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Early dynamics of T helper cell cytokines and T regulatory cells in response to treatment of active *Mycobacterium tuberculosis* infection

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Summary

Biomarkers that can identify tuberculosis (TB) disease and serve as markers for efficient therapy are requested. We have studied T cell cytokine production [interferon (IFN)-γ, interleukin (IL)-2, tumour necrosis factor (TNF)-α] and degranulation (CD107a) as well as subsets of $CD4^+$ T regulatory cells (T_{regs}) after *in-vitro Mycobacterium tuberculosis* (*Mtb*) antigen stimulation [early secretory antigenic target (ESAT)-6, culture filtrate protein (CFP)-10, antigen 85 (Ag85)] in 32 patients with active tuberculosis (TB) disease throughout 24 weeks of effective TB treatment. A significant decline in the fraction of *Mtb*-specific total IFN-γ and single IFN-γ-producing T cells was already observed after 2 weeks of treatment, whereas the pool of single $IL\text{-}2^+$ cells increased over time for both $CD4^+$ and $CD8^+$ T cells. The T_{reg} subsets $CD25^{high}CD127^{low}$, $CD25^{high}CD147^{++}$ and $CD25^{high}CD127^{low}CD161^+$ expanded significantly after *Mtb* antigen stimulation *in vitro* at all time-points, whereas the $CD25^{high}CD127^{low}CD39^+$ T_{regs} remained unchanged. The fraction of $CD25^{high}CD127^{low}$ T_{regs} increased after 8 weeks of treatment. Thus, we revealed an opposing shift of T_{regs} and intracellular cytokine production during treatment. This may indicate that functional signatures of the $CD4^+$ and $CD8^+$ T cells can serve as immunological correlates of early curative host responses. Whether such signatures can be used as biomarkers in monitoring and follow-up of TB treatment needs to be explored further.

Keywords: cytokines, T cells, treatment, T_{regs}, tuberculosis

Introduction

Mycobacterium tuberculosis (*Mtb*), the causative agent of tuberculosis (TB), kills nearly 2 million people annually and is still a major health threat [1]. As total TB cases seem to decrease slightly, the development of multi-drug resistant (MDR) TB is of increasing concern [1]. The diagnosis and treatment of active TB relies on the identification of *Mtb* by culture and resistance testing to enable the accurate choice of chemotherapy. However, surrogate biomarkers for bacterial clearance are needed for evaluation of treatment efficacy and as readouts in clinical trials evaluating new TB treatment modalities [2]. Biomarkers are especially important in the combat against MDR TB where prolonged duration of therapy with inefficient, toxic and expensive drugs is a major threat to TB cure [3].

The host defence against *Mtb* depends upon effective innate and adaptive immune responses that suppress, but generally fail to eradicate, the infection [4]. Evidence from both human and animal models suggests that $CD4^+$ and $CD8^+$ T cells play an important role in the protective immune responses against *Mtb*, where $CD4^+$ T helper type 1 (Th1) cells are of crucial importance [5]. The induction of protective interferon (IFN)-γ^+ T cell responses is interleukin (IL)-12-dependent, which is secreted mainly by activated dendritic cells and macrophages [6]. IFN-γ provides a strong effector response to *Mtb*, but tumour necrosis factor (TNF)-α is also essential to control disease progression, demonstrated by the reactivation of TB in patients given anti-TNF-α treatment [7]. Polyfunctional $CD4^+$ T cells (co-producing IFN-γ, TNF-α and IL-2) have been reported to serve as correlates of protective immunity in vaccination studies [8,9]. Harari *et al.* observed a shift from monofunctional to polyfunctional $CD4^+$ T cells in a small cohort of five patients during effective TB treatment [10], while others have shown that polyfunctional T cells correlate with active *Mtb* disease [11,12]. Thus, the role of polyfunctional T cells in TB still needs to be clarified.

T cell immunity contains *Mtb* infection *in vivo*, but may also cause inflammatory damage to the host. Regulatory T cells (T_{regs}) serve a pivotal role in controlling immune responses by preventing tissue damage due to excessive inflammation, but immune suppression may also prevent microbial eradication and facilitate chronic infection [13]. There seems to be a correlation between the severity of TB infection and T_{regs}, as studies have reported higher levels of circulating T_{regs} in patients with active TB than in subjects with latent TB infection [14–16]. The dynamics of T_{regs} during treatment of active TB is still not clarified, although reduced numbers during treatment have been described [17].

In this longitudinal prospective study we show that *Mtb*-specific IFN-γ-producing T cells already decline in TB patients during efficient TB treatment after 2 weeks, whereas the contribution of single IL-2$^+$ cells to the cytokine-producing T cell pool increases over time. We present data of exploratory T_{reg} subsets and demonstrate that *Mtb*-specific T_{reg} responses are maintained in TB disease and a transient increase in *ex-vivo* T_{reg} levels occurs during therapy. Thus, these data on the dynamics of T_{regs} and intracellular cytokine patterns may contribute to a better understanding of the role of regulatory mechanisms in TB infection and motivate further studies of biomarkers for treatment responses.

Material and methods

Study participants

Patients with active TB infection were included prospectively at the department of Infectious Diseases, Oslo University Hospital, Norway and followed longitudinally during 24 weeks of standard TB drug combination chemotherapy (Table 1). All subjects had drug-sensitive TB and were HIV-uninfected. Patients with TB disease were categorized into pulmonary TB (PTB) or extrapulmonary TB (EPTB) and in 'low symptom score' defined as either asymptomatic (detected by screening) or with only one of the following symptoms: fever >38°C, weight loss, wasting, cough or night-sweat. Patients with two or more symptoms were categorized into the 'high symptom score' group. Blood samples were obtained before treatment and after 2, 8 and 24 weeks of treatment. The study was approved by the Regional Ethics Committee and written informed consent was obtained from all participants.

Cell separation and preparation

Peripheral blood mononuclear cells (PBMC) were isolated in cell preparation tubes [CPT; Becton Dickinson (BD) Bioscience, San Jose, CA, USA] with sodium heparin, and analysed immediately or cryopreserved in 90% fetal calf serum (FCS; Sigma, St Louis, MO, USA)/10% dimethylsulphoxide (DMSO) and stored at −145°C until analysis. Frozen PBMC from 20 patients was used for the cytokine analysis and fresh PBMC from 12 patients for the T_{reg} analysis.

Table 1. Patient characteristics.

	Cytokine ($n = 20$)	T_{reg} ($n = 12$)
Median age in years (range)	29 (21–79)	26 (21–72)
Female (% of total)	12 (60)	6 (50)
Origin (% of total)		
Africa	9 (45)	5 (42)
Asia	7 (35)	4 (33)
Europe	4 (20)	3 (25)
Localization		
Pulmonary	12 (60)	6 (50)
Extrapulmonary*	8 (40)	6 (50)
IGRA assay† (positive : negative : no data)	14 : 0:6	10 : 1 : 1
ESR‡	42 (22–101)	33 (7–92)
Low : high symptom score§	5 : 15	6 : 6

*Lymph node, pericard, abdominal, cutaneous abscess, osteomyelitis. †QuantiFERON®-TB. ‡Erythrocyte sedimentation rate (ESR). §High ≥2 of the following symptoms: fever (>38C°), weight loss, wasting, cough and night-sweat. Low = 1 symptom or asymptomatic/detected by screening. T_{reg} = regulatory T cell; IGRA = interferon-γ-release assay.

