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Changes in lung immune cells related to clinical outcome during treatment with infliximab for sarcoidosis

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Summary

Pulmonary sarcoidosis is characterized by an exaggerated CD4⁺ T cell response and formation of non-necrotizing granulomas. Tumour necrosis factor α (TNF-α) is regarded as crucial for granuloma formation and TNF-α inhibitors offer a third-line treatment option for patients not responding to conventional treatment. However, not all patients benefit from treatment, and an optimal dose and treatment duration have not been established. Insight into the influence of TNF-α inhibitors on lung immune cells may provide clues as to what drives inflammation in sarcoidosis and improve our understanding of treatment outcomes. To evaluate the effects of treatment with the TNF-α inhibitor infliximab on lung immune cells and clinical features of the patients, 13 patients with sarcoidosis refractory to conventional treatment were assessed with bronchoalveolar lavage (BAL), spirometry and computerized tomography (CT) scan closely adjacent to the start of infliximab treatment. These investigations were repeated after 6 months of treatment. Treatment with TNF-α inhibitor infliximab was well tolerated with no adverse events, except for one patient who developed a probable adverse event with liver toxicity. Ten patients were classified as responders, having a reduced CD4/CD8 ratio, a decreased percentage of CD4⁺ T cells expressing the activation marker CD69 and number of mast cells ($P < 0{\cdot}05$ for all). The percentage of T regulatory cells (T_{regs}), defined as forkhead box P3⁺ CD4⁺ T cells decreased in most patients. In conclusion, six months of infliximab treatment in patients with sarcoidosis led to signs of decreased CD4⁺ T cell alveolitis and decreased mastocytosis in the lungs of responders.

Keywords: bronchoalveolar lavage, infliximab, lung immune cells, sarcoidosis

Introduction

Sarcoidosis is an inflammatory systemic disorder. The lungs and lymph nodes are most commonly affected, but any organ may be involved, resulting in organ function impairment and sometimes failure (e.g. respiratory insufficiency). The disease can be self-limiting, seen mainly in patients with the clinical phenotype Löfgren's syndrome and characterized by an acute onset, but many patients (commonly patients with non-Löfgren's syndrome, usually with a more insidious onset) experience a chronic course despite treatment. The exact nature and order of immunological events leading to formation of non-necrotizing granulomas, a pathological hallmark of the disease, remains unknown. It has been established, however, that both genetic factors and a dysregulated immune system characterized by T cell alveolitis are involved. Available data suggest that a triggering antigen is presented by human leucocyte antigen (HLA) class II molecules leading to an accumulation of CD4⁺ T cells, increased cell concentration in the lungs and production of proinflammatory cytokines [1]. Tumour necrosis factor (TNF)-α is regarded as crucial for granuloma formation, and the release from alveolar macrophages is higher in patients with active disease [2,3]. Regulatory T cells (T_{regs}) normally dampen the release of proinflammatory cytokines and thereby have the potential to control and terminate immune responses [4]. The

exaggerated inflammatory response in sarcoidosis has, at least partly, been explained by a reduced function and/or frequency of T_{regs} in bronchoalveolar fluid (BALF) and blood as well as a decreased expression of the T_{reg}-specific transcription factor forkhead box protein 3 (FoxP3), which is essential for their function [5,6].

An increased cell concentration, accumulation of $CD4^+$ T cells and a CD4/CD8 ratio exceeding 3·5 in BALF strongly support the diagnosis of sarcoidosis [7]. However, evidence indicates that not only the $CD4^+$ T cells, but also other cell types, are of importance for the sarcoid inflammation. Upon stimulation, $CD8^+$ T cells from blood and especially from BALF from patients with sarcoidosis have a higher capacity to produce interferon (IFN)-γ compared to $CD4^+$ T cells [8]. In a more recent study, blood $CD8^+$ T cells were demonstrated to have a higher cytotoxic capacity compared to healthy controls [9]. It is generally held that macrophages are the main source of TNF-α [10,11], but other cells, for example, $CD4^+$ and $CD8^+$ T cells as well as mast cells, can produce TNF- α [8,12–14]. Furthermore, the number of mast cells is higher in patients with sarcoidosis compared to healthy controls, and they are activated and more numerous in patients with high inflammatory activity and a more severe disease course [15–19].

