INCREASED EXPRESSION OF CCL4/MIP-1β IN CD8$^+$ CELLS AND CD4$^+$ CELLS IN SARCOIDOSIS

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Sarcoidosis is a granulomatous disease with an increased accumulation of T cells in lungs as a result of on-site proliferation and chemotaxis induced by chemokines. It has already been demonstrated that CCL3-5 levels were increased in BAL fluid of sarcoidosis patients. To analyze the expression of CCL3-5 chemokines by T-cell subtypes (CD4$^+$, CD8$^+$, Th1, Th2, Tc1 or Tc2) in the lungs of sarcoidosis patients, fifteen untreated sarcoidosis patients and eighteen control subjects were enrolled in this study. CD4$^+$ and CD8$^+$ cells were isolated from BAL fluid by positive magnetic selection. The expression of CCL3-5 and other cytokines in CD4$^+$ and CD8$^+$ cells were measured by flow cytometry. The percentage of CD4$^+$ or CD8$^+$ cells expressing CCL4 were significantly higher in sarcoidosis patients (22.3% and 58.1%) compared to those seen in healthy subjects (11.1% and 16.5%, P = 0.04 and P = 0.02, respectively). In addition, the expression of CCL3, CCL4 and CCL5 was significantly elevated in CD8$^+$ cells (8.9%, 58.1% and 2.1%) compared to CD4$^+$ cells (2.1%, 22.3% and 0.7%; P = 0.04, P = 0.009 and P = 0.04, respectively), whereas CCL4 was expressed by significantly more Tc1 than Th1 cells in sarcoidosis patients (P = 0.006). Our study shows the possible role of CD8$^+$ cells and CD4$^+$ cells in recruiting T cells to the site of inflammation in sarcoidosis through the release of CCL4, either alone or together with Th1/Tc1-associated cytokines.

Sarcoidosis is a granulomatous disease of unknown etiology. One of the main features of sarcoidosis is an increased accumulation of T cells and macrophages in lungs and other involved organs. The elevated number of T cells is a result of on-site proliferation (1, 2) and increased cell migration guided by chemokines produced, among others, by macrophages and epithelial cells. Apart from CXC chemokines, such as IP-10/CXCL10 (*IFN-γ-inducible protein of 10-kDa*), CC chemokines: macrophage inflammatory protein 1α (MIP-1α), macrophage inflammatory protein 1β (MIP-1β) and regulation on activation normal T-cell expressed and secreted (RANTES), play an important role in this process (3, 4). Their current official names are, respectively, CCL3, CCL4 and CCL5. They share the same receptor (CCR5) with HIV-1 virus (5). CCL3-5 chemokines have been implicated in the pathogenesis of numerous respiratory disorders. For example we have shown that levels of CCL4/MIP-1β were significantly elevated in sputum of COPD patients (6). In turn, asthmatic patients had increased levels of CCL5/RANTES in sputum (7). CCL3-5 are chemokines that facilitate the recruitment of T cells

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to the site of inflammation (8). It has been shown that CCL4 is a more potent chemoattractant for $CD4^+$ than $CD8^+$ cells, whereas CCL3/MIP-1α are more effective in attracting $CD8^+$ cells (9, 10). CCL5 may influence the migration of T cells (especially $CD45RO^+$ memory T cells) (11), increase the proliferation of T cells and stimulate cytotoxic T-cell responses. CCL3-5 chemokines may also indirectly regulate the inflammatory response by preferential recruitment of Th1 cells as CCR receptor is more frequently found on activated Th1 cells (3, 8).

