson). The proper control for affinity-purified rabbit anti-human IP-10 polyclonal Ab was represented by 100 μg/ml of rabbit IgG myeloma protein (Serotec, Oxford, U.K.). The second-step reagents were represented by $F(ab')_2$ anti-rabbit IgG (Caltag, South San Francisco, CA) for IP-10 Ab staining. To eliminate nonspecific binding of Abs to cytokines (IP-10, IFN-γ, IL-2, IL-4, IL-15) bound to the surface of cells, the cells were washed in 40 mM citrate containing 140 mM NaCl, pH 4, to remove possible bound cytokines.

The purity of the gates was always higher than 98% cells. Cells were scored using a FACScan analyzer (Becton Dickinson), and data were processed using the Macintosh CELLQuest software program (Becton Dickinson). The expression of cytoplasmic cytokines was evaluated following permeabilization of cell membranes using 1/2 diluted Permea Fix (Ortho, Raritan, NJ) for 40 min. After the permeabilization procedure, affinity-purified rabbit anti-human IP-10 polyclonal Ab, anti-IL-2, anti-IL-4, anti-IL-15, and anti-IFN-γ mAbs were added, as previously reported (5).

In vitro production of IP-10 by pulmonary cells

To verify the ability of AMs to release the chemokine, unstimulated AMs (1×10^6/ml) were isolated both from sarcoid patients and healthy subjects, resuspended in RPMI medium, and cultured for 24 h in 24-well plates at 37° in 5% CO_2. In separate experiments, AMs were stimulated with IFN-γ (100 U/ml) and LPS (10 μg/ml; Difco, Detroit, MI). Following the incubation period, supernatants were harvested, filtered through a 0.45-μm Millipore filter (Molsheim, France), and immediately stored at −80°C. At the end of the culture time, AM viability was always greater than 95%. IP-10 protein levels in supernatants were measured using the above reported double-determinant RIA.

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Biologic activity of AM supernatants and BAL fluids on the CXCR3$^+$2F12 T cell clone

The 2F12 (CD4$^+$) T cell clone (kindly provided by Dr. James Kurnick, Massachusetts General Hospital, Charlestown, MA) was used to evaluate the chemotactic activities of AM supernatants and BAL fluids. The cells were grown in RPMI 1640 supplemented with 1% glutamine, 5% human serum, 1% kanamycin, and 100 U/ml human rIL-2. Cells were periodically expanded by restimulation with PHA (1 μg/ml) in the presence of irradiated blood mononuclear cells (10:1 ratio of feeder cells:2F12) and used for experiments after a culture period of 10 to 14 days.

Cell migration was measured in a 48-well modified Boyden chamber. Chemokines were diluted in HEPES-buffered RPMI 1640 supplemented with 0.1% BSA. Supernatants from cell cultures and BAL samples were used undiluted; different concentrations of IP-10 were utilized as positive control. Polyvinylpyrrolidone-free polycarbonate membranes (pore size, 3 μm) coated with type IV collagen were used. A total of 30 μl of samples was added to the bottom wells, and 50 μl of 2F12 cells resuspended in the HEPES-buffered RPMI 1640 were added to the top wells (~100,000 cells/ml). Chambers were incubated at 37°C with 5% CO_2 for 1 h. The membrane was then removed, washed with PBS on the upper side, fixed, and stained with DiffQuik (Baxter Dasle, München, Germany). Cells were counted microscopically at ×40 magnification in four fields per well. All assays were performed in duplicate.

Statistical analysis

Data were analyzed with the assistance of the Statistical Analysis System. Data are expressed as mean ± SD. Mean values were compared using the ANOVA test. To investigate the correlation coefficients (r) between IP-10 levels and BAL cell findings, the nonparametric Spearman rank correlation test was used. A p value <0.05 was considered as significant.

Results

Cell recovery and IP-10 levels in BAL fluid

Table I summarizes the results of the BAL findings. Due to the presence of CD4$^+$ high intensity T cell alveolitis, cell recovery was significantly higher in patients with active sarcoidosis with respect to control subjects and patients with inactive disease. As a consequence of the increase in the absolute number of CD4$^+$ T cells, the BAL CD4/CD8 ratio was significantly increased in patients with active disease (6.9 ± 2.1) with respect to patients with inactive disease (1.71 ± 0.41; $p < 0.001$) and healthy controls (1.65 ± 0.35; $p < 0.001$). As previously reported (2), more than 98% of T lymphocytes recovered from the BAL of sarcoid patients were CD4$^+$/CD45R0$^+$/CD29$^+$ memory T cells (data not shown).

