T_H1/T_H2 and T_C1/T_C2 profiles in peripheral blood and bronchoalveolar lavage fluid cells in pulmonary sarcoidosis

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Background: Sarcoidosis is thought to be a type-1 cytokine-mediated disorder. However, few data are available on the profiles of cytokine expression by T_H cells at the single-cell level, as assessed by intracellular cytokine flow cytometry. Additionally, it remains to be determined whether the balance of T_C1 and T_C2 cells can be altered in sarcoidosis.
Objective: The aim of this study was to evaluate the T_H1/T_H2 and T_C1/T_C2 balances in sarcoidosis.
Methods: Using triple-color flow cytometry and phorbol 12-myristate acetate/ionomycin stimulation, we measured the production of the intracellular cytokines IFN-γ and IL-4 in $CD4^+$ and $CD8^+$ T cells separately, which were obtained from peripheral blood and bronchoalveolar lavage fluid (BALF) of 20 patients with sarcoidosis, and compared their cytokine expressions with those of 10 normal subjects.
Results: Under unstimulated conditions, there were no significant differences in the proportion of cytokine-producing $CD4^+$ or $CD8^+$ T cells in peripheral blood or BALF between patients with sarcoidosis and normal control subjects. On stimulation with phorbol 12-myristate acetate/ionomycin for 4 hours, in BALF of the patients, but not in peripheral blood, we found a significant increase in the percentage of IFN-γ–producing $CD4^+$ T cells and a decrease in the percentage of IL-4–producing $CD4^+$ T cells, resulting in a 3.5-fold higher ratio of IFN-γ/IL-4–producing $CD4^+$ T cells compared with that found in normal subjects. In contrast, no difference was found in the proportions of cytokine-producing $CD8^+$ T cells or the ratio of IFN-γ/IL-4–producing $CD8^+$ T cells in either the peripheral blood or BALF between the patients and normal subjects.
Conclusions: These findings suggest that the prominent shift toward a type-1 phenotype may occur in $CD4^+$ T-cell populations but not in $CD8^+$ T-cell populations in the affected organs of sarcoidosis. (J Allergy Clin Immunol 2001;107:337-44.)

Key words: *T_H1, T_H2, T_C1, T_C2, sarcoidosis, bronchoalveolar lavage*

Abbreviations used
BALF: Bronchoalveolar lavage fluid
MFI: Mean fluorescent intensity
NK: Natural killer
PE: Phycoerythrin
PerCP: Peridin chlorophyll protein
PMA: Phorbol 12-myristate acetate
RT: Room temperature

Cellular immune responses in lymphocyte populations provoked by antigen stimulation can be characterized by the distinct patterns of cytokine production.[1] Initially, murine $CD4^+$ T-cell clones were divided into 2 subsets on the basis of their cytokine profiles.[2,3] T_H1 cells produce type-1 cytokines (IL-2, TNF-α, and IFN-γ), whereas T_H2 cells secrete type-2 cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13). In addition, an intermediate state of a T-cell phenotype (T_H0), which is characterized by simultaneous secretion of type-1 and type-2 cytokines, has been described.[4] A T_H1 or T_H2 phenotype will ultimately dominate an immune response by amplification of a particular T_H subset and by downregulation of the opposing cell response. Comparable divisions have also been observed in human $CD4^+$ T cells.[5–7] Indeed, a variety of pathologic processes, including infections and allergic and autoimmune disorders, are linked to the predominance of T_H1- or T_H2-like cytokine expression patterns.[8–11] Recently, a more expansive division of cytokine-producing cells, including $CD8^+$ and γδ T cells, into type-1 (T_H1-like) and type-2 (T_H2-like) cells has been proposed.[12,13] In addition to their cytolytic activity, $CD8^+$ T cells (T_C cells) are further classified into 2 distinct effector cell types, T_C1 and T_C2 cells, on the basis of their cytokine profiles.[14,15] T_C1 cells characteristically produce type-1 cytokines, whereas T_C2 cells secrete type-2 cytokines.[16] The significance of the apparent domination of a type-1 or type-2 cytokine response in T_C-cell populations has also been shown to have particular relevance with respect to antigens. Elevated numbers of IL-4–producing $CD8^+$ T cells were demonstrated in atopic asthma, suggesting that T_C2 cells are one of major sources of IL-4.[17] However, the increased T_C1 cells were

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TABLE I. Characteristics of the study populations

	Control subjects (n = 10)	Patients with sarcoidosis (n = 20)
Sex, F/M	5/5	8/12
Age (y)	46.1 ± 4.3	48.4 ± 3.3
Pulmonary function		
FVC (%)	98.2 ± 13.2	89.1 ± 4.2
FEV_1 (%)	83.5 ± 4.3	80.8 ± 2.9
Serologic parameters		
ACE (IU/L)	10.8 ± 1.5	28.1 ± 3.8
γ-Globulin* (mg/dL)	1.1 ± 0.1	1.5 ± 0.1
Radiologic stages		
Stage I/II/III†		6/13/1
Disease duration before this study (y)		9.0 ± 1.8
Affected organs		
Lung		20
Eye (uveitis)		13
Skin		8
Others‡		4

