T CELL EFFECTOR AND REGULATORY SUBSETS, DIFFERENTIATING BETWEEN ACTIVE AND LATENT MTB INFECTION

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ABSTRACT

Last generation IFN gamma – based assays (IGRAs) evaluate bulk CD4 and CD8 T cell responses, and do not discriminate between latent and active *Micobacterium tuberculosis* (MTB) infection. The identification of biomarkers predicting the clinical course and specific therapy effect in latent MTB infection (LTBI) is a major contemporary challenge.

Using multicolor flow cytometry, we compared the levels of circulating CD8 and CD4 effector subsets, in relation to the levels of phenotypically defined regulatory subsets, in two groups of age- and sex-matched MTB-infected individuals: clinically and microbiologically confirmed ATB (n=15), and QFT+ stable LTBI (n=15).

As compared to LTBI subjects, ATB patients are characterized with decreased proportions of CD4 and CD8 CD45RO+CCR7- effectors (14.6% vs. 24%, and 28% vs. 40%, p <0.05 for both), decreased Th1 (10% vs. 16,5 %) and Th1/Th17 (12,5% vs. 21,5%) effector subsets. These changes are accompanied by a significantly increased share of induced (CD39+) FoxP3+CD4Treg (46% vs. 22.6%, p<0.05). The difference affected mostly the Th17-specific (CD39+CCR6+Treg) subset (10.5% vs 4.8%, p<0.05), which correlated inversely with the level of Th1/Th17 effectors (R= -0.5, p<0.05).

In conclusion, we describe a clear-cut distinction between the effector/ regulatory T subset balance in ATB and LTBI. The combined evaluation of Th17Treg and Th1/Th17 effectors in peripheral blood can be employed for MTB-infection monitoring.

***KEYWORDS**:*
LTBI, Th1/Th17, CD39+Treg, Th17Treg

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INTRODUCTION

Micobacterium tuberculosis (MTB) infection is still a major healthcare concern worldwide. About one third of world's population is estimated to be infected with MTB, while only 10% would develop active disease [1]. Latent MTB infection (LTBI) represents an enormous reservoir of the pathogen, of high concern in the ageing populations with increased risk of secondary immune deficiencies [2]. The best approach to limit the spread of the infection is to detect latent infections and prevent their activation. The recently developed interferon gamma release assays (IGRA) recommended for diagnosis of LTBI evaluate the production of IFN-γ in response to MTB-specific peptides [3]. In spite of a very high specificity and sensitivity, IGRAs do not discriminate between latent and active TB [4]. Yet, there is no consensus that the quantity of MTB-specific IFN-γ is related to the activity of pathogen and predicts disease progression or therapy effect. This requires additional and handy phenotypic and functional biomarkers discriminating between phases of TB disease [5].

A lot of evidence indicates the critical role of CD8 T cells for MTB control. On this grounds the last generation IGRA (QFT-GIT Plus) evaluates both CD4 and CD8 MTB-specific responses [6-8]. The efficiency of CD8 T cell response depends on the balanced differentiation of effector and memory pathogen-specific cells. This balance is finely regulated by induced CD4+ FoxP3+ T cells (iTreg) with inhibitory potential [9]. We and others have previously shown that in conditions of chronic infection, such as HIV, HCV or HBV, the differentiation, subset composition and function of FoxP3+Treg is also affected, which contributes to the absence of protective immune response [10]. Likewise, CD4 T effector and regulatory subsets (Th1, Th2, Th17, Treg) are of remarkable plasticity, depending on antigen burden and individual cytokine background [11]. The balance between Th effector and regulatory subsets has not been extensively studied during the different phases of MTB infection.

In this study, we compared the levels of CD8 and CD4 effector and memory subsets, in relation to the levels of circulating regulatory subsets, in two well-defined groups of MTB-infected individuals: clinically and microbiologically confirmed ATB, and QFT+ stable LTBI.

