T Cell Responses and Regulation and the Impact of In Vitro IL-10 and TGF-β Modulation During Treatment of Active Tuberculosis

S. L. Feruglio*,†, D. Kvale*,†,§ & A. M. Dyrhol-Riise*,†,‡§

Abstract

*Mycobacterium tuberculosis* (Mtb) is particularly challenging for the immune system being an intracellular pathogen, and a variety of T cell subpopulations are activated by the host defence mechanism. In this study, we investigated T cell responses and regulation in active TB patients with drug-sensitive Mtb (N = 18) during 24 weeks of efficient anti-TB therapy. T cell activation, differentiation, regulatory T cell (Treg) subsets, Mtb-induced T cell proliferation and *in vitro* IL-10 and TGF-β modulation were analysed by flow cytometry at baseline and after 8 and 24 weeks of therapy, while soluble cytokines in culture supernatants were analysed by a 9-plex Luminex assay. Successful treatment resulted in significantly reduced co-expression of HLA-DR/CD38 and PD-1/CD38 on both CD4+ and CD8+ T cells, while the fraction of CD4+CD25highCD127low Tregs (P = 0.017) and CD4+CD25highCD127low CD147+ Tregs (P = 0.029) showed significant transient increase at week 8. *In vitro* blockade of IL-10/TGF-β upon Mtb antigen stimulation significantly lowered the fraction of ESAT-6-specific CD4+CD25highCD127low Tregs at baseline (P = 0.047), while T cell proliferation and cytokine production were unaffected. Phenotypical and Mtb-specific T cell signatures may serve as markers of effective therapy, while the IL-10/TGF-β pathway could be a target for early inhibition to facilitate Mtb clearance. However, larger clinical studies are needed for verification before concluding.

Introduction

Tuberculosis (TB) remains one of the world’s most lethal communicable diseases. In 2014, 1.5 million people died from the disease, 400,000 of whom were HIV-infected [1]. Although nearly one-third of the world’s population is infected with *Mycobacterium tuberculosis* (Mtb), the majority do not develop TB disease. Key immunological mechanisms are responsible for both tissue protection and damage [2]. Thus, stage of infection as well as extent and outcome of TB disease depend on the balance between immune stimulation and immune suppression.

In murine models, it is well established that CD4+ and CD8+ T cell reactivity to Mtb antigens, with production of interferon gamma (INF-γ) and tumour necrosis factor alpha (TNF-α), is essential for TB protection [3–5]. The importance of Th1-mediated immunity is also demonstrated in humans [6, 7] and further evidenced by the increased risk of developing active TB in patient treated with TNF-α blockers and in HIV infection with impaired cellular immunity [8, 9]. However, immune regulation and recovery during anti-TB treatment have not been fully elucidated; some report restoration of *in vitro* Mtb antigen-induced T cell proliferation and INF-γ responses [10], whereas we and others have demonstrated an initial decline in Mtb-specific IFN-γ-producing T cells without full immune recovery of cytokine profiles during effective chemotherapy [11, 12].

Cytokines play an important role in the host immune response as both effectors and regulators of Mtb immunity, in innate defence against Mtb infection and in determining the subsequent adaptive T cell responses [13]. A shift in cytokine production could influence restoration of cellular responses. Interleukin 10 (IL-10) and transforming growth factor β (TGF-β) both suppress the activation of Th1 cells, and corresponding cytokines are elevated in patients with active TB and decrease during treatment [14–18]. Also, regulatory T cells (Tregs), cells that are critical in the maintenance of immune tolerance, are thought to suppress efficient protective immune responses against the pathogen hindering its effective clearance [19, 20]. Treg levels are found elevated in active TB [21, 22] and decline during
treatment [23, 24]. However, data describing Treg dynamics during the course of treatment are conflicting and need further analysis [12, 25].