Data are given as means ± SEM.
FVC, Forced vital capacity; *ACE,* serum angiotensin converting enzyme (normal range, 8.3-21.4 IU/L).
*γ-Globulin: normal range, 0.75 to 1.57 mg/dL.
†Radiologic stages: *I,* bilateral hilar lymphadenopathy; *II,* bilateral hilar lymphadenopathy plus parenchymal infiltrates; *III,* parenchymal infiltrates.
‡*Others* is defined as liver, kidney, and hypercalcemia.

shown to be primarily responsible for the protective value of T_C populations against the influenza virus.[18] Thus a polarized T_C1/T_C2 dichotomy is thought to be a critical determinant of immune responses under certain pathologic conditions, as well as an imbalance of T_H1/T_H2.

To determine the cytokine profiles of T cells, most studies have been carried out with in vitro cultured T-cell clones.[2,3,5,12,13] In these studies cytokine expression was measured at protein and mRNA levels on cloned populations but not in single cells. However, it is generally accepted that cytokine profiles of T-cell clones or cell lines do not truly reflect their characteristic expression in the original cell.[19,20] In assessing cytokine production at the single-cell level, a flow cytometric technique to detect intracellular cytokines has recently been developed.[21,22] It allows simultaneous measurement of multiple cytokines at the single-cell level and obtains fairly accurate information of the in vivo immune status. Because a polarization of the balance between type-1 and type-2 T cells has been observed in a variety of diseases,[8–11,17] this technique has the potential for exploring their pathogenesis.

Sarcoidosis is a systemic disorder of unknown cause characterized by the development of a cell-mediated immune response, which results in noncaseating granuloma formation and accumulation of activated T cells and macrophages at the sites of inflammation.[23] It was suggested that type-1 cytokines, such as IL-2 and IFN-γ, which are produced by activated $CD4^+$ T cells, are essential for developing granulomatous inflammation.[24] A recent study with $CD4^+$ T-cell clones reported the shift of T_H1/T_H2 balance toward a T_H1 phenotype in the involved organs of sarcoidosis.[25] However, there have been few studies examining intracellular cytokine production in $CD4^+$ T cells at the single-cell level by using the above flow cytometric method in sarcoidosis.[26] Additionally, among $CD8^+$ T-cell populations in sarcoidosis, it remains to be determined whether the T_C1/T_C2 balance can be altered and whether it is involved in its pathogenesis.

The present study was conducted to investigate the alteration of the T_H1/T_H2 and T_C1/T_C2 balance in sarcoidosis. To this end, using cytokine flow cytometry, we examined the production of the intracellular cytokines IFN-γ and IL-4 in $CD4^+$ and $CD8^+$ T cells separately, which were obtained from the peripheral blood and bronchoalveolar lavage fluid (BALF) of patients with sarcoidosis, and compared their cytokine expressions with those of normal subjects.

METHODS

Study population

The study population consisted of 20 patients with sarcoidosis. The diagnosis was histologically established on the basis of defined criteria, with confirmation of noncaseating granulomas on tissue biopsy. The clinical features of these patients are shown in Table I. All patients had abnormal findings on chest x-ray film and had evidence of noncaseating granulomas on transbronchial lung biopsy specimens. The most common extrathoracic disease was eye involvement (uveitis), which was found in 65% of the patients. At the time of investigation, none of the patients was undergoing systemic steroid therapy. As control subjects, 10 healthy subjects volunteered to participate in this study (Table I). All participants were nonsmokers.

Reagents and antibodies

Ionomycin, phorbol 12-myristate acetate (PMA), brefeldin-A, and BSA were purchased from Sigma Chemical Co (St Louis, Mo). The following antibodies were used: peridin chlorophyll protein (PerCP)-conjugated anti-CD4, PerCP-conjugated anti-CD8, FITC-conjugated anti-IFN-γ, and phycoerythrin (PE)-conjugated anti-IL-4 antibodies (Becton Dickinson, San Jose, Calif). Isotype-matched conjugated (FITC, PE, and PerCP) control antibodies, FACS Lysing Solution, and FACS Permeablizing Solution were obtained from Beckton Dickinson.

Preparation of peripheral blood cells

Ten milliliters of heparinized blood obtained from each patient or control subject were collected and diluted 1:20 with a culture medium consisting of RPMI-1640 supplemented with 10% FCS (Gibco), 100 U/mL penicillin (Gibco), and 100 mg/mL streptomycin (Gibco). In several experiments PBMCs were isolated by means of density centrifugation of heparinized blood on Lymphoprep (Nycomed, Oslo, Norway). To further deplete B cells, natural killer (NK) cells, and monocytes, nonadherent cells were incubated with saturating concentrations of anti-CD11b, anti-CD16, anti-CD19, anti-CD36, and anti-CD56 mAbs conjugated with superparamagnetic microbeads for 15 minutes on ice and washed in PBS containing 5 mmol/L EDTA and 0.5% human serum. Unlabeled cells were then isolated by eluting from magnetic columns, routinely resulting in greater than 98% purity of $CD3^+$ and less than 1% $CD56^+$ cells.