Antigens

Mtb-derived 10-kDa culture filtrate protein (CFP-10), 6 kDa early secretory antigenic target (ESAT-6) (both 15mer overlapping peptide pools, >80% purity; Schäfer, Hadsund, Denmark) and antigen 85 (Ag85) complex (15mer overlapping peptide, >85% purity; Genscript, Herlev, Denmark) were used alone or in combination at a final concentration of 1–1·5 μg/ml. Staphylococcal enterotoxin B (SEB), 0·5 μg/ml; Sigma-Aldrich, St Louis, MO, USA) was used as positive control.

Flow cytometric analysis

Intracellular cytokine analysis and CD107a expression. Antigen-specific responses were measured in peptide-stimulated thawed PBMC after overnight resting; $7{\cdot}5 \times 10^5$ cells/well were stimulated in sterile conditions with peptide pools; ESAT-6/CFP-10 (E6C10) or Ag85, SEB or serum-free medium (AIM V; Gibco Invitrogen, Carlsbad, CA, USA) with 0·1% highly purified human albumin in short-term (6 h) cultures. Only PBMC with viability >85% were included. Brefeldin A (BD Bioscience) (final concentration 5 μg/ml) and monensin (BD Bioscience) (final concentration 5 μg/ml) were added at the time of stimulation with anti-CD107a [18,19]. After 8 h rest at 4°C, cells were washed and stained with live/dead discriminator in azide-free and serum/protein-free phosphate-buffered saline (PBS)

followed by CD4 and CD3 staining. PBMC were then washed, permeabilized (Cytofix-Cytoperm kit; BD Bioscience) according to the manufacturer's instructions and stained for intracellular cytokines (IFN-γ, TNF-α and IL-2). The following directly conjugated monoclonal antibodies were used: anti-CD3-peridinin chlorophyll (PerCP)-cyanin 5·5 (Cy5·5), anti-CD4-V500, anti-TNF-α-phycoerythrin (PE), anti-IFN-γ-PE-Cy7, anti-IL-2-allophycocyanin (APC), anti-CD107a-fluorescein isothiocyanate (FITC) (BD Bioscience) and live/dead discriminator Fixable Viability Dye eFluor® 450 (eBioscience, San Diego, CA, USA).

T_{regs} analysis. Freshly isolated PBMC at 5×10^5 cells/well were stimulated with anti-CD28 (0·5 ug/ml), peptide pools (ESAT-6, CFP-10, ESAT-6/CFP-10 (E6C10) or AIM alone for 6 hours at 37°C in 5% CO_2 incubator. Cells were harvested after 14–18 h rest at 4°C, erythrocyte lysed, and then surface-stained in the dark. The following directly conjugated monoclonal antibodies were used: anti-CD3-Pacific Blue, anti-CD4-AmCyan, anti-CD25-Per-CP-Cy5·5, anti-CD161-PE, anti-CD39-APC, anti-CD147-FITC (BD Bioscience) and anti-CD127 PE-Cy7 (eBioscience).

Flow cytometry analyses. Flow cytometric acquisition was performed on a BD FACS Canto II. At least 10 000 $CD4^+$T and $CD8^+$ cells were analysed for the cytokine analysis; gating and analysis were made with FACS Diva software version 6 (BD Bioscience) or FlowJo version 10 (TreeStar Inc, Ashland, OR, USA). Dead cells were excluded from the lymphocyte population before gating on $CD4^+$ and $CD8^+$ cytokine-producing populations (Supporting information, Fig. S1). Frequencies (percentage of parent population) of *Mtb* antigen-stimulated cytokine-producing T cells were obtained after subtracting background values (as determined by the unstimulated control cultures). A cut-off of 0·01% was used and values below this were set to zero in accordance with other studies [20]. Total IFN-γ^+, IL-2^+ or TNF-α^+ describe all $CD4^+$ or $CD8^+$ cells positive for the cytokine measured, while Boolean gating strategy was used to create cytokine combinations defined as: polyfunctional (IFN-γ^+ IL-2^+ TNF-α^+), double-positive (IFN-γ^+ IL-2^+ or IL-2^+ TNF-α^+ or IFN-γ^+ TNF-α^+) and single-positive (IFN-γ^+ or IL-2^+ or TNF-α^+)-producing $CD4^+$ and $CD8^+$ T cells. Different T_{reg} subsets were defined as $CD3^+CD4^+CD25^{high}CD127^{low}$ [21], $CD4^+CD25^{high}CD127^{low}CD161^+$, $CD4^+CD25^{high}CD127^{low}CD39^+$ and $CD4^+CD25^{high}CD147^{++}$.

Statistics

Statistical analyses were performed by using Statistica version 7·0 (Statsoft, Tulsa, OK, USA). Non-parametric statistical methods were applied. The Mann–Whitney *U*-test was applied for groupwise comparison, and the two-tailed Wilcoxon's matched-pair test was used for dependent variables. Fisher's exact test (2×2) was used for analysis of categorical variables. A significance level of 0·05 was used. All values are presented as median and interquartile range (IQR). Graphic presentations were made using Prism version 5·04 and version 6 software (GraphPad, San Diego, CA, USA).

Results

Study participants

Thirty-two HIV-uninfected patients with culture-confirmed fully susceptible TB were followed longitudinally during 24 weeks of standard anti-TB drug chemotherapy. Demographic and clinical characteristics are summarized in Table 1. There were 18 PTB and 14 EPTB cases. Eleven patients were classified into the 'low symptom score' group (≤1 symptom) and 21 patients into the 'high symptom score' group (≥2 symptoms). A total of 78% of the PTB cases and 50% of the EPTB cases were classified in the 'high symptom score' group ($P > 0{\cdot}05$). All patients responded to treatment, PTB patients with sputum conversion and culture clearing, whereas EPTB patients demonstrated clinical improvement and normalization of inflammation parameters (ESR).

Dynamics of cytokine responses in active TB infection during TB therapy

We analysed changes of total and various combinations of $CD4^+$ and $CD8^+$ T cell cytokine production after *in-vitro Mtb* antigen stimulation [ESAT-6/CFP-10 (E6C10) and Ag85] during 24 weeks of effective TB treatment. At baseline, the majority of the patients had responses above cut-off for the different cytokine combinations (Supporting information, Fig. S2). Total IFN-γ, IL-2 and TNF-α responses were detected in 70–80%, 50–60% and 50–60% of the patients for the $CD4^+$ T cell subset and in 65–70%, 45% and 50–65% for the $CD8^+$ T cell subset, dependent on the antigen, while IFN-γ^+ IL-2^+ T cells were detected in only a few patients at baseline with levels just above cut-off.

