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# Increased frequencies of pulmonary Treg cells in latent M. *tuberculosis* infection

**Running head:** Increased BAL Treg cells in LTBI

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## Abstract

**Background:** Regulation of specific immune responses following exposure to *M. tuberculosis* in humans and the role of regulatory T ($T_{reg}$) cells in immune control of latent infection with *M. tuberculosis* are incompletely understood.

**Methods:** Latent infection was assayed by an Interferon-γ Release Assay (IGRA) in health care workers regularly exposed to tuberculosis patients and in household TB contacts in Germany. Immunophenotypes of bronchoalveolar lavage mononuclear cells and peripheral blood mononuclear cells (PBMC) were analyzed by fluorescence activated cell sorting.

**Results**: All tuberculosis contacts with latent infection (n=15) had increased ($p<0.0001$) frequencies of CD4+CD25+CD127- $T_{reg}$ cells (median 2.12%, InterQuartile Range -IQR- 1.63-3.01) among bronchoalveolar lavage (BAL) mononuclear cells compared to contacts (n= 25) with negative IGRA results (0.68%, IQR 0.32-0.96) No differences were seen when PBMC immunophenotypes of IGRA positive and negative 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 of 25 contacts with negative blood IGRA showed a positive IGRA from BAL cells, possibly indicating a limited local immune response.

**Conclusion:** In Germany, latent infection with *M. tuberculosis,* as defined by a positive *M. tuberculosis*-specific IGRA response on cells from the peripheral blood, is characterized by an increased frequency of $T_{reg}$ cells in the BAL.

## Introduction

Infection 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 Th1 cells [6]. Latent infection with *M. tuberculosis* (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 [7]. Using this definition, close recent contacts with LTBI carry a risk of approximately 2-13 % for the development of active TB within 2 years [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 of advantage 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 ($T_{reg}$ cells) are involved in the maintenance of self-tolerance and the control of immune responses to foreign antigens [10]. Several types of $T_{reg}$ cells have been described and their phenotypic and functional characterisation is under continouus scientific evaluation. A prominent yet not exclusive marker is the Interleukin-2 receptor α-chain CD25 [11]. More specific, yet not purely, is the transcription factor forkhead box P3 (FoxP3) expressed in both CD4+ and CD8+ $T_{reg}$ cells [12]. FoxP3 expression is inversely associated with the expression of the IL-7 receptor CD127 [13, 14]. In PBMC from healthy donors, Liu reported FoxP3 expression in 86.6% (range, 67.4- 93.6%) of $CD25^{+}CD127^{low/-}$ cells, while $CD127^{low/-}CD25^{-}$ contained only 25.5% (14.8%–39.5%) and $CD4^{+}CD127^{+}CD25^{+}$ contained only 22.9% (11.5%–39.2%) of FoxP3 expressing cells.[13] Similarly, Seddiki found that 87%

of $CD4^+CD127^{low}$ cells fell within the $CD25^+Foxp3^+$ gate and conversely, 84% of $CD25^+Foxp3^+$ cells were detected within the $CD4^+CD127^{low}$ gate [14]. Since even $CD4^+$ $CD25^-$ $FoxP3^+CD127^{low}$ cells were found to have Th-1 suppressive properties, the lack of CD127 surface expression is now widely accepted as a marker for $T_{reg}$ cells.

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

$T_{reg}$ 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 positive) $T_{reg}$ cells in peripheral blood mononuclear cells (PBMC) 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 $T_{reg}$ cells are similar for healthy, uninfected subjects and subjects with presumed LTBI, patients with active TB, especially with extrapulmonary disease and extensively drug resistant TB, show a higher $T_{reg}$ frequency in the peripheral blood [18-21].

However, little is known about the local involvement of pulmonary $T_{reg}$ 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 might differ in the percentage of pulmonary $T_{reg}$ cells than TB contacts with a low likelyhood of infection (IGRA negatives) [16, 18, 22]. We investigated the pulmonary and systemic role of $T_{reg}$ cells in IGRA positive (IGRA+) and IGRA negative (IGRA-) TB contact persons 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 centers (Helios Klinikum Emil von Behring Berlin, Evanglische Lungenklinik Berlin, Research Center Borstel, Klinik Donaustauf, Agaplesion Pneumologische Klinik Waldhof Elgershausen, Asklepios Fachkliniken München-Gauting, Krankenhaus Großhansdorf, Lungenfachklinik Immenhausen, Klinikum Nürnberg Nord).

Health care workers (HCWs) were enrolled if they were working in a respiratory medicine department, had regular professional contact to patients with acid-fast bacilli smear-positive TB and had no evidence of active TB. Household contacts (HHCs) who were exposed >40 hours in total were recruited through the Public Health Authority from the City of Hamburg and through the Research Center 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, Abigdon, UK ) was performed according to the manufacturers 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 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 subsegmental bronchus of the middle lobe. Bronchoalveolar lavage was performed with a total volume of 250mL sterile normal saline.