There are no sarcoidosis-specific treatments. Patients in need of treatment are eligible for third-line therapy with TNF-α inhibitors when first- and second-line therapy (mainly corticosteroids and/or methotrexate and azathioprine) have failed or when contraindications are present. Several TNF-α inhibitors are available, but infliximab seems superior [20,21]. However, approximately 20% of patients receiving TNF-α inhibitors do not seem to benefit from treatment at all, and the optimal dose and treatment duration is not established. The risk of relapse is high after cessation of therapy, as at least half the patients are reported to relapse after treatment discontinuation [20–22]. A few studies have investigated how TNF-α inhibition interferes in the sarcoid inflammation [23–27]. Notably, despite immune cells in the lung differing considerably from those in blood and that T cell activation in sarcoidosis is compartmentalized, with lung T cells disclosing a higher level of activity compared to blood [28–32], so far no one has investigated the effect of treatment on the local lung inflammation. Insight into the influence of infliximab on lung immune cells may provide clues as to what drives inflammation in sarcoidosis and how inhibition of TNF-α interferes with this process. Therefore, the current study was undertaken to analyze the effect of TNF-α inhibition on lung immune cells by performing bronchoscopy with BAL closely adjacent to start of treatment and after 6 months of infliximab therapy.

Material and methods

Study design and characterization of study subjects

Participants were identified among patients referred to the Department of Respiratory Medicine, Karolinska University Hospital, Stockholm, Sweden with deteriorating sarcoidosis, despite previous treatment with corticosteroids and/or methotrexate. They were all of Caucasian origin. None of the included patients had a history of serious infections (including tuberculosis and hepatitis), congestive heart failure or malignancy. All patients were diagnosed with sarcoidosis (non-Löfgren's syndrome) according to criteria established by the World Association of Sarcoidosis and other Granulomatous Disorders [33]. Informed consent was obtained from all subjects and ethical approval was obtained from the Stockholm County Regional Ethical Committee (approval number: 2012/2083-31/3). Information concerning the study was given, both orally and written, upon enrolment. All participants signed an informed consent according to the declaration of Helsinki. For clinical characteristics, see Table 1.

Before starting therapy with infliximab, patients were characterized with chest X-ray (classified according to Scadding's staging system), computerized tomography (CT) scan and lung function including spirometry and measurement of diffusion capacity of the lung for carbon monoxide (DLCO).

Bronchoscopy with BAL was performed on average 6 weeks before (range = 3–9 weeks) start of therapy. All investigations were repeated at follow-up after the first half-year on treatment.

To prevent antibody formation, TNF-α inhibitor treatment should be combined with a low dose of methotrexate and/or glucocorticosteroids [20]. In order to make our study population as homogeneous as possible, the intention was to use 5 mg prednisone as concomitant treatment during the whole study period. Patients who were on a higher dose before the start of treatment were told to reduce the dose to 5 mg. However, patients 2, 5 and 6 were not able to reduce the dose before infliximab therapy due to worsening symptoms, and in those patients the dose was tapered during the infliximab therapy. Patient 13 did not take any concomitant immunosuppressant at all due to misunderstanding. All patients except number 2 had a previous history of methotrexate treatment. Patients who had ongoing methotrexate treatment at inclusion were told to stop before the first bronchoscopy (patients 3, 4, 6, 7, 9 and 10). Patient 8 suffered from a psychiatric disease which had deteriorated during prednisone treatment, and therefore this patient was put on a low dose of methotrexate as concomitant treatment. For detailed information on individual treatment, see Supporting information, Table S1.