Hence, this explorative study was designed to assess T cell responses and regulation in terms of Mtb-specific activation, proliferation and cytokine production in patients with TB during the course of efficient anti-TB therapy. Also, the study aimed to explore functional regulation of Mtb-specific proliferative responses and in vitro expanded Tregs by targeting the IL-10/TGF-β pathway.

Materials and methods

Study participants. Patients with active TB infection were recruited from the Department of Infectious Diseases, Oslo University Hospital, Norway, and followed longitudinally during 24 weeks of anti-TB chemotherapy. Demographical and clinical data were as described in Table 1. Two patients were native Norwegians (age 81 and 92), and the remaining were immigrants, predominantly from Africa (N = 9) and Asia (N = 6). Patients were categorized into pulmonary TB (PTB, N = 10) or extra pulmonary TB (EPTB, N = 8). All subjects were culture positive with drug-sensitive TB, HIV-uninfected, and treated with a standard TB treatment of rifampicin, isoniazid, ethambutol and pyrazinamide for 2 months followed by additional treatment with rifampicin and isoniazid. All patients responded to treatment, PTB patient with sputum conversion and culture clearing, whereas patients with EPTB demonstrated clinical improvement and normalization of inflammation parameters. Blood samples were obtained before and after 8 and 24 weeks of treatment. The study was approved by the Norwegian Regional Ethics Committee South East, and written informed consent was obtained from all participants.

Cell separation and preparation. Peripheral blood mononuclear cells (PBMC) were isolated with cell preparation tubes (CPT™, Becton Dickinson (BD) Bioscience, San Jose, CA, USA) with sodium heparin, cryopreserved in 90% fetal calf serum (FCS; Sigma, St. Louis, MO, USA) and 10% dimethylsulphoxide and stored at -145 °C until analysis. After thawing, cells were rested overnight at 37 °C and 5% CO₂. Only samples with viability >85% were analysed.

Flow cytometry analysis. All flow cytometry data were acquired using a BD FACS Canto II™ flow cytometer with FACS DIVA software v 6.1 and analysed using Win List 6.0 (Verity Software House, Topsham, ME, USA) or FLOW jo (Version 10; TreeStar Inc., Ashland, OR, USA). Lymphocytes were gated by morphological parameters (forward and side scatter).

Phenotypic T cell analysis. Phenotypic analysis of PBMC was performed ex vivo after overnight rest in unstimulated samples. After washing with serum-free culture medium (Gibco, AIM V, Invitrogen/Life technologies, San Diego, CA, USA), pellets were resuspended and incubated in the dark for 30 min with the appropriate amount of the following monoclonal antibodies (mAbs): CD3-Pacific Blue, CD4-AmCyan, HLA-DR-FITC, PD-1-APC, CD38-PE, CCR7 PE-Cy7, CD27-Alexa Fluor 700, CD45RA- PerCP-Cy5.5, CD25-PerCP-Cy5.5, CD127-PE-Cy7 (all BD Biosciences) and CD127-PE-Cy7 (eBioscience, San Diego, CA, USA). CD4 and CD8 positive T cells (i.e. CD3+CD4+ or CD3+CD8+) were gated to PD1, CD38 and HLA-DR, respectively, and then Boolean gating strategy was used to create surface marker combinations. Various Treg phenotypes were defined as the following: CD4+CD25highCD127low, CD4+CD25highCD127lowCD161+, CD4+CD25highCD127lowCD39+ and CD4+CD25highCD127lowCD4+. T cell differentiation was defined as CD45RA−CCR7−CD27+ (naive cells), CD45RA−CCR7−CD27+ (effector memory cells) and CD45RA−CCR7−CD27+ (central memory cells). The negative control cells were stained with all the fluorescents minus the ones in question. A representative gating strategy for selected surface markers is shown in Fig. S1.