PBMCs were prepared by Ficoll-Hypaque density gradient centrifugation (465g for 42 minutes at ambient temperature) from heparinized blood. After washes with phosphate based buffer (PBS) and second centrifugation (800g for 15 minutes at 4°C), cells were again washed

with PBS and centrifuged (400g for 12 minutes at 4°C). Pellets containing large numbers of red blood cells underwent erythrocytolysis with sterile water and double PBS. PBMC flow cytometry was performed in a laboratory of the Max-Planck-Institute for Infection Biology, Berlin.

Single-cell suspensions from BAL fluid were obtained by passing the BAL fluid through a stainless steel sieve (WMF, 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 (Invitrogen, Catalogue No. MHCD0305), CD4 (Invitrogen, No. MHCD0401), CD16 (Invitrogen, No. MHCD1604), CD25 (Invitrogen, No. MHCD2505), CD40 (Invitrogen, No. CD4005), CD45RO (Invitrogen, No. MHCD45RO04), CD62L (Invitrogen, No. MHCD62L05), CD127(IL-7R)α (R&D Systems, No. FAB 306P), HLA-DR (BD Pharmingen, No. 347402), TLR-4 (NatuTec ebioscience, No. 13-9917-80) by flow cytometry (FACSCalibur; Becton Dickinson, Heidelberg, Germany). BAL cell flow cytometry was performed at a laboratory of the Research Center Borstel.

$T_{reg}$ lymphocytes were defined as CD4+CD25+CD127- as described above [13, 14].

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

## Results

Between August 2008 and May 2011, one hundred one tuberculosis contact persons were recruited for the trial. Blood samples of all participants were taken for immunological analysis prior to the BAL recruitment. BALs were used for FACS analysis, cell culture (data not

shown) and mycobacterial kill assays (data not shown). The FACS data of 47 BAL samples was available for analysis. Of these, 5 (10.6%) were HHCs recruited from the 2 centers mentioned above and 42 (89.4%) were HCWs from 9 hospitals. The BALs of 7 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 technical reasons, i.e. weak staining that prevented a clear distinction between CD127+ and CD127- cells.

Blood samples of 25 HCWs (11 LTBI, 14 non-LTBI) were tested with the QuantiFERON-TB Gold in Tube® system, with a cut off of 0.35 IU IFN-γ. All 5 HHCs blood samples (2 LTBI, 3 non-LTBI) were tested with the T-Spot.TB® system, and so were the 10 remaining blood samples of HCWs (3 LTBI, 7 non-LTBI). All BAL samples were tested with the T-Spot.TB® test.

There were 25 (62.5%) IGRA negative (21 HCWs, 4 HHCs) and 15 (37.5%) IGRA positive (14 HCWs, 1 HHCs) participants, the latter had presumed LTBI by definition. Of the 25 participants with a negative blood IGRA result, 5 (20%) had a positive BAL IGRA result, 1 (4%) BAL IGRA was indeterminate and 19 (76%) were negative. Of the 15 subjects with presumed LTBI, 8 (53.3%) BAL IGRA results were negative and 7 (46.7%) were positive (Figure 2).

The baseline criteria between the two groups were similar (Table 1). Subjects with presumed LTBI were slightly older (54.1 vs 44.4 years; p-value= 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 $T_{reg}$ cells (median, InterQuartile Range -IQR: 2.12, 1.63-3.01) in the BAL compared to unsensitised participants (median, IQR: 0.68, 0.32-0.96; $p<0.0001$) (Table 2). $T_{reg}$ frequencies were consistently

≥1.36% and ≤1.32% of BAL lymphocytes in the LTBI and IGRA negative group, respectively. (Figure 3).

When the subjects were stratified according to their BAL-based IGRA rather than the PBMC IGRA, the differences in $T_{reg}$ frequencies in the BAL remained significant (Figure 4). Higher BAL $T_{reg}$ cell frequencies were associated with a positive BAL IGRA (median, IQR: 1.42; 0.99-2.39) compared to a negative BAL IGRA (0.91, 0.35-1.41). No differences were found for blood $T_{reg}$ cells (9.1, 6.3-9.85 vs. 7.75, 5.05-11.65). Correlating individual $T_{reg}$ cell frequencies from BAL and PBMC for subjects in which both measurements were available, did not reveal any new insights (Figure 5).

No differences in the BAL cells or PBMC were found between contacts with presumed LTBI and IGRA negative subjects analysing several other immunophenotypes (Table 2) of lymphocytes (HLA-DR, CD62L, CD3CD4, CD3CD8) or monocytes (HLA-DR, CD40, TLR-4).