Table 1. Baseline characteristics when infliximab therapy was initiated

Patient	Gender	Smoking	Age	Years	Scadding stage	EPM	Treatment indication
1	M	No	53	20	IV	Peripheral lymph nodes	Pulmonary
2	M	No	40	5	I	Ocular	Fatigue, joint pain
3	M	No	46	3	II	0	Pulmonary
4	M	No	42	2	II	0	Pulmonary
5	F	No	42	12	IV	0	Pulmonary
6	F	No	55	3	I	Skin	Fatigue
7	M	No	44	9	III	Hypercalciuria	Pulmonary
8	M	No	44	4	II	0	Pulmonary
9	M	No	50	4	IV	0	Pulmonary
10	M	No	55	2	II	Skin	Pulmonary
11	M	No	51	6	IV	0	Pulmonary
12	M	No	51	7	IV	0	Pulmonary
13	M	Yes	49	4	II	0	Pulmonary

F = female; M = male; smoking = current smoking habits; no = not a current smoker; yes = current smoker; Stage = radiographic extent of sarcoidosis assessed by chest X-ray using the Scadding staging system (0–4); EPM = extrapulmonary manifestations.

Bronchoscopy with BAL and lung function

Bronchoscopy with BAL was performed as previously described [34]. Patient 4 became obstructive after the first bronchoscopy and patient 5 was excluded due to a possible adverse event and the procedure was, therefore, not repeated in these two patients. Cells in BALF were separated from recovered fluid by centrifugation, fixed on cytospin slides and stained with Giemsa for calculation of the leucocyte differential count. Mast cells were counted in 10 visual fields at ×16 magnification. The percentage of $CD4^+$ and $CD8^+$ T cells, respectively, were measured by triple-laser, eight-colour flow cytometry using a FACS Fortessa X-20 (Becton-Dickinson, Franklin Lakes, NJ, USA). The following antibodies were used: CD3-fluorescein isothiocyanate (FITC) clone UCHT1 (BD Pharmingen, San Diego, CA, USA); CD4-allophycocyanin (APC)-H7 clone SK3, CD8-AmCyan clone SK1, CD69-PE-cyanin 7 (Cy7), clone FN50 (BD Biosciences, San Jose, CA, USA); and FoxP3-phycoerythrin (PE) clone PCH 101 (eBioscience, San Diego, CA, USA). Spirometry was performed using a SensorMedics system (SensorMedics, Yorba Linda, CA, USA).

Infliximab therapy

Infliximab (3–5 mg/kg body weight) was administered intravenously every 4–8 weeks after an initial induction phase. During the study period, a consensus document on infliximab therapy was published [20] recommending a dose of 5 mg/kg body weight and infusion every fourth week after the initial induction phase. Patients included thereafter were therefore treated according to these recommendations; that is, given a higher dose and more frequently than patients included in the beginning of the study. For individual total doses, see Supporting information, Table S2. During the study period, biosimilars appeared on the market. Patients 1–4 and 12–13 received Remicade®, Merck Sharpe & Dome AB (Stockholm, Sweden) while the rest received the biosimilar Inflectra®, Pfizer AB (Sollentuna, Sweden).

Evaluation of response

CT scans were independently evaluated by a radiologist and one of the researchers in the study (S. K.). Response was defined as either improved radiographic changes compatible with sarcoidosis or stable radiographic changes despite reduction in concomitant immunosuppressant therapy, while non-response was defined by increased radiographic changes and/or a need to increase concomitant immunosuppressant therapy.

Data analysis

Power analysis was not performed, as this is a pilot study. Statistical analysis was performed using the free software R (www.r-project.org). The Wilcoxon rank sum test was used for comparisons between pre- and post-treatment values. *P*-value significance was set at $< 0{\cdot}05$. Patient 4 was excluded from analysis of BALF data, as he did not undergo the follow-up bronchoscopy. Patient 5 was excluded from all analyses, as follow-up data were missing due to a possible adverse event.