T cell proliferation assay. PBMCs (250,000 in 200 μl AIM) were pulse labelled with carboxyfluorescin succinimidyl ester (CFSE) (Invitrogen Molecular Probes, Eugene, OR, USA) at 2 μM for 5 min before stimulation in sterile condition with peptide pools [Mtb-derived 6-kDa early secretory antigenic target (ESAT-6) and 10-kDa culture filtrate protein-10 (CFP-10), both 15-mer overlapping by six amino acids peptide pools, >80% purity (Schafer, Copenhagen, Denmark) at a final concentration of 1.5 μg/ml] alone, peptide pools with inhibitory mAbs to IL-10 (MAB217 Clone 23738) and TGF-β (MAB1837 Clone 1D11) at 10 μg/ml final concentration (both R&D Systems Europe, Abingdon, UK), Staphylococcal Enterotoxin B (SEB) (Sigma-Aldrich, Oslo, Norway) at 0.5 μg/ml as positive control and serum-free culture medium as negative test control (background proliferation) and cultured at 37°C and 5% CO₂ in 96-well tissue culture plates (Nunc, Roskilde, Denmark) for 6 days. Cell supernatants from

### Table 1 Demographical and clinical characteristics of study participants (N = 18).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age median years (range)</td>
<td>33 (21–92)</td>
</tr>
<tr>
<td>Female (%)</td>
<td>10 (56)</td>
</tr>
<tr>
<td>Origin (%)</td>
<td></td>
</tr>
<tr>
<td>Africa</td>
<td>9 (50)</td>
</tr>
<tr>
<td>Asia</td>
<td>6 (33)</td>
</tr>
<tr>
<td>Europe</td>
<td>3 (17)</td>
</tr>
<tr>
<td>Localization (%)</td>
<td></td>
</tr>
<tr>
<td>Pulmonary</td>
<td>10</td>
</tr>
<tr>
<td>Extrapulmonary</td>
<td>8</td>
</tr>
<tr>
<td>ESR median mm/h (range)b</td>
<td>46 (6–101)</td>
</tr>
<tr>
<td>Culture positive (%)</td>
<td>18 (100)</td>
</tr>
</tbody>
</table>

*a*Lymph node, pericard, abdominal, abscess, osteomyelitis

*b*Erythrocyte sedimentation rate
ESAT-6- and CFP-10-stimulated wells with and without inhibitory mAbs to IL-10/TGF-β were harvested after 20 h (100 μl) and kept at −80°C until required for multiplex analyses. Cells were harvested after 6 days and stained with CD3 Pacific Blue, CD4 AmCyan, CD25 PE, HLA-DR APC (all BD Biosciences), CD127 Pe-Cy7 (eBioscience) and 7-Aminoactinomycin (7-AAD) (BD Biosciences) to exclude nonviable cells.

*Mtb* Ag-specific proliferative T cell responses were defined as percentages of live (7AAD−) CFSEdimCD3+ in the CD4+ or CD8+ (i.e. CD4+) population. Net proliferative responses were calculated by subtracting background proliferation in corresponding unstimulated control cultures. The cut-off for proliferation (CFSEdim) was set by the median fluorescence intensity equal to or below the second proliferated generation. T regulatory cells were analysed in 6 days cultures and defined as CD4+CD25highCD127dim T cells.

An exploratory assessment of *Mtb* antigen-induced regulation mediated by IL-10/TGF-β was defined as the difference in 6-day proliferative responses with or without the presence of IL-10/TGF-β blocking mAbs above background proliferation as described elsewhere [26].

**Multiplex assay.** The levels of IL-1β, IL-8, IL-10, IFN-γ, interferon gamma inducible protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1), RANTES and TNF-α were measured in supernatants from *Mtb* antigen-stimulated and inhibitory IL-10/TGF-β cell cultures using Bio-Plex XMap technology with a Luminex IS 100 instrument (Bio-Rad, Hercules, CA, USA) and BIO-PLEX MANAGER Software v6 (Bio-Rad, Hercules, CA, USA). The STATLIA software package v3 (Brendan Scientific Inc., Carlsbad, CA, USA) was used to calculate sample cytokine concentrations. The limit of detection was calculated as the concentration of analyte on the standard curve for which the corresponding MFI value is two standard deviations above the background measured in the blank. IFN-γ was below the lower detection level (LDL) or detected at very low levels in many samples (<80% below the LDL of the assay) and excluded from further statistical analysis. Remaining occasional values (<20%) below the LDL were replaced by a defined common value (0.001), which allowed nonparametric statistical analysis. To calculate *Mtb*-specific levels of cytokines and chemokines, background responses from unstimulated cell culture supernatants were subtracted.