No differences were seen in Bacille Calmette-Guérin (BCG) vaccinated and non-vaccinated subjects. The differences in $T_{reg}$ cell frequencies remained significant when HIV-infected individuals were excluded from the analysis.

## Discussion

$T_{reg}$ 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]. Although our hypothesis may have been influenced by a potential publication bias because non-significant results may not have been published, we postulated that TB contacts with presumed LTBI would differ in the percentage of pulmonary $T_{reg}$ cells compared to non-LTBI contacts.

Higher frequencies of $T_{reg}$ cells were found in the BAL of all subjects with presumed LTBI compared to IGRA negative individuals. This is potentially an indication of ongoing

inflammation triggered by a regional mycobacterial infection. In analogy, recruitment of $T_{reg}$ cells to the site of infection has been described in patients with active TB [18, 27, 30, 31]. Furthermore, the predominant production of both, pro-inflammatory IFN-γ and anti-inflammatory IL-10 by $CD4^+$ cells in the BAL of patients with pulmonary TB has been noted more than 10 years ago [32]. It is therefore possible that the recruitment of $T_{reg}$ 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 $T_{reg}$ cytokine, TGF-$\beta_1$ 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 $T_{reg}$ cells. This contradicts *in vitro* experiments where the presence of $T_{reg}$ cells reduced IFN-γ production and cellular immune responses to MTB antigens in nearly all studies [18, 19, 27, 28, 34, 35]. However, *in vitro* experiments measure an immune response before and after $T_{reg}$ cell arrival in a pre-defined setting with constant numbers of effector T cells and mycobacteria. *In vivo*, the recruitment of $T_{reg}$ 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 and after the advent of $T_{reg}$ cells challenging, if not impossible. Unfortunately, the observational character of our data does not allow a closer characterization of pulmonary $T_{reg}$ cells. Their role as suppressor cells or bystanders ought to be investigated in further studies. We cannot exclude that the phenomenon reflects inflammation-induced secondary recruitment of $T_{reg}$ cells to the lungs with the aim to control excessive inflammatory processes but without effect on mycobacterial load. On the other hand, $T_{reg}$ cells seem to play a key role for the balance between tissue integrity and anti-mycobacterial immune responses, as seen in a macaque model, in which higher post-infection $T_{reg}$ cell frequencies were found in the BAL of animals who developed LTBI rather than active TB [22]. Further studies are required to investigate whether $T_{reg}$ 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 $T_{reg}$ cells. In active TB, the immunological balance fails, inflammation increases and larger numbers of $T_{reg}$ cells are recruited to the site of infection, indicated by higher frequencies of circulating $T_{reg}$ cells [18, 19, 28, 29, 36]. Supporting this finding, infected macaques show decreasing numbers of circulating $T_{reg}$ cells during the first weeks post infection as these are recruited to the lung. After about 5-6 months however, peripheral $T_{reg}$ cells increase only in animals that develop active disease [22].

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

High frequencies of $T_{reg}$ 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 $T_{reg}$ cells decrease after resection of pulmonary cavities [36]. Therefore, $T_{reg}$ cells or their surrogate markers (TGF-β1, IL-10) merit further investigation for the monitoring of TB treatment response.

Five 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 addressed this issue. In one study on household contacts with presumed LTBI (defined by a positive TST), 50% (5 of 10 subjects) showed a ≥3-fold frequency of IFN-γ secreting cells in BAL compared to PBMC upon stimulation with the mycobacterial antigen Ag85 [40].

Apparently, this compartmental enrichment was associated with recent MTB exposure as it was found in only 12% (1 of 13) of TST positive control subjects without recent exposure. In contrast to our own study, Schwander et al. did not include *M. tuberculosis* -exposed, TST-negative subjects preventing a direct comparison.

The phenomenon of localised specific immune responses in healthy TB contacts remains elusive. Dheda and colleagues found a positive MTB specific IGRA in in the BAL of 5 out of 48 non-TB lung disease patients but of these only 2 had a negative blood IGRA result (one patient with a carcinoma, one with a lymphocytic interstitial pneumonitis) [41]. BAL IGRAs were also assessed in two studies of our own group. In a large European study, Jafari al. found a negative blood IGRA in 118 of 250 non-TB patients, and 14 (12%) of them had a positive BAL IGRA [42]. In a smaller trial, 40 out of 84 subjects with a confirmed non-TB diagnosis had a negative blood IGRA, and 6 (15%) of them had a positive BAL IGRA [43]. This trial included 10 subjects with a non-tuberculous mycobacterial infection, 4 of which had a negative blood IGRA but none had a positive BAL IGRA. The non-TB patients in all three studies were suspected to have active tuberculosis but eventually diagnosed with another active respiratory disease. This is a very different setting to our current investigation adressing healty individuals. Their findings however underline that in patients without active tuberculosis, the correlation between systemic and local immune response to *M. tuberculosis* varies.