CCL4/MIP-1β levels were increased in BAL fluid of sarcoidosis patients compared to controls (3). Capelli at al. also showed a correlation between levels of CCL4 and the number of $CD4^+$ and $CD8^+$ cells in BAL fluid (3). Elevated levels of CCL3/MIP-1α were only found in sarcoidosis patients, stages 2 or 3 (3). BAL fluid CCL3 levels were found to be inversely correlated to diffusing capacity for carbon monoxide (DL_{CO}) and the partial pressure of oxygen in blood (PaO_2) in patients with fibrotic sarcoidosis (3). CCL3 levels in BAL or blood were also increased in other studies (12, 13). However, no differences were present in the Iida et al. study, in which only sarcoidosis patients with good prognosis were recruited (14). Similarly, increased CCL5 levels were reported in BAL fluid of sarcoidosis patients (11, 14-16). Particularly high levels of CCL5 were observed in patients who require treatment and in those with active disease (16). Additionally, CCL5 levels correlated with the number of lymphocytes and $CD4^+$ cells in BAL fluid (14-16). However, to date, it is not known whether T lymphocytes are a source of increased production of CCL3-5 chemokines in sarcoidosis. We only know, based on immunohistochemical and cell culture *in vitro* studies, that macrophages are rich producers of CCL3 and CCL5 in sarcoidosis (12, 17, 18). CCL5 has been recently linked with increased expression of the Th1 transcription factor T-bet in pulmonary sarcoidosis (19).

The aim of our study was to assess the production of CCL3-5 chemokines by T cells and their subtypes ($CD4^+$, $CD8^+$, Th1, Th2, Tc1 or Tc2) in the lungs of sarcoidosis patients. Additionally, we sought to examine the relationship between the expression of CCL4 in T cells and levels of this chemokine in BAL.

MATERIALS AND METHODS

Subjects

The study protocol was approved by the ethics committee of the Silesian Medical University. All study participants provided written consent before enrolment in the study. Fifteen untreated sarcoidosis patients

Table I. *Characteristics of study subjects.*

Group	Patients with sarcoidosis	Control subjects
N	15	18
Age (y)	37 (27-53)	50 (41-58)
Sarcoidosis type (I/II)	8/7	-
Sex (M/F)	9/6	15/3
Cigarette smokers (Y/Ex/N)	2/6/7	6/5/7
Pack years	2 (0-10)	7 (0-11)
FEV1 (% predicted)	94.7 (81.5-104.3)	103.6 (100.1-119.7) *
FEV1%FVC	83.7 (81-85.1)	87.9 (81.5-90.2)
VC (% predicted)	96.7 (79.6-102.5)	-
TLC (% predicted)	94.2 (82.8-94.9)	-
DLCO (% predicted)	81.2 (67.5-85.1)	-

*Data are presented as median (upper and lower quartiles) or as the number of groups. M: Male; F: Female; Y: current smokers; Ex: ex-smokers; N: never smokers; FEV_1: forced expiratory volume in 1 sec; FVC: forced vital capacity; VC: vital capacity; TLC: total lung capacity; DLCO: diffusion lung capacity for carbon monoxide. *P < 0.05 compared with the control group.*

with a median age of 37 years (range 27-53 years) were recruited to the study. All patients fulfilled the criteria for sarcoidosis according to international guidelines (20). The diagnosis was made when typical clinical manifestations, radiological findings and BAL cell profiles were supported by positive histological results from transbronchial biopsy (8 patients), mediastinoscopy (5 patients) and biopsy of peripheral lymph nodes (2 patients). Additionally, clinical and laboratory findings were negative for tuberculosis.

The control group consisted of eighteen healthy subjects with a median age of 50 years (range 41-58 years) with no symptoms or history of any respiratory disease. The age did not show any significant differences between the sarcoidosis and control group. The characteristics of the subjects are shown in Table I.

Bronchoscopy and BAL

The tip of the bronchoscope was wedged into the orifice of a subsegmental bronchus of the middle lobe. Bronchoalveolar lavage (BAL) was performed by sequential installation and aspiration of 8 separate aliquots of 25 mL of warm 0.9% normal saline. BAL fluid was centrifuged and the supernatant was frozen at -70°C until used for enzyme-linked immunosorbent assay (ELISA). A differential cell count was performed on cytospins of BAL cells using May-Grunwald-Giemsa stain.