MATERIAL AND METHODS

Samples were collected during the routine diagnostic activity of NRLI from 40 men, and 52 women, average (min-max) age 52 (18-76) years. Additional samples of 4 ml peripheral blood were drawn, PMNC were isolated, and frozen after informed consent, approved by the local ethics committee. IGRAs were performed using QFT-GIT-Plus (Qiagen) and T-spot (T.SPOT.TB Oxford Immunotec) tests, according to manufacturer's instructions. ATB status was defined based on a combination of clinical, microbiological and radiological data; LTBI was defined as asymptomatic MTB infection, detected by immunological test, in the absence of clinical, microbiological or radiological data for tuberculosis [3]. Percentage and absolute count (AC) of CD4+CD3+ and CD8+CD3+T cells were determined by direct flow cytometry platform, using MultiTest and TRUCount standard tubes (BD Biosciences). Based on the co-expression of CD45RO and CCR7, naïve (RO-R7+), central-memory (RO-R7-), effector-memory (RO+R7-) and terminally differentiated (RO-R7-) CD4 and CD8 T were defined. Co-expression of CD196 (CXCR6) and CD183 (CXCR3) was used to define Th1 (CXCR3+CCR6-), Th2 (CXCR3-CCR6-), Th1/Th17 (CXCR3+CCR6+), and Th17 (CXCR3-CCR6+) effector subsets. T-regulatory CD4 FoxP3+ cells were defined as CD4+ $CD25^{hi}CD127^{lo}$. Within Treg, the share of CD39+Treg and CD39+CD196+Treg was determined. All flow cytometry analyses were performed with 6 to 8-color combinations and FACSDiva 6.1.2 software (FACSCanto II, BD Biosciences). Statistical analysis was performed with parametric T-test or Spearman's rank correlation test for quantitative data (GraphPad v.6).

RESULTS

1.**Bulk MTB-specific interferon-gamma responses of CD4 and CD8 T cells does not differentiate between ATB and LTBI.**

During the period 01.2018 – 04.2019, 92 consecutive patients were tested for MTB infection with the last generation IGRA QFT-GIT-Plus. ATB cases (n=28) and LTBI cases (n=27) were defined as stated in M&M section. In 8 (27.5%) ATB cases QFT-GIT-Plus results were negative (TB1–Nil< 0.35 and TB2–Nil < 0.35) and 2 were indeterminate (6.9%). The average interferon-gamma secretion (IU/ml) in response to TB1 and TB2 was as follows: 2.29 and 2.40, respectively, in ATB; 3.62 and 3.57 - in LTBI. No significant differences were established between CD4 and CD8 specific responses within each group (paired T-test $p>0.05$), or between the two groups (non-paired T-test $p>0.05$), (Fig.1). Further on, no domination of TB1 (8/55) or TB2 (12/55) responses could be attributed to either ATB or LTB. Therefore, we concluded that analyzing separately bulk CD8

and CD4 T cell responses did not contribute to the discrimination between ATB and LTBI.

2. ATB patients are in shortage of CD4 Th1, Th1/Th17 and CD8 effector T cells.

In order to compare the proportions of phenotypically defined CD4 and CD8 effector and memory subsets in ATB and LTBI, we retrospectively selected age- and sex-matched subgroups. The absence of known secondary immune deficiency or autoimmune condition was additional criterion.

A significantly lower proportion of EM CD4 and CD8 T cells was observed in ATB as compared to LTBI (14.6% vs. 24%, and 28% vs. 40%, p <0.05 for both comparisons), **Fig.2A.** While the level of Th17 (CD183-CD196+) cells seemed unaffected (25 vs. 29 % in LTBI), the Th1 (CD183+CD196-) subset was decreased (10% vs. 16,5 % in LTBI, p<0.05). A significantly lower Th1/Th2 ratio (0,34 vs. 0,54) reflected an additional increase of Th2 cells in ATB (49,5% vs. 34% in LTBI). Finally, the Th1/Th17 (CD196+CD183+) subset that was shown to contain most of the MTB-specific effectors [12], was importantly decreased in ATB, as compared to LTBI (12,5% vs. 21,5%, p<0.05), Fig.2B. The changes observed in ATB corresponded to a prominent humoral response and deficient cellular immunity in the presence of the vigorously replicating pathogen.