In all BAL fluid from patients with active sarcoidosis, detectable levels of IP-10 were demonstrated (range, 45–2639 pg/ml of BAL fluid) (Table I). They were significantly higher than in the BAL of patients with inactive disease (range, 0–128 pg/ml of BAL fluid). Only three of the six control subjects showed detectable levels of IP-10 in the BAL fluid (18, 14, and 16 pg/ml of BAL fluid, respectively). Spearman rank correlation coefficients between BAL cell findings and levels of IP-10 were also calculated. A positive significant correlation was demonstrated between the concentrations of IP-10 and the percentages and absolute numbers of BAL lymphocytes (r, 0.845 and $p < 0.001$; and r, 0.816 and $p < 0.001$, respectively) and the percentages and absolute numbers of CD4$^+$ T cells (r, 0.815 and $p < 0.001$; and r, 0.825 and $p < 0.001$, respectively). As a consequence of the high intensity alveolitis, AMs and CD8 T cells significantly increased in their absolute number (Table I); however, correlation coefficients between IP-10 levels and alveolar macrophages and CD8$^+$ T cell numbers were −0.802 and −0.693, respectively, indicating a negative correlation. Most likely, other chemokines are involved in the recruitment of these cell subsets as well as in the mechanisms leading to the increase of neutrophils (Table I) that have been involved in the mechanisms leading to fibrogenic events occurring in the lungs of patients with sarcoidosis (1, 2).

Table I. *Summary of cell findings and IP-10 levels in BAL of 24 patients with active and inactive sarcoidosis and six healthy subjects.*[a]

Patients	Cell Recovery ($\times 10^3$/ml)	Lymphocytes		Alveolar Macrophages		Neutrophils		Eosinophils		CD4 T Cells		CD8 T Cells		IP-10 Levels in BAL Fluid (pg/ml)
		%	$\times 10^3$/ml	%	$\times 10^3$/ml	%	$\times 10^3$/ml	%	$\times 10^3$/ml	%	$\times 10^3$/ml	%	$\times 10^3$/ml	
Sarcoidosis														
Active ($n = 17$)	317.7 ± 43.2	41.6 ± 2.3	135.2 ± 22.0	57.6 ± 2.4	180.1 ± 24.4	0.8 ± 0.5	2.1 ± 1.6	0.2 ± 0.4	0.7 ± 0.2	70.4 ± 1.9	96.5 ± 17.5	10.2 ± 1.3	11.0 ± 1.9	528.2 ± 139.0
Inactive ($n = 7$)	119 ± 25.5	6.0 ± 1.7	6.9 ± 3.3	92.3 ± 2.6	93.3 ± 23.2	0.5 ± 0.4	0.6 ± 0.4	0.2 ± 0.2	0.3 ± 0.2	47.2 ± 4.6	3.1 ± 1.5	25.9 ± 1.6	1.8 ± 1.0	39.0 ± 18.1
Controls ($n = 6$)	108.5 ± 24.9	4.5 ± 1.6	4.9 ± 1.4	95.8 ± 1.6	103.8 ± 24.4	0.3 ± 0.4	0.5 ± 0.9	0.1 ± 0.4	0.2 ± 0.2	44.5 ± 1.6	2.1 ± 0.9	24.5 ± 0.5	1.1 ± 0.3	7.5 ± 3.4
Probability														
AS[a] vs C	0.01	0.001	0.001	0.001	NS	NS	NS	NS	NS	NS	0.001	0.01	0.01	0.01
AS vs IS	0.01	0.001	0.001	0.001	NS	NS	NS	NS	NS	NS	0.001	0.01	0.01	0.05
IS vs C	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

[a] Abbreviations used: AS, active sarcoidosis; C, controls; IS, inactive sarcoidosis.

