Increased frequencies of pulmonary regulatory T-cells in latent *Mycobacterium tuberculosis* infection

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ABSTRACT: Regulation of specific immune responses following exposure to *Mycobacterium tuberculosis* in humans and the role of regulatory T (Treg) cells in the immune control of latent infection with *M. tuberculosis* are incompletely understood.

Latent infection was assayed by an interferon-γ release assay (IGRA) in healthcare workers regularly exposed to tuberculosis (TB) patients and in household TB contacts in Germany. Immunophenotypes of bronchoalveolar lavage (BAL) mononuclear cells and peripheral blood mononuclear cells (PBMCs) were analysed by fluorescence-activated cell sorting.

All TB contacts with latent infection (n=15) had increased (p<0.0001) frequencies of CD4+ CD25+ CD127- Treg cells (median 2.12%, interquartile range (IQR) 1.63–3.01%) among BAL mononuclear cells compared with contacts (n=25) with negative IGRA results (median 0.68%, IQR 0.32–0.96%) No differences were seen when PBMC immunophenotypes of IGRA+ and IGRA- TB contacts were compared (IGRA+ median 9.6%, IQR 5.9–10.1%; IGRA- median 7.7%, IQR 4.6–11.3%; p=0.47). Five out of 25 contacts with negative blood IGRAs showed a positive IGRA from BAL cells, possibly indicating a limited local immune response.

In Germany, latent infection with *M. tuberculosis*, as defined by a positive *M. tuberculosis*-specific IGRA response on cells from the peripheral blood, is characterised by an increased frequency of Treg cells in the BAL.

KEYWORDS: Bronchoalveolar lavage, healthcare workers, latent tuberculosis infection, regulatory T cells, tuberculosis, tuberculosis exposure

nfection with *Mycobacterium tuberculosis* is acquired by aerosol inhalation, and subsequent phagocytosis of the bacteria by alveolar macrophages and dendritic cells [1, 2]. An adaptive immune response against mycobacteria is generated when antigen-presenting cells stimulate T-lymphocytes in the draining lymph node [3]. In mouse models, this occurs 8–10 days after infection [4, 5]. Protective immunity is generally associated with interferon (IFN)-γ-secreting T-helper (Th) type 1 cells [6]. Latent infection with *M. tuberculosis* (latent tuberculosis infection; LTBI) is defined by the presence of an adaptive immune response against *M. tuberculosis* in the tuberculin skin test (TST) or in a blood-based IFN-γ release assay (IGRA) in the absence of clinical signs of tuberculosis (TB) [7].

Using this definition, close recent contacts of LTBI cases carry a risk of approximately 2–13% for the development of active TB within 2 yrs [8]. However, IGRAs are not useful for the diagnosis of active TB due to a high percentage of false-negative results [9]. Mechanisms that govern local containment *versus* the development of active disease are still incompletely understood. It would be advantageous if biomarkers that correlate either with maintenance of effective immune control of the mycobacteria or with progression to active disease could be identified.

Regulatory T-lymphocytes (Treg cells) are involved in the maintenance of self-tolerance and the control of immune responses to foreign antigens [10]. Several types of Treg cells have been described and their phenotypic and functional characterisation is under continuous scientific evaluation. A prominent, yet not exclusive, marker is the interleukin (IL)-2 receptor α-chain, CD25 [11]. More specific, yet not purely so, is the transcription factor forkhead box P3 (FoxP3) expressed in both CD4+ and CD8+ Treg cells [12]. FoxP3 expression is inversely associated with the expression of the IL-7 receptor CD127 [13, 14]. In peripheral blood

mononuclear cells (PBMCs) from healthy donors, LIU *et al.* [13] reported FoxP3 expression in 86.6% (range 67.4–93.6%) of CD25+ CD127low/- cells, while CD127low/CD25- cells contained only 25.5% (14.8–39.5%) and CD4+ CD127+ CD25+ cells contained only 22.9% (11.5–39.2%) of FoxP3 expressing cells. Similarly, SEDDIKI *et al.* [14] found that 87% of CD4+ CD127low cells fell within the CD25+ Foxp3+ gate and conversely, 84% of CD25+ Foxp3+ cells were detected within the CD4+ CD127low gate. Since even CD4+ CD25- FoxP3+ CD127low cells were found to have Th1-suppressive properties, the lack of CD127 surface expression is now widely accepted as a marker for Treg cells.

Treg cells play an important role in modulating *M. tuberculosis*-specific immune responses [2, 15]. In mice infected with *M. tuberculosis*, Treg cells start to expand once phagocytosed mycobacteria have been transported to mediastinal lymphnodes [16]. Treg cells are also recruited to the site of infection in active extrapulmonary TB [17, 18].