3. The subsets of CD39+ and CCR6+Treg contribute to the exhaustion of T cell effector functions in ATB

Since the balance between antigen-specific effector and memory subset differentiation depends on iTreg, we further evaluated their proportions and subset composition in the ATB and LTBI subgroups. While the difference between the levels of FoxP3+ CD4 in ATB and LTBI did not reach statistical significance (6,6% vs. 4,9% and 38 vs. 40 cells/µl), ATB was characterized with a domination of CD39+Treg, corresponding to induced Treg with strong inhibitory activity (46% vs. 22.6%, p<0.001), **Fig. 3A, B**. In addition CD196+ CD39+ Treg that were proposed to inhibit specifically Th17 [13] were importantly increased in the settings of ATB as compared to LTBI (10.5% vs 4.8%), **(Fig. 4A,B)** These distinct Treg proportions corroborated with the skewed differentiation of CD4 and CD8 effector cells in ATB. We concluded that the increased bacterial load in ATB drives the differentiation of Treg subsets that affect the balanced differentiation of effector and memory pools, and of Th1 and Th17 responses. Indeed, a significant inverse correlation was established between the level of Th1/Th17 effectors, and the Th17-specific Treg (R=-0.5, p<0.01, **Fig. 4C**. In a limited number of ATB patients (n=5) who had completed a specific therapy course, the proportion of CD39+Treg significantly decreased in successfully treated patients (35% vs. 46%), accompanied with consequent increase of Th17 effectors (43 vs. 25%).

DISCUSSION:

Adequate monitoring and control of LTBI are important to prevent ATB and limit the spread of infection. Our results show that the proportions of Th1/Th17 effectors and the lineage-specific CD196+CD39+Treg (Th17Treg) subset change reciprocally in relation to bacterial burden and may differentiate between stages of MTB-infection.

It is well recognized that adaptive immune response to MTB relies mostly on T cells. MTB antigens are primarily presented to CD4 T cells, and elicit a Th1 response dominated by IFN-γ production. Current immune tests rely exclusively on MTB-specific IFN-γ detection. However, a number of large studies and meta-analyses have suggested that the predictive value of IGRAs for progression to TB disease, and for benefit from specific therapy remains unsatisfactory and not significantly higher than that of the TST. [14-17].

We analyzed results obtained with last generation IGRA (QFT-GIT), containing additional CD8 T cell-stimulating MTB antigens (TB2). Indeed, CD8 T cells can also release IFN-γ after stimulation with MTB antigens, even without

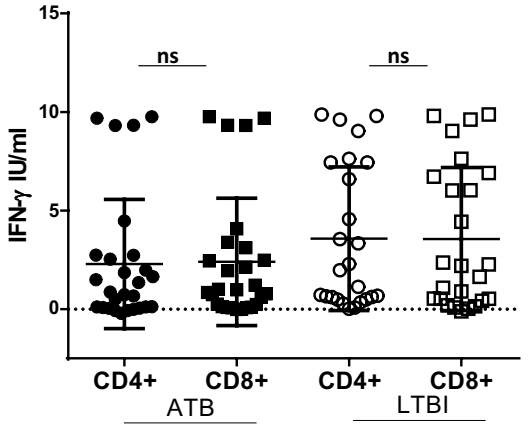

Fig.1 Individual data about MTB-specific IFN-γ secretion by CD4 T (A) and CD8 T (B) in patients with ATB (black symbols) and LTBI (open symbols). Non-significant differences are indicated (ns, p>0.05)

