Expanded lung T-bet$^+$RORγT$^+$ CD4$^+$ T-cells in sarcoidosis patients with a favourable disease phenotype

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ABSTRACT Disease phenotypes of pulmonary sarcoidosis are distinguished by clinical rather than immunological criteria. We aimed to characterise patterns of CD4$^+$ T-cell lineage plasticity underlying the differences in clinical presentation and disease course between the acute form, Löfgren's syndrome, and the heterogeneous, potentially progressive "non-Löfgren" form.

33 pulmonary sarcoidosis patients and nine controls underwent bronchoscopy with bronchoalveolar lavage. CD4$^+$ T-cell transcription factor, chemokine receptor and T-cell receptor expression, proliferation and cytokine production were assessed in the lavage fluid and peripheral blood using flow cytometry and multicolour FluoroSpot.

CD4$^+$ T-cells simultaneously expressing the T-helper cell (Th)1 and Th17 transcriptional regulators T-bet and RORγT (T-bet$^+$RORγT$^+$) were identified in the lavage, but not blood, of all subjects, and to a significantly higher degree in Löfgren's patients. T-bet$^+$RORγT$^+$ cells proliferated actively, produced interferon (IFN)γ and interleukin (IL)-17A, co-expressed the chemokine receptors CXCR3 and CCR6, and correlated with nonchronic disease. T-cell receptor-restricted Vα2.3$^+$Vβ22$^+$ T-cells strongly co-expressed T-bet/RORγT and CXCR3/CCR6. Cytokine production was more heterogeneous in Löfgren's patients, with significantly higher IL-17A, IL-10, IL-22 and IL-2, but lower IFNγ.

Here we demonstrate the presence of lung T-bet$^+$RORγT$^+$CXCR3$^+$CCR6$^+$ CD4$^+$ T-cells and Th17-associated cytokines especially in sarcoidosis patients with a favourable prognosis, suggesting a Th1/Th17-permissive environment in the lung with implications for disease resolution.

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This article has supplementary material available from erj.ersjournals.com

Received: Jan 13 2016 | Accepted after revision: March 29 2016 | First published online: May 26 2016

Support statement: Financial support in the form of research grants was received from The Swedish Heart Lung Foundation, The Swedish Research Council, The Mats Kleberg Foundation, The King Oscar II Jubilee Foundation, The Stockholm County Council, The Swedish Association for Chest Physicians, and Karolinska Institutet. Funding information for this article has been deposited with FundRef.

Conflict of interest: None declared.

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Introduction

Sarcoidosis is a granulomatous interstitial lung disorder of unknown aetiology, characterised at the cellular level by accumulations of activated $CD4^+$ T-cells in the lungs [1]. Although the triggering antigen(s) have not yet been identified, $CD4^+$ T-cells are generally acknowledged to be key players in driving disease progression. Our group has previously established a connection between certain *human leukocyte antigen (HLA)-DRB1* alleles, accumulation of T-cell receptor (TCR)-restricted $CD4^+$ T-cell clones and a good prognosis [2], implicating specific antigen recognition and differentiated effector T-cells in disease resolution.

In recent years, $CD4^+$ T-cell plasticity has become an area of increasing interest and importance for understanding the dynamics of the immune system throughout disease progression. Subset "switching", *e.g.* between regulatory T-cells (Treg) and T-helper (Th)17 cells, or cells with a Th1/Th17 "hybrid" phenotype has been reported both in experimental settings and human chronic inflammatory diseases [3–7]. Although sarcoidosis has traditionally been regarded as a Th1-driven disease, Th17 cells have recently also been implicated in the disease course [8–12]. To date, few studies have been able to address the subject of $CD4^+$ T-cell plasticity in the context of pulmonary inflammation in sarcoidosis, which motivated us to perform a detailed phenotypic and functional characterisation of $CD4^+$ T-cells from the perspective of lineage-specific transcription factors and cytokines. By paired comparisons of peripheral blood and bronchoalveolar lavage fluid (BALF) cells, this study contributes valuable information regarding immune cell function and compartmentalisation to the lungs.

While sarcoidosis patients are commonly treated as one group, detailed studies have revealed distinct differences between patients with the specific disease phenotype Löfgren's syndrome (LS) and those without, henceforth referred to as "non-LS" [13]. LS patients typically experience acute disease with presentation of distinct clinical symptoms, and usually, a favourable disease course [13, 14]. Non-LS patients, however, more often demonstrate a chronic disease progression and are at higher risk of developing pulmonary fibrosis. Despite clinical discrepancies, the exact immunological mechanism underlying this subgrouping remains elusive. Here, we focused on comparing LS and non-LS patients at the level of $CD4^+$ T-helper subsets and their regulatory elements, in order to better understand the immunological background of the two different disease forms. We show, for the first time, significant differences between the LS and non-LS forms at the transcription factor level *ex vivo*. Functionally, $CD4^+$ T-cells simultaneously expressing Th1 and Th17 regulators T-bet and RORγT, respectively, demonstrate significantly higher proliferative capacity and chemokine receptor expression than $T\text{-bet}^-ROR\gamma T^-$ cells, and have the capacity to produce multiple cytokines. Production of a significantly wider range of Th17-associated cytokines in LS further suggests heterogeneity in immune regulation that may positively influence disease outcome.

Materials and methods

Study subjects, bronchoscopy and bronchoalveolar lavage

Bronchoscopy with bronchoalveolar lavage (BAL) was performed as previously described [15]. 33 newly diagnosed sarcoidosis patients (seven females) with a median age of 46.2 years were included in the study (table 1). All patients were HLA-typed and diagnosed with sarcoidosis according to criteria established by the World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) [16]. Specifically, these included typical clinical and radiographic manifestations, findings at bronchoscopy with BAL including an elevated CD4/CD8 ratio and, if required, positive biopsies, as well as exclusion of other diagnoses. Eight patients were diagnosed with LS, defined as an acute onset, usually with fever, chest radiographic findings of bilateral hilar lymphadenopathy, sometimes with pulmonary infiltrates, and erythema nodosum and/or bilateral ankle arthritis. 25 patients had non-LS. At the time of BAL, one non-LS patient received treatment with prednisolone. In addition, BALF cells from four healthy volunteers and five patients with other pulmonary disorders (nonspecific lung fibrosis and allergic alveolitis) were analysed for comparison. Patients with alternative diagnoses were included in part to complement the low number of healthy subjects, as well as with the aim of distinguishing any cell characteristics inherent to pulmonary inflammation, as opposed to those specific for sarcoidosis. Informed consent was obtained from all subjects and ethical approval obtained from the Stockholm County Regional Ethical Committee (approval numbers: 2005/1031-31/2, 2009/20-32 and 2011/35-32).

Flow cytometry, intracellular staining and multicolour FluoroSpot

A detailed description of protocols and antibodies used for surface and intracellular staining by flow cytometry (figure S1), cell stimulation and assessment of cytokine production by multicolour FluoroSpot and flow cytometry can be found in the supplementary material.