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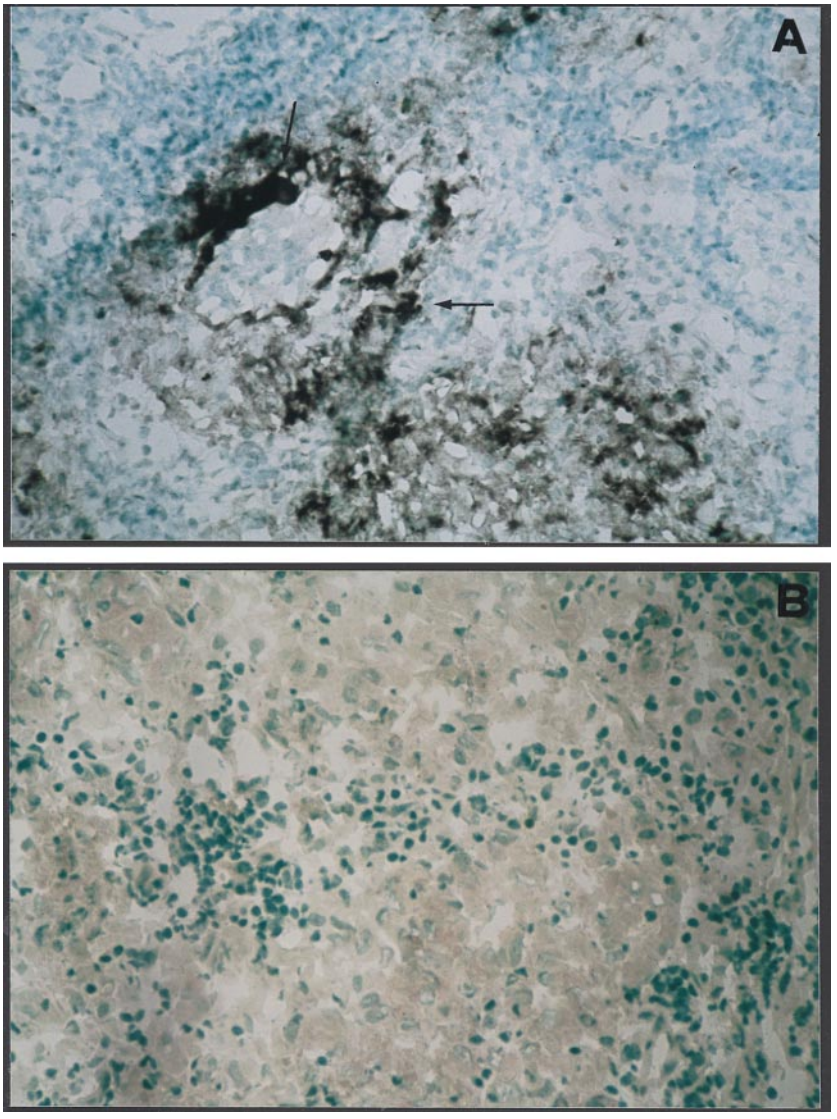

FIGURE 1. IP-10 expression was studied in 11 lymph nodes with sarcoid granulomas. The morphology and distribution of cell populations were studied by immunochemistry. IP-10 expression with an affinity-purified rabbit anti-human IP-10 polyclonal Ab (arrows) (*A*) or with a control Ab (*B*). Original magnification: ×400.

IP-10 expression by immunoinflammatory cells in sarcoid granuloma

Immunohistochemical analysis was used with an Ab recognizing IP-10 to investigate the membrane interactions occurring among macrophages within granulomas (Fig. 1). The central core of sarcoid granuloma was made up of a number of monocytes/macrophages at various states of activation and differentiation, as well as epithelioid cells and multinucleated giant cells, while a number of CD4 lymphocytes and plasma cells surrounded the central core of the granuloma. IP-10 was preferentially expressed by macrophages and epithelioid cells located inside the granuloma, whereas giant cells were mainly nonreactive.

IP-10 expression by bronchoalveolar cells

Immunochemical analysis of IP-10-producing cells in the BAL demonstrated that the majority of AMs retrieved from the lungs of patients with sarcoidosis showed a strong expression of this chemokine (Fig. 2*A*).

Furthermore, flow cytometry analysis confirmed that AMs bore IP-10 protein (Fig. 2, *B–E*). In particular, as demonstrated by the Kolmogorov-Smirnov analysis, lung macrophages expressed IP-10 since the peak of positive cells was significantly shifted with respect to the negative controls (percentage ranging from 51–78% of AMs bore IP-10). Profiles shown in Fig. 2 are representative of membrane IP-10 staining in 17 patients with active sarcoidosis (nonpermeabilized AMs). Histogram profiles of cytoplasmic IP-10 following cell permeabilization were superimposable (data not shown). Pulmonary macrophages from two of the seven patients with inactive sarcoidosis (41 and 49%, respectively) and two of the six controls expressed low levels of cytoplasmic IP-10 (35 and 27%, respectively), but not membrane IP-10. Fig. 2 shows pulmonary macrophages from two representative control subjects who express low levels of IP-10 (*G*) or do not (*F*). BAL T cells from both sarcoid patients and controls did not show IP-10 expression.