TB antigen-specific $CD4^+$ T cell cytokine responses. Within the $CD4^+$ T cell subset, the fraction of total IFN-γ-producing cells declined from baseline to week 2 (Ag85; $P = 0{\cdot}013$) and week 8 (E6C10; $P = 0{\cdot}004$) (Fig. 1a,b). A corresponding decline was seen for the single IFN-γ-producing cells from baseline to week 2 (E6C10; $P = 0{\cdot}047$ and Ag85; $P = 0{\cdot}013$) and week 8 (E6C10; $P = 0{\cdot}033$) (Fig. 2a,b). An initial reduction, although not significant, was also seen for total IL-2 and TNF-α-producing $CD4^+$ T cells. This was followed by increased responses towards week 24 for all cytokines, although only significant for total IFN-γ after Ag85 stimulation ($P = 0{\cdot}043$) (Fig. 1a,b). Similar changes were found for the $CD4^+$ IFN-γ^+ TNF-α^+ T cells, which first decreased at week 2 followed by a significant increase between weeks 2 and 24 (Ag85; $P = 0{\cdot}037$) (Fig. 2b). In

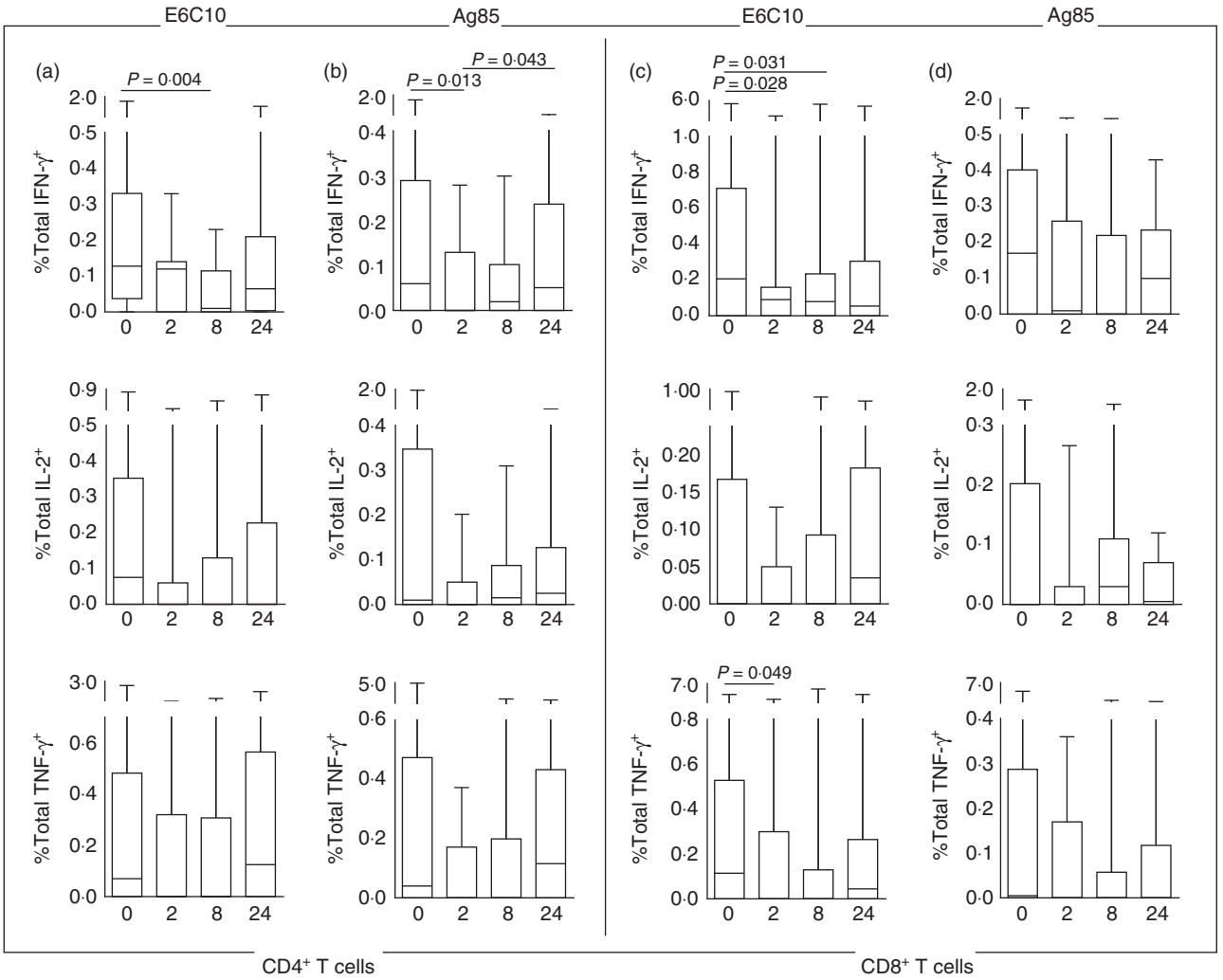

Fig. 1. Total *Mycobacterium tuberculosis* (*Mtb*) antigen-specific interferon (IFN)-γ^+, interleukin (IL)-2^+ or tumour necrosis factor (TNF)-α^+ T cell responses at different time-points of tuberculosis (TB) treatment: baseline ($n = 20$), week 2 ($n = 11$), week 8 ($n = 18$), week 24 ($n = 20$). (a) Early secretory antigenic target (ESAT)-6/culture filtrate protein (CFP)-10 (E6C10)-stimulated $CD4^+$ T cells. (b) Antigen 85 (Ag85)-stimulated $CD4^+$ T cells. (c) E6C10-stimulated $CD8^+$ T cells. (d) Ag85-stimulated $CD8^+$ T cells. *P*-values calculated by Wilcoxon's matched-pairs test. Plots are shown with median, interquartile range (IQR) and minimum/maximum values.

contrast, no significant changes were observed for the $CD4^+$ TNF-α^+ IL-2^+ T cells. Polyfunctional (IFN-γ^+ IL-2^+ TNF-α^+) $CD4^+$ T cell responses were detected in 70% (E6C10) and 45% (Ag85) of the patients at baseline, but in only 60% and 35%, respectively, after 24 weeks (Supporting information, Fig. S2a,b). Further, there were no significant changes observed in the fractions of polyfunctional $CD4^+$ T cells during treatment (Fig. 2a).