Treg cells counteract the Th1 adaptive immune response and delay the priming of effector CD4+ and CD8+ T-cells and their subsequent accumulation in the lung [16]. In humans with presumed LTBI (*i.e.* TST+ or IGRA+) Treg cells in PBMCs expand *ex vivo* in cultures infected with *M. tuberculosis* [19]. Their presence reduces the frequency of *M. tuberculosis*-responsive CD4+ and CD8+ T-cells producing IFN-γ [19]. While the frequencies of circulating Treg cells are similar for healthy, uninfected subjects and subjects with presumed LTBI, patients with active TB, especially those with extrapulmonary disease and extensively drug-resistant TB, show a higher Treg frequency in the peripheral blood [18–21].

However, little is known about the local involvement of pulmonary Treg cells in human TB *in vivo*. Their regional presence and Th1-suppressive function may contribute to the early stages of mycobacterial infection in the lung. In accordance with studies in animal and *in vitro* studies with human cells, we hypothesised that recent TB contacts with presumed LTBI cases might differ in the percentage of pulmonary Treg cells from those with a low likelihood of infection (IGRA-) [16, 18, 22]. We investigated the pulmonary and systemic role of Treg cells in IGRA+ and IGRA- TB contacts in order to identify associations with local and systemic *M. tuberculosis*-specific immune responses.

METHODS

The study was conducted by the German research consortium on ''Pulmonary Tuberculosis – Host and Pathogen Determinants of Resistance and Disease Progression'', and was approved by the ethics committees of all participating German centres (Helios Klinikum Emil von Behring, Berlin; Evanglische Lungenklinik, Berlin; Research Center Borstel, Borstel; Klinik Donaustauf, Donaustauf; Agaplesion Pneumologische Klinik Waldhof, Elgershausen; Asklepios Fachkliniken München-Gauting, Munich; Krankenhaus Großhansdorf, Großhansdorf; Lungenfachklinik, Immenhausen; and Klinikum Nürnberg Nord, Nuremburg, Germany).

Healthcare workers (HCWs) were enrolled if they were working in a respiratory medicine department, had regular professional contact with patients with acid-fast bacilli smear-positive TB and had no evidence of active TB. Household contacts (HHCs) who were exposed for >40 h in total were recruited through the public health authority of the City of

Hamburg and through the Research Center Borstel, Borstel. Demographic and health-related data were collected using a standardised questionnaire.

An IGRA (QuantiFERON®-TB Gold In-Tube (Cellestis Ltd, Carnegie, Australia) or T-SPOT.*TB*® (Oxford Immunotec, Abingdon, UK)) was performed according to the manufacturer's instructions, with the exception that for the T-SPOT.*TB*®, 200,000 mononuclear cells (instead of 250,000 mononuclear cells) were plated per well for improved visibility of the spots [23, 24] TSTs were not performed.

A flexible bronchoscopy was performed according to German guidelines with intravenous and local anaesthesia at the physicians' discretion [25]. The bronchoscope was wedged into a sub-segmental bronchus of the middle lobe. Bronchoalveolar lavage (BAL) was performed with a total volume of 250 mL sterile normal saline.

PBMCs were prepared by Ficoll–Hypaque density gradient centrifugation ($465 \times g$ for 42 min at ambient temperature) from heparinised blood. After washes with PBS and a second centrifugation ($800 \times g$ for 15 min at 4°C), cells were washed with PBS and centrifuged again ($400 \times g$ for 12 min at 4°C). Pellets containing large numbers of red blood cells underwent erythrocytolysis with sterile water and $2\times$ PBS. PBMC flow cytometry was performed in a laboratory of the Max Planck Institute for Infection Biology, Berlin, Germany.

Single-cell suspensions from BAL fluid were obtained by passing the BAL fluid through a stainless steel sieve (Teesieb Profi Plus, WMF, Geislingen, Germany) with a mesh aperture of 0.5 mm.

Lymphocyte and mononuclear cell subsets were defined in cells from BAL fluid, using monoclonal antibodies against CD3 (MHCD0305; Invitrogen, Carlsbad, CA, USA), CD4 (MHCD0401, Invitrogen), CD16 (MHCD1604, Invitrogen), CD25 (MHCD2505, Invitrogen), CD40 (CD4005, Invitrogen), CD45RO (MHCD45RO04, Invitrogen), CD62L (MHCD62L05, Invitrogen), CD127α (FAB 306P; R&D Systems, Minneapolis, MN, USA), HLA-DR (347402; BD Pharmingen, Heidelberg, Germany), Toll-like receptor (TLR)4 (13-9917-80; NatuTec ebioscience, Vienna, Austria) by flow cytometry (FACSCalibur; Becton Dickinson, Heidelberg, Germany). BAL cell flow cytometry was performed at a laboratory of the Research Center Borstel.

Treg were defined as CD4+ CD25+ CD127- as described above [13, 14].

Chi-squared or Fisher's exact test was used to compare categorical data; Wilcoxon–Mann–Whitney and unpaired t-tests were used for continuous non-normally and normally distributed variables, respectively. Statistical significance was defined as a two-tailed p-value <0.05. Analyses were performed using the Stata software (version 9.0, StataCorp, College Station, TX, USA).