To determine whether increased IP-10 expression was associated with an enhanced expression of cytokines that are actively released in sarcoid lung (4, 5), BAL cells were analyzed for the expression of IL-2, IL-4, IFN-γ, and IL-15. Fig. 3 shows cytoplasmic expression of these cytokines by sarcoid AMs and T cells. A percentage ranging from 47–58% and from 55–84% of AMs from five patients with active sarcoidosis expressed cytoplasmic IFN-γ and IL-15, respectively (mean 45.1% ± 6.3 and 66.5% ± 8.1, respectively). By contrast, less than 5% of AMs isolated from patients with inactive sarcoidosis and normal AMs showed cellular IFN-γ or IL-15. Concerning pulmonary T cells, BAL T cells from patients with active sarcoidosis bore IL-2 (percentage ranging

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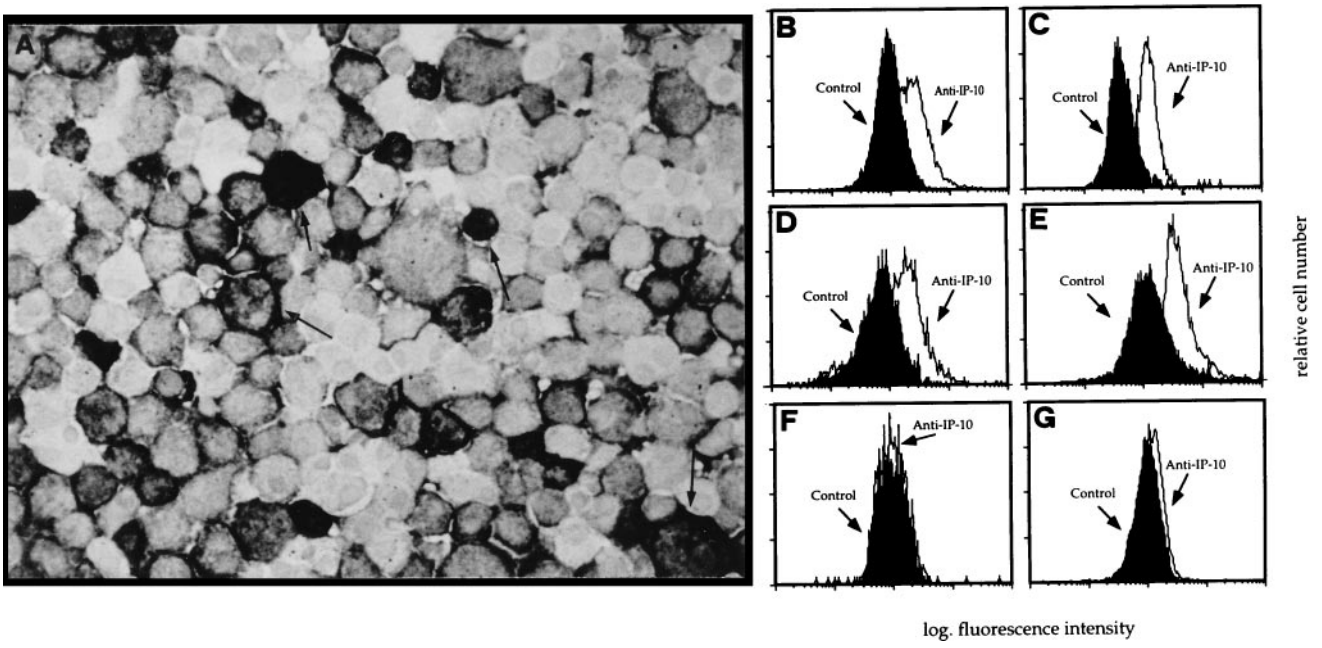

FIGURE 2. Expression of IP-10 by sarcoid alveolar macrophages recovered from the BAL. Immunochemical analysis of cytocentrifuged smears (*A*) demonstrated that the majority of AMs retrieved from the lungs of sarcoid patients showed a strong expression of IP-10 (arrows). Flow cytometry analysis confirmed the IP-10 expression by sarcoid AMs. To remove possible bound IP-10, the cells were washed in 40 mM citrate containing 140 mM NaCl, pH 4. Statistical analysis demonstrated that AMs recovered from four representative patients with active sarcoidosis bore IP-10 protein (*B–E*). The IP-10 histograms were completely shifted with respect to the control histograms, indicating that the entire macrophage population expressed the cytokine. Pulmonary macrophages from two representative control subjects expressed low level (*G*) or did not express IP-10 (*F*).

from 17–35%; mean 29% ± 13.8) and IFN-γ (percentage ranging from 21–39%; mean 32.3% ± 11.7), but did not express IL-4 (data not shown). Less than 1% of normal BAL T cells express this cytokine. Thus, sarcoid T cells show a striking polarization of Th1-type immune response that parallels IP-10 expression.