TB antigen-specific $CD8^+$ T cell cytokine responses. $CD8^+$ T cell cytokine responses also decreased after 2 weeks of treatment followed by an increase at week 24, although these findings were not consistent for all combinations of cytokines. The fraction of total IFN-γ-producing $CD8^+$ T cells decreased significantly from baseline to week 2 (E6C10; $P = 0{\cdot}028$) and week 8 (E6C10; $P = 0{\cdot}031$) (Fig. 1c) and single IFN-γ-producing cells was also reduced at week 2 (E6C10; $P = 0{\cdot}028$) (Fig. 2c). We observed a corresponding decline for the total TNF-α-producing $CD8^+$ T cells from baseline to week 2 (E6C10; $P = 0{\cdot}049$), whereas for the total IL-2-producing $CD8^+$ T cells the reduction was not significant (Fig. 1c). Similarly, the $CD8^+$ IFN-γ^+ TNF-α^+ T cells decreased from baseline to weeks 2 and 24 (E6C10; $P = 0{\cdot}035$ and $P = 0{\cdot}025$) (Fig. 2c). As observed in the $CD4^+$ population, single IL-2-producing $CD8^+$ T cells showed a significant increase from weeks 2 to 24 (E6C10; $P = 0{\cdot}046$) (Fig. 2c). Polyfunctional $CD8^+$ T cell responses were detected only in 65% (E6C10) and 25% (Ag85) of the patients at baseline, and even fewer patients elicited a polyfunctional response after 24 weeks of treatment (45% for E6C10 and 10% for Ag85) (Supporting information, Fig. S2c,d). In patients with detectable responses, the

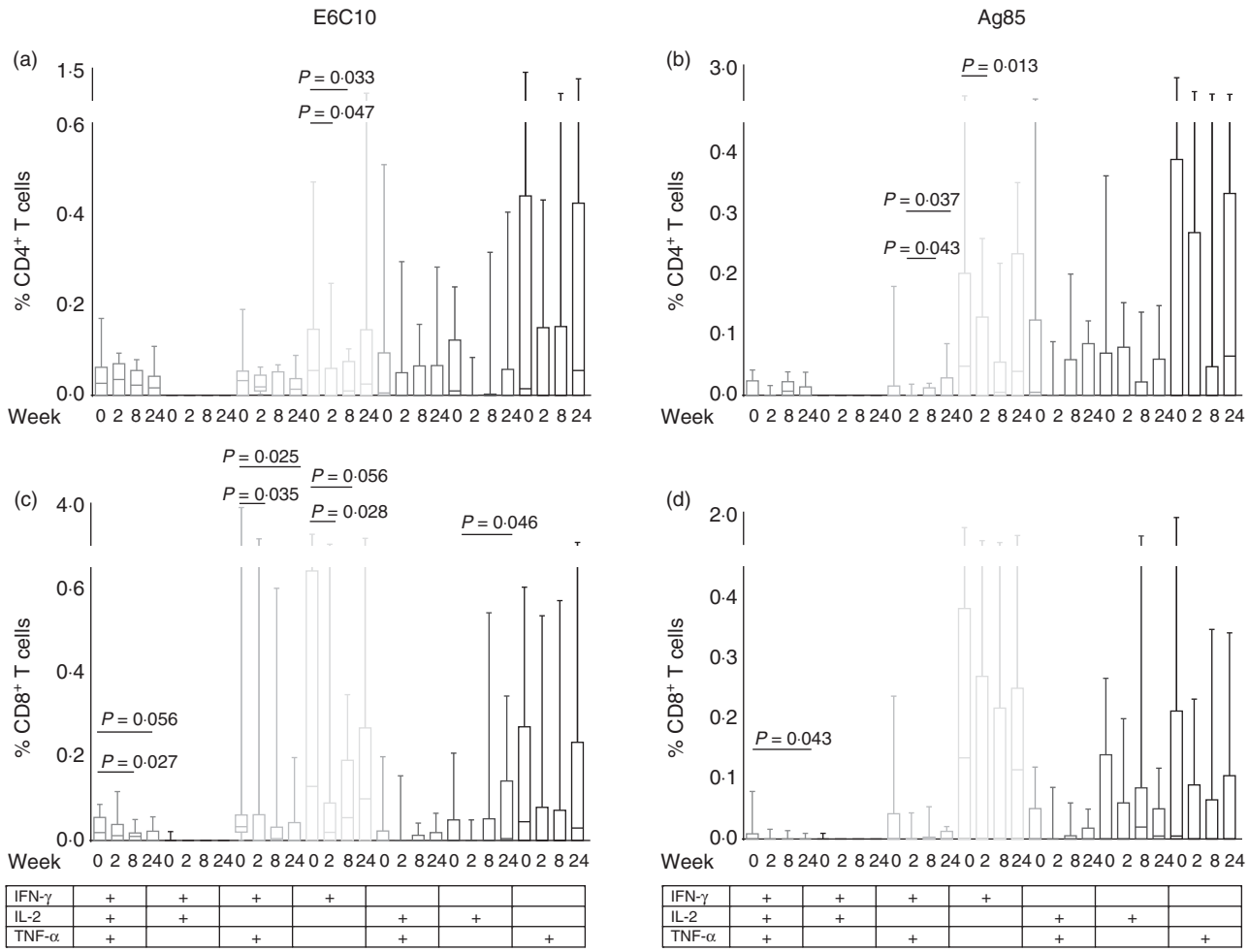

Fig. 2. *Mycobacterium tuberculosis* (*Mtb*) antigen-specific cytokine T cell subsets at different time-points of tuberculosis (TB) treatment: baseline ($n = 20$), week 2 ($n = 11$), week 8 ($n = 18$), week 24 ($n = 20$). Boolean gating strategy was used to create cytokine combinations of single-producing, duo-producing and polyfunctional T cells. (a) Early secretory antigenic target (ESAT)-6/culture filtrate protein (CFP)-10 (E6C10)-stimulated $CD4^+$ T cells. (b) Antigen 85 (Ag85)-stimulated $CD4^+$ T cells. (c) E6C10 stimulated $CD8^+$ T cells. (d) Ag85-stimulated $CD8^+$ T cells. *P*-values calculated by Wilcoxon's matched-pairs test. Plots are shown with median, interquartile range (IQR) and minimum/maximum values.

fraction of polyfunctional $CD8^+$ T cells declined from baseline to weeks 8 (E6C10; $P = 0{\cdot}027$) and 24 (Ag85; $P = 0{\cdot}043$) (Fig. 2c,d).

Degranulating CD107a$^+$ T cells. Analyses were also performed to explore changes of degranulating $CD107a^+$ T cells in response to TB therapy. In both $CD4^+$ and $CD8^+$ T cells, a down-regulation of CD107a after 2 weeks of treatment followed by an increase towards week 24 was observed, although not significant (data not shown).

Dynamics in cytokine patterns. We then compared the contribution of the various cytokine combinations to the overall pool of cytokine-producing T cells during the treatment period (Fig. 3). The majority of $CD4^+$ and $CD8^+$ T cells were single-cytokine producers at all time-points, while the fraction of polyfunctional T cells was low. However, we detected an increased contribution of polyfunctional and duo-cytokine-producing T cells at week 2 followed by a decrease at week 24, while an opposite pattern was observed for single-cytokine-producing T cells, most evident in the E6C10-stimulated cells.

Among the single-cytokine-producing $CD4^+$ T cells, the TNF-α^+ subset and, to a lesser extent, the IFN-γ^+ cells, dominated. This was consistent over time, but there was a decrease in TNF-α^+ cells throughout therapy (Fig. 3b). In contrast, the proportion of the single IL-2^+ cells, although less dominant at baseline, increased towards week 24 for both *Mtb* antigens (Fig. 3a,b). The opposite was seen for the TNF-α^+ IL-2^+ cells, indicating a shift from duo to single IL-2-producing cells.