Results

Study subjects

The majority of patients tolerated the treatment well and no adverse events were recorded. Patients 1–4 and 6–11

Table 2. Patients 1–4 and 6–11 were classified as responders (R), 12–13 as non-responders (N)

Patient	FVC% predicted change between baseline and follow-up	Change in concomitant immunosuppressants	CT scan	Response
1	10%	n.c.	↓	R
2	7%	↓	↔	R
3	14%	↓	↓	R
4	21%	↓	↓	R
5	nd	n.c.	n.d.	n.d.
6	−1%	↓	↓	R
7	−2%	↓	↓	R
8	34%	n.c.	↓	R
9	16%	↓	↓	R
10	−6%	↓	↓	R
11	7%	n.c.	↓	R
12	0%	↑	↑	N
13	−4%	↑	↑	N

The symbols ↓ and ↑ denote decreasing and increasing sarcoidosis related changes on computerized tomography (CT) scan, respectively, while ↔ denotes stable disease assessed with CT. The symbols ↓ and ↑ in the column 'Change in concomitant immunosuppressants' denote if concomitant immunosuppressant therapy was decreased or increased between inclusion and just after the second bronchoscopy/follow-up; n.c. = no change; n.d. = not determined; FVC = forced vital capacity.

were classified as responders (n = 10) and 12 and 13 as non-responders (n = 2); see Table 2. Both patients with stage I disease (patients 2 and 6) had lung parenchymal nodules, but they were only visible on CT scan, not on chest X-ray.

The non-responders had a clearly deteriorating disease as assessed by CT scan and prednisone dose was increased (patient 12) or started (patient 13) after the follow-up bronchoscopy. Patient 5 developed a slight increase of liver enzymes after the third infusion, but they returned to just above normal without any specific treatment, and therefore infliximab infusions were continued. However, after the fifth infusion the liver enzymes began to increase again. Autoantibodies and hepatitis serology were negative and ultrasound of the liver disclosed no abnormalities. A liver biopsy showed chronic inflammation and a slight fibrosis. The prednisolone dose was increased and thereafter liver enzymes normalized. Due to the adverse event the patient was excluded from the study and did not take part in the follow-up.

Lung function

Responders disclosed an increase in mean percentage of predicted forced vital capacity (FVC) from 70 to 80% (Fig. 1), and mean percentage of predicted forced expiratory volume in 1 s (FEV_1) from 59 to 67% at follow-up ($P < 0.05$ for both). The mean percentage of DLCO increased from 69 to 74% in responders (Fig. 2), but this did not reach significance.

BALF cells

In responders, the mean CD4/CD8 ratio decreased from 9·2 to 3·0 (P = 0·008), while both non-responders disclosed an increase; individual data are shown in Fig. 3. The mean

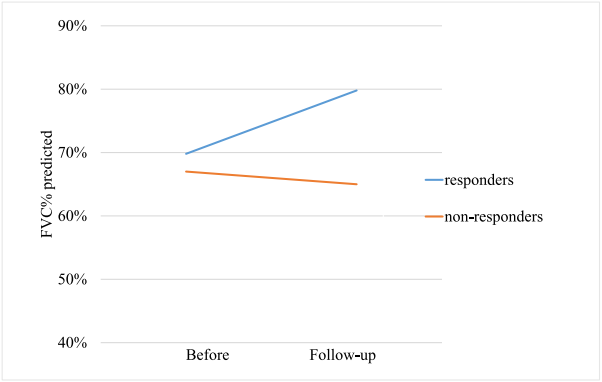

Fig. 1. Mean percentage of predicted forced vital capacity (FVC) before infliximab treatment and at follow-up in responders (n = 10) and non-responders (n = 2).

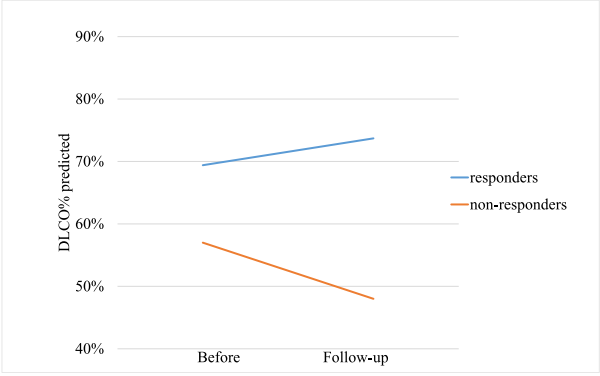

Fig. 2. Mean percentage of predicted diffusion capacity of the lung for carbon monoxide (DLCO) before infliximab treatment and at follow-up in responders (n = 10) and non-responders (n = 2).