Statistical analysis

Overall differences in cell populations (outlined in detail in the supplementary material) between patient groups were assessed by one-way ANOVA, the non-parametric Mann–Whitney U-test, Wilcoxon's signed

TABLE 1 Clinical characteristics of sarcoidosis patients and controls

	All sarcoidosis patients	LS patients	Non-LS patients	Healthy controls	Patients with other lung diseases
Subjects	33	8	25	4	5
Male/females	26/7	7/1	19/6	3/1	3/2
Age years	46.2 (36.0–51.0)	39.0 (33.0–43.8)	49.0 (40.0–55.0)	28.5 (21.5–35.3)	58.0 (50.0–66.0)
Chest radiographic stage 0/I/II/III/IV#	0/7/22/3/1	0/5/3/0/0	0/2/19/3/1	N/A	N/A
Non-/former/current smoker	17/11/5	3/4/1	14/7/4	4/0/0	2/3/0
VC % predicted	90.0 (80.0–98.0)	101.5 (95.8–102.8)	87.0 (76.0–97.0)	N/A	84.5 (69.3–100.5)
FEV_1 % predicted	83.0 (73.0–88.0)	85.0 (77.0–85.0)	82.0 (71.5–91.0)	107.5 (102.5–110.0)	83.5 (68.3–95.5)
D_{LCO} % predicted	92.0 (83.0–100.0)	104.0 (96.0–107.5)	89.0 (80.8–98.8)	N/A	78.0 (76.0–82.5)
% BAL recovery	59.0 (51.0–66.0)	65.5 (57.0–71.3)	57.5 (48.8–64.0)	64.0 (59.0–69.5)	38.0 (36.0–40.0)
BALF cell concentration $\times 10^6$ cells·L^{-1}	172.2 (119.2–279.8)	237.4 (178.4–313.1)	154.3 (115.1–275.4)	135.0 (109.4–155.5)	427.1 (163.4–538.2)
BALF macrophages %¶	76.9 (66.7–86.7)	78.0 (62.8–83.9)	76.5 (66.7–86.7)	86.1 (79.9–89.8)	56.0 (16.2–72.2)
BALF lymphocytes %	20.3 (9.9–30.2)	18.4 (11.6–35.5)	20.5 (9.4–29.7)	11.0 (7.5–17.2)	33.2 (20.6–82.2)
BALF neutrophils %	1.5 (0.8–2.6)	0.9 (0.8–2.2)	1.6 (1.0–2.6)	2.4 (2.2–2.7)	2.0 (1.4–5.0)
BALF eosinophils %	0.2 (0.0–0.8)	0.0 (0.0–0.3)	0.2 (0.0–1.0)	0.0 (0.0–0.2)	0.4 (0.2–1.0)
BAL CD4/CD8 ratio	6.7 (3.4–12.2)	14.1 (11.1–17.2)	5.2 (3.3–9.5)	3.4 (2.9–3.8)	6.7 (2.4–9.6)
HLA-DRB1*03$^+$/ DRB1*03$^-$DRB3*01$^+$/ DRB1*03$^-$DRB3*01$^-$	10/4/19	6/1/1	4/3/18	1/1/2	N/A
$V\alpha 2.3^+V\beta 22^+$ $CD4^+$ T-cells in BALF %	0.4 (0.1–2.5)	4.0 (2.5–5.6)	0.3 (0.1–0.5)	0.2 (0.1–0.2)	0.2 (0.0–0.2)

Data are presented as n or median (25–75th percentile). LS: Löfgren's syndrome; VC: vital capacity; FEV_1: forced expiratory volume in 1 s; D_{LCO}: diffusing capacity of the lung for carbon monoxide; BAL: bronchoalveolar lavage; BALF: bronchoalveolar lavage fluid; HLA: human leukocyte antigen; N/A: not applicable. #: Chest radiography staging was as follows. Stage 0: normal chest radiography; stage I: enlarged lymph nodes; stage II: enlarged lymph nodes with parenchymal infiltrates; stage III: parenchymal infiltrates without enlarged lymph nodes; stage IV: signs of pulmonary fibrosis. ¶: BALF basophils and mast cells were excluded from the cell differential counts.

rank test for paired comparisons and the nonparametric Spearman rank test. FluoroSpot cytokine production was compared using two-way ANOVA and the unpaired t-test. Analysis was performed using GraphPad Prism v.5.02 software (GraphPad Software, Inc., La Jolla, CA, USA). $p<0.05$ was considered significant.

Results

Increased $CD4^+$ T-cell proliferation and cytokine production in BALF

In all sarcoidosis patients, we found $CD4^+$ T-cell proliferation to be significantly higher in BALF than in blood (figure S2a). $CD4^+$ T-cells stimulated with anti-CD3/anti-CD28 antibodies also showed significantly higher total cytokine production (interferon (IFN)γ, interleukin (IL)-2 and IL-17A combined) in BALF (figure S2b), further indicating a generally higher activation state compared with blood.

BALF $CD4^+$ T-cells express T-bet and RORγT

In all sarcoidosis patients as well as controls, a majority of $CD4^+$ T-cells in BALF expressed the Th1 regulator T-bet (figure 1a). LS patients showed a significantly higher proportion of BALF T-bet$^+$ $CD4^+$ T-cells than non-LS or healthy individuals (figure 1b). RORγT$^+$ $CD4^+$ T-cells were also identified in BALF (figure 1c), and were significantly more frequent in LS patients than in any of the other groups (figure 1d). Furthermore, a large proportion of T-bet$^+$ $CD4^+$ T-cells were found to also express RORγT (figure S3). In turn, almost all RORγT$^+$ $CD4^+$ T-cells were found within the T-bet$^+$ $CD4^+$ T-cell compartment (figure S3).

T-bet$^+$RORγT$^+$ $CD4^+$ T-cells are present in BALF, but not blood, and are most prominent in LS

We found $CD4^+$ T-cells simultaneously expressing T-bet and RORγT in BALF of both sarcoidosis patients and healthy individuals, and to some degree in patients with other lung diseases (figure 2a–c, e), while they were virtually absent in blood (figure 2d, f). The proportion of T-bet$^+$RORγT$^+$ T-cells was significantly larger in BALF from LS patients (median 65.2% of all $CD4^+$ T-cells) than in non-LS patients (median 38.7%; $p=0.0078$ *versus* LS), patients with other lung diseases (median 19.5%; $p=0.0062$ *versus* LS) or healthy individuals (median 23.4%; $p=0.0040$ *versus* LS) (figure 2a–c, e). No significant correlation of T-bet/RORγT expression with either patient age, sex or chest radiographic stage at the time of BAL was found, neither for the patient cohort as a whole nor when LS and non-LS were considered separately.