**Statistics.** Statistical analysis and graphics were performed using GRAPHPAD PRISM v5.04 software (GraphPad, San Diego, CA, USA). Nonparametrical statistical methods were applied. Group-wise comparison was analysed by the Mann–Whitney *U*-test, while dependent variables were analysed by the two-tailed Wilcoxon matched-pair test. Correlations were assessed using Spearman’s rank. *P*-values <0.05 were considered significant. All patients were included and analysed at all time points. However, some data were excluded on criteria such as viability <85%, less than 10,000 events in the lymphocyte gate and lack of SEB responses prior to statistical calculations, hence leading to different number of patient samples included in the different subanalyses (Fig. 1).

**Results**

**Changes in T cell activation during TB therapy**

T cell activation, differentiation and senescence were longitudinally evaluated on PBMC by surface expression of the activation markers HLA-DR and CD38, naïve and memory markers and programmed death protein (PD-1). The co-expression of HLA-DR/CD38, indicating activated T cell subsets, decreased after 8 weeks of effective treatment for the CD4+ T cells (*P* = 0.032) and after 24 weeks for the CD8+ T cells (*P* = 0.042) (Fig. 2A,B). Similarly, an early decrease in co-expression of PD1/CD38 was observed for the CD4+ T cells (*P* = 0.004) at week 8 and later at week 24 also for the CD8+ T cell subsets (*P* = 0.013) (Fig. 2C,D). In contrast, the fractions of naïve, effector memory and central memory T cells did not change during the treatment period, nor did stratification of patients in PTB versus EPTB alter the results (data not shown).

Figure 1 Patients included in the study. All patients samples included were analysed at all time points. Samples were excluded from further statistical analysis due to *lack of SEB response (N = 1) and viability <85% (N = 1), **less than 10,000 events in the CD3+ lymphocyte gate (N = 4) and technical problems (N = 1). SEB, Staphylococcal Enterotoxin B.
Changes of CD4^{high}CD127^{low} Tregs and explorative Treg subsets during TB treatment

Phenotypic ex vivo analysis of Tregs in blood revealed a transient increase in the fraction of CD4^{+}CD25^{high}CD127^{low} Tregs after 8 weeks of therapy ($P = 0.029$) followed by a significant decrease at week 24 ($P = 0.048$) (Fig. 4A). A similar pattern was also observed in the fraction of CD4^{+}CD25^{high}CD127^{low}CD14^{+} T cells with a significant increase from baseline to week 8 ($P = 0.017$) followed by a modest decrease at week 24 ($P = 0.015$) (Fig. 4B). In contrast, no statistical significant changes were seen for the CD3^{+} (Fig. 4C) or the CD161^{+} Treg subsets (Fig. 4D). Stratification of patients in PTB versus EPTB did not alter the results (data not shown).

We next investigated changes in Tregs in vitro after 6-day stimulation by Mtb antigens. The fraction of CD4^{+}CD25^{high}CD127^{low} Tregs increased significantly by Mtb antigen stimulation compared to the unstimulated cells both at week 0 ($P = 0.002$) and 24 ($P = 0.012$) (Fig. 5A). When Tregs were quantified in parallel cultures that in addition provided functional blockade of the inhibitory cytokines TGF-β and IL-10, this Treg subset decreased significantly ($P = 0.047$) at baseline but not during therapy (Fig. 5B).