Isolation of $CD4^+$ and $CD8^+$ T cells from the BAL fluid

$CD4^+$ and $CD8^+$ cells were isolated from BAL fluid according to previously described procedures (21). In brief, BAL cells were spun, washed and resuspended in the culture medium [RPMI-1640, 10% (vol/vol) FCS, 2 mM l-glutamine, 100 IU/mL penicillin, and 100 mg/mL streptomycin]. Macrophages were depleted by adhesion to plastic plates. The resulting non-adherent cells consisted mainly of lymphocytes, but variable amounts of dead cells, granulocytes, and epithelial cells were also present. Dead cells were eliminated with the Dead Cell Removal Kit (Miltenyi Biotec, Bergisch Gladbach, Germany). $CD8^+$ cells and then $CD4^+$ cells were isolated by positive selection using first MACS CD8+ MicroBeads and later MACS CD4+ MicroBeads (Miltenyi Biotec). The purity of isolated $CD4^+$ and $CD8^+$ cells were consistently >95% as assessed by flow cytometry (FACS).

Flow cytometry

$CD4^+$ and $CD8^+$ cells were washed and stimulated for 6 h with phorbol 12-myristate 13-acetate (PMA; 10ng/mL) and ionomycin (400ng/mL) in the presence of brefeldin A (5mg/mL). When a sufficient number of cells were obtained, part of them were incubated only in the presence of brefeldin A to check the expression of chemokines in unstimulated cells. Subsequently, the cells were washed twice in wash buffer, fixed in cold 4% formaldehyde, washed in PBS and frozen at -70°C for later use as previously described (21). Cells were then thawed, washed, permeabilized, washed again and stained with a cocktail of antibodies (described in detail in Table II) or respective mouse isotype control antibodies for 30 min at room temperature in the dark (all antibodies originated from BD Pharmingen, Oxford, UK). After being washed twice, cells were resuspended in 1% formaldehyde in PBS and analyzed within 24 h by four-colour flow cytometry (FACSCanto flow cytometer, BD Biosciences). $CD4^+$ cells and $CD8^+$ cells expressing IFN-γ, but not IL-4 were defined as Th1 and Tc1 cells, respectively. Similarly, $CD4^+$ cells and $CD8^+$ cells positive for IL-4 but not for IFN-γ were described as Th2 and Tc2, respectively.

ELISA for CCL4/MIP-1β

CCL4/MIP-1β expression in BAL fluid was measured by sandwich ELISA as recommended by the manufacturer (McKinley Place N.E., Minneapolis, USA). The lower limit of detection was 4 pg/ml.

Statistical analysis

Statistical analysis was performed with the use of software packages (GraphPad Prism 5.0; Statistica 9.0).

Table II. *Combinations of antibodies used in the study.*

Fluorochrome	Antibodies
FITC	Anti-IFN-γ
PE	Anti-CCL3/MIP-1α; anti-CCL4/MIP-1β; anti-CCL5/RANTES
APC	Anti-TNF-α; anti-IL-10
PE-Cy7	Anti-IL-4

FITC: Fluorescein isothiocyanate; PE: R-phycoerythrin; PE-Cy7: a tandem fluorochrome composed of R-phycoerythrin (PE) coupled to the cyanine dye Cy7; APC: Allophycocyanin.

Data are presented as median (interquartile range) except where stated otherwise. A Gaussian distribution was tested with D'Agostino and Pearson omnibus normality test. Between-group comparisons were assessed with a Mann-Whitney *U* test for non-parametric data. Regression analysis was performed with the Spearman rank correlation test. *P* values of less than 0.05 were accepted as significant.

RESULTS

BAL cell characterization

BAL fluid recovery ($P < 0.05$), the number of isolated $CD4^+$ ($P < 0.001$) and $CD8^+$ cells ($P < 0.01$), as well as the percentage of lymphocytes in BAL fluid ($P < 0.001$) of sarcoidosis patients were significantly higher compared to control subjects. On the contrary, the percentage of macrophages demonstrated significant decrease in BAL fluid of sarcoidosis patients ($P < 0.001$). A full characteristic of BAL cells is provided in Table III.