Foxp3 is expressed by T-bet$^+$ but not RORγT$^+$ $CD4^+$ T-cells

To verify that T-bet/RORγT co-expression in $CD4^+$ T-cells was not random or unspecific, forkhead box protein 3 (FoxP3) was included in the intracellular staining panel. Almost all FoxP3$^+$ cells were found within the T-bet$^+$ population (figure S4a), while RORγT$^+$ T-cells by and large did not express FoxP3

 DOI: 10.1183/13993003.00092-2016

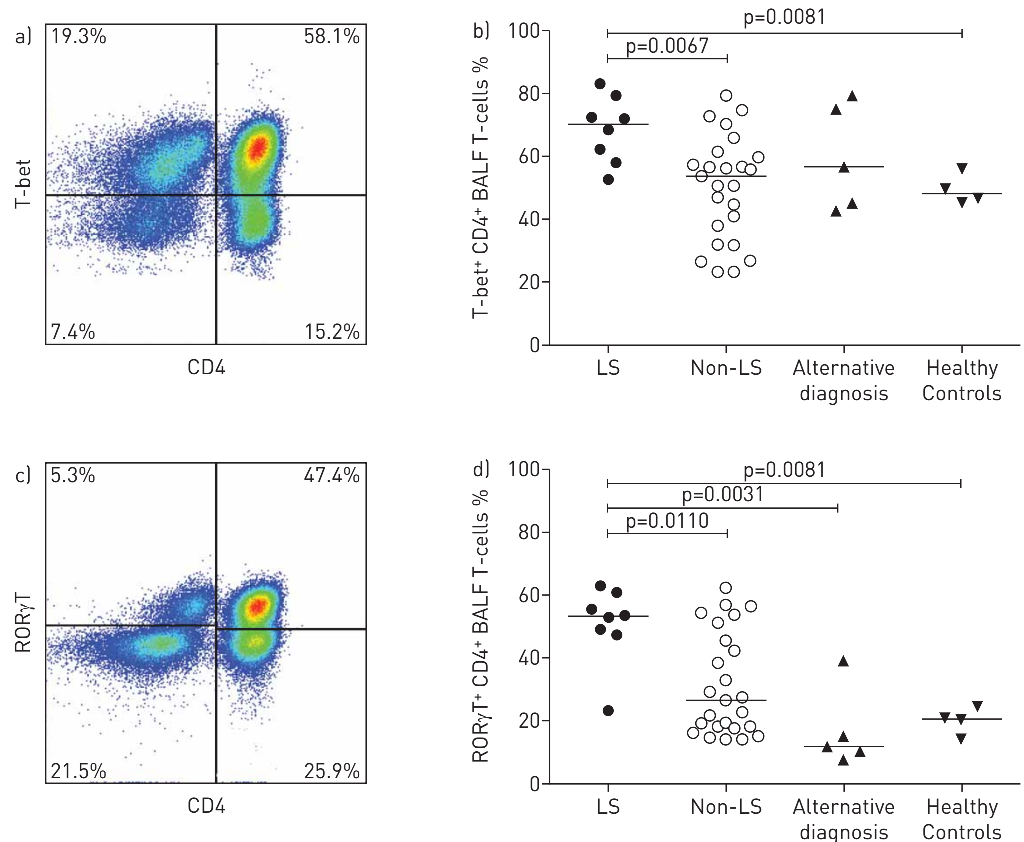

FIGURE 1 Higher proportions of T-bet and RORγT expression in the bronchoalveolar lavage fluid (BALF) of Löfgren's syndrome (LS) patients. Representative flow cytometry scatter plots are shown for a) the T-bet$^+$ CD4$^+$ and c) the RORγT$^+$ CD4$^+$ T-cell populations in BALF from an LS patient. b) LS patients presented with significantly higher levels of T-bet$^+$ CD4$^+$ T-cells in BALF than non-LS patients and healthy controls. d) A pronounced difference was also observed for RORγT$^+$ CD4$^+$ T-cells (all comparisons were made using the two-tailed Mann–Whitney U-test).

(figure S4b). Overall, only a few per cent of CD4$^+$ T-cells in BALF expressed FoxP3 (median 6.1% in LS, 6.8% in non-LS, 4.7% in patients with other diseases and 5.6% in healthy individuals), and there were no significant differences between patient groups (figure S4c).

Simultaneous expression of CXCR3 and CCR6 in BALF CD4$^+$ T-cells

To strengthen our findings that CD4$^+$ T-cells in the lungs of sarcoidosis patients share features of different lineages, we analysed expression of the Th1- and Th17-associated chemokine receptors CXCR3 and CCR6, respectively, along with Th2-associated CCR4. We found that a majority of CD4$^+$ T-cells in BALF (median 75.3%), but only a fraction in blood (median 19.1%) co-expressed CXCR3 and CCR6 (figure 3a–c). Neither CXCR3 nor CCR6 were expressed in conjunction with CCR4 (figure S5a, b). Simultaneous expression of CXCR3/CCR6 correlated with T-bet/RORγT co-expression, and was especially pronounced in LS patients (figure 3d). Moreover, co-expression of CXCR3 and CCR6 was significantly higher in T-bet$^+$RORγT$^+$ than in T-bet$^-$RORγT$^-$ CD4$^+$ T-cells (figure 3e).

T-bet$^+$RORγT$^+$ CD4$^+$ T-cells have a higher proliferative capacity

For all sarcoidosis patients, the proportion of proliferating (Ki-67$^+$) cells was dramatically increased in the T-bet$^+$RORγT$^+$ compartment (figure 4a, c), with a median of 18.1% Ki-67$^+$ cells compared with 1.3% for T-bet$^-$RORγT$^-$ CD4$^+$ T-cells (figure 4b, c). This pattern was also consistent for healthy individuals (data not shown). In addition, proliferation was observed in a significantly higher percentage of T-bet$^+$FoxP3$^+$ than T-bet$^+$FoxP3$^-$ CD4$^+$ T-cells (figure S4d). No significant differences could be observed between LS, non-LS and healthy controls in terms of proliferating T-bet$^+$RORγT$^+$ CD4$^+$ T-cells (data not shown).

TCR-restricted Vα2.3$^+$Vβ22$^+$ CD4$^+$ T-cells co-express T-bet/RORγT and CXCR3/CCR6

Due to our previous findings of accumulated BALF CD4$^+$ T-cells carrying the Vα2.3/Vβ22 TCR in patients positive for HLA-DRB1*03 or HLA-DRB3*01 [2], which associate with LS and a good prognosis [17],