Since BAL cells are strikingly different from PBMCs cells regarding 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 peripheral blood–derived monocytes, with potential implications for antigen presentation [44]. In patients with active TB, cultured lymphocyte proliferation and IFN-γ production in response to CFP-10 and other antigens is more pronounced in BAL cells than PBMC. 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 PBMC and BAL. Other hypothetical explanations include a compartmentalization effect or a recent MTB exposure triggering a localised pulmonary IFN-γ response first, followed by a systemic response.

Our study has got several limitations. Firstly, we investigated a heterogeneous cohort. For professionally active HCWs, the timepoint and quantity of exposure to *M. tuberculosis* is not clearly defined. In contrast, our HHCs were exposed very closely to one contagious patient only. Secondly, the number of available blood samples for $T_{reg}$ analysis was small due to technical difficulties, i.e. indistinctive or weak CD127 staining and sample failure (Figure 1). Thirdly, we could not establish the role of BAL $T_{reg}$ cells in subjects with presumed LTBI. As there is no established method for depletion or enrichment of functional $T_{reg}$ 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 $T_{reg}$ cells in the BAL compared to IGRA negative 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 $T_{reg}$ cells as markers for the progression to active disease and as markers for treatment response merits further investigation.

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**Table 1. Demographic, epidemiological and clinical characteristics of the enrolled individuals.**

| Variables | | | Groups | | p value |
|---|---|---|---|---|---|
| | | | IGRA-negative (n= 25) | IGRA-positive (n= 15) | |
| ***Mean age (SD), years*** | | | 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 |
| Duration of profession (%) | | <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 hours of exposure to TB patients (%) | | 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 |
| Median (IQR) number of TB patients per year | | | 50 (22.5 - 60.0) | 50 (45.0-150.0) | 0.65 |
| N. 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-positives, (%) | | | 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 from former German territory, (%) | | | 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 |
| IVDUs, (%) | | | 3/25 (12.0) | 3/12 (25.0) | 0.32 |
| Iatrogenic immunosuppression, (%) | | | 4/25 (16.0) | 2/12 (16.7) | 0.96 |
| Smoking, (%) | | Lifelong no smoker | 9/22 (40.9) | 4/12 (33.3) | 0.66 |
| | | Current | 3/22 (13.6) | 5/12 (41.7) | 0.07 |

| | | | | |
|---|---|---|---|---|
| | Previously | 9/22 (40.9) | 5/11 (45.5) | 0.80 |
| TB in blood relations, (%) | | 4/24 (16.7) | 2/15 (13.3) | 0.78 |
| TB in partner, (%) | | 3/24 (12.5) | 1/15 (6.7) | 0.56 |

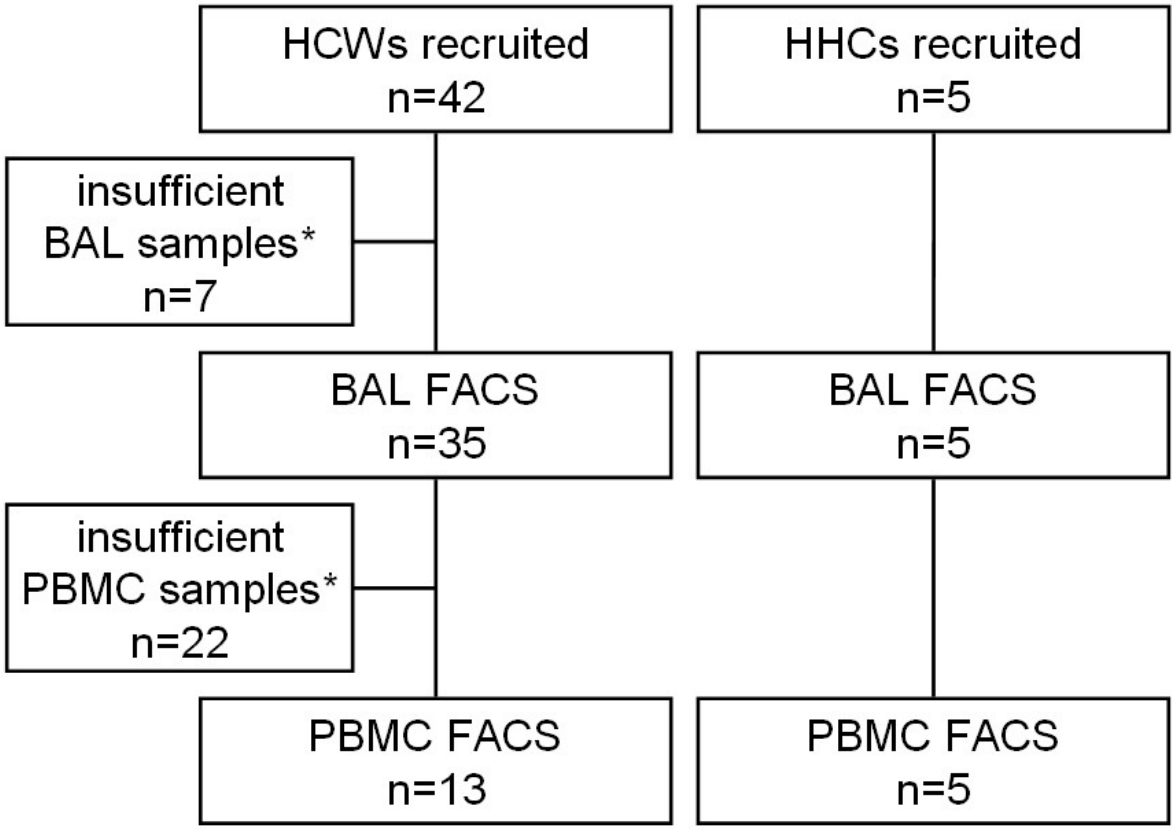
BCG: Bacille Calmette-Guérin
FFP: Filtering Face Piece
HIV: Human Immunodeficiency Virus
IGRA: Interferon-γ Release Assay
IQR: InterQuartile Range
IVDU: Intra-Venous Drug User
SD: Standard Deviation
TB: Tuberculosis
TST: Tuberculin Skin Testing