RESULTS

Between August 2008 and May 2011, 101 TB contacts were recruited for the trial. Blood samples were taken from all participants for immunological analysis prior to the BAL recruitment. BALs were used for fluorescence-activated cell sorter (FACS) analysis, cell culture (data not shown) and mycobacterial kill assays (data not shown). The FACS data of 47 BAL samples
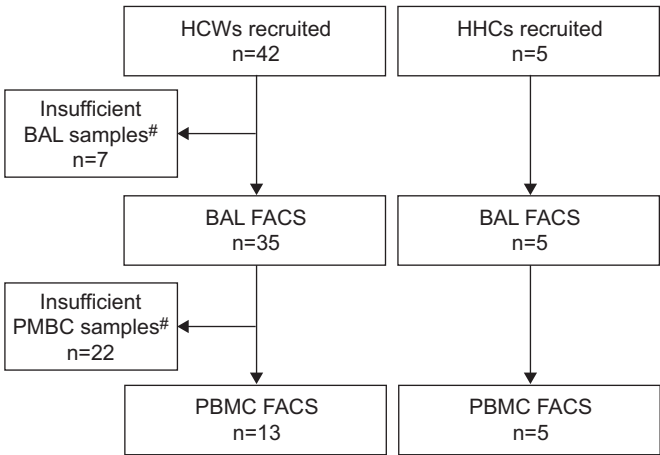
FIGURE 1. Recruitment flowchart and fluorescence-activated cell sorter (FACS) sample processing. HCW: healthcare worker; HHC: household contact; BAL: bronchoalveolar lavage; PBMC: peripheral blood mononuclear cell. #: number of regulatory T-cells undeterminable due to indistinctive CD127 staining or sample failure.

were available for analysis. Of these, five (10.6%) were from HHCs recruited from the two centres mentioned above and 42 (89.4%) were from HCWs from nine hospitals. The BALs of seven HCWs did not contain enough mononuclear cells for immunophenotyping. Phenotyping with CD127 was available in 45% (18 out of 40) of the blood samples from TB contacts, mainly for
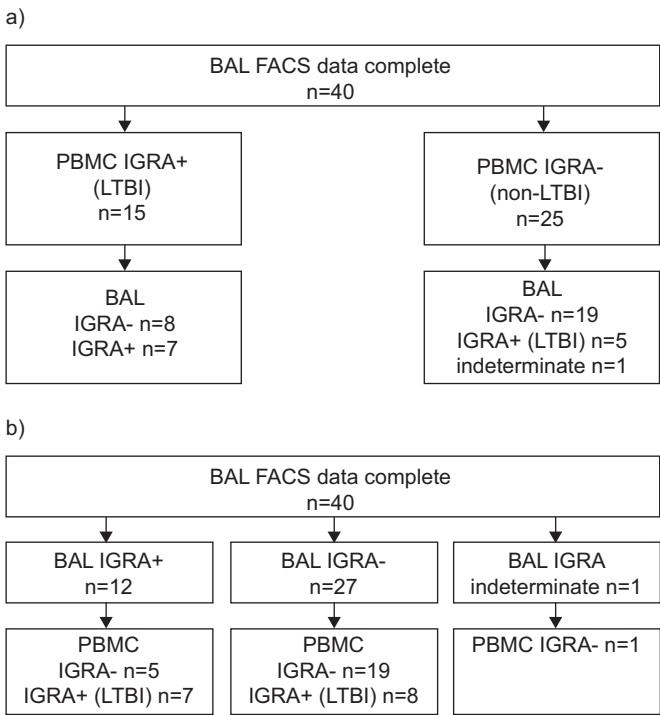

FIGURE 2. a) Distribution of interferon-γ release assay (IGRA) results stratified by peripheral blood mononuclear cell (PBMC) IGRA; b) distribution of IGRA results stratified by bronchoalveolar lavage (BAL) IGRA. FACS: fluorescence-activated cell sorter; LTBI: latent tuberculosis infection.