In the $CD8^+$ T cell population, the single IFN-γ^+ cells contributed as much as the TNF-α^+ subset (Fig. 3c,d). Throughout the treatment period the most prominent finding was a decline in single producing TNF-α^+ cells, while IL-2^+ cells increased correspondingly. The single

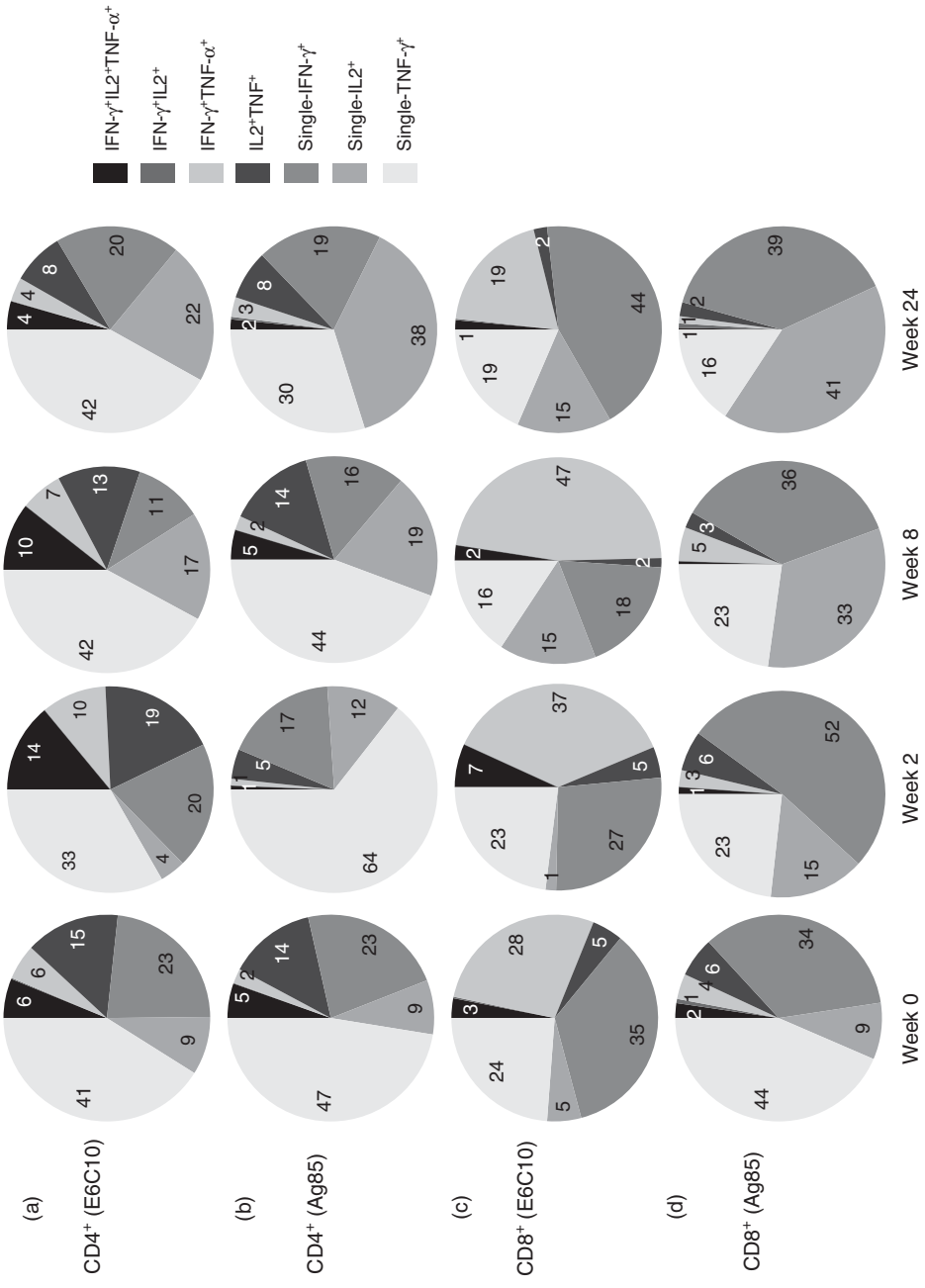

Fig. 3. Piecharts representing percentages of the various cytokine combinations to the overall pool of cytokine producing $CD4^+$ and $CD8^+$ T cells at different time-points during treatment: week 0 ($n = 20$), week 2 ($n = 11$), week 8 ($n = 18$) and week 24 ($n = 20$). (a) Early secretory antigenic target (ESAT)-6/culture filtrate protein (CFP)-10 (E6C10)-stimulated $CD4^+$ T cells. (b) Antigen 85 (Ag85)-stimulated $CD4^+$ T cells. (c) E6C10-stimulated $CD8^+$ T cells. (d) Ag85-stimulated $CD8^+$ T cells.

IFN-γ^+ cells were reduced at week 2 followed by an increase at week 24, in contrast to the IFN-γ^+ TNF-α^+ subset that increased initially followed by a decline at week 24, most noticeable for E6C10-stimulated cells for both cytokine populations (Fig. 3c).

Cytokine profiles related to symptoms and localization of disease during TB therapy

We studied T cell responses after *in-vitro Mtb* stimulation in patients with EPTB compared to PTB and found a tendency of overall higher levels of cytokine production in the EPTB group compared to the PTB group both at baseline and during therapy, but only significant, however, for polyfunctional CD4$^+$ T cells (E6C10, week 24, $P = 0{\cdot}031$) (Supporting information, Fig. S3).

In the CD8$^+$ T cell population, we found significant higher levels of both total and single IL-2-producing cells in the EPTB group compared to the PTB group at baseline (E6C10; $P = 0{\cdot}034$ and $P = 0{\cdot}011$, respectively). Further, single IL-2-producing CD8$^+$ T cells increased significantly (E6C10; $P = 0{\cdot}004$) after 8 weeks of therapy in the EPTB group (data not shown).

We did not detect any significant differences between the 'low symptom score' and 'high symptom score' groups, either at baseline or during therapy (data not shown). However, within the group with 'high symptom score' the frequencies of total IFN-γ- and total IL-2-producing cells decreased from baseline to week 2 for both the CD4$^+$ T cells (Ag85; $P = 0{\cdot}015$ and $P = 0{\cdot}027$, respectively) and the CD8$^+$ T cells (E6C10; $P = 0{\cdot}035$ and Ag85; $P = 0{\cdot}043$, respectively). These changes were not seen for the 'low symptom score' group.

