Sarcoidosis is a Th1/Th17 multisystem disorder

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ABSTRACT

Background and aims Sarcoidosis is characterised by a compartmentalisation of CD4+ T helper 1 (Th1) lymphocytes and activated macrophages in involved organs, including the lung. Recently, Th17 effector CD4+ T cells have been claimed to be involved in the pathogenesis of granuloma formation. The objective of this study was to investigate the involvement of Th17 cells in the pathogenesis of sarcoidosis.

Methods Peripheral and pulmonary Th17 cells were evaluated by flow cytometry, real-time PCR, immunohistochemistry analyses and functional assays in patients with sarcoidosis in different phases of the disease and in control subjects.

Results Th17 cells were detected both in the peripheral blood (4.72±2.27% of CD4+ T cells) and in the bronchoalveolar lavage (BAL) (8.81±2.25% of CD4+ T lymphocytes) of patients with sarcoidosis and T cell alveolitis. Immunohistochemical analysis of lung and lymph node specimens showed that interleukin 17 (IL-17)+/CD4+ T cells infiltrate sarcoid tissues surrounding the central core of the granuloma. IL-17 was expressed by macrophages infiltrating sarcoid tissue and/or forming the granuloma core (7.88±2.40% of alveolar macrophages). Analysis of some lung specimens highlighted the persistence of IL-17+/CD4+ T cells in relapsed patients and their absence in the recovered cases. Migratory assays demonstrated the ability of the Th17 cell to respond to the chemotactic stimulus CCL20—that is, the CCR6 ligand (74.8±8.5 vs 76.±2.8 migrating BAL lymphocytes/high-powered field, with and without CCL20, respectively).

Conclusions Th17 cells participate in the alveolitic/granuloma phase and also to the progression towards the fibrotic phase of the disease. The recruitment of this cell subset may be driven by CCL20 chemokine.

INTRODUCTION

Sarcoidosis is a multisystemic granulomatous disease of unknown aetiology characterised by a compartmentalisation of CD4+ T helper 1 (Th1) lymphocytes and activated monocyte/macrophages in involved organs, including the lung, lymph nodes and skin.1 In ~60% of patients the disease spontaneously resolves, but in some subjects the persistence of the antigenic stimulus favours a chronic inflammatory state, granuloma lung formation and, in some cases, an evolution towards fibrosis.2 A complex network of cytokines and chemokines orchestrates the pathogenesis of sarcoidosis: early phases are characterised by a local overproduction of Th1 cytokines, such as interleukin 2 (IL-2) and interferon γ (IFNγ),3 associated with the high expression of macrophage-derived molecules such as IL-15,4 CXCL10,5 CXCL16,6 CCL57 and CCL20.8

Th17, a new CD4+ effector T cell population, has been recently described.3 Initially identified for its ability to produce IL-17A, IL-17F and IL-22, it is now known that human Th17 cells develop in response to IL-23 and IL-1β. These cells express CD4, CD45RO, CCR6 (actually, the unique known receptor of the chemokine CCL20), CCR4 and the subunit IL-25R (the specific receptor for the p19 chain of IL-23). In addition, Th17 lymphocytes express a specific master transcription factor known as retinoic acid-related orphan receptor (ROR)γt10 11 and release an array of cytokines, including proinflammatory molecules such as tumour necrosis factor α (TNFα) and IL-6.12 Recent studies have highlighted the effector role of Th17 cells in pathological conditions, including autoimmunity and inflammation. In particular, the Th17 subset has been linked to Th1 chronic inflammatory diseases, such as psoriasis and inflammatory bowel diseases, and also lung fibrosis.13–15

Herein, we investigate the hypothesis that Th17 cells are involved in the pathogenesis of sarcoidosis. Forty patients were stratified in different phases of the disease and compared with healthy controls. Our results clearly demonstrate that Th17 cells infiltrate the sarcoid lung and that CCL20 chemokine contributes to the recruitment of these proinflammatory cells from the blood into the lung.