TABLE 1	Demographic, epidemiological and clinical characteristics of the enrolled individuals		
Variables	**Groups**		**p-value**
	IGRA-	**IGRA+**	
Subjects n	25	15	
Age yrs	44.4±14.3	54.1±8.2	0.02
Males	6/25 (24.0)	3/15 (20.0)	0.77
Individuals born in Germany	22/25 (88.0)	11/15 (73.3)	0.23
Migration background	7/25 (28.0)	3/15 (20.0)	0.57
Grown up in urban area	10/24 (41.7)	8/15 (53.3)	0.42
Current residence in urban area	10/25 (40.0)	8/15 (53.3)	0.41
Previous history of TB	3/25 (12.0)	1/14 (7.14)	0.63
BCG vaccination	11/22 (50.0)	7/13 (53.9)	0.82
Previous TST	17/24 (70.8)	14/15 (93.3)	0.09
Previous positive TST	8/15 (53.3)	12/13 (92.3)	0.02
Previous IGRA	6/23 (26.1)	1/15 (6.7)	0.13
Previous positive IGRA	1/6 (16.7)	1/1 (100.0)	0.09
Type of contact			
Nursing	16/25 (64.0)	11/15 (73.3)	0.54
Medical	3/25 (12.0)	2/15 (13.3)	0.90
Household	4/25 (16.0)	1/15 (6.7)	0.39
Time in profession yrs			
<5	5/21 (23.8)	2/14 (14.3)	0.49
5–10	4/21 (19.1)	1/14 (7.1)	0.32
10–20	6/21 (28.6)	5/14 (35.7)	0.66
>20	6/21 (28.6)	6/14 (42.9)	0.38
Weekly exposure to TB patients h			
Unknown/<1	2/21 (9.5)	2/13 (15.4)	0.60
1–10	12/21 (57.1)	9/13 (69.2)	0.48
11–20	1/21 (4.8)	1/13 (7.7)	0.73
>20	6/21 (28.6)	1/13 (7.7)	0.14
TB patients per yr	50 (22.5–60.0)	50 (45.0–150.0)	0.65
Clean room available	2/18 (11.1)	0/10 (0.0)	0.27
Use of FFP masks	10/17 (58.8)	4/12 (33.3)	0.18
HIV+	3/23 (13.0)	3/10 (30.0)	0.24
Close contact definition			
Close	1/4 (25.0)		
Very close	3/4 (75.0)	1/1 (100.0)	0.58
Resettler#	5/25 (20.0)	4/12 (33.3)	0.38
Organ transplantation	1/24 (4.2)	1/12 (8.3)	0.61
Diabetes mellitus	4/25 (16.0)	3/12 (25.0)	0.51
Pulmonary silicosis	0/25 (0.0)	2/12 (16.7)	0.04
Gastrectomy	2/25 (8.0)	0/12 (0.0)	0.31
Renal failure	2/25 (8.0)	1/12 (8.3)	0.98
Malignancy	4/25 (16.0)	1/12 (8.3)	0.52
IVDU	3/25 (12.0)	3/12 (25.0)	0.32
Iatrogenic immunosuppression	4/25 (16.0)	2/12 (16.7)	0.96
Smoking			
Never-smoker	9/22 (40.9)	4/12 (33.3)	0.66
Current smoker	3/22 (13.6)	5/12 (41.7)	0.07
Ex-smoker	9/22 (40.9)	5/11 (45.5)	0.80
TB in blood relatives	4/24 (16.7)	2/15 (13.3)	0.78
TB in partner	3/24 (12.5)	1/15 (6.7)	0.56

Data are presented as mean±SD, n (%) or median (interquartile range), unless otherwise stated. IGRA: interferon-γ release assay; TB: tuberculosis; BCG: bacille Calmette–Guérin; TST: tuberculin skin test; FFP: filtering face piece; IVDU: intravenous drug user. #from former German territory.

TABLE 2	Frequencies of lymphocyte and monocyte immunophenotypes in bronchoalveolar lavage (BAL) and among peripheral blood mononuclear cells (PBMCs)		
Phenotype	**IGRA-**	**IGRA+ (LTBI)**	**p-value**
	Median (IQR)	**Median (IQR)**	
BAL samples			
Lymphocytes[#]	9.9 (3.815–19.83) (n=25)	9.37 (3.87–27.7) (n=15)	0.77
Macrophages[#]	70 (55.85–82.85) (n=25)	70.12 (57.9–80.68) (n=15)	0.89
Monocytes[#]	5.8 (3.45–7.28) (n=25)	5.4 (4.43–7.84) (n=15)	0.56
HLA-DR[¶]	18.84 (9.98–23) (n=24)	19.86 (6.69–30.68) (n=15)	0.44
CD16[¶]	2.485 (1.71–6.355) (n=24)	3.61 (2.04–7.16) (n=15)	0.64
CD62L[¶]	5.37 (3.345–8.23) (n=25)	8.03 (3.87–13.42) (n=15)	0.18
CD25[¶]	2.22 (0.79–4.09) (n=11)	4.25 (2.83–5.48) (n=11)	0.048
CD127[¶]	43.15 (27.03–50.68) (n=11)	24.45 (15.52–34.92) (n=11)	0.03
CD3+ CD4+[¶]	34.55 (28.19–50.45) (n=25)	47.22 (31.77–59.18) (n=15)	0.18
CD3+ CD8+[¶]	27.2 (13.03–37.36) (n=25)	25.07 (13.85–40.65) (n=15)	0.74
CD4+ CD3+ CD45R0[¶]	31.32 (27.37–42.05) (n=25)	46.1 (31.28–57.58) (n=15)	0.10
CD8+ CD3+ CD45R0[¶]	22 (11.79–27.92) (n=25)	18.1 (14.63–22.55) (n=15)	0.74
CD4+ CD25+ CD127-[¶]	0.68 (0.32–0.955) (n=25)	2.12 (1.63–3.01) (n=15)	<0.0001
HLA-DR+[§]	284 (198–445.1) (n=23)	285.1 (208.5–1106) (n=14)	0.56
TLR4[§]	70 (49–91) (n=23)	69.65 (42.58–114) (n=14)	0.89
PBMC samples			
CD16+[ƒ]	12.75 (10.5–15.15) (n=20)	12.55 (9.4–19.4) (n=14)	0.96
CD62L+[ƒ]	55.4 (39.5–67.25) (n=20)	32.1 (27.65–57.6) (n=14)	0.08
CD25+[ƒ]	20.8 (13.9–50.75) (n=10)	27.6 (15.35–33.05) (n=10)	0.91
CD127+[ƒ]	58.25 (37.25–68.65) (n=10)	51.95 (42.6–68.4) (n=10)	0.85
CD40+ HLA-DR+[ƒ]	10.55 (5.8–14.95) (n=20)	11.1 (9.1–18.55) (n=14)	0.47
CD3+ CD4+[ƒ]	42.1 (34.45–52.7) (n=20)	44.9 (29.1–54.3) (n=14)	0.90
CD3+ CD8+[ƒ]	20.65 (13.4–27.65) (n=20)	16.7 (10.25–21.45) (n=14)	0.17
CD4+ CD3+ CD45R0[ƒ]	16.46 (7.961–25.16) (n=25)	21.57 (11.2–33.81) (n=15)	0.71
CD8+ CD3+ CD45R0[ƒ]	4.542 (1.78–9.806) (n=25)	4.744 (3.752–8.051) (n=15)	0.68
CD4+ CD25+ CD127-[ƒ]	7.7 (4.6–11.3) (n=11)	9.6 (5.9–10.1) (n=7)	0.47
CD40+ HLA-DR[##]	83.5 (78.2–86.8) (n=20)	78.65 (60.8–87.75) (n=14)	0.38