CD25^{high}CD127^{low} T_{regs} and explorative T_{reg} subsets during TB therapy

T_{regs} were analysed in fresh PBMC from 12 TB patients before and after 8 and 24 weeks of therapy. The fraction of CD4$^+$CD25^{high}CD127^{low} T_{regs} increased significantly after *in-vitro Mtb* antigen stimulation throughout the whole observation period (Fig. 4a). Further, there was a significant increase of CD4$^+$CD25^{high}CD127^{low} T_{regs} from baseline to week 8 in both unstimulated [7·1% (5·4–8·3) *versus* 8·2% (7–10·6), $P = 0{\cdot}032$] (Fig. 4a) and *Mtb* antigen-stimulated [CFP-10; 10·9% (8·1–16·4) *versus* 12·1% (9·2–16·6),

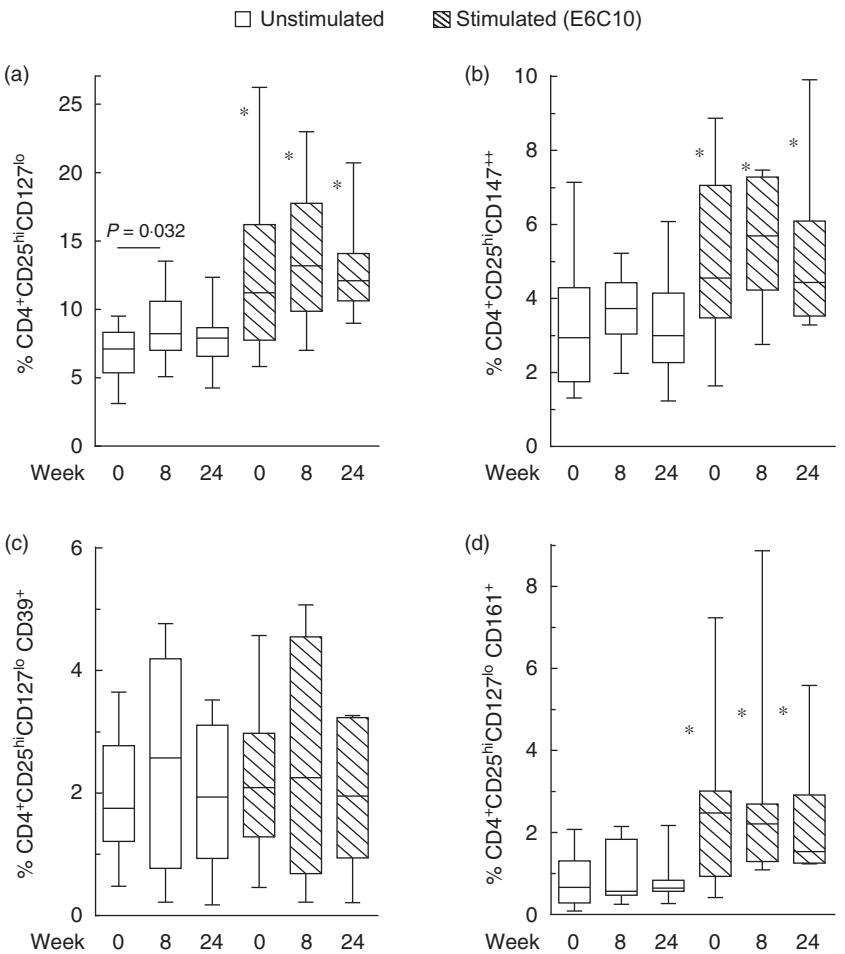

Fig. 4. The fraction of regulatory T cell (T_{reg}) subsets in tuberculosis (TB) patients before ($n = 12$) and after 8 ($n = 11$) and 24 weeks ($n = 11$) of TB treatment in unstimulated (white bars) and early secretory antigenic target (ESAT)-6/culture filtrate protein (CFP)-10 (E6C10)-stimulated peripheral blood mononuclear cells (PBMC) (hatched bars). (a) CD4$^+$CD25^{hi}CD127^{low}, (b) CD4$^+$CD25^{hi}CD127^{low}CD147^{++}, (c) CD4$^+$CD25^{hi}CD127^{low}CD39$^+$ and (d) CD4$^+$CD25^{hi}CD127^{low}CD161$^+$. *Significant changes ($P < 0{\cdot}005$) between unstimulated and E6C10-stimulated samples at corresponding time-points. *P*-values calculated by Wilcoxon's matched-pairs test. Plots are shown with median, interquartile range (IQR) and minimum/maximum values.

$P = 0{\cdot}039$] $CD4^+$ T cells with high levels even after 24 weeks of treatment.

We further analysed the dynamics of different explorative T_{reg} markers throughout therapy. CD147 has been suggested as a marker of activated and highly suppressive T_{regs} [22]. Before therapy, 2·9% (1·7–4·3) of the $CD4^+$ cells were $CD25^{high}CD147^{++}$ (Fig. 4b). We observed similar trends as described for the $CD4^+CD25^{high}CD127^{low}$ population, with higher levels of $CD4^+CD25^{high}CD147^{++}$ T cells after *in-vitro Mtb* antigen stimulation. An increase at week 8 followed by a slight decrease towards week 24 was also seen for both unstimulated and *Mtb* peptide-stimulated T_{regs} expressing CD147, although not significantly. CD161 characterizing a suppressive subpopulation of T_{regs} that produces IL-17 [23] was expressed on 8·6% (2·6–16·4) of the $CD4^+CD25^{high}CD127^{low}$ T cells at baseline, and this T_{reg} subset constituted 0·7% (0·3–1·3) of the $CD4^+$ T cells (Fig. 4d). CD161 expression also became significantly up-regulated after *in-vitro Mtb* antigen stimulation, but no significant changes were detected during treatment. Finally CD39, an ectoenzyme associated specifically with T_{regs} [24], was expressed on 32·6% (5·6–41·2) of the $CD25^{high}CD127^{low}$ T_{regs} at baseline. The decline in CD39 expression on T_{regs} in response to *Mtb* antigen stimulation was compensated for by a corresponding increase in CD39-negative cells (Supporting information, Fig. S1). Thus, 1·7% (1·2–2·7) of the $CD4^+$ cells were $CD25^{high}CD127^{low}CD39^+$ with no significant changes over time (Fig. 4c).

Dynamics of T_{regs} related to symptoms and localization of disease during TB therapy

The possible association between $CD4^+CD25^{high}CD127^{low}$ T_{regs}, symptoms and disease localization was also studied. There were no significant differences in *ex-vivo* T_{reg} levels at baseline between the 'low symptom score' and 'high symptom score' groups (Fig. 5a). However, in the 'low symptom score' group we observed a significant increase in the fraction of T_{regs} in response to *in-vitro Mtb* antigen stimulation, both at baseline (E6C10; $P = 0{\cdot}015$) and during therapy (E6C10; week 8, $P = 0{\cdot}032$; week 24, $P = 0{\cdot}008$). A corresponding increase in T_{regs} was seen in the 'high symptom score' group only at the end of treatment (E6C10; week 24, $P = 0{\cdot}026$).

When grouping the patients according to disease localization we found comparable proportions of T_{regs} in the PTB and EPTB groups (Fig. 5b). The fraction of T_{regs} increased after *in-vitro Mtb* peptide stimulation both before and throughout therapy, most pronounced and only significant for the EPTB group (E6C10; baseline $P = 0{\cdot}009$ and week 24 $P = 0{\cdot}004$).