Discussion

In this study, we present data on T cell responses and regulation during the course of effective anti-TB treatment. We show that levels of T cell activation declined, while the fraction of Treg subsets displayed an early increase with a subsequent decline to baseline levels in response to 24 weeks of anti-TB therapy. In vitro blockade of IL-10 and TGF-β inhibited the expansion of Mtb-specific Tregs seen in untreated TB infection. Still, Mtb-specific proliferative responses remained unaltered during therapy with no further effect seen neither on proliferation nor on Tregs when blocking the inhibitory cytokines.

Overall we found decreasing levels of CD38 and HLA-DR in both CD4^{+} and CD8^{+} T lymphocytes during TB treatment. These are markers that reflect T cell activation in response to different pathogens [27, 28] and are found to be elevated in patients with TB compared to subjects with latent TB or uninfected controls [21, 29, 30]. Our findings indicate a decrease in immune activation throughout effective TB therapy that might mirror reduced levels of bacterial burden. Our data also support...
a recent study concluding that activated HLA-DR⁺ CD4⁺ T cells are associated with increased TB disease risk both in adolescence and BCG-vaccinated infants [31]. In addition, we found decreasing levels of PD-1 expression on both T cell subsets, most prominent at the end of treatment. PD-1 is a co-inhibitory receptor that is...
upregulated on activated antigen-specific effector cells [32]. PD-1 and its ligands are exploited by a variety of micro-organisms to attenuate antimicrobial immunity and hence may facilitate chronic infection [33]. In TB infection, PD-1 is suggested to have a key regulatory role during the immune response of the host to the pathogen [34–36] and is expressed at higher levels in latent TB compared to healthy controls [37]. In accordance with our findings, Singh et al. also describe decreasing numbers of PD-1 expressing T cells during therapy. Thus, PD-1 may be potentially used to monitor host immunity among patients with TB during treatment [36].

In the present study, we also assessed Mtb-specific proliferative responses of CD4+ and CD8+ T cells, as they are reported to be impaired in patients with active TB compared to latent TB and may serve as a biomarker of TB disease [38, 39]. In our cohort, Mtb-specific CD4+ T cells proliferation was transiently reduced after 8 weeks of treatment, which coincides with the early reduction of bacterial load during the initial phase of treatment, and may thus reflect efficient treatment. At end of treatment however, the proliferative responses were again comparable to baseline values. This observation is in accordance with findings of Day et al. who did not detect any evidence of restoration of CFP-10/ESAT-6-specific CD4+ or CD8+ T cell proliferative capacity, even in subjects evaluated up to 12 months after diagnosis and successful completion of TB treatment [11]. However, others have described improved cellular responses in patients with TB following effective chemotherapy [10]. These contrasting findings might be explained by different patient population, methodology and variability of T cell responses due to the use of different antigens [10, 40]. The implication for the individual patient is hard to assess. Still, even though some T cell functions are not completely restored after successful TB treatment, it is assumed that recurrence of active disease after effective treatment is mainly caused by reinfection [41] or associated with immunosuppression [41, 42]. This may indicate that the functional restoration of memory effector T cells is at least partially effective and probably depends on a complex orchestration of the immune system.

Active TB is characterized by a relative depression of Th1 type cytokines and overproduction and/or enhanced effect of immunosuppressive molecules such as TGF-β and IL-10 [14, 43–45]. Immunotherapy in TB has gained renewed interest with the emergence and spread of drug resistant and extensively drug resistant TB [46], and experimental animal models have demonstrated effect of these strategies [47]. It has been argued that the presence of IL-10 producing T cells might result in sustained survival of the infectious organisms and that methods to reverse this Mtb-induced anergy should be investigated as an integral part of TB treatment strategies [44].

On this background, we studied the effects mediated by blocking of the IL-10/TGF-β pathway on Mtb-induced proliferation, cytokine production and T cell regulation during TB treatment. Given that Mtb antigens induce a predominance of these inhibitory cytokines, we expected reinforced proliferation and secretion of proinflammatory cytokines [48, 49]. However, no such effects were observed throughout the treatment period. Instead, we found a significant decrease in levels of Mtb-stimulated Tregs after blocking IL-10/TGF-β, but only at baseline and not later.