Fluorescence-activated cell sorter analysis of lymphocytes and monocytes in BAL or PBMC were performed due to the respective gates in the forward/side-scatter pattern. HLA: human leukocyte antigen; TLR: Toll-like receptor; IGRA: interferon-γ release assay; LTBI: latent tuberculosis infection. [#]: % of all somatic BAL cells; [¶]: % of all BAL lymphocytes; [§]: median fluorescence intensity (MFI) of all BAL monocytes; [ƒ]: % of all PBMC lymphocytes; [##]: MFI of all PBMC monocytes.

technical reasons, *i.e.* weak staining that prevented a clear distinction between CD127+ and CD127- cells (fig. 1).

Blood samples of 25 HCWs (11 LTBI and 14 non-LTBI) were tested with the QuantiFERON®-TB Gold In-Tube system, with a cut-off of 0.35 IU IFN-γ. All five HHC blood samples (two LTBI and three non-LTBI) were tested with the T-SPOT.*TB*® system, and so were the 10 remaining blood samples of HCWs (three LTBI and seven non-LTBI). All BAL samples were tested with the T-SPOT.*TB*® test.

There were 25 (62.5%) IGRA- (21 HCWs and four HHCs) and 15 (37.5%) IGRA+ (14 HCWs and one HHC) participants; the latter had presumed LTBI by definition. Of the 25 participants with a negative blood IGRA result, five (20%) had a positive BAL IGRA result, one (4%) had an indeterminate BAL IGRA and 19 (76%) were BAL IGRA-. Of the 15 subjects with presumed LTBI, eight (53.3%) BAL IGRA results were negative and seven (46.7%) were positive (fig. 2).

The baseline criteria between the two groups were similar (table 1). Subjects with presumed LTBI were slightly older (54.1 *versus* 44.4 yrs; p=0.02), but no other significant differences were noted regarding the exposure duration, intensity and protective measures used. Common risk factors for the development of TB were distributed equally (HIV infection, malignancy, immunosuppression, diabetes mellitus, gastrointestinal surgery, *etc.*).

Subjects with presumed LTBI had a significantly higher frequency of Treg cells (median 2.12%, interquartile range (IQR) 1.63–3.01%) in the BAL compared to unsensitised participants (median 0.68%, IQR 0.32–0.96%; p<0.0001) (table 2). Treg frequencies were consistently ≥1.36% and ≤1.32% of BAL lymphocytes in the LTBI and IGRA- group, respectively (fig. 3).