The percentage of BAL fluid $CD4^+$ or $CD8^+$ cells, stimulated in vitro with PMA/ionomycin, expressing CCL4/MIP-1β or other cytokines

Representative examples of CCL4/MIP-1β expression together with IFN-γ, IL-4 and TNF-α co-expression in $CD8^+$ cells isolated from BAL fluid of sarcoidosis patients are shown in Fig. 1. The percentage of $CD4^+$ or $CD8^+$ cells expressing CCL4/MIP-1β were significantly higher in sarcoidosis patients [22.3% (15-45.8%) and 58.1% (35.1-65.7%), respectively] than in those seen in healthy subjects [11.1% (4.2-24.2%), P = 0.04 and 16.5% (12.4-57.6%), P = 0.02, respectively; Fig. 2]. In addition, $CD8^+$ cells expressing CCL4/MIP-1β were present to a significantly greater extent compared to $CD4^+$ cells expressing this chemokine within the sarcoidosis group (P = 0.009, Fig. 2).

CCL4 and IFN-γ were co-expressed by a significantly higher percentage of $CD4^+$ or $CD8^+$ cells in sarcoidosis patients compared to healthy subjects [14.3% (6.6-26.7%) *vs* 6.7% (0.3-14.1%), P = 0.04 and 34.1% (17.5-47.8%) *vs* 3.1% (0.224.7%), P = 0.008, respectively; Fig. 3]. In addition, there was a significantly higher percentage of $CD8^+$ cells co-expressing CCL4 and IFN-γ compared to $CD4^+$ cells co-expressing CCL4 and IFN-γ in sarcoidosis patients (P = 0.03). In contrast, no significant difference was detected between the two studied groups in the percentage of Th1 or Tc1 cells

Table III. *Characteristics of BAL cells from the study population.*

Group	Patients with sarcoidosis	Control subjects
Recovery (ml)	138 (130-145)*	124.5 (110-133)
Total cell number (x 10^6)	14.4 (8.9-27.6)	10.5 (4.5-15)
Total cell number per volume of BAL recovered (x 10^4/ml)	10.3 (6-19.7)	8.4 (3.8-11.9)
The number of isolated $CD4^+$ cells (x10^4)	40.5 (21-134)‡	4.8 (2-11.2)
The number of isolated $CD8^+$ cells (x10^4)	20 (10-50)†	7.4 (3.5-12.5)
The ratio of isolated $CD4^+$ cells to isolated $CD8^+$ cells	2.3 (1.7-3.6)‡	0.7 (0.4-1.0)
Macrophages (%)	79 (61.7-86.6)	94.4 (88.7-96.3)‡
Neutrophils (%)	0.7 (0.2-1.7)	0.4 (0.3-1)
Eosinophils (%)	0 (0-1)	0 (0-0.2)
Lymphocytes (%)	18.7 (10-36.3)‡	5 (3.5-10.7)

Data are expressed as median (upper and lower quartiles).
**P < 0.05, †P < 0.01, and ‡P < 0.001 compared with the control group.*

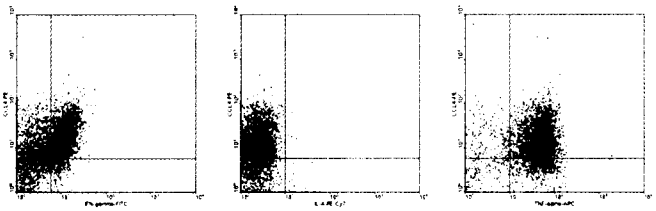

Fig. 1. *Representative examples of flow cytometric analysis of sarcoidosis patient. CD8⁺ cells co-expressing CCL4/MIP-1β with IFN-γ, IL-4 and TNF-α.*