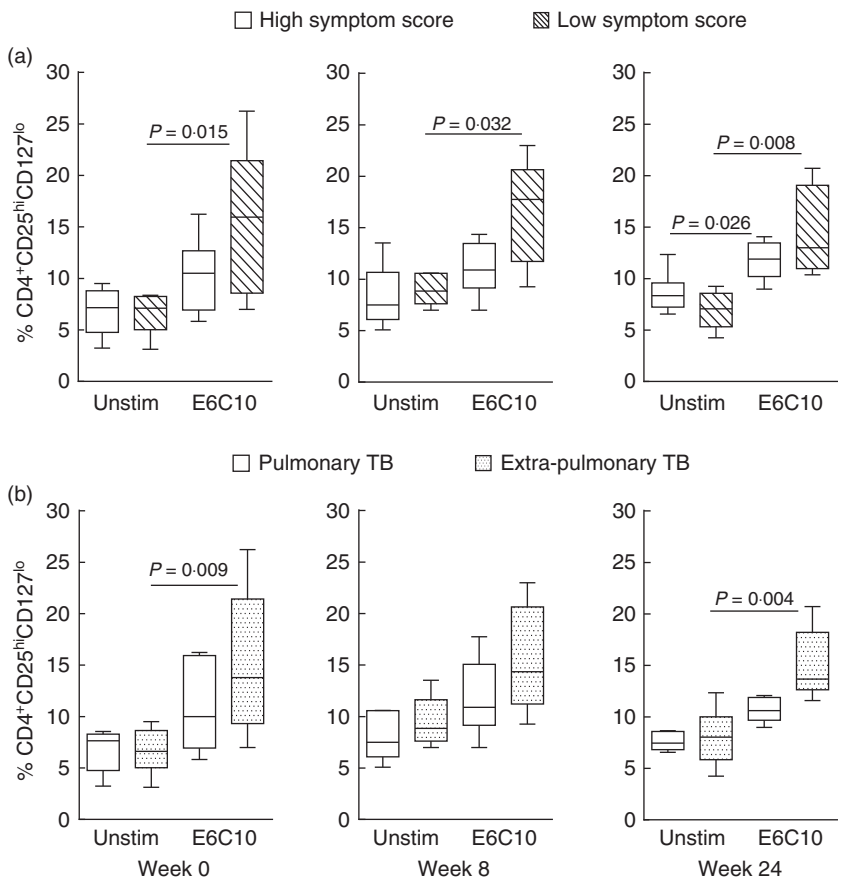

Fig. 5. Fraction of regulatory T cells (T_{regs}) ($CD4^+CD25^{hi}CD127^{low}$) in unstimulated and early secretory antigenic target (ESAT)-6/culture filtrate protein (CFP)-10 (E6C10)-stimulated peripheral blood mononuclear cells (PBMC) during treatment at baseline (week 0), week 8 and week 24 in (a) high (white bars, $n = 6$) *versus* low (hatched bars, $n = 6$) symptom score and (b) pulmonary tuberculosis (PTB) (white bars, $n = 6$) *versus* extrapulmonary tuberculosis (EPTB) (dotted bars, $n = 6$). Plots are shown with median, interquartile range (IQR) and minimum/maximum values. *P*-values were calculated by Mann–Whitney *U*-test.

Discussion

This study presents data on cytokine production, T_{regs} and cytotoxicity in order to explore kinetics and quantitative changes in TB-specific $CD4^+$ and $CD8^+$ T cell responses in patients with TB disease during effective TB treatment. We document dynamic changes of TB-specific immune activation and T cell regulation in response to treatment.

Reductions in the frequencies of *Mtb* antigen-specific total and single IFN-γ-producing $CD4^+$ and $CD8^+$ T cells, already significant after 2 weeks, were overall the best markers associated with successful therapy. We found support in both human studies [25–27] and animal TB models [28,29], where reductions in the proinflammatory cytokines IFN-γ and TNF-α were described after few weeks of treatment. Day *et al.* observed a decline in PPD-specific TNF-α^+ and IFN-γ^+ single-positive T cells in a cohort of pulmonary TB patients during 6 months of anti-TB treatment with a concomitant increase in the polyfunctional T cells [27]. In contrast, Jackson-Sillah *et al.* showed a significant increase in IFN-γ^+ $CD4^+$ T cells in patients with PTB as early as 2 weeks of treatment [30]. These conflicting results could be explained by differences in patient populations, choice of stimuli or methods used. Although accumulating studies conclude that the potential role of plasma-based IFN-γ-release assays (IGRAs) for assessment of response to anti-TB therapy is questionable [31], our data highlight that the prognostic potential of cellular IFN-γ measured early after initiation of TB therapy needs to be elucidated further.

We further studied the dynamics of cytokine patterns in response to therapy by analysing the contribution of single-producing, duo-producing and polyfunctional T cells to the total cytokine pool over time. Prior to TB treatment, single TNF-α-producing and, to a lesser extent, single IFN-γ-producing $CD4^+$ T cells dominated, whereas both cell subsets contributed equally to the total pool of $CD8^+$ T cell cytokine-producing cells. These findings are in accordance with studies reporting a predominance of single IFN-γ [32] or single TNF-α production [10,12,33] in $CD4^+$ T cells from patients with active TB compared to latent infection. Elevated levels of $CD4^+$ TNF-α^+ IFN-γ^+ T cells with an effector memory phenotype have also been described as a feature of active TB disease [12,27,34]. In a recent study of TB in HIV-infected patients, $CD4^+$ TNF-α^+ IFN-γ^+ T cells were associated with active TB, whereas IL-2^+ TNF-α^+ cells predominated in latent TB [35]. However, no differences in cytokine profiles between the groups were observed for the $CD8^+$ T cells. In the current study we observed a moderate decrease in TNF-α^+ IFN-γ^+ T cells for both the $CD4^+$ and $CD8^+$ subsets in the E6C10-stimulated cells, possibly indicating a decrease in the effector memory T cells in response to TB treatment.

We detected low proportions of single IL-2-producing T cells at baseline with an increase during TB treatment for both T cell subsets. Defective *Mtb*-specific IL-2 responses seem to have a major role in TB pathogenesis [12,35,36]. TB cases have a lower proportion of IL-2-producing T cells compared to household contacts [12]. Further, smear-positive TB patients have decreased fractions of IL-2-producing $CD4^+$ T cells when compared with both smear-negative TB and latent TB cases [27]. Improved IL-2 production during TB therapy has been suggested to represent central memory T cells persisting after antigen clearance, as Millington *et al.* observed a shift from co-dominance of single IFN-γ^+ and IFN-γ^+ IL-2^+ T cells to IFN-γ^+ IL-2^+ and single IL-2^+ T cells [37]. In our study, however, IFN-γ^+ IL-2^+ T cell responses were weak and detected in only a few patients throughout treatment. Nevertheless, IFN-γ- and IL-2-producing T cells may have potential as therapy efficacy markers.