When the subjects were stratified according to their BAL-based IGRA rather than the PBMC IGRA, the differences in Treg frequencies in the BAL remained significant (fig. 4). Higher
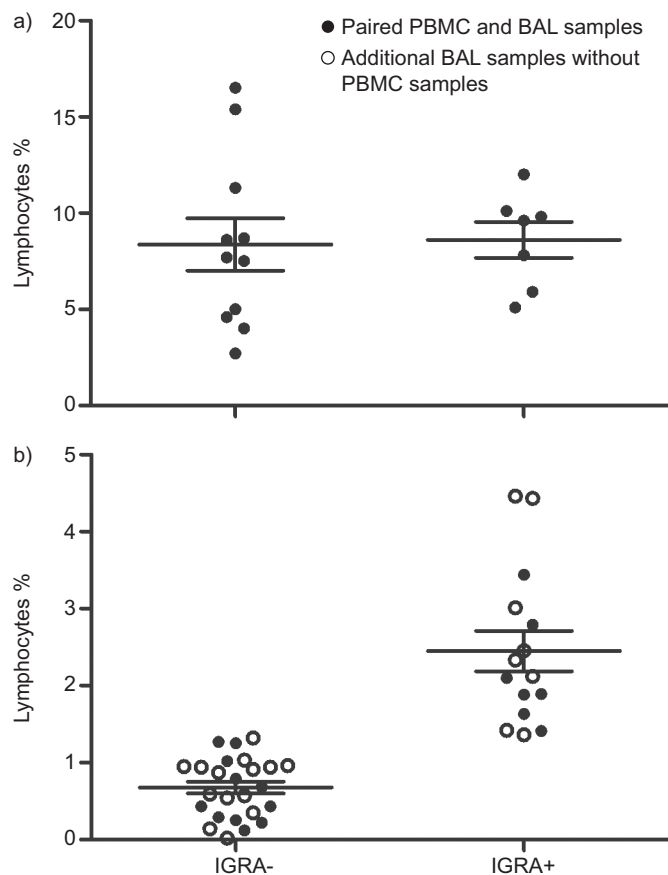
FIGURE 3. Frequency of regulatory T-cells (Treg) stratified by interferon-γ release assay (IGRA) status from peripheral blood mononuclear cells (PBMCs). Tregs percentages of lymphocytes in a) PBMC and b) BAL. Bars represent median and interquartile range.

FIGURE 4. Frequency of regulatory T-cells (Treg) stratified by interferon-γ release assay (IGRA) status from bronchoalveolar lavage cells (BAL). Tregs percentages of lymphocytes in a) PBMC and b) BAL. Bars represent median and interquartile range. [#]: p=0.96; [¶]: p=0.02.

BAL Treg cell frequencies were associated with a positive BAL IGRA (median 1.42%, IQR 0.99–2.39%) compared to a negative BAL IGRA (median 0.91%, IQR 0.35–1.41%). No differences were found for blood Treg cells (median 9.1%, IQR 6.3–9.85% *versus* median 7.75%, IQR 5.05–11.65%). Correlating individual Treg cell frequencies from BAL and PBMC for subjects in which both measurements were available did not reveal any new insights (fig. 5).

No differences in the BAL cells or PBMC were found between contacts with presumed LTBI and IGRA- subjects on analysis of several other immunophenotypes (table 2) of lymphocytes (human leukocyte antigen (HLA)-DR, CD62L, CD3 CD4 and CD3 CD8) or monocytes (HLA-DR, CD40 and TLR4).

No differences were seen in bacille Calmette–Guérin-vaccinated and -nonvaccinated subjects. The differences in Treg cell frequencies remained significant when HIV-infected individuals were excluded from the analysis.

DISCUSSION

Treg cells seem to be involved in the regulation of *M. tuberculosis*-specific immune responses [2, 15, 26]. Their role in active TB has been described but their function during the early and latent stages of *M. tuberculosis* infection is unknown [18, 27–31]. Althou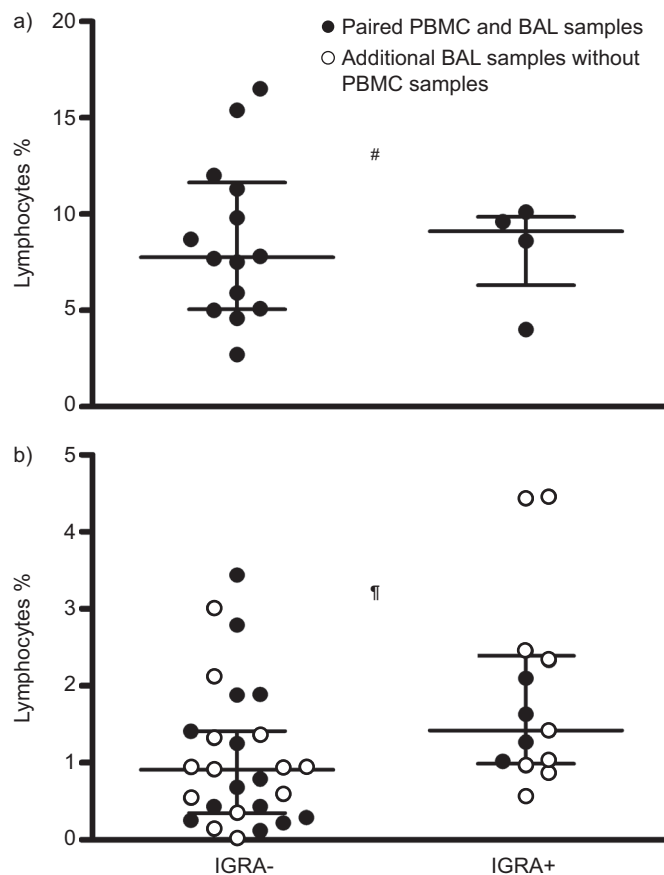gh our hypothesis may have been influenced by a potential publication bias because nonsignificant results may not have been published, we postulated that TB contacts with presumed LTBI would differ in the percentage of pulmonary Treg cells compared with non-LTBI contacts.