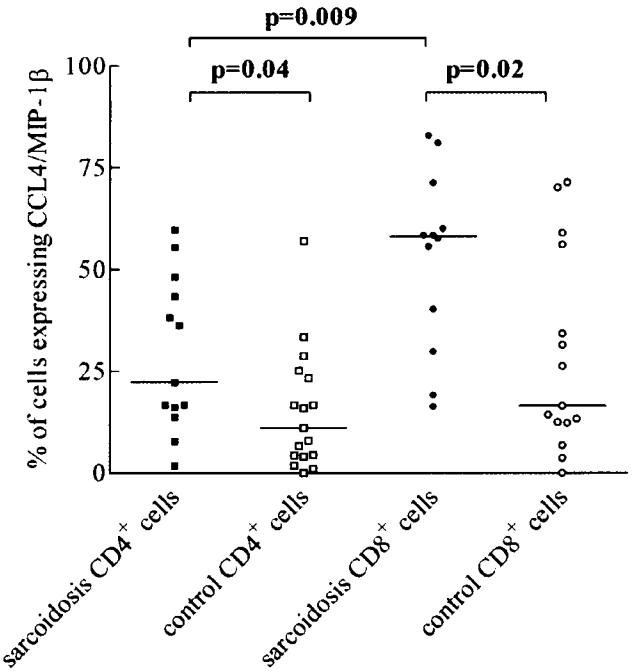

Fig. 2. *Percentage of BAL CD4⁺ cells and CD8⁺ cells expressing CCL4/MIP-1β.*

expressing CCL4. However, CCL4 was expressed by significantly more Tc1 than Th1 cells in sarcoidosis patients (P = 0.006; Fig. 4A).

The percentage of CD4⁺ or CD8⁺ cells co-expressing CCL4 and TNF-α were significantly higher in sarcoidosis patients [14.6% (6.1-32.7%) and 32.2% (13.8-42.3%), respectively] than in healthy subjects [5.5% (0.513.6%), P = 0.03 and 1.4% (0.5-9.7%), P = 0.005, respectively]. There were low levels of CD4⁺ or CD8⁺ cells co-expressing CCL4 and IL-4 and no differences were observed with respect to the percentages of those cells between the two studied groups. Similarly, the expression of CCL4 was low in Th2 and Tc2 cells and did not show any considerable difference between the sarcoidosis and control group.

The percentage of BAL fluid CD4⁺ or CD8⁺ cells, stimulated in vitro with PMA/ionomycin, expressing CCL3/MIP-1α or other cytokines

The percentage of CD4⁺ or CD8⁺ cells expressing CCL3/MIP-1α did not demonstrate any significant difference between the two studied groups. However, the expression of CCL3/MIP-1α was significantly elevated in CD8⁺ cells compared to CD4⁺ cells within the sarcoidosis group [8.9% (2.6-16.2%) *vs*

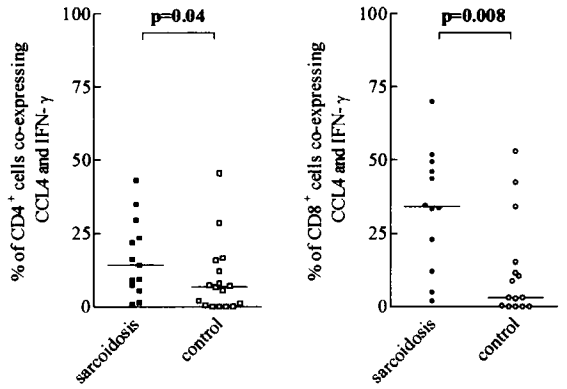

Fig. 3. *Percentage of BAL CD4⁺ cells and CD8⁺ cells co-expressing CCL4 and IFN-γ.*