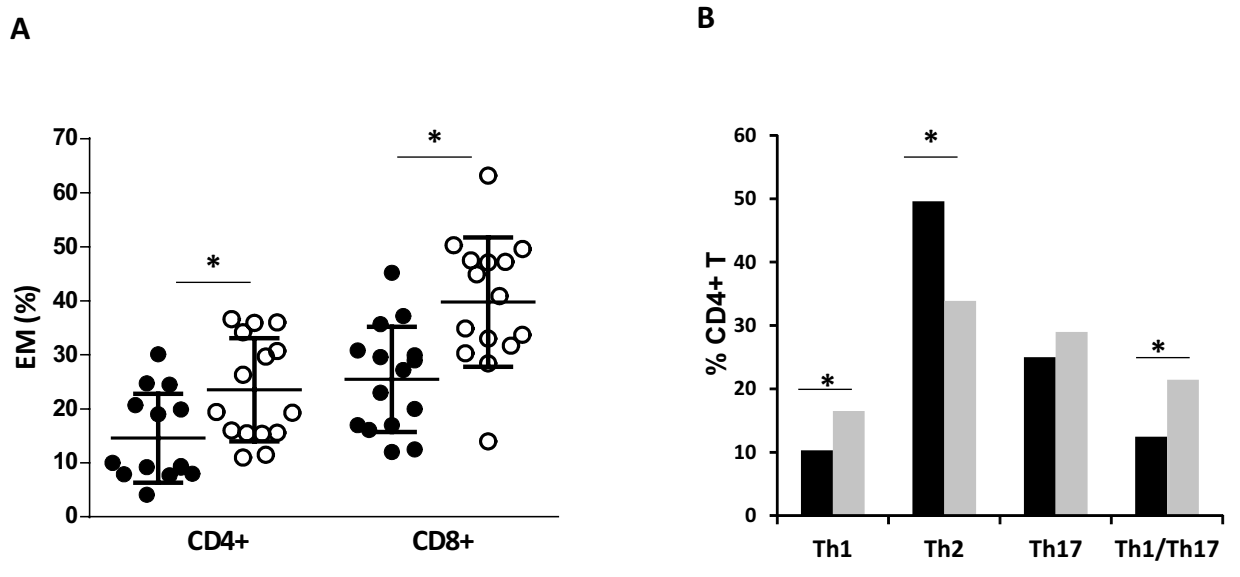

Fig.2 Proportions of EM (CD45RO+R7+) CD4 and CD8 T cells (A), Th1, Th2 and Th17 (B) in ATB (black) and LTBI. Mean values are presented. Significant differences are indicated (* p<0.05).

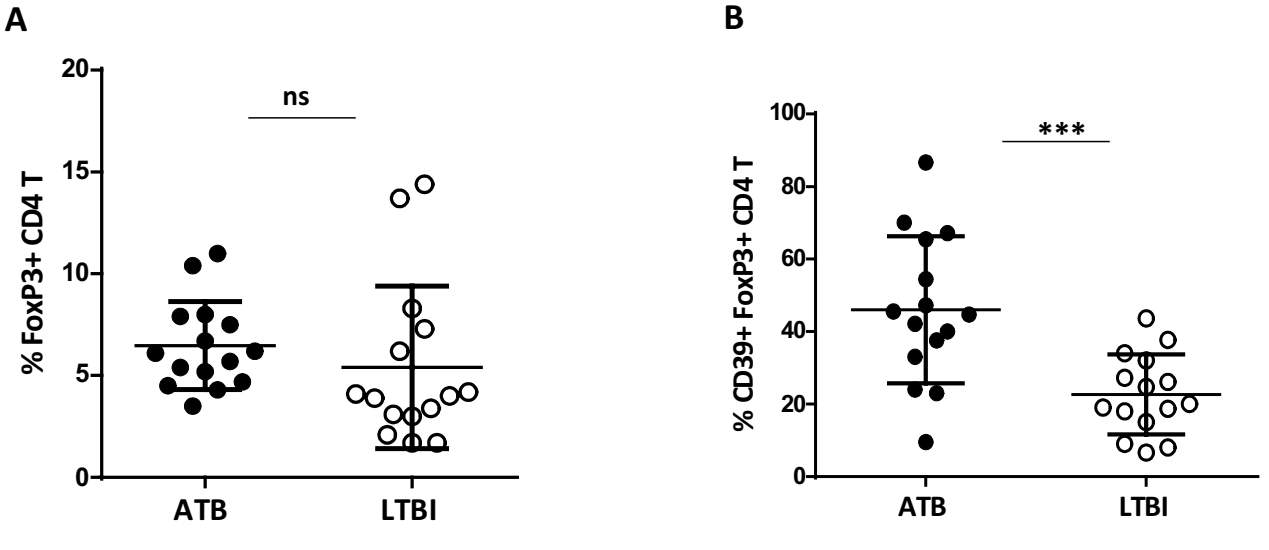

Fig.3 Individual data about the proportions of T regulatory subsets in ATB (black symbols) and LTBI (open symbols): A. Total FoxP3+CD4 Treg and B. CD39+ FoxP3+CD4 in ATB and LTBI. Significant differences are indicated (*** p<0.001).