MATERIALS AND METHODS

Study population Forty patients with sarcoidosis were analysed. In all cases, the diagnosis was made from a biopsy obtained either from the lungs or from lymph nodes and showing non-caseating epithelioid granulomas with no evidence of inorganic material known to cause granulomatous diseases. Thirty-six patients underwent bronchoalveolar lavage (BAL) fluid analysis. In particular, 25 patients with sarcoidosis presenting with an episode of pulmonary involvement were evaluated at the onset of the disease. They were defined as having a high intensity alveolitis (ie, the active form of the disease) on the basis of the following characteristics: lymphocytic alveolitis (>30×106 lymphocytes/L) and lung CD4 to CD8 ratio >4.0. The assessment of disease activity included BAL, clinical features, chest radiograph, lung function tests, high-resolution CT and routine blood studies. BAL samples were also obtained from 11 patients with previously diagnosed pulmonary sarcoidosis who repeated BAL fluid analysis during their follow-up period. These patients had normal lung function, normal BAL fluid
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with controls (0.43±0.48% of CD4+ T lymphocytes; p<0.001 vs active disease; analysis of variance (ANOVA) p<0.001).

As far as IL-23R is concerned, a significantly higher percentage of lung CD4+ T cells obtained from patients with active sarcoidosis expressed the receptor (9.01±2.10%) with respect to pulmonary CD4+ T cells of patients with inactive sarcoidosis (2.38±1.96%; p<0.001 vs active disease) and controls (0.64±0.73%; p<0.001 vs active disease; ANOVA p<0.001; figure 2B).

The results drawn from the peripheral blood parallel the data we obtained from the lung and confirm the multisystemic nature of the disease. In fact, the mean percentage of freshly isolated IL-17+ T cells in the CD4+ T cell subset was 4.72±2.27%...
in patients with the active form of the disease, whereas the percentages of the IL-17+/CD4+ T cell subset in patients with the inactive disease and in controls were 0.63±0.44% (p=0.001 vs active disease) and 0.67±0.45% (p=0.001 vs active disease), respectively (ANOVA p<0.001) (figure 2A). The IL-23R expression was more pronounced on peripheral blood CD4+ T cells of patients with active sarcoidosis (3.22±0.48%) than on CD4+ T lymphocytes isolated from patients with the inactive form of the disease (0.81±0.81%; p<0.001 vs active disease) and from the controls (0.75±0.21%; p<0.001 vs active disease; ANOVA p<0.001; figure 2B).

Stimulation of pulmonary and peripheral blood CD4+ T cells with PMA and ionomycin induced a strong downmodulation of CD4 expression.18 19 Therefore, flow cytometric analysis was restricted to CD8−/IL-17+ T cells, that we regarded as Th17 cells. Stimulated lung T cells isolated from patients with active sarcoidosis showed an increased percentage of intracellular IL-17 (10.35±5.84%; p=0.055 vs unstimulated cells). In parallel, the mean percentage of peripheral blood stimulated IL-17+ T cells rose to 8.43±1.65% (p=0.082 vs unstimulated cells).

Molecular analysis of RORγt and IL-17 expression by lung T cells
As shown in figure 2C, real-time PCR evaluation of RORγt gene expression by highly purified BAL T lymphocytes demonstrated that mRNA expression of RORγt was higher in lung CD4+ T lymphocytes of patients with active sarcoidosis (48.80±14.07) than in CD8+ T cells obtained from the lung of patients with
inactive disease and control subjects (inactive, 2.80±0.6; p<0.001 vs active disease; controls, 1.45±1.05; p<0.001 vs active disease; ANOVA p<0.001). Similarly, a real-time PCR evaluation of IL-17 gene expression by pulmonary T cells demonstrated that IL-17 mRNA was higher in lung CD4+ T lymphocytes of active sarcoidosis (38.53±18.41) than in CD3+ T cells from the lung of patients with inactive disease and control subjects (inactive, 0.78±0.73; p=0.002 vs active disease; controls, 0.49±0.47; p=0.002 vs active disease; ANOVA p=0.001; figure 2D).