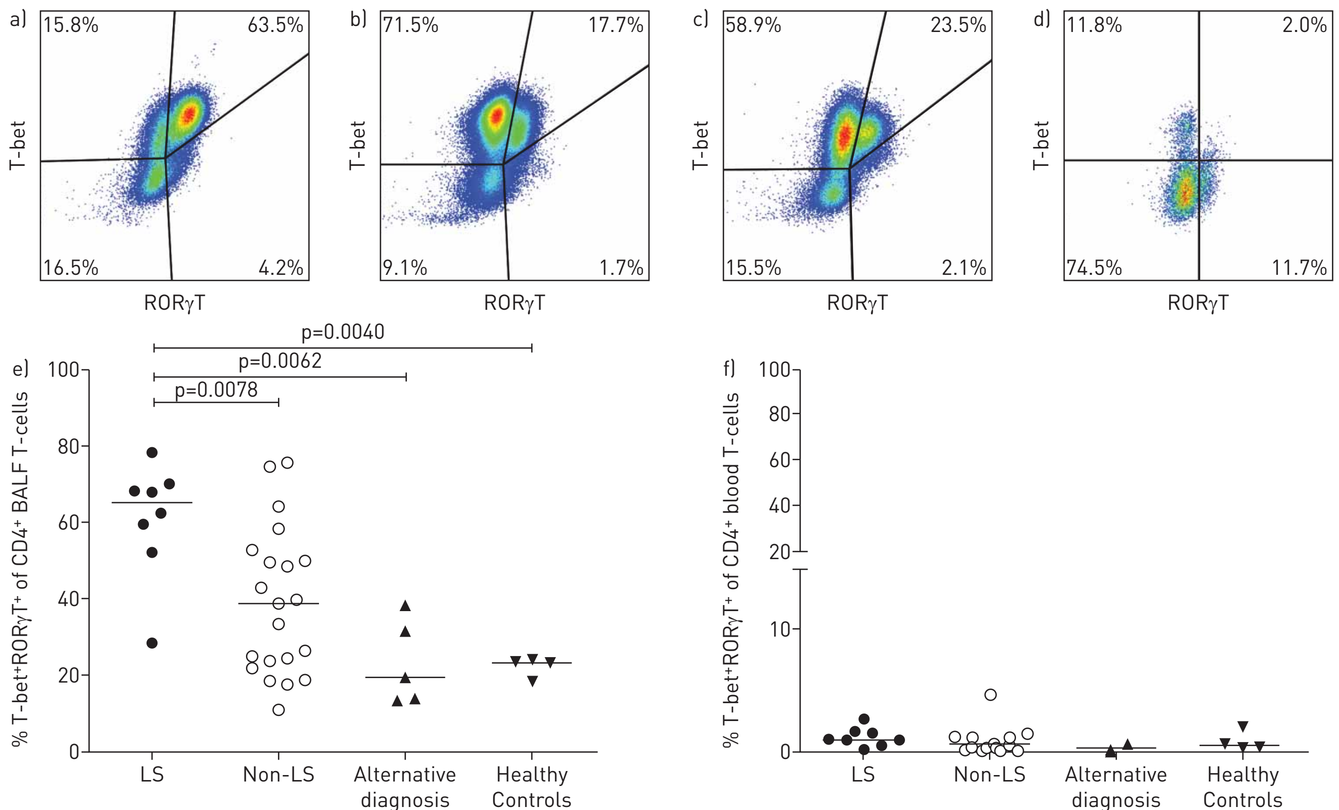

FIGURE 2 T-bet and RORγT are co-expressed in the bronchoalveolar lavage fluid (BALF), but not blood, of all subjects, and this is most pronounced in Löfgren's syndrome (LS). Representative flow cytometry scatter plots are shown for the simultaneous expression of T-bet and RORγT in BALF $CD4^+$ T-cells from a) an LS patient, b) a non-LS patient, c) a healthy individual and d) in the blood of a sarcoidosis patient. e) LS patients showed a significantly higher degree of T-bet/RORγT co-expression than any of the other groups (comparisons were made using the two-tailed Mann–Whitney U-test). f) In contrast, no distinct double-positive population could be discerned in the blood of either patient group.

we also investigated T-bet and RORγT expression in this specific subpopulation. Of the patients included in this study, 10 (six LS and four non-LS) had expansions of $V\alpha2.3^+V\beta22^+$ T-cells (defined as >3 times the median percentage of $V\alpha2.3^+V\beta22^+$ $CD4^+$ T-cells in a healthy individual, *i.e.* >0.5% of all $CD4^+$ T-cells [2]). Of these, a median of 85.0% expressed both T-bet and RORγT, compared with 71.4% in corresponding $V\alpha2.3^-V\beta22^-$ $CD4^+$ T-cells (figure S6a, b). Moreover, co-expression of CXCR3 and CCR6 was observed in a significantly higher proportion of $V\alpha2.3^+V\beta22^+$ T-cells than $V\alpha2.3^-V\beta22^-$ T-cells (figure S6a, c). $V\alpha2.3^+V\beta22^+$ T-cells also showed significantly higher expression of T-bet (figure S7a) and particularly RORγT (figure S7b), when considered separately.

Higher IL-17A, IL-10, IL-22, IL-2 and lower IFNγ production in LS BALF cells

IFNγ was the dominant cytokine produced by BALF cells from both LS and non-LS patients, as measured by FluoroSpot in response to anti-CD3/anti-CD28 stimulation (figure 5a, b), but the percentage of IFNγ-producing cells was significantly lower in LS than in non-LS patients ($p=0.0205$). In contrast, LS patients showed a significant increase in the proportion of cells producing IL-17A ($p=0.0087$), IL-10 ($p=0.0220$) and IL-2 ($p=0.0277$), as well as a trend towards increased IL-22 production ($p=0.1865$) (figure 5a). IL-17A, IL-22 and IL-10 constituted a markedly larger fraction of the total cytokine pool in LS (an average of 5%, 11% and 25%, respectively, of total production) than in non-LS (2%, 4% and 4%, respectively) (figure 5c–f). The trend of increased IL-17A, IL-10, IL-22 and IL-2, and reduced IFNγ production in LS compared with non-LS was also consistent when considering absolute numbers of spot-forming cells, although the differences did not reach statistical significance (figure S8a). No significant difference could be observed between LS and non-LS in terms of simultaneous production of IFNγ and IL-2 (figure S8b).

$T\text{-bet}^+ROR\gamma T^+$ $CD4^+$ T-cells produce IFNγ, IL-17A or both

In accordance with FluoroSpot data, anti-CD3/anti-CD28-stimulated BALF $CD4^+$ T-cells expressing T-bet and RORγT produced mainly IFNγ, but also IL-17A, as measured by flow cytometry (figure 6a–e). Aside from the expected pattern of IFNγ production by $T\text{-bet}^+$ cells (figure 6a) and IL-17A by $ROR\gamma T^+$ (figure 6b),