Bronchoalveolar lavage procedures and preparation of BALF cells

Bronchoalveolar lavage was performed as previously described.[27] Briefly, with topically administered lidocaine (Xylocaine), both the upper and lower respiratory tracts were anesthetized. The BF-240 Olympus fiberoptic bronchoscope (Olympus Co, Tokyo, Japan) was passed transorally and wedged in a segmental or subsegmental bronchus of the middle lobe. Three 50-mL aliquots of sterile 0.9% saline were instilled, and the returns were gently aspirated through the side channel of the bronchoscope. BALF cells were separated by means of centrifugation at 500*g* for 10 minutes at 4°C and washed with PBS containing 0.5% BSA. Total cell count was determined by using a hemocytometer, and cells were resuspended in the culture medium at 1×10^6 cells/mL. A differential cell count was taken on Giemsa-stained cytocentrifuged preparations. In several experiments we purified $CD3^+$ T cells from BALF by using the same procedure used in peripheral blood cells.

Stimulation of cells and intracellular cytokine staining

One milliliter of peripheral blood cells diluted 1:20 in the culture medium or BALF cell suspension were placed in each well of a 24-well culture plate. They were then stimulated with PMA (25 ng/mL) and ionomycin (1 μg/mL) in the presence of brefeldin-A (10 μg/mL) for 4 hours at 37°C in a humidified 5% CO_2 incubator. Brefeldin-A was used to interrupt intracellular transport mechanisms, thereby leading to accumulation of cytokines in the cells.

After stimulation with PMA/ionomycin, the cells were incubated with either PerCP-conjugated anti-CD4 or anti-CD8 antibody for 15 minutes at room temperature (RT). In the preparation of peripheral blood cells and BALF cells, erythrocytes were lysed by adding FACS Lysing Solution. After centrifugation, the cells were washed with 0.1% BSA-PBS and subsequently incubated in FACS Permeabilizing Solution for 10 minutes at RT. After washing with 0.1% BSA-PBS, the permeabilized cells were then incubated with FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4 antibodies for 30 minutes at RT. After staining, they were washed with 0.1% BSA-PBS, fixed in 1% paraformaldehyde, and stored at 4°C. The cells were analyzed within 12 hours after storage. Negative controls consisted of unstimulated cells. We confirmed that the viability of the cells freshly obtained from peripheral blood and BALF was greater than 97% by using trypan blue exclusion. After PMA/ionomycin activation, the viability of each cell population was greater than 95%.

Flow cytometric analysis

The samples were analyzed on a FACScan flow cytometer (Beckton Dickinson) equipped with a 15-mW argon ion laser and appropriate filters for FITC (530 nm), PE (585 nm), and PerCP (>650 nm) by using the Cell Quest Software (Beckton Dickinson). Lymphocytes were first gated on the basis of forward and side light scatter. Subsequently, within this gated area, in which $CD3^+$ cells fell, a second gate was further selected. The following analyses were done on cells in the second gate. For 3-color analysis, we measured the number of positive cells for anti-IFN-γ (FITC), anti-IL-4 (PE), or both antibodies simultaneously in gated cell populations that were stained with either anti-CD4 or anti-CD8 antibody (PerCP). The $CD8^+$ cells in the gated area contained less than 1% $CD56^+$ cells. The number of positive cells for each cytokine was expressed as a percentage of $CD4^+$ or $CD8^+$ T cells. In all experiments parallel incubations were performed with FITC- or PE-conjugated irrelevant antibodies matched for the isotypes of the anticytokine antibodies. The cutoff level for definition of positive cells was thus set so that less than 1% of irrelevant-antibody stained cells were positive.

In both peripheral blood and BALF cells, preliminary experiments revealed that the cytokine profiles in the purified $CD3^+$ T-cell populations were basically comparable with those in the unseparated whole cells (data not shown). These findings are consistent with those of a recent study on intracellular cytokine expression with unseparated whole blood cells.[28] Therefore we measured the number of intracellular cytokine–positive cells in the unseparated cells.

Because PMA/ionomycin stimulation has been shown to downregulate CD4 expression, there may be difficulty in recognizing a clear $CD4^+$ population with anti-CD4 mAb,[29,30] resulting in the misreading of the true population of $CD4^+$ T cells. Indeed, we found that the mean fluorescent intensity (MFI) of CD4 expression by blood cells was decreased by 62% after 4 hours of stimulation with PMA/ionomycin (MFI of unstimulated cells, 601; MFI of stimulated cells, 226), although there was a negligible change in $CD8^+$ expression (data not shown). In our assays, however, we could still detect clearly bimodal profiles for CD4 after the stimulation, and there was no difference in the proportion of $CD4^+$ cells between unstimulated and stimulated cells (36.0% vs 35.4%). Thus we used the above protocol for measurement of cytokine expression in $CD4^+$ T cells, as well as $CD8^+$ T cells.