**Table 2. Frequencies of lymphocyte and monocyte immunophenotypes in bronchoalveolar lavage and among peripheral blood mononuclear cells**

| Phenotype | | IGRA negative | | | IGRA positive (LTBI) | | | p-value |
|---|---|---|---|---|---|---|---|---|
| **BAL samples** | | **n** | **median** | **IQR** | **n** | **median** | **IQR** | |
| Lymphocytes | % of all somatic BAL cells | 25 | 9.9 | 3.815-19.83 | 15 | 9.37 | 3.87-27.7 | 0.77 |
| Macrophages | | 25 | 70 | 55.85-82.85 | 15 | 70.12 | 57.9-80.68 | 0.89 |
| Monocytes | | 25 | 5.8 | 3.45-7.28 | 15 | 5.4 | 4.43-7.84 | 0.56 |
| HLA-DR | % positive of all BAL lymphocytes | 24 | 18.84 | 9.98-23 | 15 | 19.86 | 6.69-30.68 | 0.44 |
| CD16 | | 24 | 2.485 | 1.71-6.355 | 15 | 3.61 | 2.04-7.16 | 0.64 |
| CD62L | | 25 | 5.37 | 3.345-8.23 | 15 | 8.03 | 3.87-13.42 | 0.18 |
| CD25 | | 11 | 2.22 | 0.79-4.09 | 11 | 4.25 | 2.83-5.48 | 0.048 |
| CD127 | | 11 | 43.15 | 27.03-50.68 | 11 | 24.45 | 15.52-34.92 | 0.03 |
| CD3+CD4+ | % of all BAL lymphocytes | 25 | 34.55 | 28.19-50.45 | 15 | 47.22 | 31.77-59.18 | 0.18 |
| CD3+CD8+ | | 25 | 27.2 | 13.03-37.36 | 15 | 25.07 | 13.85-40.65 | 0.74 |
| CD4+CD3+CD45R0 | | 25 | 31.32 | 27.37-42.05 | 15 | 46.1 | 31.28-57.58 | 0.10 |
| CD8+CD3+CD45R0 | | 25 | 22 | 11.79-27.92 | 15 | 18.1 | 14.63-22.55 | 0.74 |
| CD4+CD25+CD127- | | 25 | 0.68 | 0.32-0.955 | 15 | 2.12 | 1.63-3.01 | <0.0001 |
| HLA-DR | MFI of all BAL monocytes | 23 | 284 | 198-445.1 | 14 | 285.1 | 208.5-1106 | 0.56 |
| TLR-4 | | 23 | 70 | 49-91 | 14 | 69.65 | 42.58-114 | 0.89 |
| | | | | | | | | |
| **PBMC samples** | | **n** | **median** | **IQR** | **n** | **median** | **IQR** | **p-value** |
| CD16+ | % positive of all PBMC lymphocytes | 20 | 12.75 | 10.5-15.15 | 14 | 12.55 | 9.4-19.4 | 0.96 |
| CD62L | | 20 | 55.4 | 39.5-67.25 | 14 | 32.1 | 27.65-57.6 | 0.08 |
| CD25 | | 10 | 20.8 | 13.9-50.75 | 10 | 27.6 | 15.35-33.05 | 0.91 |
| CD127 | | 10 | 58.25 | 37.25-68.65 | 10 | 51.95 | 42.6-68.4 | 0.85 |
| CD40+HLA-DR+ | % of all PBMC lymphocytes | 20 | 10.55 | 5.8-14.95 | 14 | 11.1 | 9.1-18.55 | 0.47 |
| CD3+CD4+ | | 20 | 42.1 | 34.45-52.7 | 14 | 44.9 | 29.1-54.3 | 0.90 |
| CD3+CD8+ | | 20 | 20.65 | 13.4-27.65 | 14 | 16.7 | 10.25-21.45 | 0.17 |
| CD4+CD3+CD45R0 | | 25 | 16.46 | 7.961-25.16 | 15 | 21.57 | 11.2-33.81 | 0.71 |
| CD8+CD3+CD45R0 | | 25 | 4.542 | 1.78-9.806 | 15 | 4.744 | 3.752-8.051 | 0.68 |
| CD4+CD25+CD127- | | 11 | 7.7 | 4.6-11.3 | 7 | 9.6 | 5.9-10.1 | 0.47 |
| CD40+HLA-DR | MFI of all PBMC monocytes | 20 | 83.5 | 78.2-86.8 | 14 | 78.65 | 60.8-87.75 | 0.38 |