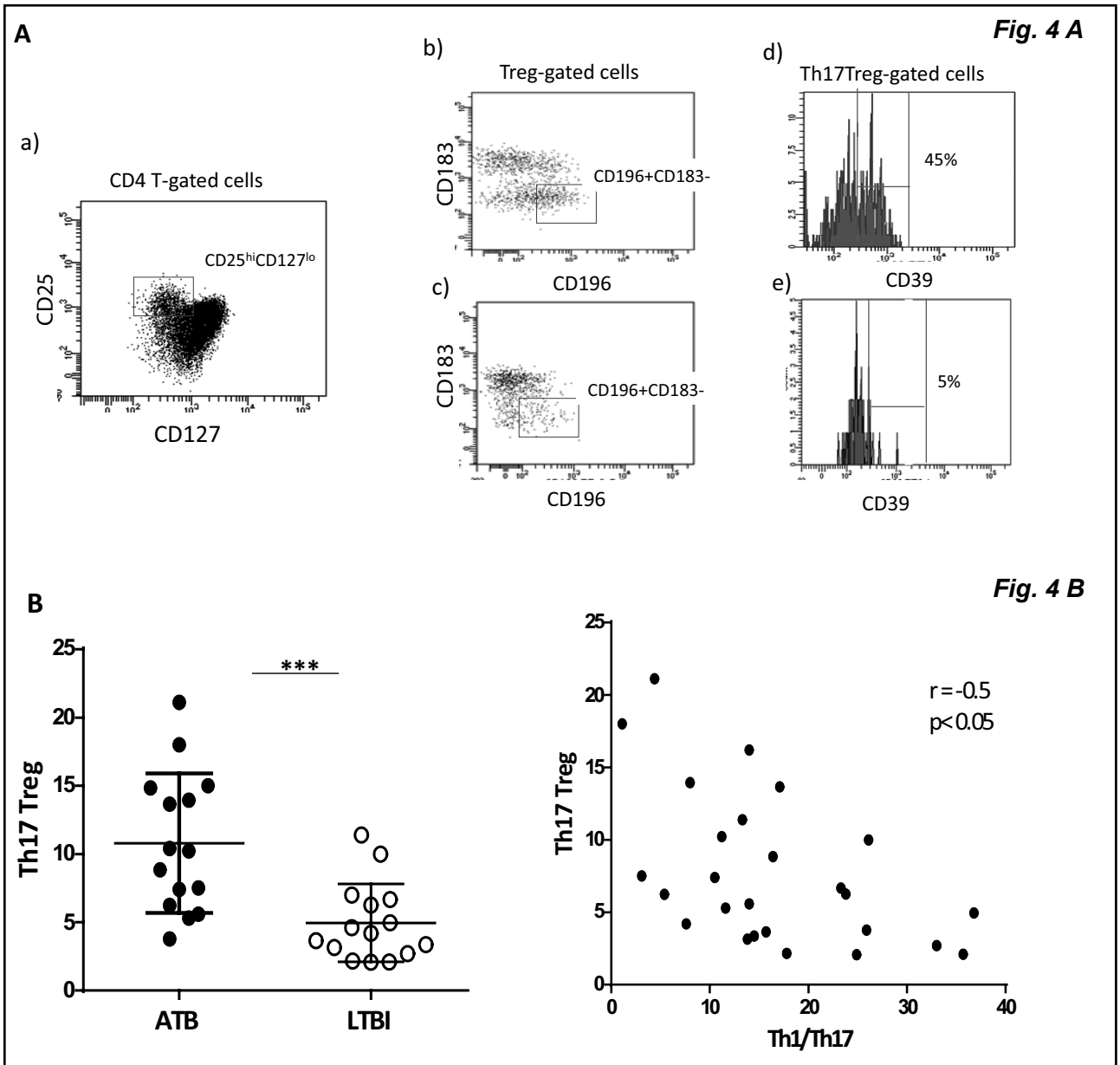

Fig.4. Th17Treg in ATB and LTBI.A: Gating strategy for evaluation of Th17Treg in ATB (a, b, d) and LTBI (a, c, e); B. Individual data about the proportions of Th17Treg in ATB (black symbols) and LTBI (open symbols). Significant differences are indicated (*** p<0.001); C. Correlation between the levels of Th1/Th17 effectors, and Th17-specific Treg (R= -0.5, p<0.01)