TABLE II. Characteristics of bronchoalveolar lavage

	Control	Sarcoidosis
Recovery rate (%)	59.20 ± 3.06	63.80 ± 1.74
Total cell counts (1×10^5)	1.89 ± 0.53	2.62 ± 0.49
Macrophages (1×10^5)	1.78 ± 0.49	2.01 ± 0.30
Lymphocytes (1×10^5)	0.06 ± 0.02	0.56 ± 0.17†
Neutrophils (1×10^5)	0.03 ± 0.02	0.02 ± 0.08
Eosinophils (1×10^5)	0.01 ± 0.01	0.04 ± 0.02
Macrophages (%)	94.42 ± 1.27	78.90 ± 3.88†
Lymphocytes (%)	3.74 ± 1.09	19.85 ± 3.53†
Neutrophils (%)	0.52 ± 0.22	0.90 ± 0.44
Eosinophils (%)	1.08 ± 0.68	0.20 ± 0.13
CD4 (%)	55.34 ± 3.35	77.41 ± 3.51†
CD8 (%)	24.66 ± 3.89	13.26 ± 2.99*
CD4/CD8	2.55 ± 0.52	9.05 ± 2.80*

Data are shown as means ± SEM.
*$P < .05$ compared with control subjects.
†$P < .01$ compared with control subjects.

Statistics

For statistical analysis, the Mann-Whitney *U* test was used. A *P* value of less than .05 was considered significant. All values are expressed as means ± SEM.