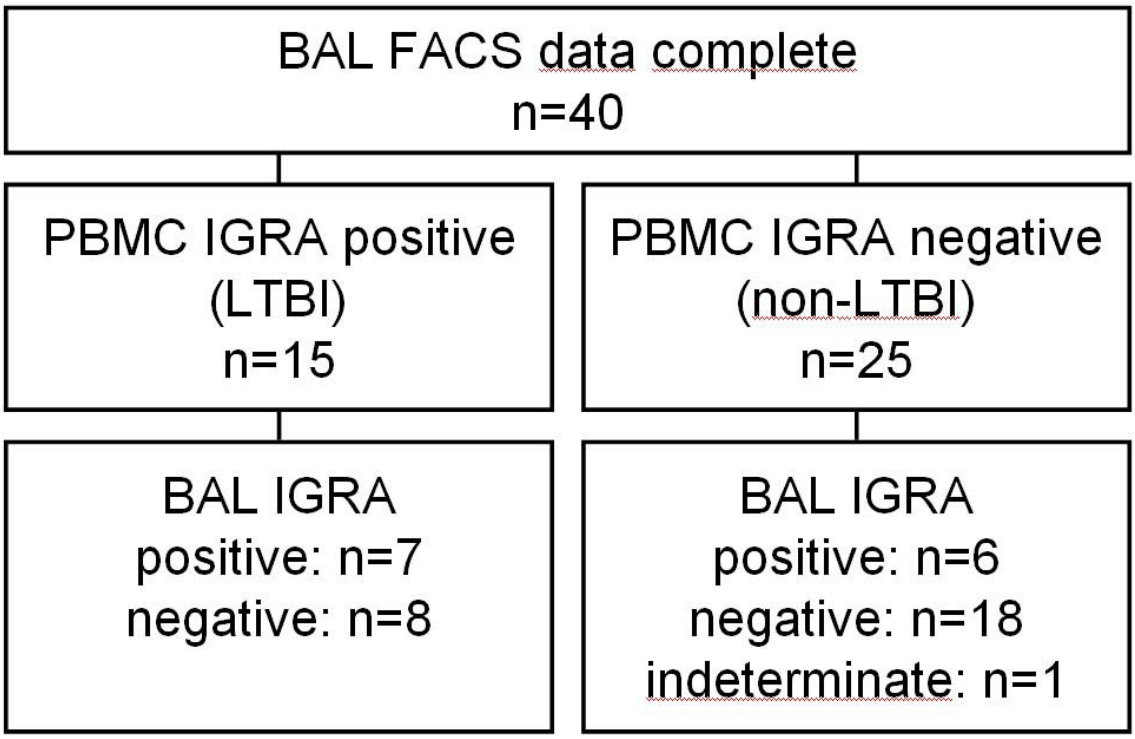
FACS analysis of lymphocytes and monocytes in
BAL or PBMC were performed due to the respective gates in the forward/side-scatter pattern
BAL: Bronchoalveolar Lavage; IGRA: Interferon-γ release assay; MFI: Median fluorescence intensity; LTBI: Latent Tuberculosis Infection; PBMC: Peripheral Blood Mononuclear Cells