help from CD4+ T-cells, preferentially recognize cells from a host heavily infected with MTB, and the magnitude of their response correlates well with the bacterial load and smear-positivity [18 – 21]. Using a different approach (flow cytometry ICS) we have previously compared CD8 T cell responses elicited against RD-1 antigens, and reported differences between patients with active TB, LTBI, and recent MTB infection [22]. Therefore, it may be expected that evaluation of CD8 T response would not only increase the sensitivity of the assay, but also - its ability to differentiate between phases of the latent and active MTB "spectrum". However, the reports on QFT-GIT-Plus are rather contradictory. Petruccioli et al. found that the majority of LTBI subjects simultaneously responded to both TB1 and TB2 antigens, and that an "only to TB2" response was associated with active TB [7]. Another study among older adults categorized most of those with "only to TB2" response as definite LTBI [8]. Although in a limited number of patients, our results did not establish an association between a predomi-

nant TB1 or TB2 response and the activity of MTB infection. Most probably, specific CD4 and CD8 T cell subsets are associated with the activity and stage of MTB infection.

In a simplified scheme, antigen-specific T-cell response leads to differentiation of both effector cells, eliminating antigen burden, and long-living memory cells, preventing new infections. This process is strictly regulated by FoxP3+ T cells with inhibitory potential, induced simultaneously with the effectors. The balance memory/effector/regulatory cells is crucial for a protective immune response [10]. In chronic infections, this balance is skewed, either towards accumulation of immature and poorly functional effectors, [23, 24] or - in excessive differentiation and exhaustion of the effector potential [25].

According to our results, ATB was distinguished by decreased shares of EM (CD45RA-CCR7-) CD4 and CD8 T cells. In addition, ATB was associated with accumulation of Th2-like effectors at the expense of Th1, and Th17 cells.

We have previously described accumulation of CD45RA-CD27+ CD8 T cells in stable LTBI as compared to ATB [26]. Our present results are not contradictory, since the CD45RA-CD27+ subset is contained within the CD45RA/CCR7-defined EM population. Phenotypically defined T cell differentiation stages correlate with certain functional capacities. The EM subset contains mostly IFN-γ and IL-2 producing cells, while TE (CD45RA+CCR7-) cells have decreased IFN-γ and increased perforin and TNF- α secretion potential [27]. In line with our results, Sutherland et al. reported a significantly higher proportion of cells single-positive for TNF-α, but a lower proportion of cells producing IL-2 alone in TB cases compared with contacts, for both CD4(+)and CD8(+) T cells [28].

Th1, Th2, and Th17 are of major importance for protection against MTB [29]. The role of Th1-type cytokines, including IL-12, IFN-γ, IL-2, and TNF-α in MTB-specific response is well recognized. Genetic defects leading to IL-12/IL-12R deficiency are associated with a high susceptibility to MTB infection [30]. Multi-functional (IFNγ+ TNFα+ IL-2+) MTB-specific CD4+ T cells have been defined as a correlate of protective immune response [28, 31]. The role of Th17-related cytokines is not so evident. They were shown to contribute to immune protection against primary MTB infection: suppressing IL-17A production increases TB susceptibility, since IL-17A is involved in the formation and stability of granulomas; IL-17 expression levels were found decreased in TB patients by different studies [32, 33]. At the same time, Th17 responses in TB may also participate in pathology through recruitment of neutrophils and promotion of inflammatory responses, and some studies find significantly higher percentages of Th17 in active TB patients than in latent TB or control groups [34, 35].