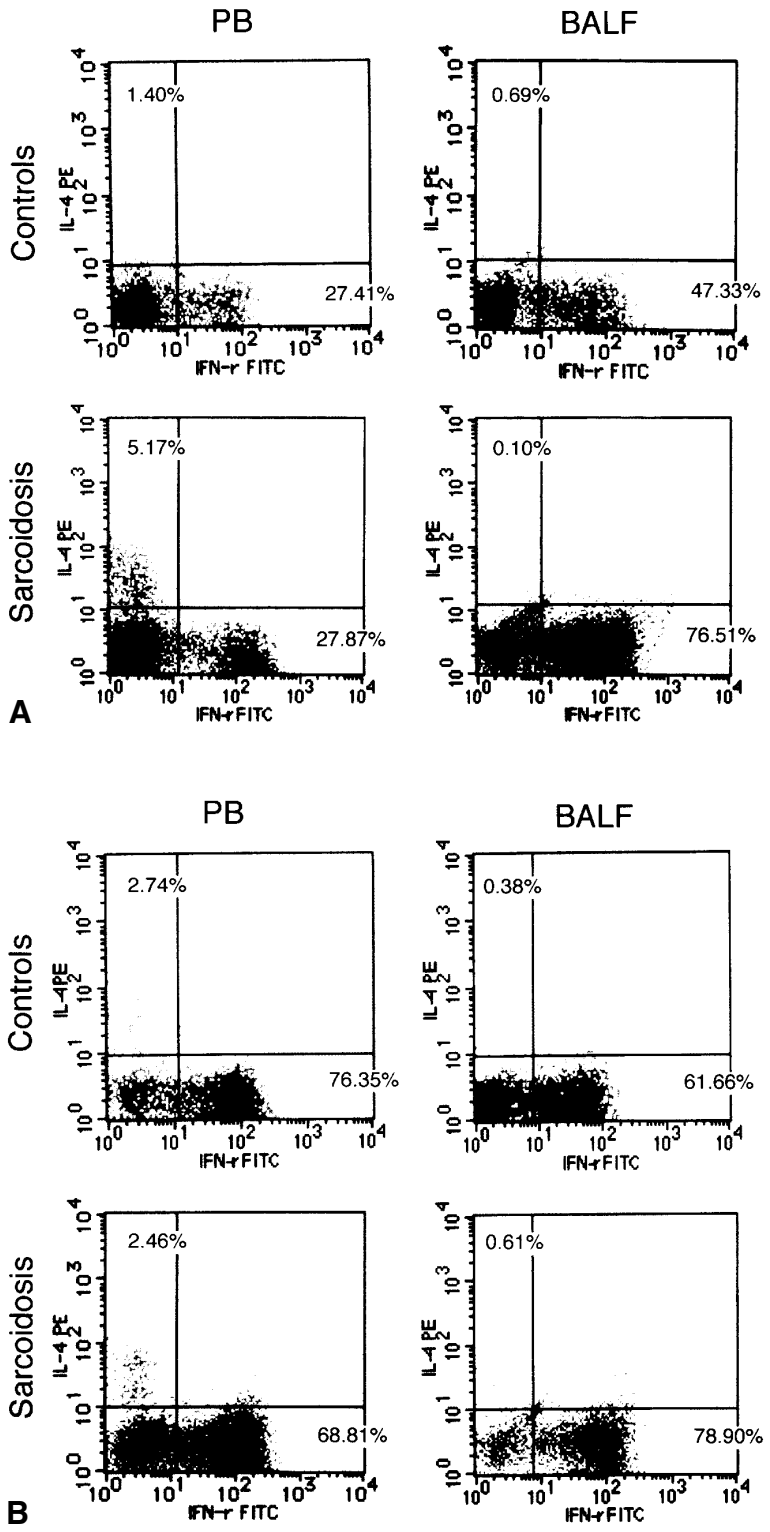

FIG 1. Flow cytometric detection of intracellular cytokine production in $CD4^+$ **(A)** and $CD8^+$ **(B)** T cells obtained from peripheral blood and BALF after stimulation with PMA/ionomycin. Cells were stimulated with PMA (25 ng/mL) and ionomycin (1 μg/mL) in the presence of brefeldin-A (10 μg/mL) for 4 hours at 37°C. They were stained with either PerCP-conjugated anti-CD4 or anti-CD8 antibody. After permeabilization, the cells were then incubated with FITC-conjugated anti-IFN-γ and PE-conjugated anti-IL-4 antibodies. A total of 10,000 events gated on either $CD4^+$ or $CD8^+$ cells are shown in each plot. Percentages in *upper* and *lower quadrants* represent percentage of cells expressing IFN-γ or IL-4 in either $CD4^+$ or $CD8^+$ cells.

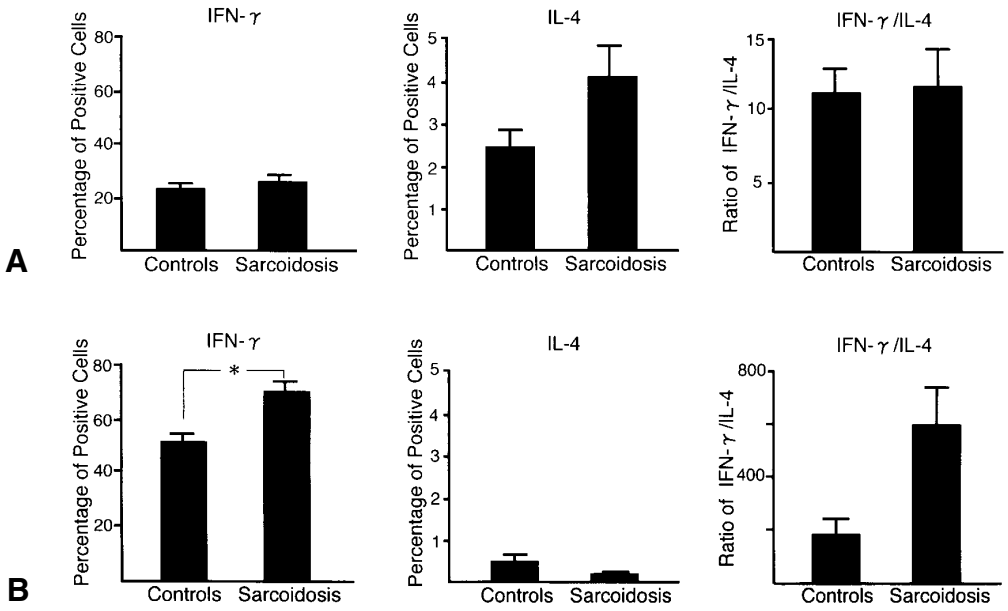

FIG 2. Percentages of IFN-γ– and IL-4–producing CD4+ T cells and the ratio of IFN-γ/IL-4–producing CD4+ T cells obtained from peripheral blood and BALF (**A** and **B**, respectively) after simulation with PMA/ionomycin. Values are expressed as means ± SEM. The percentages of IFN-γ–producing CD4+ T cells in BALF are significantly higher in patients with sarcoidosis than in normal subjects. *$P = .0192$.

RESULTS

Characteristics of BALF cells

As shown in Table II, patients with sarcoidosis had significant increases in the percentage of lymphocytes ($P = .0034$) and a significant decrease in the percentage of macrophages ($P = .0034$) compared with normal subjects. There were no significant differences in volume recovered, total cell number, and the percentages of other cell types between the patients and control subjects. A phenotypic study revealed that the percentage of CD4+ T cells ($P = .0084$) and the ratio of CD4+/CD8+ T cells ($P = .0128$) were significantly higher in patients than in normal subjects.

Intracellular cytokine production in CD4+ T cells

Under unstimulated conditions, we could find no cytokine-expressing cells in the CD4+ T-cell population of peripheral blood in patients or normal subjects. In BALF small percentages of the cytokine-expressing cells were detected, but no significant differences in the percentages of cytokine-expressing cells were observed between patients with sarcoidosis (IFN-γ–producing CD4+ T cells, 8.8% ± 4.8%; IL-4–producing CD4+ T cells, 0.3% ± 0.1%; IFN-γ/IL-4–producing CD4+ T cells, 102.7 ± 55.4) and normal control subjects (IFN-γ–producing CD4+ T cells, 7.6% ± 1.5%; IL-4–producing CD4+ T cells, 0.3% ± 0%; IFN-γ/IL-4–producing CD4+ T cells, 26.7 ± 5.2). Fig 1, *A*, shows representative profiles of intracellular cytokine staining for IFN-γ and IL-4 in CD4+ T cells in a patient with sarcoidosis and a normal subject after PMA/ionomycin stimulation.