IP-10 production by pulmonary macrophages

Alveolar macrophages have never been reported to release IP-10 in vitro. To confirm our immunochemical and flow cytometry data, we evaluated whether sarcoid AMs can produce IP-10 both at resting conditions and following stimulation.

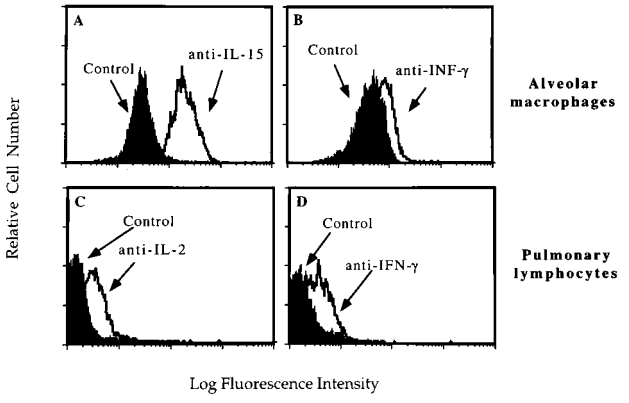

FIGURE 3. The flow cytometry profile of cytoplasmic cytokines in alveolar macrophages and T cells from a representative sarcoid patient with lymphocytic alveolitis. Forty-four percent of BAL cells were lymphocytes, as determined by morphologic evaluation of the cytospin, and the CD4:CD8 ratio was 7.9. The proportion of cytoplasmic cytokines on AMs (IL-15 and IFN-γ; *A* and *B*, respectively) and T cells (IL-2 and IFN-γ; *D* and *E*, respectively) was determined as specified in *Materials and Methods*. Statistical analysis demonstrated that AMs recovered from five representative patients with active sarcoidosis bore IL-15 and, to lesser extent, IFN-γ. BAL CD4 T cells showed a Th1-type phenotype since they bore IL-2 and IFN-γ.

Fig. 4 shows the in vitro production of IP-10 by AMs. Cell-free supernatants were obtained from AMs cultured in different experimental conditions and tested for the presence of IP-10. After 24 h of culture, unstimulated AMs from patients with active sarcoidosis were able to produce higher amounts of IP-10 (mean 354.7 ± 146.9 pg/ml) than AMs isolated from patients with inactive disease (108.3 ± 75.6 pg/ml; $p < 0.05$) and healthy subjects (27.6 ± 12.1 pg/ml; $p < 0.001$). Both AMs from patients with active and inactive disease increased IP-10 release following LPS stimulation, and there were no significant differences in the production of IP-10 between patients with active and inactive disease (1714 ± 1330 pg/ml vs 710.5 ± 624.6 pg/ml, respectively; p = NS). In contrast, normal LPS-stimulated AMs showed a slight increase in the release of IP-10 (54.4 ± 9). When AMs were cultured in the presence of IFN-γ, the amount of IP-10 in cell-free supernatants from patients with active disease was 10-fold higher (69,571 ± 25,255 pg/ml) than the levels obtained from IFN-γ-stimulated AMs from controls (6,722 ± 2,323 pg/ml, $p < 0.001$). Patients with inactive sarcoidosis released intermediate levels of IP-10 (27,671 ± 5,459 pg/ml).

Expression of CXCR3 by sarcoid and normal pulmonary T cells

Data reported above clearly demonstrated that sarcoid AMs release IP-10 that interacts with a G protein-coupled cell surface receptor (CXCR3) expressed on activated T cells and may induce T cell inflammation in peripheral tissues (13, 18, 25). To evaluate whether T cells accumulating in the lungs of patients with sarcoidosis express CXCR3, flow cytometry analysis experiments were performed using a mAb against CXCR3. Fig. 5 shows that alveolar $CD4^+$ T cells of patients with active sarcoidosis are CXCR3 positive (Fig. 5, *A* and *B*). Interestingly, BAL T cells of patients with active sarcoidosis (*A* and *B*) showed enhanced levels of CXCR3