**Sarcoid AMs express IL-17 and IL-23R**

The flow cytometric analysis of freshly isolated AMs obtained from the BAL of the lungs of patients with active sarcoidosis showed the intracellular expression of IL-17 (7.88±2.40%) and the surface presence of IL-23R (8.13±3.47%). In contrast, a very low expression of IL-17 and IL-23R was detected on the AMs from patients with inactive sarcoidosis (IL-17, 0.39±0.34%; p<0.001 vs active disease; IL-23R, 0.74±0.80%; p<0.001 vs active disease) and from controls (IL-17, 0.48±0.46%; p<0.001 vs active disease; IL-23R, 0.64±0.56%; p<0.001 vs active disease; ANOVA p<0.001 for both; figure 3A).

**Immunohistochemical analysis of IL-23R and IL-17 expression**

Immunohistochemical analysis confirmed the expression of IL-23R and IL-17 by sarcoid lung T cells infiltrating surgical pulmonary biopsies obtained from four patients with active sarcoidosis (figure 4A,B). When the cell sources of IL-17 in sarcoid tissue was investigated, we showed that the cytokine was preferentially expressed by macrophage multinucleated giant cells and T cells localised inside the granuloma. We identified IL-17+ T cells infiltrating lymph node granulomas of patients with active sarcoidosis (figure 4E,1–2), thus documenting the presence of Th17 cells at the extrapulmonary active sites of the disease. Immunohistochemical analysis of native lungs obtained from three patients with refractory sarcoidosis...
and pulmonary fibrosis who underwent lung allograft transplantation showed that in the fibrotic phase of the disease, lung T cells were mainly non-reactive for IL-23R and IL-17, and AMs were not stained with anti-IL-17 mAb (figure 4C,D).

To investigate the role of Th17 cells in different phases of the disease, we analysed surgical pulmonary biopsies obtained from two patients with sarcoidosis relapse and from two subjects whose sarcoidosis underwent resolution (figure 5). Interestingly, we identified IL-17+ T cells only in the relapsed patient, suggesting the critical role of Th17 cells in sarcoidosis progression.

**Pulmonary Th17 cells expressed a functional CCR6 receptor**

Th17 cells are characterised by the expression of CCR6, the receptor of the chemokine CCL20.13 By flow cytometric analysis, we showed that this receptor is highly expressed by Th17 cells of patients with sarcoidosis (79.3%±11.1% of IL-17+/CD4+ T cells) (figure 6). In a previous work,8 we demonstrated the involvement of the CCR6/CCL20 axis in the lung microenvironment of patients with sarcoidosis. Herein, we confirmed that sarcoid lung T lymphocytes bear CCR6 (48.6%±7.8% of CD4+ T cells; 52.3%±7.1% of CCR6+CD4+ T cells were CXCR3 negative) and migrate in the presence of CCL20 (74.8%±8.5 vs 7.6%±2.8 migrating BAL lymphocytes/high-powered field, in the presence and absence of CCL20, respectively; p<0.001). It is noteworthy that we showed that sarcoid macrophages release CCL20 and that detectable levels of CCL20 protein can be demonstrated in the BAL fluid components.5

**DISCUSSION**

In this report we have provided evidence that Th17 cells infiltrate sarcoid lung, localising around and inside the granuloma, at the sites of disease activity, not only in the early phase, but also in the progression towards the fibrotic phase of the disease.