In response to PMA/ionomycin, in the peripheral blood there were still no significant differences in the proportion of IFN-γ– or IL-4–producing CD4+ T cells or in the ratio of IFN-γ/IL-4–producing CD4+ T cells between patients (IFN-γ–producing CD4+ T cells, 26.0% ± 2.4%; IL-4–producing CD4+ T cells, 4.1% ± 0.9%; IFN-γ/IL-4–producing CD4+ T cells, 11.7 ± 2.7) and normal control subjects (IFN-γ–producing CD4+ T cells, 23.0% ± 2.3%; IL-4–producing CD4+ T cells, 2.4% ± 0.4%; IFN-γ/IL-4–producing CD4+ T cells, 11.3 ± 1.9; Fig 2, *A*). However, in BALF the percentage of IFN-γ–producing CD4+ T cells was significantly higher in patients than in normal control subjects (72.0% ± 4.4% vs 53.0% ± 3.4%, respectively; $P = .0192$; Fig 2, *B*) after the stimulation. In addition, there was a trend that patients had a lower proportion of IL-4–producing CD4+ T cells compared with normal subjects (0.2% ± 0.2% vs 0.5% ± 0.2%, respectively; $P = .0790$) in BALF, resulting in a 3.5-fold higher ratio of IFN-γ/IL-4–producing CD4+ T cells in sarcoidosis than that of the control subjects (592.4 ± 154.1 vs 178.7 ± 64.0, respectively; $P = .0790$).

Compared with peripheral blood cells, BALF cells showed a significant increase in the percentage of IFN-γ–producing CD4+ T cells (patients with sarcoidosis, $P < .001$; control subjects, $P = .0027$) and a significant decrease in the percentage of IL-4–producing CD4+ T cells (patients with sarcoidosis, $P < .001$; control subjects, $P = .027$) together with a more than 50-fold higher ratio of IFN-γ/IL-4–producing CD4+ T cells (patients with sarcoidosis, $P < .001$; control subjects, $P = .027$) in both patients and normal control subjects.

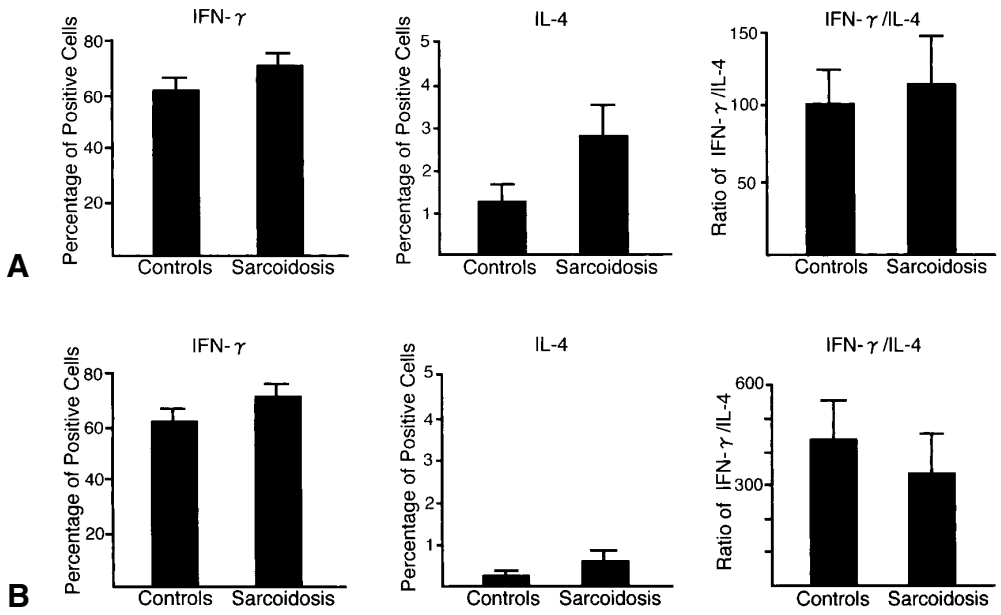

FIG 3. Percentages of IFN-γ– and IL-4–producing CD8+ T cells and the ratio of IFN-γ/IL-4–producing CD8+ T cells obtained from peripheral blood and BALF (**A** and **B**, respectively) after stimulation with PMA/ionomycin. Values are expressed as means ± SEM. There are no differences in the percentages or the ratio of cytokine-producing CD8+ T cells between patients with sarcoidosis and normal subjects.