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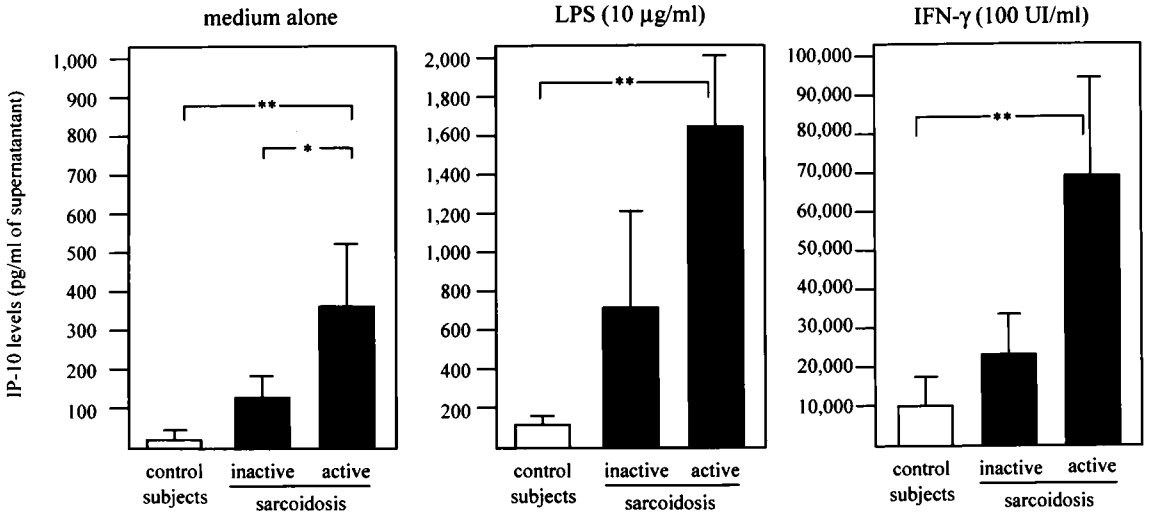

FIGURE 4. IP-10 levels obtained using a RIA assay in cell-free supernatants conditioned by 24-h cultured AMs. AMs were cultured in medium alone, with LPS and with IFN-γ. Sarcoid AMs both at resting conditions and following stimulation produced significantly higher levels of IP-10 than normal AMs. Bar represents the SD of the mean. Significance as follows: *, $p < 0.05$; **, $p < 0.001$.

expression with respect to BAL T cells of patients with inactive sarcoidosis (*C*) and normal BAL T cells (*C* and *D*) ($p < 0.001$, as determined by the Kolmogorov-Smirnon analysis). However, the small number of $CD8^+$ T cells found in the BAL of patients with active sarcoidosis also expressed CXCR3, albeit at lower density than $CD4^+$BAL T cells (data not shown).

Biologic activity of AM supernatants and BAL fluids on the $CXCR3^+$2F12 T cell clone

The possibility that the increased levels of IP-10 in AM supernatants might account for the recruitment of $CXCR3^+$ sarcoid T cells was also investigated by an in vitro chemotaxis assay that uses a T cell clone (2F12) expressing high levels of CXCR3 (Fig. 6*A*). As shown in Fig. 6*B*, supernatants obtained by IFN-γ-stimulated AMs isolated from six sarcoid patients with T cell alveolitis exerted significant chemotactic activity on the $CXCR3^+$2F12 clone. As determined by the RIA test, IP-10 levels ranged from 11,073 to 40,125 pg/ml in these supernatants. However, on the basis of number of migrating $CXCR3^+$ T cells, the levels of the in vitro chemotaxis assay seen with purified protein (*D*) correlated with IP-10 levels in the supernatants.

By contrast, BAL samples did not exhibit biologic activity on the $CXCR3^+$2F12 clone (data not shown). In fact, IP-10 levels in the fluid component of the BAL using the RIA test ranged from 45 to 2632 pg/ml, which is below the range in which significant chemotaxis was seen for the 2F12 clone (*D*).

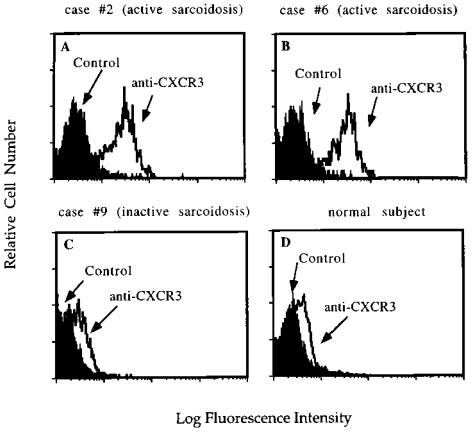

FIGURE 5. Comparison of CXCR3 expression by pulmonary $CD4^+$ T recovered from two representative patients with active sarcoidosis presenting with lymphocytic alveolitis (cases 2 and 6; 44 and 52% of BAL cells were lymphocytes, respectively), a representative patient with inactive sarcoidosis (case 9; 14% of BAL cells were lymphocytes), and a normal subject (6% of BAL cells were lymphocytes). Pulmonary $CD4^+$ T cells from sarcoid patients with high intensity lymphocytic alveolitis expressed the CXCR molecule at higher levels than $CD4^+$ T cells of patients with inactive disease or control subjects (as compared by the Kolmogorov-Smirnov analysis).