**Figure 1. Recruitment flowchart and fluorescence-activated cell sorter sample processing**



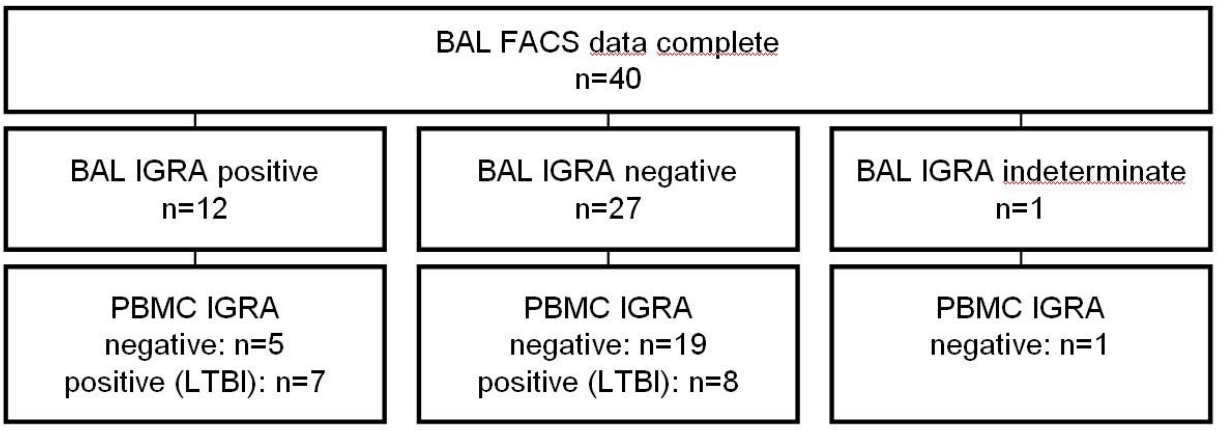

* Number of $T_{reg}$ cells undeterminable due to indistinctive CD127 staining or sample failure
*BAL: BronchoAlveolar Lavage*
*FACS: Fluorescence-Activated Cell Sorter*
*HCWs: Health-Care Workers*
*HHCs: HouseHold Contacts*
*PBMC: Peripheral Blood Mononuclear Cells*

**Figure 2a. Distribution of IGRA results stratified by PBMC IGRA**



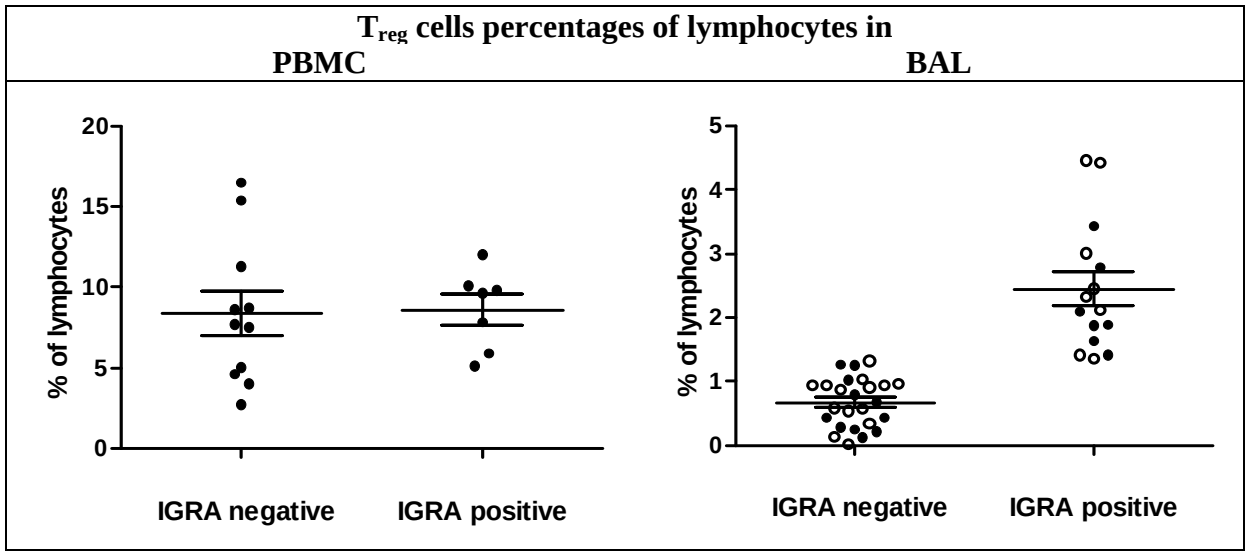

*BAL: BronchoAlveolar Lavage*
*FACS: Fluorescence-Activated Cell Sorter*
*IGRA: Interferon-γ release assay*
*LTBI: Latent Tuberculosis Infection*
*PBMC: Peripheral Blood Mononuclear Cells*

**Figure 2b. Distribution of IGRA results stratified by BAL IGRA**




*BAL: BronchoAlveolar Lavage*
*FACS: Fluorescence-Activated Cell Sorter*
*IGRA: Interferon-γ release assay*
*LTBI: Latent Tuberculosis Infection*
*PBMC: Peripheral Blood Mononuclear Cells*