Intracellular cytokine production in CD8+ T cells

Representative profiles of intracellular cytokine staining for IFN-γ and IL-4 in CD8+ T cells in a patient with sarcoidosis and a normal subject after stimulation with PMA/ionomycin are shown in Fig 1, *B*. Without activation, we could detect no cytokine-expressing cells in CD8+ T-cell populations of peripheral blood in patients or normal subjects. In BALF no significant difference in the cytokine profiles was observed between patients with sarcoidosis (IFN-γ–producing CD8+ T cells, 17.7% ± 6.8%; IL-4–producing CD8+ T cells, 0.9% ± 0.4%; IFN-γ/IL-4–producing CD8+ T cells, 29.5 ± 9.5) and normal control subjects (IFN-γ–producing CD8+ T cells, 11.0% ± 4.5%; IL-4–producing CD8+ T cells, 0.8% ± 0.5%; IFN-γ/IL-4–producing CD8+ T cells, 27.5 ± 10.2).

Even after stimulation with PMA/ionomycin, there was no significant difference in the proportion of cytokine-producing CD8+ T cells or in the ratio of IFN-γ/IL-4–producing CD8+ T cells in the peripheral blood in patients and normal control subjects, respectively (IFN-γ–producing CD8+ T cells, 69.5% ± 3.2% vs 59.2% ± 5.8%; IL-4–producing CD8+ T cells, 2.7% ± 0.8% vs 1.1% ± 0.5%; IFN-γ/IL-4–producing CD8+ T cells, 112.8 ± 41.3 vs 97.9 ± 25.2; Fig 3, *A*). In stimulated cells of BALF, the percentage of cytokine-producing CD8+ T cells or the ratio of IFN-γ/IL-4–producing CD8+ T cells was not statistically different between patients and normal subjects, respectively (IFN-γ–producing CD8+ T cells, 76.3% ± 3.3% vs 74.1% ± 1.4%; IL-4–producing CD8+ T cells, 0.6% ± 0.3% vs 0.2% ± 0.1%; IFN-γ/IL-4–producing CD8+ T cells, 336.7 ± 113.4 vs 440.5 ± 121.8; Fig 3, *B*).

In comparison of the cytokine profiles between BALF and peripheral blood cells, the BALF cells of patients and normal subjects showed a significant decrease in the percentage of IL-4–producing CD8+ T cells ($P = .0118$ and $P = .0233$, respectively) and a significant increase in the ratio of IFN-γ/IL-4–producing CD8+ T cells ($P = .0082$ and $P = .0233$, respectively).

DISCUSSION

By using flow cytometry to detect intracellular cytokines, the present study for the first time demonstrated the T_C1/T_C2 balance and the T_H1/T_H2 balance in peripheral blood and BALF cells in pulmonary sarcoidosis. We found a significantly higher percentage of IFN-γ–producing CD4+ T cells together with a markedly increased ratio of IFN-γ/IL-4–producing CD4+ T cells in BALF, although not in peripheral blood, after PMA/ionomycin stimulation in patients with sarcoidosis compared with normal subjects. In contrast, there were no differences in the percentages of IFN-γ– or IL-4–producing CD8+ T cells in either the peripheral blood or BALF between patients and control subjects. These findings suggest that the prominent shift toward a type-1 phenotype, relative to normal subjects, may occur in CD4+ T cells, although not in CD8+ T cells, in the affected organs of sarcoidosis.

In this study we used triple-color flow cytometry for measuring intracellular cytokine proteins. This technique permits the simultaneous detection of multiple cytokines at the single-cell level in different cell populations, contributing to the clinical evaluation of immune

status in terms of the cytokine profiles. In sarcoidosis, most studies of balance of cytokine production in T cells have used in vitro prepared T-cell clones.[25,31] However, it is well recognized that there is a wide difference in the patterns of cytokine synthesis among cloned T cells obtained from the same individual, indicating that functional responses of cloned T cells may not reflect physiologic responses occurring in vivo.[19,20] In contrast, the patterns of cytokine expression in T cells activated with a combination of PMA and ionomycin used in this study were shown to generally agree with their physiologically regulated expression,[32] except certain types of cytokines, such as IL-10.[33] Indeed, our results indicated that PMA/ionomycin stimulated the cytokine production equally among $CD4^+$ and $CD8^+$ T cells. Moreover, recent study reported that there was no difference in the profiles of cytokine expression among activating signals, such as PMA/ionomycin, superantigens, and anti-CD3 plus anti-CD28 mAbs, because the profiles could reflect intrinsic characteristics of the T cells in vivo after fully activating them.[30] Thus unlike T-cell cloning, this method enabled us to study cells with little in vitro incubation, decreasing the potential for artifacts arising from prolonged culture. Although PMA/ionomycin stimulation downregulated CD4 expression,[29,30] we could clearly recognize the $CD4^+$ cell population in our assays. There may still be the possibility that we lost $CD4^+$ dim T cells, in which its expression was decreased by PMA/ionomycin, but the number of these cells appeared to be small. Another possibility may exist that NK $CD8^+$ dim cells expressing IFN-γ were identified in the $CD8^+$ cell population. However, we found that $CD56^+$ NK cells in the CD8-gated cells were less than 1%. In addition, highly purified $CD3^+$ T cells, not including NK cells, showed comparable profiles of cytokine expression to those of unseparated cells. Collectively, it is likely that NK $CD8^+$ dim cells expressing IFN-γ were very few in the $CD8^+$ cell population. Thus the analysis of intracellular cytokine production with multiple-color cytometry in combination with PMA/ionomycin stimulation can provide precise information on the immune status of patients with sarcoidosis.