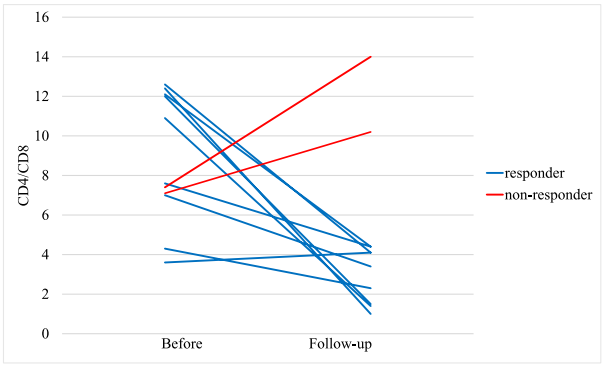

Fig. 3. CD4/CD8 before infliximab treatment and at follow-up. Each line denotes one patient.

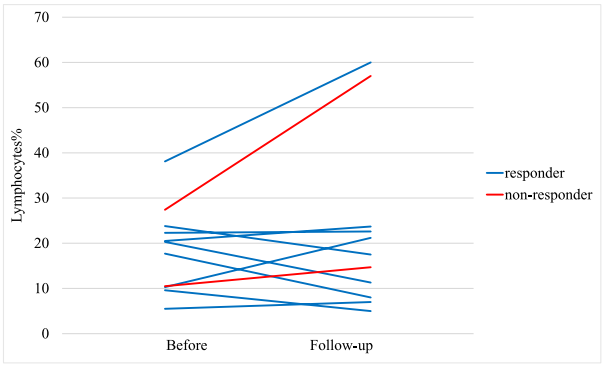

Fig. 6. Percentage of lymphocytes before infliximab treatment and at follow-up. Each line denotes one patient.

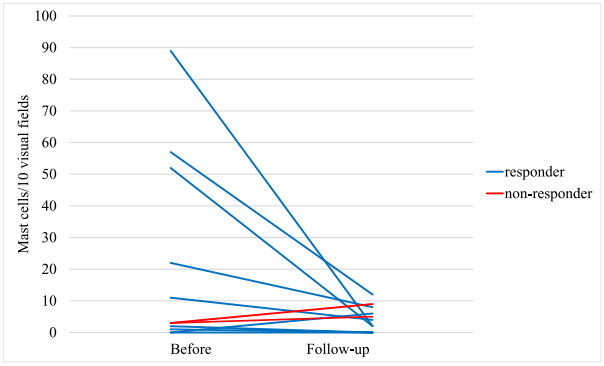

Fig. 4. Number of mast cells per 10 visual fields at ×16 magnification before infliximab treatment and at follow-up. Each line denotes one patient.

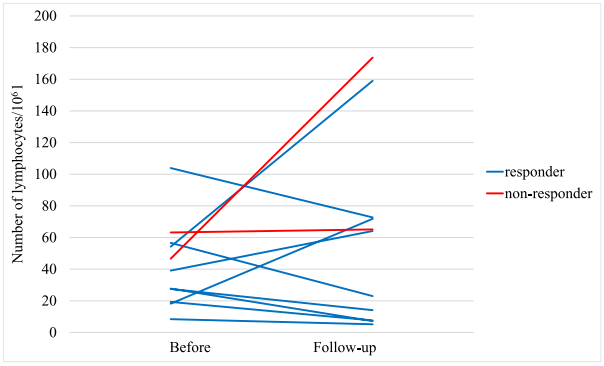

Fig. 7. Number of lymphocytes/10^6 l before infliximab treatment and at follow-up. Each line denotes one patient.