DOI: 10.1183/13993003.00092-2016

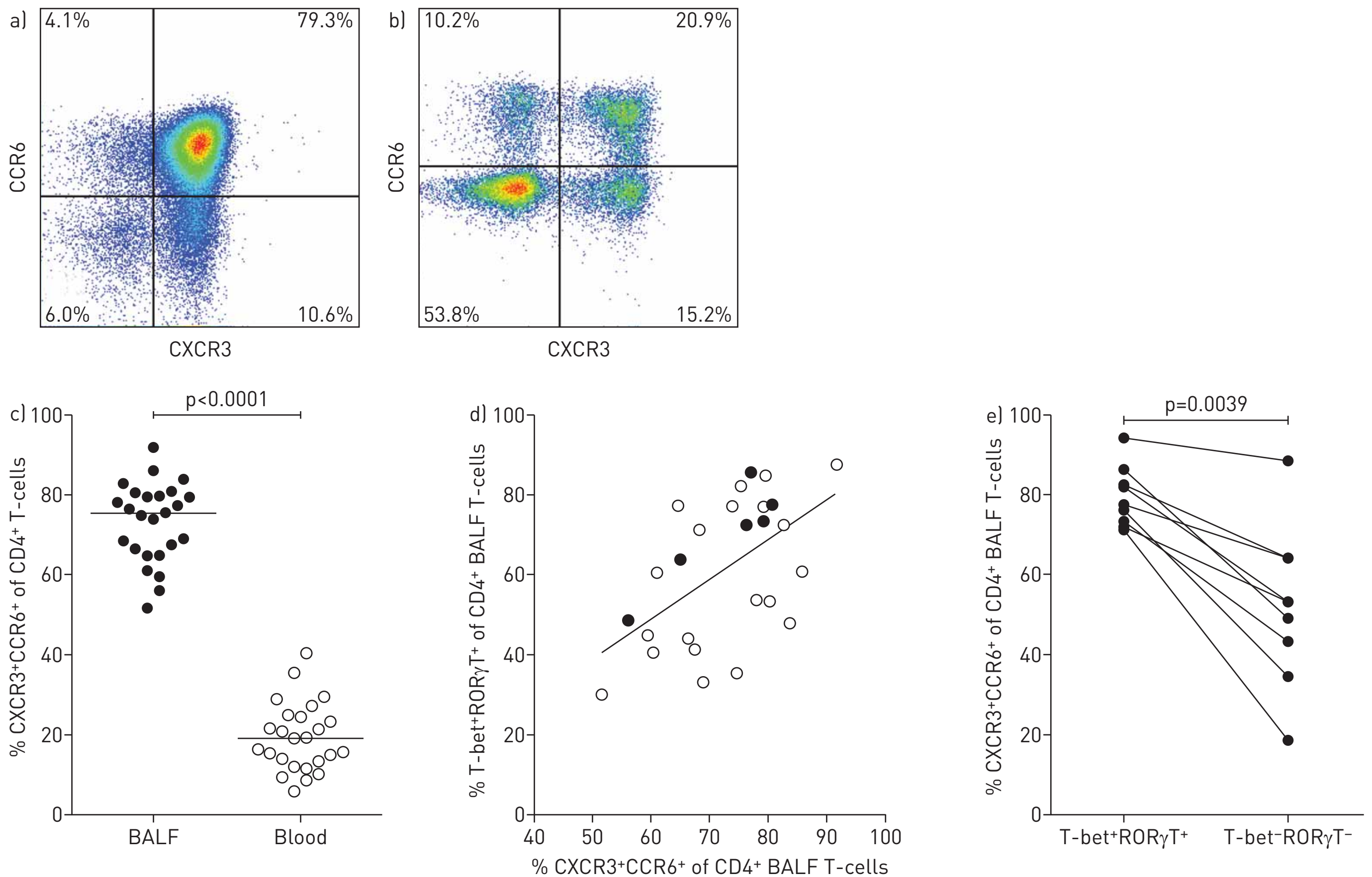

FIGURE 3 Chemokine receptors CXCR3 and CCR6 are co-expressed to a higher degree in the bronchoalveolar lavage fluid (BALF) than in blood, and correlate with T-bet/RORγT expression. Representative flow cytometry scatter plots are shown for simultaneous expression of the T-helper (Th)1- and Th17-associated chemokine receptors CXCR3 and CCR6, respectively, in a) BALF and b) blood of a sarcoidosis patient. c) CXCR3/CCR6 expression was significantly higher in $CD4^+$ T-cells in BALF than in blood for all sarcoidosis patients (compared using the two-tailed Mann–Whitney U-test). The pattern was also consistent in healthy individuals, albeit with a lower average simultaneous expression. d) In both Löfgren's syndrome (LS) (black dots) and non-LS patients (open dots), co-expression of CXCR3 and CCR6 correlated with co-expression of T-bet and RORγT in BALF $CD4^+$ T-cells (r=0.4593, p=0.0209 using non-parametric Spearman rank test; linear regression R^2=0.2756, p=0.0070). e) CXCR3/CCR6 co-expression was also significantly higher in BALF T-bet^+RorγT^+ than in T-bet^-RORγT^- $CD4^+$ T-cells from the same patient (compared using the two-tailed Wilcoxon's signed rank test).

all IL-17A-producing cells were found in the T-bet^+ compartment (figure 6c) and virtually all IFNγ-producing cells were RORγT^+ (figure 6d). Moreover, IFNγ^+IL-$17A^+$, IFNγ^+IL-$17A^-$ and IFNγ^-IL-$17A^+$ cells were all observed within the T-bet^+RORγT^+ T-cell population (figure 6e).

Higher levels of T-bet⁺RORγT⁺ CD4⁺ T-cells associate with nonchronic disease

Of the 33 patients enrolled in the study, 12 patients could be clearly defined as "nonchronic" (the absence of pathological changes and clinical symptoms within 2 years of disease onset) or "chronic" (with persistence of disease more than 2 years after the first radiographic changes were observed). Patients with nonchronic disease showed a significantly higher percentage of BALF T-bet^+RORγT^+ $CD4^+$ T-cells than patients with chronic disease (figure 7). While information on cytokine production was available for too few of these subjects for reliable statistical analysis, a trend towards lower IFNγ and higher IL-17A, IL-22, IL-10 and IL-2 production in BALF of nonchronic patients could still be discerned (data not shown).

Discussion

Despite similarities in key characteristics of sarcoidosis such as granuloma formation and an elevated BALF CD4/CD8 ratio, LS and non-LS patients differ markedly in symptomatic disease presentation and progression. In fact, LS has even been suggested for consideration as a separate disease [13]. The strength of the present study is thus the discrimination between the two patient subgroups, as well as the comprehensive analyses performed *ex vivo*, both at the intracellular protein level and through functional studies of cytokine production and proliferation of cells derived from the lung. Here, we show the concomitant expression of the Th1 and Th17 transcriptional regulators T-bet and RORγT in $CD4^+$ T-cells

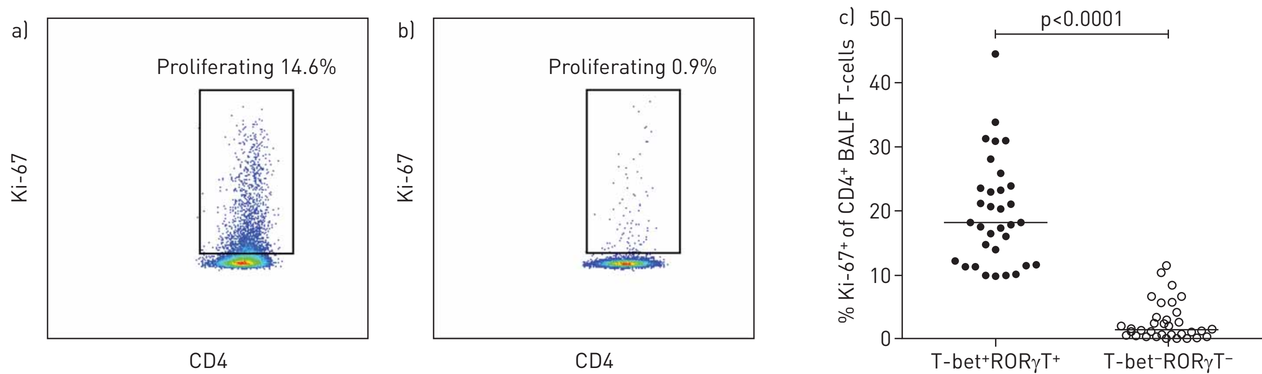

FIGURE 4 T-bet+RORγT+ CD4+ T-cells have a higher proliferative capacity than the corresponding T-bet−RORγT− CD4+ T-cells. Representative flow cytometry scatter plots depict Ki-67 expression in a) T-bet+RORγT+ and b) T-bet−RORγT− CD4+ T-cells in a sarcoidosis patient. c) T-bet+RORγT+ CD4+ T-cells demonstrated significantly higher proliferative capacity than T-bet−RORγT− CD4+ T-cells (compared using the two-tailed Mann–Whitney U-test). T-bet+RORγT− CD4+ T-cells on average showed an intermediate Ki-67 expression, *i.e.* lower than T-bet+RORγT+ and higher than T-bet−RORγT− CD4+ T-cells (data not shown).