Under unstimulated condition, we found no cytokine-expressing cells in peripheral blood. In BALF, however, we could detect small percentages of the cytokine-producing cells, the proportions of which were not statistically different between patients with sarcoidosis and normal subjects. On stimulation with PMA/ionomycin, in BALF of patients, although not in peripheral blood, a significant increase in the percentage of IFN-γ–producing $CD4^+$ T cells and a decrease in the percentage of IL-4–producing $CD4^+$ T cells were found, resulting in a 3.5-fold higher ratio of IFN-γ/IL-4–producing $CD4^+$ T cells compared with normal subjects. These findings indicate that the T_H1/T_H2 balance was primed toward a T_H1 phenotype in the lungs of patients with pulmonary sarcoidosis. Even in normal subjects, BALF cells had significant increased proportions of IFN-γ–producing $CD4^+$ T cells together with a decrease in the percentage of IL-4–producing $CD4^+$ T cells compared with peripheral blood cells, implying that a more prominent shift of $CD4^+$ T cells to a T_H1 phenotype exists in BALF than in peripheral blood under normal conditions. However, the increase of IFN-γ–producing $CD4^+$ T cells in BALF was more noticeable in patients with sarcoidosis. Previous studies on pulmonary sarcoidosis demonstrated an elevated concentration of the type-1 cytokines IL-2 and IFN-γ in BALF,[34] which suggested that activated T_H1-like cells accumulated at the affected sites. However, increases of T_H1-associated cytokines in BALF, as measured by such bulk-release detection, does not accurately reflect the immune status in the sarcoid lungs in terms of the T_H1/T_H2 balance because the sources of cytokines varied, including T_C1- and T_H1-like cells. Thus it is not clearly determined whether T_H1-like cells predominate over T_H2-like cells or, alternatively, whether the T_H1/T_H2 balance moved toward T_H1 in the lungs of patients with pulmonary sarcoidosis. In this respect the present study clearly demonstrated a pronounced shift toward a T_H1 phenotype in BALF $CD4^+$ T cells of patients with sarcoidosis, as determined by the cytokine profiles at the single-cell level.

Recent studies provided new insight into the function of T_C cells, revealing that they possess the capacity to produce a variety of cytokines in addition to their cytotoxic function, which in some instances exceed that of T_H cells.[12–15] Increasing evidence suggests that the alteration of the T_C1/T_C2 balance can be implicated in the pathogenesis of several diseases.[17,18] Possibly, an imbalance of T_C1/T_C2 may also be involved in sarcoidosis, which is a well-known type-1 cytokine–associated disorder. The present study showed that even in normal subjects the percentage of IFN-γ–producing cells was considerably higher in $CD8^+$ T cells than in $CD4^+$ T cells in peripheral blood, resulting in an approximately 10-fold increase in the ratio of IFN-γ/IL-4–producing cells in $CD8^+$ T cells compared with that of $CD4^+$ T cells. This indicates that $CD8^+$ T cells have a more favorable type-1 phenotype than $CD4^+$ T cells in peripheral blood, even under normal conditions, which is consistent with a previous report.[35] In comparison between the patients with sarcoidosis and normal subjects, there was no significant difference in the ratio of IFN-γ/IL-4–producing $CD8^+$ T cells in either peripheral blood or BALF. Recently, Baumer et al[25] reported that cloned $CD8^+$ T cells obtained from lung parenchyma of patients with pulmonary sarcoidosis had no prevalence of type-1 or type-2 cytokine profiles, whereas $CD4^+$ T-cell clones shifted to a type-1 phenotype. They concluded that the significant alteration toward a type-1 phenotype was present in the $CD4^+$ T-cell population but not in $CD8^+$ T cells in sarcoidosis. In agreement with this, the present study showed that the T_C1/T_C2 balance was not changed in patients with sarcoidosis. Thus unlike the modification in the T_H1/T_H2 balance observed in sarcoidosis, the T_C1/T_C2 balance may not be altered, even in affected tissues. To confirm this, further studies will be required in a larger series of patients with sarcoidosis.

In conclusion, the $CD4^+$ T-cell population in the BALF of patients with pulmonary sarcoidosis had a prominent shift to a T_H1 phenotype compared with those of normal subjects, supporting the concept that the dominant activation of a T_H1 phenotype is associated with pathogenesis of sarcoidosis. In contrast, an alteration in the T_C1/T_C2 balance in BALF did not occur, suggesting the possibility that T_C1 cells might not play a major role in sarcoidosis.

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