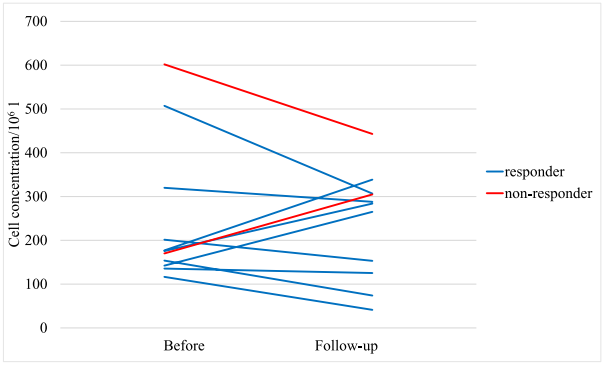

Fig. 5. Cell concentration before infliximab treatment and at follow-up. Each line denotes one patient.

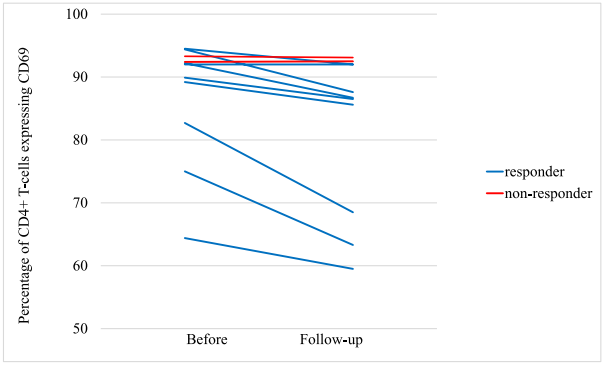

Fig. 8. Percentage of $CD4^+$ T cells expressing CD69 before infliximab treatment and at follow-up. Each line denotes one patient.

number of mast cells per 10 visual fields at ×16 magnification decreased from 26·0 to 3·8 in responders (P = 0·04) but increased in both non-responders; see Fig. 4. The change in cell concentration, percentage and absolute numbers of macrophages, lymphocytes, eosinophils and basophils between baseline and follow-up disclosed individual differences, but no significant changes were observed. Cell concentration and lymphocyte data are shown in Figs. 5–7. In responders, the mean percentage of $CD4^+$ T cells expressing CD69 decreased from 86·0 to 80·2 (P = 0·01), while no change was seen in the two non-responders, shown in Fig. 8. No significant change was seen in the percentage of $CD8^+$ T cells expressing CD69. The percentage of $CD4^+$ T cells expressing FoxP3 decreased in all patients except

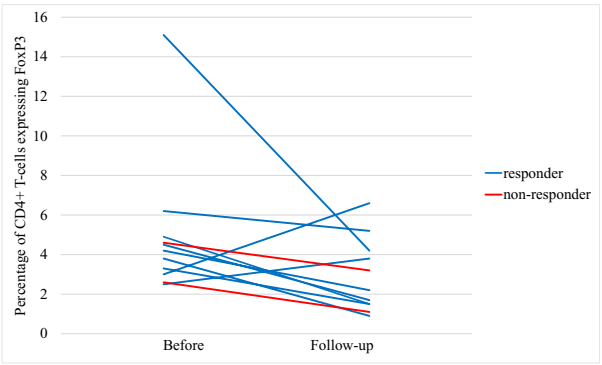

Fig. 9. Percentage of CD4$^+$ T cells expressing forkhead box protein 3 (FoxP3) before infliximab treatment and at follow-up. Each line denotes one patient.

two responders, patients 7 and 8 (P = 0·07); see Fig. 9. We did not find anything in clinical phenotype or BALF data differentiating these from the other patients. BALF data at follow-up did not correlate with the time-span between last infusion of infliximab and bronchoscopy. Detailed individual information on BALF data is given in Supporting information, Table S3a–c.

Discussion

To date, few studies have repeatedly examined the local inflammation in the lungs in patients with sarcoidosis and, to our knowledge, this is the first study exploring the effect of infliximab on BALF cells.