in BALF, but not blood, of sarcoidosis patients, and delineate the preferential co-expression of CXCR3 and CCR6 in pulmonary CD4+ T-cells. Moreover, we demonstrate significantly higher levels of T-bet+RORγT+ CD4+ T-cells in LS compared with non-LS, a broader cytokine distribution in LS, and a significant association of elevated T-bet/RORγT expression with nonchronic disease.

We found proliferative capacity and overall cytokine production to be elevated in BALF compared with blood in all sarcoidosis patients, demonstrating activation of CD4+ T-cells specifically in the lungs, as previously reported [18].

Previous studies have described elevated T-bet expression in sarcoidosis patients compared with healthy controls, but noted no difference between LS and non-LS, or in terms of disease stage [19]. Here, the slight but significant increase in T-bet protein expression in LS was coupled with a pronounced difference in RORγT expression, as well as simultaneous expression of T-bet and RORγT. Stable concomitant expression of T-bet and RORγT in response to *in vitro* IFNγ/IL-12 stimulation has been reported [7], but here we show marked co-expression of these transcription factors in a tissue-specific *ex vivo* setting without prior stimulation, indicating previous activation by triggering factor(s) in the lung. The Th1/Th17 phenotype appears more pronounced in LS patients, who generally have a favourable disease phenotype, and higher T-bet/RORγT expression correlated with a nonchronic disease course. Lexberg *et al.* [7] previously suggested that combined Th1 and Th17 effector repertoires confer a physiological advantage at the single-cell level, and that such cells may be more efficient in orchestrating disease resolution. Our findings parallel this line of reasoning, as the high degree of proliferation and elevated co-expression of CXCR3 and CCR6 in T-bet+RORγT+ cells signifies a potent effector subset. Recently, gene profiles associated with the Th17 and Th22 differentiation pathways were identified in LS patients, but not in those with non-LS [12], alluding to a larger influence of “Th17-like” responses in LS. Non-LS patients were also found to be genetically more heterogeneous than LS patients [12], which coincides well with the variability in non-LS transcription factor expression presented here.

Interestingly, a recent report identified IFNγ-producing Th17-polarised (Th17.1) cells in BALF of sarcoidosis patients based on chemokine receptor expression [11]. Our study confirms that Th1/Th17 hybrid cells are abundant in the lungs of sarcoidosis patients, and that IFNγ dominates the BALF cytokine profile, while IL-17A is produced to a lesser degree. However, our distinction between LS and non-LS patients provides an opportunity for comparisons that were not previously possible, for example, in the study by Ramstein *et al.* [11] all patients either had established chronic disease or were defined as non-LS. Likewise, our analyses of a broader range of Th17-associated cytokines, as well as of both transcription factor and chemokine receptor expression, probably contribute to differences in interpretation. Rather than a pathogenic, IFNγ-induced Th17-to-Th1 transitional T-cell phenotype, we propose a potentially protective role of Th1/Th17 cells and Th17-associated cytokines in the lung, and active involvement of a plastic hybrid subset with functional characteristics of both Th1 and Th17 cells.

In this study, we found a significantly higher proportion of Th17-related cytokines and a lower frequency of IFNγ in LS. Reduced IFNγ and elevated IL-10 levels in LS are consistent with previous studies of mRNA expression [20, 21], as is the increased IL-17A production [8]. Considering the disease course in LS, the observed patterns of cytokine production indicate that a combination of cytokines with different