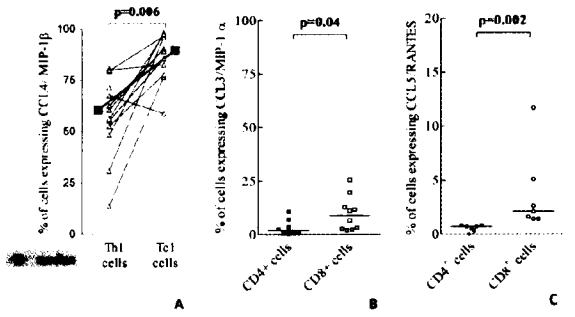

Fig. 4. *Expression of CCL3-5 chemokines in CD8⁺ cells or Tc1 cells compared to CD4⁺ cells or Th1 cells in sarcoidosis patients. **A)** Expression of CCL4 in BAL Tc1 cells vs BAL Th1 cells in sarcoidosis; **B)** Expression of CCL3 in BAL CD8⁺ cells vs BAL CD4⁺ cells in sarcoidosis; **C)** Expression of CCL5 in BAL CD8⁺ cells vs BAL CD4⁺ cells in sarcoidosis.*

2.1% (0.7-5.4%), P = 0.04; Fig. 4B]. There were no material differences in the co-expression of CCL3 with IFN-γ, TNF-α, IL-4 or IL-10 in $CD4^+$ or $CD8^+$ cells between the sarcoidosis and control group.

The percentage of BAL fluid CD4⁺ or CD8⁺ cells, stimulated in vitro with PMA/ionomycin, expressing CCL5/RANTES or other cytokines

No significant changes in the expression of CCL5/RANTES in $CD4^+$ or $CD8^+$ cells were observed between the two groups studied. However, the percentage of $CD4^+$cells expressing CCL5/RANTES showed considerable decrease compared to the percentage of $CD8^+$ cells expressing this chemokine in sarcoidosis patients [0.7% (0.4-0.8%) *vs* 2.1% (1.5-8.4%), P = 0.002; Fig. 4C]. In addition, CCL5 and IL-4 were co-expressed by a significantly higher percentage of $CD8^+$ cells in sarcoidosis patients compared to healthy subjects [0.4% (0.1-1%) *vs* 0% (0-0.2%), P = 0.02]. In contrast, there were no significant differences between the two studied groups in the percentage of $CD8^+$ cells co-expressing CCL5 and IFN-γ or the percentage of $CD4^+$ cells co-expressing CCL5 with IL-4, IL-10 or IFN-γ.

The expression of CCL3-5 chemokines in CD4⁺ cells isolated from sarcoidosis patients and not stimulated with PMA/ionomycin

The production of CCL4 by unstimulated $CD4^+$ cells was very low and showed significant increase after PMA/ionomycin stimulation. On the contrary, the expression of CCL3 or CCL5 in $CD4^+$ cells did not change after stimulation (Fig. 5).

Levels of CCL4/MIP-1β in the BAL fluid

BAL fluid levels of CCL4/MIP-1β were significantly elevated in sarcoidosis patients compared to control subjects [0 pg/ml (0-12 pg/ml) *vs* 0 pg/ml (0-0 pg/ml), P = 0.03]. Levels of CCL4 in BAL fluid showed a weak but still significant correlation with the percentage of $CD4^+$ cells expressing CCL4 (R = 0.37, P = 0.049), although not with the percentage of $CD8^+$ cells expressing CCL4. However, the levels of CCL4 in BAL fluid correlated with the percentage of: Tc1 cells (R = 0.4, P = 0.03), $CD8^+$ cells expressing TNF-α (R = 0.51, P = 0.005), $CD8^+$ cells co-expressing TNF-α and CCL4 (R = 0.47, P = 0.02) and $CD8^+$ cells co-expressing TNF-α and IFN-γ (R = 0.44, P = 0.02).

Correlations with BAL fluid cells

The percentage of lymphocytes in BAL fluid showed a positive correlation with the percentage of $CD8^+$ cells expressing CCL4 (R = 0.47, P = 0.01) and the levels of CCL4 in BAL fluid (R = 0.55, P = 0.002). In contrast, the percentage of eosinophils in BAL fluid correlated with: the percentage of Tc2 cells expressing CCL3 (R = 0.46, P = 0.02) and the percentage of $CD4^+$ cells co-expressing IL-4 and CCL5 (R = 0.53, P = 0.04).