Production of multiple cytokines has been associated with protection from disease progression [10]. In our study, we observed a modest reduction in the fraction of polyfunctional T cells during therapy, significant only for the $CD8^+$ subset. However, polyfunctional cells contributed significantly less to the overall cytokine pool after therapy both for $CD4^+$ and $CD8^+$ T cells in keeping with previous studies [11,38], and in contrast to studies including only a few cases where polyfunctional cells increased during treatment [10,27]. Whether polyfunctional T cells measured in *in-vitro Mtb* antigen-stimulated cultures represent a population of primed T cells returned to a resting state due to lack of *Mtb* antigen stimulation *in vivo* during therapy or, rather, recovery of beneficial *Mtb*-specific T cell responses, is unknown. Thus, the role of polyfunctional T cells in TB is still unclear [39].

We analysed cytokine production according to TB disease localization and degree of symptoms. In general, T cells from patients with EPTB seemed to be more able to produce all combinations of cytokines compared to PTB both at baseline and during therapy, especially IL-2-producing $CD8^+$ T cells and polyfunctional $CD4^+$ T cells. An inverse correlation has been reported between *Mtb* load and the levels of both polyfunctional and IL-2-producing T cells [27]. Taken together, this may indicate that T cells in PTB with high *Mtb* load are more anergic, while patients with pauci-bacillary EPTB maintain favourable immune responses. However, cytokine production may also depend upon the degree of systemic inflammation in the individual patient regardless of clinical localization [40]. Finally, we found no significant difference in cytokine patterns between patients with high and low symptom scores, possibly explained by the high proportion of patients with symptoms in both groups.

We also analysed the degranulating capacity of T cells by measuring CD107a expression [41]. While cytotoxicity and degranulation are related commonly to $CD8^+$ T cells, cytotoxic $CD4^+$ cells may also play a role in TB immunity [42,43]. In our study there was a tendency of decreased CD107a expression in both $CD4^+$ and $CD8^+$ cells during the

first 2 weeks followed by an increase towards the end of treatment. Young *et al*. found higher expression of CD107a in both T cell subsets pretreatment compared with after TB therapy [38], whereas Mueller *et al*. detected long-lived granulysin-expressing $CD4^+$ T cells in patients with treated TB [43]. Thus, the role of cytotoxic and degranulating T cells as a correlate of protection in TB is still unclear.

$CD4^+$ regulatory T cells suppress TB antigen-specific Th1 cell responses [14,44,45]. Expansion of T_{regs} has been observed both at organ-specific sites [16,46] and in blood in active TB [47]. In our TB cohort the fraction of $CD25^{high}CD127^{low}$ T_{regs} increased in response to *in-vitro Mtb* antigen stimulation both before and during therapy. However, the transient increase in T_{regs} observed in both unstimulated and *Mtb* antigen-stimulated $CD4^+$ cells after 8 weeks of TB treatment has not, to our knowledge, been described previously. In contrast, decreasing levels of T_{regs} have been reported in response to TB treatment [17,30]. However, high T_{reg} levels are also seen in previously treated EPTB [48] and in response to preventive treatment for latent TB [15]. We could not confirm previous reports demonstrating higher *ex-vivo* levels of T_{regs} in extrapulmonary compared to pulmonary disease [16,48]. Nevertheless, after *in-vitro Mtb* antigen stimulation the increase in T_{regs} was most pronounced in the groups with EPTB or 'low-symptom score' throughout the whole observation period. Thus, preactivated *Mtb*-specific effector T cells seem to have a greater capacity to induce T_{regs} at local sites of infection in these patient groups [49]. Whether increased T_{regs} seen in EPTB could predispose to further bacillary dissemination is still unknown.

Interestingly, we document for the first time in TB disease a similar response pattern as seen for the $CD25^{high}CD127^{low}$ T_{regs} both in the $CD147^{++}$ T_{reg} subset, a recently described highly suppressive and activated subset of human T_{regs} [22], and in the $CD161^+$ T_{reg} subset, capable of producing proinflammatory cytokines [23]. Finally, CD39 is also suggested as a marker for T_{reg} identification [50]. Chiacchio *et al*. showed that the fraction of $CD39^+$ T_{regs} in active TB increased after *in-vitro* stimulation and that depletion of CD39 improved TB-specific responses [24]. In our cohort, although a decline in CD39 expression after *Mtb* antigen stimulation was seen, the $CD39^+$ T_{reg} levels did not change throughout therapy due to a corresponding increase in CD39 negative T_{regs}. Whether this reflects a beneficial qualitative shift in T_{reg} subsets supporting TB immunity remains to be established.

Taken together, our study revealed an opposing shift in the dynamics of T_{regs} and *in-vitro Mtb*-specific T cell responses during treatment, an initial transient increase of T_{regs} followed by a decrease towards baseline levels at week 24, while the majority of cytokine combinations decreased during the first part and then increased at the end of treatment. Reduced T cell effector cytokine production and an early and transient redistribution of redundant T_{regs} to peripheral blood from local sites of infection in response to reduced *Mtb* load and inflammation during TB therapy could explain our data. In support, an animal model shows that the initial *Mtb*-induced inflammatory response promotes proliferation of short-lived pathogen-specific T_{regs} from the pre-existing pool of thymus-derived T_{reg} cells [51]. After the first intensive phase of *Mtb* killing, a more functional immune response with single IL-2-producing cells is established. Thus, our data support a transient change in dynamics of regulatory immune mechanisms after initiation of treatment with a subsequent restoration of immune homeostasis [52].

We acknowledge the limitation of exploring T_{reg} and cytokine responses in peripheral blood, as TB is normally a localized and organ-specific disease. However, both human studies [53,54] and mouse models [51] suggest that effector T cells found in peripheral blood are capable of migrating to sites of active TB infection. Thus, changes in blood may mirror local immunity and blood is a relevant compartment for biomarkers.

In conclusion, the initial reduction of *Mtb* antigen-specific IFN-γ^+ T cells with a gradual increase in single IL-2^+ cells were the strongest indicators of successful TB treatment and may have potential as biomarkers of treatment efficacy. Our results provide evidence for a more dynamic shift of cytokine patterns and T_{regs} than reported previously. Future longitudinal studies may provide further insight into the dynamics of immune responses and regulation during TB treatment.

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Disclosure

The authors declare no financial or commercial conflicts of interest.

Author contributions

S. F., K. T., A. M. D. R. and D. K. conceived and designed the experiments; S. F. and K. T. recruited the patients and performed the experiments; S. F., K. T. and A. M. D. R. analysed the data; S. F, K. T., A. M. D. R. and D. K. contributed reagents/materials/analysis tools; S. F., K. T., A. M. D. R. and D. K. drafted and reviewed the manuscript.

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Supporting information

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

Fig. S1. Flow cytometry gating strategy on $CD4^+$ and $CD8^+$ T cells and subpopulations.
Fig. S2. Overview tuberculosis (TB)-specific T cell responses in TB patients.
Fig. S3. Comparisons of cytokine responses in pulmonary and extrapulmonary tuberculosis (TB) during TB therapy.