 DOI: 10.1183/13993003.00092-2016

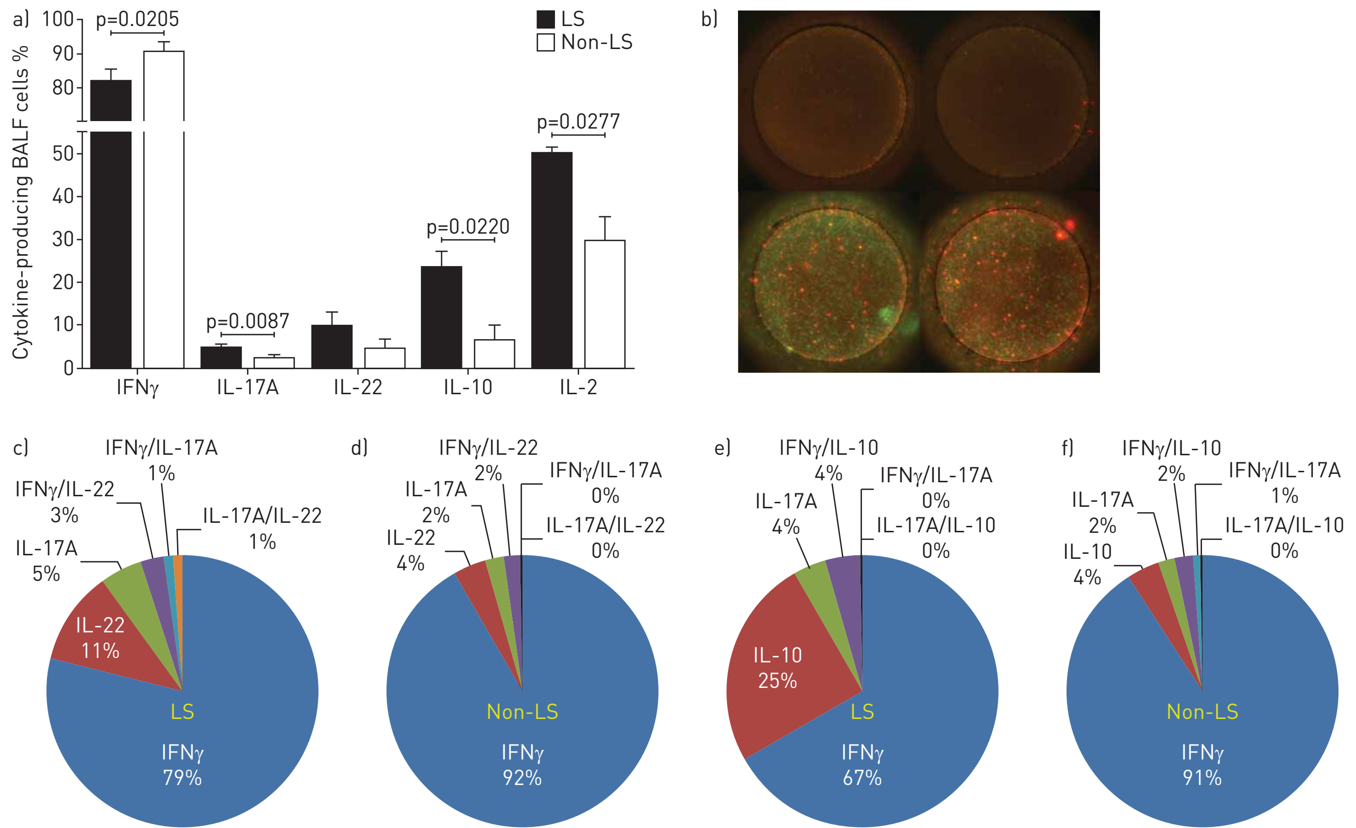

FIGURE 5 Löfgren's syndrome (LS) bronchoalveolar lavage fluid (BALF) cells produce a significantly broader array of T-helper (Th)17 cytokines and lower levels of interferon (IFN)γ than non-LS BALF cells. a) FluoroSpot analysis of BALF cell cytokine production showed a significantly lower percentage of IFNγ-producing cells in LS (n=4) compared with non-LS patients (n=6), and a significantly higher percentage of interleukin (IL)-17A-, IL-10- and IL-2-producing cells (all comparisons using the two-tailed t-test). Data are presented as mean±SEM. b) Representative well images showing duplicates of unstimulated BALF cells from an LS patient, as well as following stimulation with anti-CD3/anti-CD28. IFNγ production is shown in green, IL-17A in red and IL-22 in orange (shown as blue during analysis in AID ELISpot v.7.0 iSpot software (Autoimmun Diagnostika GmbH, Strassberg, Germany)). The average distribution of cytokine-producing cells in the two patient groups are shown as pie charts for the assay combinations c, d) IFNγ/IL-17A/IL-22 and e, f) IFNγ/IL-17A/IL-10. Production of each cytokine is expressed as a percentage of all cytokine-producing cells, amounting to a total of 100%.

properties is beneficial in terms of disease resolution compared with IFNγ alone. The increased IL-2 expression in LS patients may be related to modulation of Th cell differentiation and proliferation, and elevated T-bet expression [22], as well as expansion of already established Th17 responses [23], thus contributing to the implied favourable role of a heterogeneous cytokine profile in the lung.

Current knowledge of the Th17 subset outlines a plastic, unstable phenotype that is strongly influenced by factors in the microenvironment. Studies in mice have identified "pathogenic" and "nonpathogenic" Th17 cells [24], where the latter comprises RORγT^{+} cells that preferentially produce IL-10 and have a more regulatory role, especially in mucosal tissues [25]. In the lung, IL-17- and IL-22-producing cells have been associated with protective immune responses against several pathogens [26, 27]. Increased frequencies of antigen-specific IL-17A- and IL-22-producing CD4^{+} memory T-cells were also observed in healthy individuals exposed to *Mycobacterium tuberculosis* compared with infected patients [28]. Moreover, IL-22 levels were reduced in patients with chronic sarcoidosis and idiopathic pulmonary fibrosis [29]. Although anti-IL-17 treatment has shown promise in chronic inflammatory diseases such as rheumatoid arthritis [30] and psoriasis [31], the heterogeneity of Th17 responses complicates prediction of any potential therapeutic benefits in sarcoidosis. While IL-17 is associated with granuloma formation [10], elevated levels of other Th17-associated cytokines such as IL-10 and IL-22 in nonchronic disease suggest the tissue localisation of and balance between these cytokines influence disease outcome and thereby response to therapy.

In humans, multifunctional Th1/Th17 effector CD4^{+} T-cells have been described in several autoimmune conditions [3–5]. Intriguingly, the clinical presentation of LS shows traits of autoimmunity, and recent data from our group suggest vimentin as a potential autoantigen [2]. In this context, TCR Vα2.3/Vβ22-expressing

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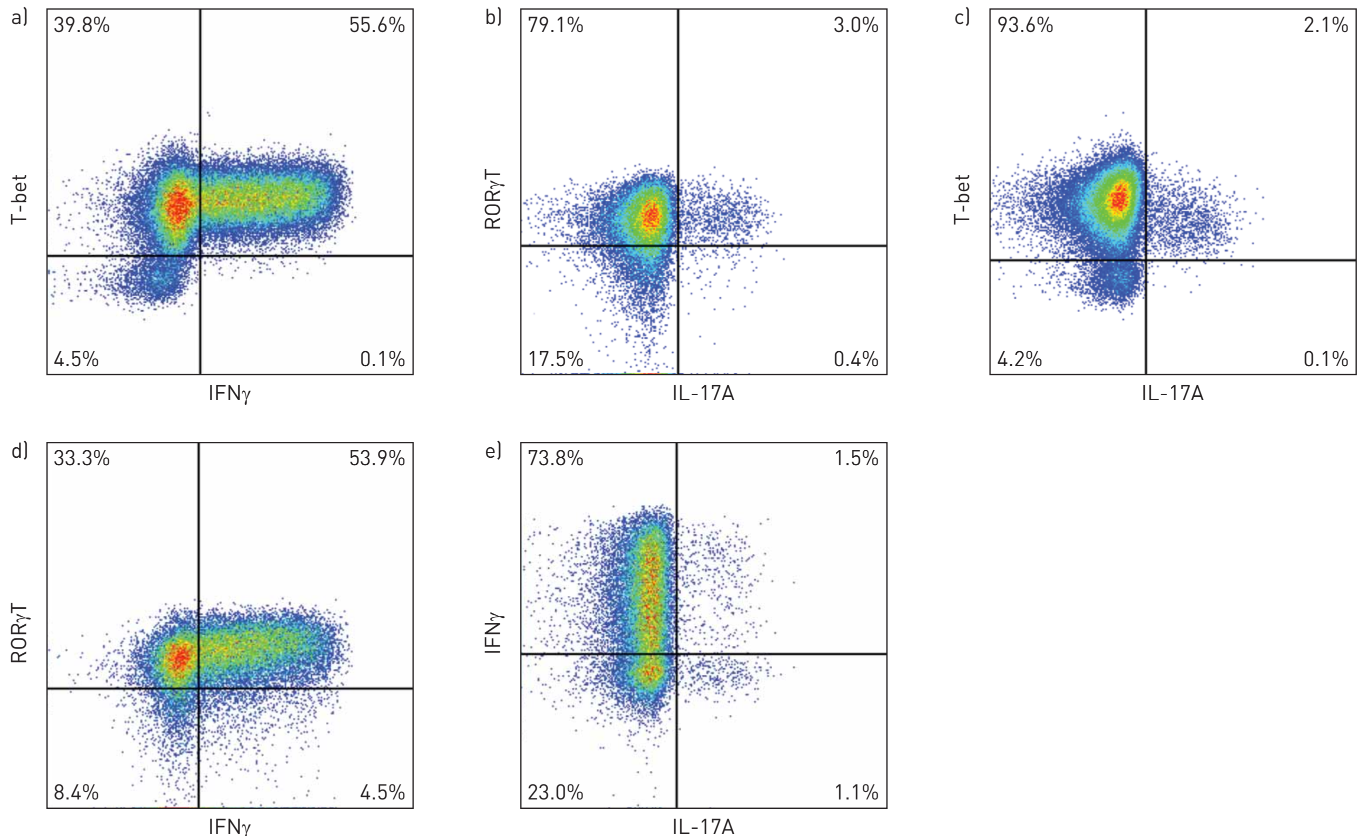