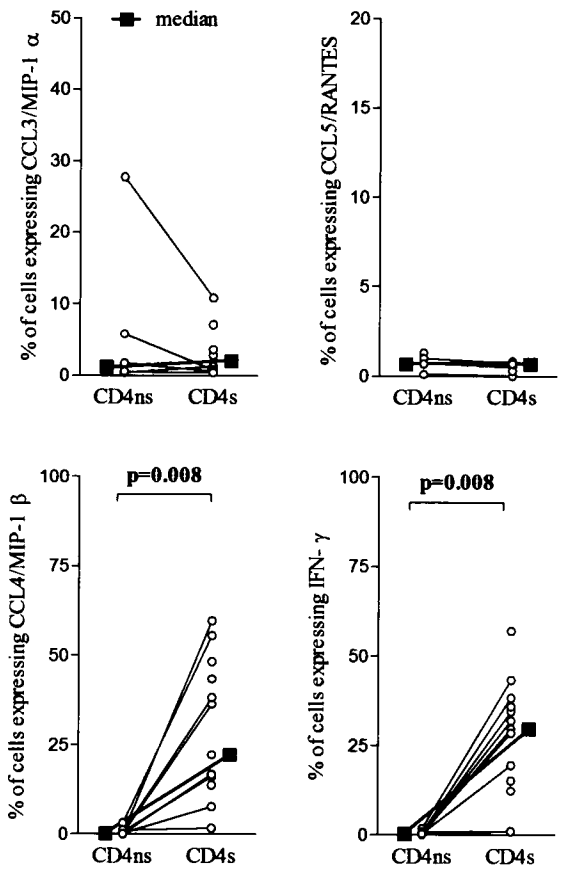

Fig. 5. *The effect of PMA/ionomycin stimulation on the expression of CCL3/MIP-1α, CCL5/RANTES, CCL4/MIP-1β and IFN-γ in CD4⁺ cells in sarcoidosis. The results are presented as changes for individual patients; CD4ns: non-stimulated CD4⁺ cells; CD4s: stimulated CD4⁺ cells.*

DISCUSSION

To our knowledge, this is the first study showing that CD8⁺ and CD4⁺ cells isolated from BAL fluid of sarcoidosis patients release increased amounts of CCL4/MIP-1β either alone or together with IFN-γ or TNF-α compared to control subjects. In addition, we observed that CD8⁺ cells compared to CD4⁺ cells of sarcoidosis patients were highly efficient producers of CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CCL4 together with IFN-γ. Similarly, the expression of CCL4 showed an increase in Tc1 cells in comparison with Th1 cells in sarcoidosis patients. Finally, the levels of CCL4 in BAL fluid correlated with the percentage of CD4⁺ cells, Tc1 cells, CD8⁺ cells expressing TNF-α and CD8⁺ cells co-expressing TNF-α together with CCL4.