Higher frequencies of Treg cells were found in the BAL of all subjects with presumed LTBI compared with IGRA- individuals. This is potentially an indication of ongoing inflammation triggered by a regional mycobacterial infection. Recruitment of Treg cells to the site of infection has been described in patients with active TB [18, 27, 30, 31]. Furthermore, the predominant production of both proinflammatory IFN-γ and anti-inflammatory interleukin (IL)-10 by CD4+ cells in the BAL of patients with pulmonary TB was noted over 10 yrs ago [32]. It is therefore possible that the recruitment of Treg cells results in a situation where, despite the prevention of inflammation-induced tissue damage, mycobacteria may escape the control of the immune system [16]. Supporting this notion, a pivotal Treg cytokine, transforming growth factor (TGF)-β$_1$ promotes intracellular growth of *M. tuberculosis in vitro* [33].

Notably, a positive *M. tuberculosis*-specific IGRA from the BAL was also associated with a higher frequency of BAL Treg cells. This contradicts *in vitro* experiments where the presence of Treg cells reduced IFN-γ production and cellular immune responses
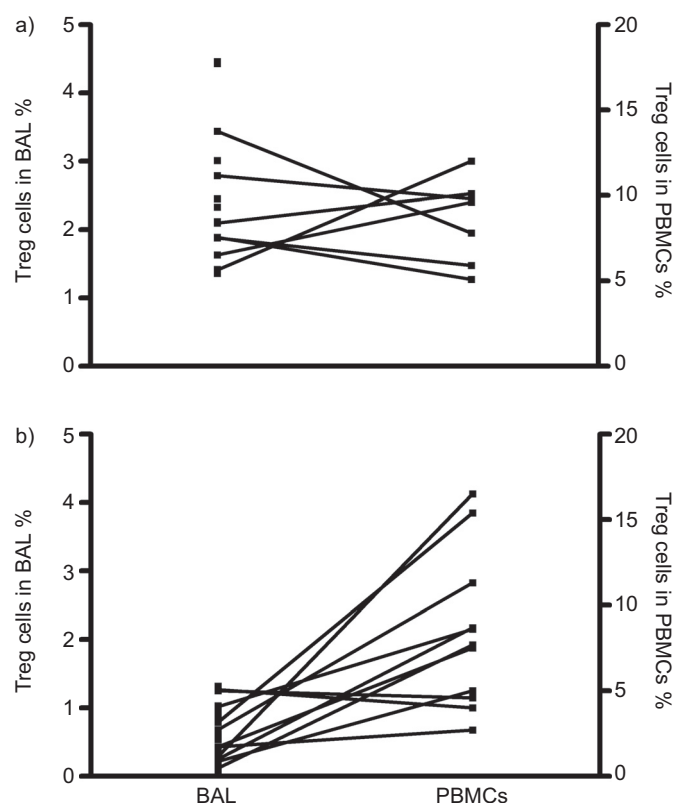
FIGURE 5. Relationship between regulatory T-cells (Treg) frequencies in bronchoalveolar lavage (BAL) and peripheral blood mononuclear cells (PBMCs) in subjects with a) positive PBMC interferon-γ release assay (IGRA) status and b) negative PBMC IGRA status.

to *M. tuberculosis* antigens in nearly all studies [18, 19, 27, 28, 34, 35]. However, *in vitro* experiments measure an immune response before and after Treg cell arrival in a predefined setting with constant numbers of effector T-cells and mycobacteria. *In vivo*, the recruitment of Treg cells is secondary to regional inflammation triggered by a changing *M. tuberculosis* burden and dynamic cellular immune responses. This lack of standardisation renders the assessment of local immune responses prior to and after the advent of Treg cells challenging, if not impossible.

Unfortunately, the observational character of our data does not allow a closer characterisation of pulmonary Treg cells. Their role as suppressor cells or bystanders should be investigated in further studies. That the phenomenon reflects inflammation-induced secondary recruitment of Treg cells to the lungs with the aim to control excessive inflammatory processes but without effect on mycobacterial load cannot be excluded. Conversely, Treg cells seem to play a key role in the balance between tissue integrity and antimycobacterial immune responses, as seen in a macaque model, in which higher post-infection Treg cell frequencies were found in the BAL of animals who developed LTBI rather than active TB [24]. Further studies are required to investigate whether Treg cells are associated with the risk of developing active disease in humans. Using this approach, the proportion of individuals with positive *M. tuberculosis*-specific immune responses who benefit from preventive antibiotic therapy could possibly be identified more clearly.