FIGURE 6 T-bet⁺RORγT⁺ CD4⁺ T-cells produce interferon (IFN)γ, interleukin (IL)-17A or both. Representative flow cytometry scatter plots gated on bronchoalveolar lavage fluid CD4⁺ T-cells show a) IFNγ production by T-bet⁺ cells, b) IL-17A production by RORγT⁺ cells, as well as c) IL-17A production by T-bet⁺ cells and d) IFNγ production by RORγT⁺ cells. e) Furthermore, when gating on T-bet and RORγT followed by subsequent gating on IFNγ and IL-17A, IFNγ⁺IL-17A⁺, IFNγ⁺IL-17A⁻ as well as IFNγ⁻IL-17A⁺ cells were all observed within the T-bet⁺RORγT⁺ CD4⁺ T-cell population.

CD4⁺ T-cells are of particular interest as they appear to have undergone clonal expansion following recognition of a specific antigen presented on HLA-DRB1*03 [2]. The finding that Vα2.3⁺Vβ22⁺ cells co-express T-bet and RORγT as well as CXCR3 and CCR6 to a higher degree than corresponding Vα2.3⁻Vβ22⁻ CD4⁺ T-cells strengthens previous reports of these cells as differentiated effector cells [2, 32, 33]. The high expression of T-bet and RORγT in Vα2.3⁺Vβ22⁺ T-cells also supports the involvement of T-bet⁺RORγT⁺ cells in disease resolution, as higher frequencies of TCR-restricted cells correlate with more rapid recovery [17].

It was also intriguing to find virtually all BALF FoxP3⁺ cells within the T-bet⁺ compartment. T-bet expression has been observed in Tregs selectively regulating Th1-driven inflammation [34, 35], as well as during effector T-cell activation [36]. Tregs deficient in both T-bet and GATA-3 also lack suppressive capacity, resulting in autoimmunity [37]. The high proliferation state of T-bet⁺FoxP3⁺ cells indicates an activated cell population, and further implicates T-bet not only in effector responses, but also in lung-specific immune regulation and prevention of harmful autoimmune responses.

We propose that in activated CD4⁺ T-cells in sarcoidosis, cytokine identity is partially determined by regulatory elements distinct from the transcriptional “master regulators”, and that the T-bet and RORγT transcriptional programmes have downstream functions of relevance for disease outcome that are not solely dependent on cytokine production. In some cases, such functions could be restricted to the lungs and related to, for example, migration and cytokine delivery (as indicated by CXCR3/CCR6 co-expression) or survival [38]. Moreover, the presence of proliferating T-bet⁺RORγT⁺ and T-bet⁺FoxP3⁺ CD4⁺ T-cells in BALF of healthy individuals, as well as elevated T-bet/RORγT expression in patients with resolving disease, suggests an active contribution to pulmonary tissue homeostasis.

An important limitation to this study is the incomplete longitudinal patient follow-up in order to verify the role of T-bet⁺RORγT⁺ T-cells in the disease course. Following all patients over a 2-year interval from disease onset is, however, beyond the scope of this particular study, but is a subject that should be revisited in the future. Still, the current clinical assessment of 12 patients provides an indication of these cells’

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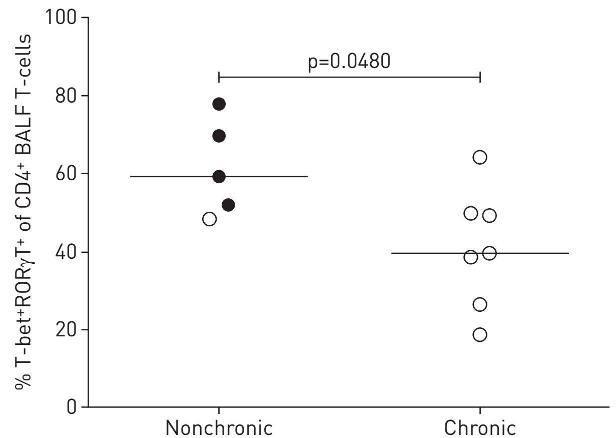

FIGURE 7 T-bet$^+$RORγT$^+$ CD4$^+$ T-cells associate with nonchronic disease. Stratification of Löfgren's syndrome (LS) (black dots) and non-LS patients (open dots), in whom the disease course could be ascertained (n=12), into nonchronic or chronic disease showed significantly higher co-expression of T-bet and RORγT in patients with resolving disease (compared using the two-tailed Mann–Whitney U-test).

possible role in promoting disease resolution. An ideal second study should be to address long-term disease development, with the aim of correlating immunological parameters at onset with time until resolution, responsiveness to treatment (if applicable) and a predisposition towards chronicity.

In terms of *ex vivo* analyses, the process of intracellular staining confers an expected limitation, as viable cells cannot be sorted based on transcription factor expression for further functional investigation. Moreover, the lack of an established animal model for sarcoidosis limits the ability to track the origin, migration, functional plasticity and lifespan of T-bet$^+$RORγT$^+$ T-cells *in vivo*, or at the very least *ex vivo* over the course of disease, which would provide the most accurate representation of how these cells influence disease outcome.

Concluding remarks

Our findings indicate that the lung microenvironment favours a Th1/Th17 phenotype, and that these cells, as well as a broad array of Th17 cytokines, associate with a beneficial disease phenotype in pulmonary sarcoidosis. These results strive to increase understanding of clinical and immunological differences between LS and non-LS, and could potentially have implications for future diagnostic and prognostic applications in a clinical setting.

Acknowledgements

The authors thank research nurses Gunnel de Forest, Margitha Dahl, Heléne Blomqvist and biomedical analyst Benita Dahlberg for excellent assistance in sample collection and preparation (all at the Respiratory Medicine Unit, Karolinska University Hospital Solna, Stockholm, Sweden). Thanks also to Christian Smedman, Staffan Paulie and Theresa Bengtsson at Mabtech AB (Nacka Strand, Sweden) for fruitful discussions, protocol modifications and valuable assistance in reading FluoroSpot plates.

Author contributions were as follows. Conception and study design: Y. Kaiser, R. Lepzien, A. Smed-Sörensen and J. Grunewald. Data collection: Y. Kaiser, R. Lepzien, S. Kullberg and A. Eklund. Performing experiments: Y. Kaiser and R. Lepzien. Analysis and interpretation: Y. Kaiser, R. Lepzien, S. Kullberg, A. Smed-Sörensen and J. Grunewald. Manuscript preparation: Y. Kaiser, R. Lepzien, A. Smed-Sörensen and J. Grunewald. Critical reading and intellectual assessment of manuscript: Y. Kaiser, R. Lepzien, S. Kullberg, A. Eklund, A. Smed-Sörensen and J. Grunewald. All authors read and approved the final manuscript.

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DOI: 10.1183/13993003.00092-2016