In patients with sarcoidosis, the pulmonary microenvironment is characterised by a well-known highly polarised Th1 profile.6 7 20 Despite the in vitro inhibition of Th17 development by Th1-derived IFNγ,21 22 the presence in vivo of a Th1/Th17 population has been extensively demonstrated in different pathological conditions characterised by an exaggerated inflammatory response, including psoriasis,13 rheumatoid arthritis,23 Crohn’s disease,24 hypersensitivity pneumonitis25 and tuberculosis.26 We detected high levels of IL-17+/CD4+ T lymphocytes among cells retrieved from the BAL of patients with sarcoidosis. In particular, patients with a marked alveolitis showed the highest amounts of Th17 cells, both in the lung and in the peripheral blood, suggesting an overall increase of this subset, spreading from the periphery to the sites of active disease. In a previous report, Meloni et al27 assessed the frequency of Th1, Th2 and Th17 lymphocytes in the BAL of patients with interstitial lung diseases, including sarcoidosis (15 cases), describing no variation of Th17 clones in the patients compared with controls. The different technical approaches (an IL-17Elispot and a different stimulation of T cells) may explain the contradictory results they obtained. In addition, the reported absence of any increase in the basal frequency of lung IFNγ-producing cells in the patients with sarcoidosis included in this study is a feature conflicting with the classical, active Th1 pulmonary microenvironment.

The evidence that Th17 cells are increased in the lung and the peripheral blood of patients with active sarcoidosis supports the multisystem nature of the disease but raises the issue of the diminished cellular immune response in the blood of patients with sarcoidosis. Regulatory T lymphocytes (Tregs) of patients with sarcoidosis deeply affect the concomitant presence of an intense immune response in the affected organs and a reduced/suppressed antigen immune response in the periphery. Actually, the interactions between Tregs and Th17 cells are not completely understood. Intriguingly, recent data demonstrated the plasticity/instability of Tregs, mainly in the presence of some proinflammatory cytokines, such as IL-6, that converted T cell receptor (TCR)-stimulated Tregs into Th17 lymphocytes in vitro.28

Sarcoid AMs produce elevated levels of IL-17. Recent studies showed the relevant role of monocytes/macrophages in the
promotion of the Th17 response. In fact, activated monocytes obtained from the synovial fluid of patients with rheumatoid arthritis specifically induce the Th17 response via cell contact signals and IL-1β/TNFα cytokines. Our data favour the hypothesis that a Th17 response may be driven by AMs since IL-1β- and TNFα-producing AMs are widely detectable in sarcoid lung.

IL-17+/CD4+ T cells were further characterised for the expression of IL-23R, the master gene RORγt and the CCR6 receptor, a recently recognised Th17 marker. IL-23R binds IL-23, a cytokine involved in human Th17 cell differentiation. The overexpression of this molecule on CD4+ T cells parallels the increase in IL-17+/CD4+ T lymphocytes, both in the lung and in the blood of patients with active sarcoidosis. RORγt and CCR6 are two strictly linked molecules: RORγt directs Th17 cell differentiation and specifically controls the differentiation of CD4+ T cells towards CCR6+ Th17 cells, as demonstrated by RORγt transduction experiments in CCR6−/CD4+ T cells. The pulmonary and peripheral Th17 cells of our patients over-expressed the RORγt mRNA and showed a strong positivity for CCR6 protein. Actually, CCL20 is the only chemokine produced driving the development/progress of fibroblasts and epithelioid cells, forming the central core of the sarcoid lung. CCL20 expression of IL-23R, the master gene RORγt, requires transforming growth factor-beta and induction of the nuclear receptor factor C and ligand CCL20 regulate Th17 cell trafficking. This study was conducted with the approval of the Padova Hospital Ethic Committee. Our data highlight the presence of Th17 cells in the lung and the blood of patients with sarcoidosis, particularly in those patients with the active form of the disease, suggesting a role for the Th17 subset in sarcoidosis progression. Further studies are needed to better understand the role of Th17 cells in the various stages of the disease and to validate the possibility of a treatment based on Th17 and/or IL-17 neutralisation.

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Competing interests None.

Ethics approval This study was conducted with the approval of the Padova Hospital Ethics Committee.

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In conclusion, our data highlight the presence of Th17 cells in the lung and the blood of patients with sarcoidosis, particularly in those patients with the active form of the disease, suggesting a role for the Th17 subset in sarcoidosis progression. Further studies are needed to better understand the role of Th17 cells in the various stages of the disease and to validate the possibility of a treatment based on Th17 and/or IL-17 neutralisation.
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