In this study, individuals with or without presumed LTBI showed similar frequencies of circulating Treg cells. In active TB, the immunological balance fails, inflammation increases and larger numbers of Treg cells are recruited to the site of infection, indicated by higher frequencies of circulating Treg cells [18, 19, 28, 29, 36]. Supporting this finding, infected macaques show decreasing numbers of circulating Treg cells during the first weeks following infection, as these are recruited to the lung. After about 5–6 months, however, peripheral Treg cells increase only in animals that develop active disease [24].

These results contrast the results of BURL *et al.* [21], who reported a lower expression of FoxP3 mRNA, a surrogate marker of Treg cells, in the blood of TB contacts with presumed LTBI compared with IGRA- subjects. According to the macaque model, however, the time since *M. tuberculosis* exposure determines the numbers of circulating Treg cells. This interval is often unknown in humans.

High frequencies of Treg cells are also found in the BAL and blood of patients with other granulomatous lung diseases, such as sarcoidosis and Churg–Strauss syndrome, where they also correlate with disease activity [37–39]. In TB, the numbers of circulating Treg cells decrease after resection of pulmonary cavities [36]. Therefore, Treg cells or their surrogate markers (TGF-β1 and IL-10) merit further investigation for the monitoring of TB treatment response.

Five out of 25 *M. tuberculosis*-exposed subjects without LTBI showed a specific immune response in the BAL. The interpretation of this finding is challenging since no published trial has addressed this issue. In one study on HHCs with presumed LTBI (defined as a positive TST), 50% (five out of 10 subjects) showed a ⩾3-fold frequency of IFN-γ-secreting cells in BAL compared with PBMCs upon stimulation with the mycobacterial antigen Ag85 [40]. Apparently, this compartmental enrichment was associated with recent *M. tuberculosis* exposure as it was found in only 12% (one of 13) of TST-positive control subjects without recent exposure. In contrast to our own study, SCHWANDER *et al.* [40] did not include *M. tuberculosis*-exposed, TST-, subjects, preventing a direct comparison.

The phenomenon of localised specific immune responses in healthy TB contacts remains elusive. DHEDA *et al.* [41] found a positive *M. tuberculosis*-specific IGRA in the BAL of five out of 48 non-TB lung disease patients, but of these only two had a negative blood IGRA result (one patient with a carcinoma and one with a lymphocytic interstitial pneumonitis). BAL IGRAs were also assessed in two studies from our own group. In a large European study, JAFARI *et al.* [42] found a negative blood IGRA in 118 out of 250 non-TB patients and 14 (12%) of them had a positive BAL IGRA; in a smaller trial, 40 out of 84 subjects with a confirmed non-TB diagnosis had a negative blood IGRA and six (15%) of them had a positive BAL IGRA [43]. This trial included 10 subjects with a non-tuberculous mycobacterial infection, four of which had a negative blood IGRA, but none had a positive BAL IGRA. The non-TB patients in all three studies were suspected of having active TB but were eventually diagnosed with another active respiratory disease. This is a very different setting to our current investigation addressing healthy individuals. Their findings, however, underline that in patients without active TB, the correlation between systemic and local immune response to *M. tuberculosis* varies.

Since BAL cells are strikingly different from PBMCs cells in terms of functional capabilities and phenotype, one potential reason includes the discordant recognition of antigens [44]. Mycobacterial uptake, accessory function and infectability of alveolar macrophages are greater than that of PBMCs, with potential implications for antigen presentation [44]. In patients with active TB, cultured lymphocyte proliferation and IFN-γ production in response to the 10-kDa culture filtrate protein and other antigens is more pronounced in BAL cells than PBMCs. No such differences were seen in response to ESAT-6 [45]. Both antigens are used in commercially available IGRAs and may, in part, explain the discordance between IGRA results from PBMCs and BAL. Other hypothetical explanations include a compartmentalisation effect or a recent *M. tuberculosis* exposure triggering a localised pulmonary IFN-γ response first, followed by a systemic response.

Our study has several limitations. First, we investigated a heterogeneous cohort. For professionally active HCWs, the timepoint and amount of exposure to *M. tuberculosis* is not clearly defined. In contrast, our HHCs were exposed very closely to one contagious patient only. Second, the number of available blood samples for Treg analysis was small due to technical difficulties, *i.e.* indistinctive or weak CD127 staining and sample failure (fig. 1). Third, we could not establish the role of BAL Treg cells in subjects with presumed LTBI. As there is no established method for depletion or enrichment of functional Treg cells from BAL fluid, we were unable to investigate their influence on IGRA results.

To our knowledge, this study represents the largest cohort of BAL sampling in healthy TB contacts. Subjects with presumed LTBI showed increased frequencies of Treg cells in the BAL compared with IGRA- subjects. We speculate in accordance with other studies and animal models that their recruitment to the lung reflects the attempt of the immune system to prevent tissue damage by reducing inflammation. The role of Treg cells as markers for the progression to active disease and as markers for treatment response merits further investigation.

STATEMENT OF INTEREST
A statement of interest for C. Lange can be found at www.erj.ersjournals.com/site/misc/statements.xhtml

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