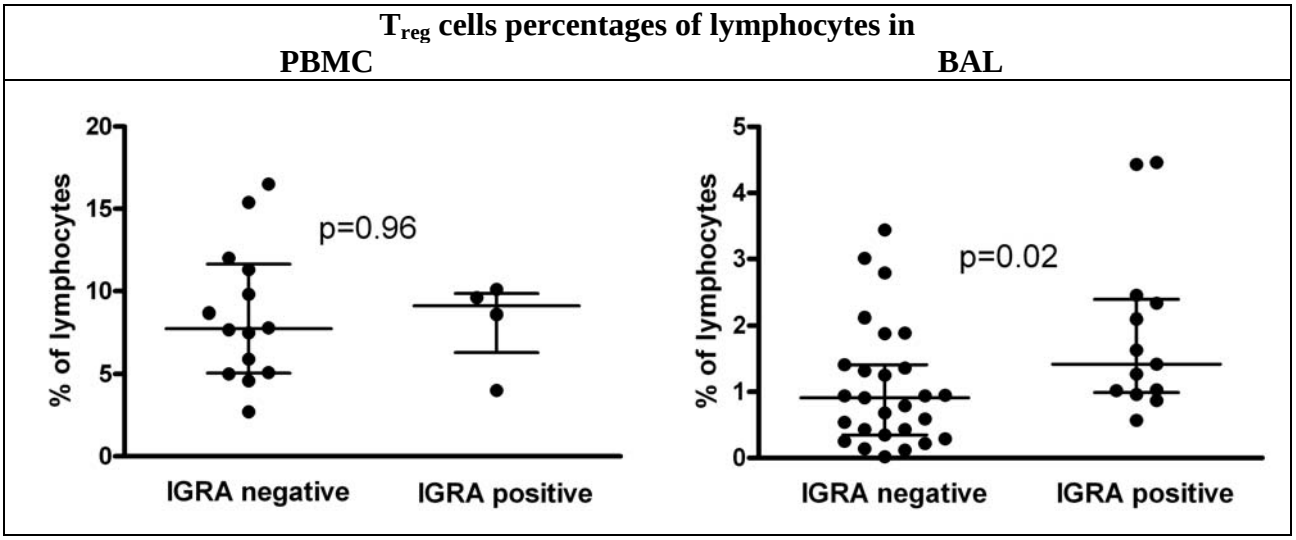
**Figure 3. Frequency of $T_{reg}$ cells stratified by PBMC IGRA status**




IGRA was performed in PBMCs. Black dots: paired PBMC and BAL samples available. White dots: additional BAL samples without PBMC samples. Bars: median and interquartile range

*BAL: BronchoAlveolar Lavage*
*IGRA: Interferon-γ release assay*
*PBMC: Peripheral Blood Mononuclear Cells*

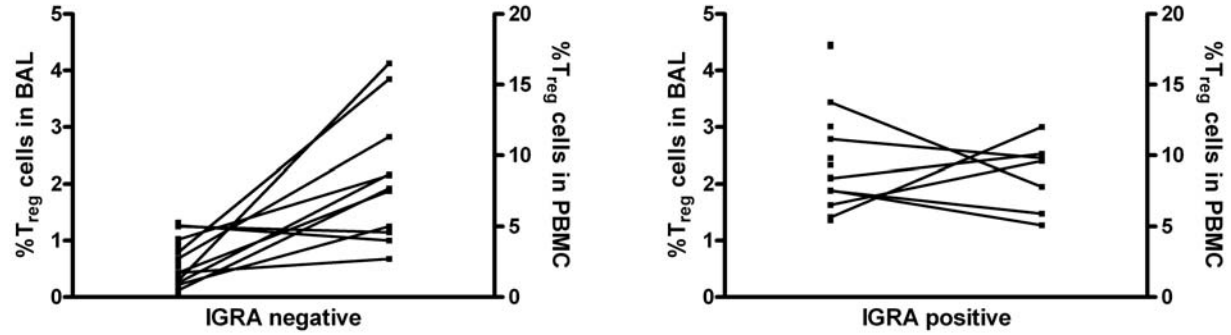
**Figure 4. Frequency of $T_{reg}$ cells stratified by BAL cell IGRA status**




IGRA was performed on BAL cells. Black dots: paired PBMC and BAL samples available. White dots: additional BAL samples without PBMC samples. Bars: median and interquartile range

*BAL: BronchoAlveolar Lavage*
*IGRA: Interferon-γ release assay*
*PBMC: Peripheral Blood Mononuclear Cells*

**Figure 5. Relation between Treg cell frequencies in BAL and PBMC in subjects with negative or positive PBMC-IGRA.**



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