Discussion

The sarcoid process is characterized by a persistent accumulation of macrophages and activated $CD45R0^+$Th1-type T cells mostly bearing helper CD4 phenotype. Our findings that 1) IP-10 is strongly expressed in sarcoid tissues; 2) sarcoid macrophages represent a source for IP-10 production and exert chemotactic activity on a $CXCR3^+$ T cell clone; and 3) there is a positive relationship between BAL levels of IP-10, the degree of CD4 alveolitis, the expression of Th1 cytokines, and CXCR3R by sarcoid T cells, suggest a role for IP-10 in the mechanisms that account for sarcoid CD4 T cell activation and the development of sarcoid granuloma.

Most of the CXC and CC chemokines that have been involved in lymphocyte chemotaxis are not selective for lymphocytes since they are active also on monocytes and granulocytes. The scenario is quite different for IP-10. As recently demonstrated by Loetscher et al. (17), this cytokine favors the selective recruitment of IL-2-activated T cells, which exclusively express the IP-10 receptor. The observations that sarcoid AMs abundantly express IP-10 and the significant correlation demonstrated between the concentration of IP-10 and the number of BAL $CD4^+$ T lymphocytes indicate the role of macrophage-derived IP-10 in regulating the migration of IL-2-activated T cells toward sites of the sarcoid inflammatory process. Most likely, the fact that sarcoid BAL cells express a number of Th1-type cytokines, including IL-2 and IL-15, suggests

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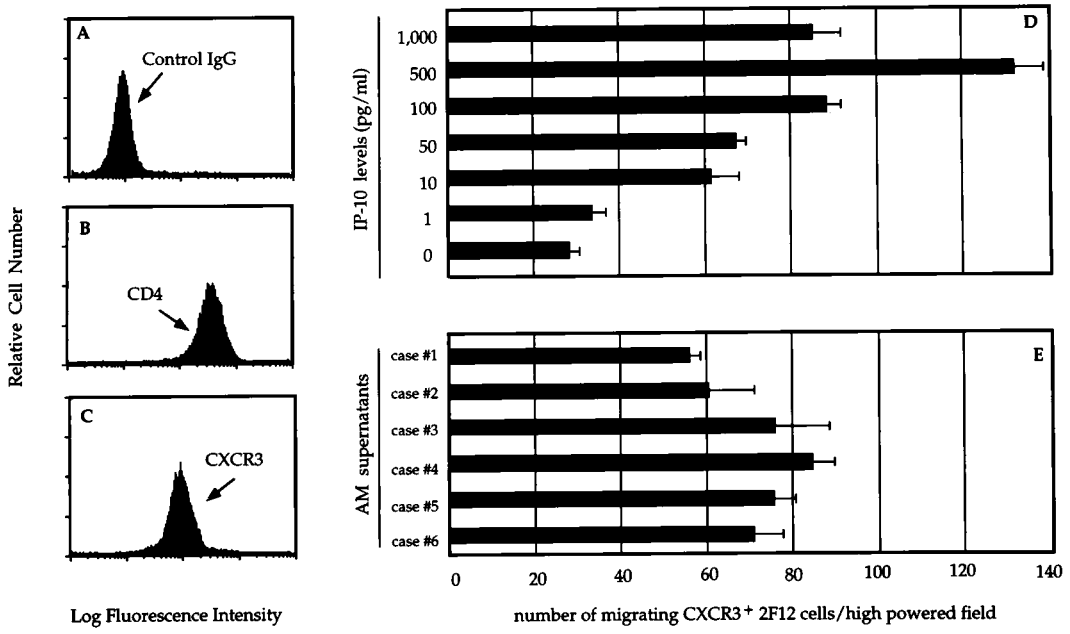

FIGURE 6. Chemotactic activity of AM supernatants on the $CD4^+/CXCR3^+$2F12 T cell clone. Flow cytometry reveals that the 2F12 clones express CD4 (*B*) and high levels of CXCR3 (*C*); *A* shows the negative IgG control. *D* shows dose-response chemotactic activity of increasing concentrations of IP-10 on the 2F12 T cell clone using a modified Boyden chamber. Data are given as mean + SD; highest and lowest counts were eliminated before the data were averaged to produce the values depicted in the graph. *E* shows that supernatants obtained by sarcoid AMs and cultured for 24 h in absence of stimulation exerted significant chemotactic activity on the $CXCR3^+$2F12 T cell clone.

that IP-10 cooperates with Th1-type cytokines that act as local factors for T cell activation and proliferation (5).