We found that 10 of 13 patients with sarcoidosis refractory to conventional treatment benefited from therapy with either improvement of radiographic extent of sarcoidosis-related changes or stabilization, while concomitant immunosuppressant therapy could be decreased. This was paralleled by a statistically significant reduction of CD4/CD8 ratio and expression of the activation marker CD69 on CD4$^+$ T cells, indicating a decreased CD4$^+$ T cell alveolitis. Furthermore, the number of mast cells decreased significantly in responders, but no significant changes were seen in cell concentration, percentage and absolute numbers of lymphocytes. The percentage of FoxP3$^+$CD4$^+$ T cells decreased in all patients except for two.

There is still controversy regarding how to define response in pulmonary sarcoidosis. In a consensus document from 2012 [35] it was suggested that disease progression or regression is defined as a ≥ 15% change in FVC or a 5–15% change in FVC in association with a definite change in chest radiographic extent. However, the largest clinical trial on infliximab therapy reported an average improvement of only 2·5% from baseline in the percentage of predicted FVC [36]. Later studies have used different end-points. One study from 2014 used improvement of > 10% in either FVC, FEV_1 or DLCO, but found that only 56·5% of 69 patients with respiratory functional impairment fulfilled that criterion after 1 year or longer with TNF-α inhibitor treatment (infliximab or adalimumab) [37]. Another study from 2015 used improvement of ≥ 5% FVC percentage of predicted, a 40% reduction in biomarker levels or maximum standardized uptake value on ^{18}F-fluorodeoxyglucose positron emission tomography (^{18}F-FDG PET) and assessment of quality of life as markers for response in a composite index. The majority of 48 included patients fulfilling 26 weeks of infliximab therapy had a pulmonary treatment indication and a mean FVC percentage of predicted improved 6·64% [23]. In our study population four of 10 responders do not fulfil the criteria suggested by the 2012 consensus [35]. However, considering that the included patients had a deteriorating disease despite conventional treatment, that several previous studies on infliximab therapy have found improvements smaller than suggested in the 2012 consensus and that the infliximab treatment in the four not fulfilling these criteria led to a stabilization of the disease despite reduction in concomitant immunosuppressant treatment, we believe it is reasonable to define them as responders. Similarly, the non-responders did not decrease greatly in FVC, but disclosed clear progress on CT and needed more immunosuppressant; hence, we believe they should be defined as non-responders.

Sarcoidosis is characterized by an exaggerated T cell immune response, which is believed, at least to some extent, to be the result of dysfunctional T_{regs}. However, high numbers of mast cells in the lavage fluid have been connected to a more active and severe sarcoidosis, and mediators released from activated mast cells have been suggested to be involved in the development of fibrosis [15,17]. Interestingly, mast cells can be activated by activated but not resting T cells, and this has been suggested to be a mechanism underlying the propagation of T cell-mediated inflammatory processes [38]. Also, T_{regs} can interact with mast cells, but normally in order to dampen inflammation. However, if the T_{regs} are defective, mast cell-mediated inflammation can be enhanced [39]. The importance of mast cells is further strengthened by the fact that they are capable of producing TNF-α [12,13] and they can also induce CD8$^+$ T cell activation, proliferation and cytotoxicity [40]. Furthermore, a persistent increase of mast cells on serial BALF measurements has been associated with active disease [19].

Taken together with our finding that the mean number of mast cells decreased in responders but increased in both non-responders, it is tempting to speculate that the CD4$^+$ T cell alveolitis, including T_{reg} dysfunction, can lead to an increase in mast cell numbers and activity, further propagating the inflammation by secretion of cytokines;

that is, TNF-α and activation of $CD8^+$ T cells. Mast cell-secreted TNF-α can also be chemotactic for T cells [41] and thereby contribute to a propagating T cell alveolitis in the lungs of patients with non-resolving sarcoidosis.

Only a few studies have examined how TNF-α exerts its effects in sarcoidosis and whether lung immune cells change during the natural course of the disease. A previous study showed that patients who had recovered from Löfgren's syndrome disclosed a termination of the $CD4^+$ T cell alveolitis, demonstrated by a normalized CD4/CD8 ratio, cell concentration and percentage of lymphocytes [42].