CCL3-5 are implicated in the pathogenesis of sarcoidosis because the levels of those chemokines were found to be elevated in BAL fluid of sarcoidosis patients (3, 11, 14-16). Additionally, it is suggested, based on immunohistochemical and cell culture in vitro studies, that CCL3 and CCL5 are likely to be secreted by macrophages (12, 17, 18). However, it was unclear whether BAL T cells are effective producers of CCL3-5 chemokine in sarcoidosis. The results of our study show that CCL4 is expressed in high amounts in T cells, especially in CD8⁺ cells. This may imply that CD8⁺ cells, and to a lesser extent CD4⁺ cells, may recruit new T cells to the site of inflammation in the lung of sarcoidosis patients. On the basis of the other studies, it seems probable that CCL4 attracts more CD4⁺ cells than CD8⁺ cells (9, 10). The results of our study implicate a potentially new mechanism in the pathogenesis of sarcoidosis. The increased population of T cells, mostly CD4⁺ cells, present in the lung of sarcoidosis patients, are not only the result of T-cell proliferation at the site of the inflammation or migration of those cells caused by macrophages and other cells but also the result of T-cell chemotaxis induced by T cells. In this mechanism, activated CD8⁺ cells, and to a lesser degree CD4⁺ cells, would attract more T cells by producing large amounts of CCL4, especially CD4⁺ cells but also CD8⁺ cells, to the lung of sarcoidosis patients. A correlation analysis provided further support for the significance of T cells as an important source of CCL4 because the percentage of CD4⁺ cells expressing CCL4 correlated with the levels of CCL4 in BAL fluid. While no significant correlation was found between the percentage of CD8⁺ cells expressing CCL4 and the levels of CCL4 in BAL fluid, the percentage of CD8⁺ cells expressing CCL4 correlated with the percentage of lymphocytes in BAL fluid. The levels of CCL4 in BAL fluid correlated with different subsets of CD8⁺ cells such as Tc1 cells and CD8⁺ cells expressing TNF-α either alone or together with CCL4 or IFN-γ.

Although we did not find that Tc1 cells or Th1 cells of sarcoidosis patients produced more CCL4 compared to control subjects, we observed that CD8⁺ cells and CD4⁺ cells of sarcoidosis patients secreted

more CCL4 together with Tc1/Th1 cytokines (IFN-γ and TNF-α) than in control subjects. Additionally, it is known from previous studies that CCL3-5 chemokines recruit Th1/Tc1 cells preferentially (22). It seems therefore that in sarcoidosis not only CCL4 attract more Tc1/Th1 cells but also that CCL4 is to a greater extent co-expressed with Tc1/Th1 cytokines in T cells. This new mechanism may also be partially responsible for the increased Th1 cytokine pattern observed in the lungs of sarcoidosis patients without lung fibrosis.

Another new finding of our study is the importance of $CD8^+$ cells for the production of CCL3-5 chemokines. We showed that $CD8^+$ cells produced significantly more CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES and CCL4 together with IFN-γ, compared to $CD4^+$ cells of sarcoidosis patients. Additionally, when compared to Th1 cells, Tc1 cells expressed significantly more CCL4 in the sarcoidosis group. These data indicate a possible role of $CD8^+$ cells and Tc1 cells for the recruitment of T cells (especially $CD4^+$ cells and Th1 cells) to the site of inflammation in the lung of sarcoidosis patients.

The significance of the increased co-expression of CCL5 and IL-4 in $CD8^+$ cells in sarcoidosis patients compared to control subjects is uncertain because of the low percentage of such cells in BAL fluid and the absence of other relationships between CCL3-5 chemokines and Th2/Tc2-associated cytokines in our study. However, this observation may require further studies as the association between $CD8^+$ cells, CCL5 and Th2/Tc2-related cytokines may potentially be of importance, especially for the patients with lung fibrosis due to sarcoidosis.

In our study, we observed different patterns of expression of CCL4 versus CCL3 or CCL5 in unstimulated and stimulated T cells. The production of CCL3 or CCL5 was at the same level before and after stimulation, whereas the expression of CCL4 in unstimulated T cells was low but, similar to IFN-γ and TNF-α (Fig. 5) (21), it increased many times after stimulation. Low basal expression of CCL4 in T cells indicates that it may be difficult or even impossible to demonstrate an association between T cells and CCL4 with the use of immunohistochemistry in lung sections of sarcoidosis patients.

In summary, the results of our study show the possible role of $CD8^+$ cells and $CD4^+$ cells in recruiting T cells to the site of inflammation in sarcoidosis through the release of CCL4 either alone or together with Th1/Tc1-associated cytokines. Additionally, our data suggest that $CD8^+$ cells, rather than $uCD4^+$ cells, are involved in the migration of T cells to the lung of sarcoidosis patients as $CD8^+$ cells produced more CCL3-5 chemokines compared to $CD4^+$ cells.

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