Although our study did not clarify factors regulating the in situ IP-10 production by sarcoid macrophages, we postulate that the chemokine release is induced by the chronic IFN-γ-dependent activation of sarcoid AMs and T cells. AMs from sarcoid patients differ from normal AMs in a series of parameters related to their functional properties, such as their phenotypic characteristics, accessory cell function, and secretory capabilities (26 and our results). The hypothesis being proposed is that, as a result of the chronic IFN-γ-dependent stimulation, AMs release IP-10. In this scenario, it is possible that Th1-type sarcoid T cells that, as herein demonstrated, express IFN-γ, IL-2, and CXCR3, could be considered inducers of production as well as targets of IP-10. IFN-γ/IP-10 axis might contribute to the migration of Th1-type sarcoid T cells to the site of sarcoid reaction, inducing self-perpetuating immune mechanisms that drive the development of sarcoid granuloma.

Another interesting question is whether IP-10 acts in concert with other chemokines in regulating the inflammatory infiltrate of sarcoid lesions. It has been demonstrated previously that RANTES, a CC chemokine, is highly expressed in sarcoid tissues (27). Like IP-10, RANTES is known to recruit and activate specific T cell subsets (28). Furthermore, immunocytochemical staining of sarcoid granuloma in lymph node and lung revealed that a chemoattractant factor for $CD4^+$ T cells, i.e., IL-16, is expressed in areas in which there is perivascular accumulation of lymphocytes (29). Finally, our preliminary data have shown the presence of Mig in sarcoid granulomas, a related member of the CXC chemokine family that targets lymphocytes via CXCR3 (30). We have also seen that that small number of $CD8^+$ T cells found in the BAL of sarcoid patients with high intensity alveolitis also express CXCR3, albeit at lower density than $CD4^+$ T cells. This suggests that different T cell-attracting cytokines might play a role in the regulation of the sarcoid granuloma development, perhaps by controlling the trafficking of discrete CD4 and CD8 T cell subsets at sites of sarcoid inflammatory response.

In this regard, data in animal models have shown that Th cells facilitate, while suppressor T cells down-modulate the growth of the granulomatous process (2). Furthermore, while $CD4^+$ T cells predominate in the inner area of sarcoid granulomas, $CD8^+$ T cells predominate in the outer margin of the lymphocyte rim (1, 2). From our observations, it may be inferred that IP-10 release is higher in sarcoid patients characterized by a massive $CD4^+$ T cell infiltrate than in patients with inactive disease. Sarcoid $CD4^+$ T cells produce Th1-type cytokines, including IL-2 and IFN-γ (our data and 4, 5, 11, 31); the net effect of the Th1-type response is the development of the hypersensitivity granuloma (32). However, depending on the host susceptibility, a switch to Th2-type T cells may occur in sarcoid patients evolving toward lung fibrosis with concomitant release of cytokines, including IL-4, which stimulates the production of extracellular matrix proteins and/or are chemoattractants for fibroblasts (32). Since IL-4 has also been shown to down-regulate chemokine production (33), studies on the Th1/Th2 pattern during the different phases of the sarcoid inflammatory process could clarify whether the Th2 shift has a suppressive effect on the in situ production of IP-10 and, thus, on the formation of granuloma and the evolution toward fibrosis.

The present study has shown that sarcoid T cells express CXCR3 at high levels. Five human CXC chemokine receptors (CXCR1 through CXCR5), nine human CC chemokine receptors (CCR1 through CCR9), and one CXXXC chemokine receptor (CX_3R1) have been identified to date (13). Since the increase in the secretion of different chemokines and the overexpression and/or down-modulation of some chemokine receptors might have dramatic effects in regulating Th1/Th2 pattern during inflammation (34), further studies are in progress in our labs to verify the importance of other chemokines and chemokine receptors in the pathogenesis of sarcoid inflammatory process.

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In conclusion, this study supports the hypothesis that IP-10 participates in the complex network between pulmonary immunocompetent cells and cytokines, which sets the stage for the development of the sarcoid granuloma. A greater understanding of local mechanisms controlling IP-10 release will clarify the sequel of events that lead to sarcoid-associated pulmonary fibrosis. Furthermore, our findings suggest that the in situ release of IP-10 mirrors the development of the Th1 sarcoid inflammatory process. In this context, the use of IP-10 levels in BAL fluid as biologic markers for disease activity in sarcoidosis needs to be validated by a wide and long-term follow-up study.

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