Regarding the effects of TNF-α on immune cells, most studies have focused on T_{regs}. In cell culture from mice, exposure to exogenous TNF-α promotes T_{reg} expansion [43], but it has also been shown in patients with rheumatoid arthritis (RA) that FoxP3 is dephosphorylated, leading to suppression of T_{reg} function [44]. Treatment with infliximab restored the suppressive capacity of peripheral T_{regs} and gave rise to a newly differentiated T_{reg} population in RA after 4–6 months [45]. One study investigated the effect of infliximab on peripheral T_{regs} in sarcoidosis and, in contrast to the situation in RA, the relative frequencies of T_{reg} population decreased in both responders and non-responders after 26 weeks [26], which is in line with our results from BALF, as we saw a decrease in most patients, including the two non-responders. The effect on T_{regs} of infliximab therapy thus remains elusive.

It should also be mentioned that the response of infliximab has been suggested to be not solely due to an effect on T_{regs}. Both changes in soluble TNF receptor 2 expression, functionality of T effector cells and activity of monocytes/macrophages in blood [26,46] have been associated with response to treatment. Also, $CD8^+$ T cells are influenced by infliximab treatment, and this might explain the increased risk for tuberculosis during anti-TNF-α treatment [47]. Interestingly, $CD8^+$ T cell activities can also modulate mast cell activities [40].

We did not detect any significant changes in cell concentration, percentage and absolute numbers of lymphocytes between baseline and follow-up, as seen in patients having recovered from Löfgren's syndrome [42]. Considering the remaining lymphocytosis despite treatment, and the high risk of relapse after cessation of treatment with infliximab, the treatment does not seem to interfere with the primary event causing the inflammation but, rather, blocking downstream events in the inflammatory cascade.

Major limitations of this study include a relatively small study sample with a gender imbalance. In particular, as there were only two non-responders, results from the non-responders must be interpreted with caution. We cannot be sure that we properly identified the T_{reg} population using FoxP3, as no definitive surface marker exists that uniquely isolates T_{reg} cells from other T cell populations. However, FoxP3 is essential for the function and has been widely used for identification of T_{regs} [48,49]. Furthermore, the extent of parenchymal infiltrates, and most probably also disease activity, differed between patients, which also may have influenced the results. As we did not use ^{18}F-FDG PET, we could not assess differences in disease activity between patients.

Major strengths include exploration of the lung inflammation with bronchoscopy before and after treatment and the homogeneity of the subjects: all having a non-Löfgren's syndrome with pulmonary involvement and several years of disease duration.

Conclusions

In conclusion, we observed a significant decrease in CD4/CD8 ratio, percentage of $CD4^+$ T cells expressing the activation marker CD69 and number of mast cells in patients responding to 6 months infliximab therapy. Our results also indicate that the percentage of T_{regs} decreases in most patients irrespective of response.

Given the knowledge that T cells and mast cells may interact and propagate inflammatory processes, we speculate that the T cell alveolitis and T_{reg} dysfunction in sarcoidosis may lead to activation of mast cells, further promoting the inflammation. Infliximab treatment seems to interfere in this process in a positive way, reflected by less pronounced chest radiographic changes and improved lung function. In future studies, as we continue to collect patient data we need to study these cells in more detail in relation to disease activity to gain knowledge concerning the way in which they contribute to the sarcoid inflammation, which may also have implications for altered therapy with regard to optimal dose, treatment interval and duration.

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Disclosures

The authors have no conflicts of interest to declare.

Author contributions

S. K., J. G. and A. E. contributed to the conception and design of the study, the acquisition, analysis and interpretation of data and drafting the article. N. V. R. and M. A. A. H. contributed to the analysis of data and revising the article.

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Supporting Information

Additional supporting information may be found in the online version of this article at the publisher's web site:

Table S1. Detailed information on immunosuppressant therapy in study subjects.

Table S2. Doses of infliximab at 1st and 2nd bronchoscopy/follow-up, nd = not determined.

Table S3. Individual BALF data.