Figure 5 Blocking of IL-10/TGF-β pathway affects in vitro expansion of Tregs. Fraction of T regulatory cells (CD4+CD25hiCD127low) in patients with TB (N = 16) at different time points of anti-TB treatment. (A) Unstimulated (white bars) and ESAT-6 (hatched bars)-stimulated PBMCs. (B) ESAT-6-stimulated PBMCs with (hatched) or without (grey) IL-10/TGF-β blocking mAbs. Values in (B) were calculated by subtracting background responses in corresponding unstimulated controls. Box-whisker plots are shown with median, IQR and minimum/maximum values. P-values were calculated by Mann–Whitney U-test for group-wise comparison and by Wilcoxon’s matched-pairs test for dependent variables. PBMC, peripheral blood mononuclear cell.

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during TB treatment. To our knowledge, this has not been previously described. IL-10 has been shown to play a primary role in the generation of Tregs in vitro [50]. In animal models, rapid expansion of different Treg subsets has caused a decline in early immune responses and failure in achieving clearance of \textit{Mtb} [51], while depletion of FoxP3$^+$ cells has been associated with reduction of bacterial load in mice [52]. Our findings support the notion that the IL-10 and/or TGF-$\beta$ pathway upregulates the Treg compartment in active TB disease.

In accordance with our recent report where Tregs in blood were analysed in another cohort of active patients with TB [12], we again observed an initial but transient increase in CD25$^{\text{high}}$CD127$^{\text{low}}$ Tregs during effective TB treatment. This might be due to a transient redistribution of redundant Tregs from local sites of infection to peripheral blood in response to reduced bacterial load during TB therapy, as supported by animal models [53]. It may also reflect increased expansion of Tregs as a response to generalized immune activation during TB, which might persist for up to several months [54]. Similar patterns were also found for the CD147$^+$ Tregs, a marker expressed on a highly suppressive and activated subset of human Tregs [55]. As the Tregs immune suppressive effect may hamper mycobacterial eradication [56], interventions that lead to early downregulation and enhanced Th1 responses might accelerate disease resolution. Future studies should address the effect of strategies that target Tregs, as well as the IL-10/TGF-$\beta$ pathway, to evaluate their potential adjuvant use in TB treatment regimens.

The limitation of this study is the small sample size which increases the risk of type II statistical errors. Secondly, the heterogeneous patient population consisting of both PTB and EPTB could potentially influence upon data. However, when looking at these patient groups separately, no differences could be found in the various immune markers. Thirdly, only patients with active TB were included; hence, we have not been able to compare our findings with healthy individuals or in patients with latent TB. Finally, we did not use FoxP3 as a Treg marker or analysed functional parameters as cytokine production due to limited number of cells. However, we have recently shown a significant decline in the fraction of \textit{Mtb}-specific IFN-$\gamma$ producing T cells after 2 weeks of treatment in a comparable cohort of patients with TB [12].

In conclusion, a transient increase in CD4$^+$ Tregs together with decreased expression of CD38/HLA-DR and CD-38/PD-1 on both CD4$^+$ and CD8$^+$ T cells was found during the course of effective TB therapy, regardless of TB localization. In addition, our data suggest that the IL-10/TGF-$\beta$ pathway may upregulate the Treg compartment during \textit{Mtb} antigen stimulation. Thus, one may speculate whether early inhibition of Tregs followed by an immune enhancement might favour a more rapid clearance of \textit{Mtb}.

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Author contributions

SF, DK and AMDR conceived and designed the experiments. SF recruited the patients and performed the experiments. SF, DK and AMDR analysed the data. SF, DK and AMDR contributed reagents/materials/analysis tools. SF, DK and AMDR drafted and reviewed the manuscript. The authors declare no financial or commercial conflict of interests.

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2012;7:e44728.


Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Figure S1 Flow cytometry gating strategy on CD4 + and CD8 + T cells and subpopulations.