The Th1/Th17 (CXCR3+CCR6+) population producing both IL-17 and IFN-γ was only recently identified. Th1/Th17 cells were shown to include more than 80% of the MTB-reactive memory CD4 T cell response, and were reported as the main source of IL-17 in MTB infection, regulated by IFN-γ [11, 36, 37]. According to our results, the most prominent difference regarding T cell effector subsets in LTBI and ATB subjects was a decreased level of Th1/Th17 cells. Data about the dynamics of this subset during the phases of MTB infection are scarce. Unlike us, Jurado et al. reported markedly augmented levels of IL-17 in ATB patients and concluded that the ratio of antigen-expanded CD4+IFN-γ+IL-17+ lymphocytes correlated directly with disease severity [36]. However, the comparison was with BCG-vaccinated healthy donors and not with LTBI. These contradictory results only underline the critical importance of Th1/Th17 cytokine balance in MTB infection, provided by Treg and the necessity to evaluate effector and regulatory subsets in parallel.

Our study shows that the changes in effector subsets during ATB are accompanied with significant increase in particular Treg subsets, and these changes are transient after treatment.

The physiological role of Treg is to fine-tune the memory-effector cell balance. In condi-

tions of high antigen load Treg would prevent the extreme differentiation of effectors and eventual loss of important specificities, and also prevent collateral damage [22]. A number of studies reported elevated levels of circulating iTreg in the settings of chronic infection, including MTB. Expansion of Treg has been observed both at organ-specific sites and in blood in active TB. [34, 36, 38] Feruglio et al. demonstrated a transient increase in ex-vivo Treg levels during therapy [38], while decreasing levels of Tregs have been reported in response to TB treatment by others [35, 39]. High Treg levels are also seen in response to preventive treatment for latent TB [40]. However, all these studies have compared ATB with healthy controls, and not with LTBI patients. We did not find a significant difference between the levels of the entire FoxP3+ population in LTB and ATB. Therefore, we supposed that specific subsets as CD39/Treg and CCR6+Treg might be significantly associated with infection activity.

CD39 is the dominant immune system ectonucleotidase that hydrolyses extracellular ATP and ADP, and in concert with CD73 generates highly suppressive adenosine monophosphate at the sites of immune activation. In humans, CD39 expression is highly variable and restricted to a subset of Foxp3+ Treg [41]. Increased CD39+Treg subset has been reported in chronic HIV,HBV and HCV infection, as a direct sequence of pathogen-induced response. Therefore, it is legitimate to consider CD39+Treg as iTreg. In the long term, CD39-mediated inhibition of T cell proliferation might exert an adverse effect on the immediate generation of T-cell immune responses, thus contributing to disease progression. Most available data about CD39+Treg in MTB infection are based on *in vitro* stimulation studies. While Feruglio documented a decline in CD39 expression after MTB antigen stimulation [38], Chiacchio et al. showed that the fraction of CD39+ Treg in active TB increased after in-vitro stimulation and that depletion of CD39 improved TB-specific responses [42]. Aggrawal found a markedly higher expression for CD39 (in terms of MFI) when compared between ATB and healthy controls. Higher frequencies of CD39+Treg were observed in [TST+, QTF+] individuals when compared with [TST-, QTF-] control group [40, 43]. In our hands, CD39+T-cells were markedly increased in ATB as compared to LTBI, and readily decreased upon treatment. In line with our results, Kim et al. concluded that MTB-reactive CD39+CD4+ T cells were more abundant in active TB than LTBI [39].

Recently, a new subset of RORγt+Foxp3+ Treg (Tr17) cells was described, generated in an antigen-specific manner through Stat3 signaling. These effector Treg express CCR6, and hence can be recruited within the inflammatory milieu and regulate Th17-dependent immune responses [44, 45]. In fact, only activated CD39+Tregs co-expressing Th17-associated markers CCR6 and IL-23R, phosphorylate Stat3, and not the CD39-negative ones. Thus the CD39+CCR6+Treg subset is the first lineage-specific Treg subset in human [46]. Our results show that Th17-specific Treg are the regulatory subset that differs most significantly between ATB and LTBI, correlates best with the level of Th1/Th17 effectors, and may be used to monitor the course of infection.

The balance Th1/Th17/Treg determines the clinical course of MTB infection. This balance is based on the possibility of Th1, Th17 and Treg cells to transdifferentiate, one into each other, including the Th1/Th17 stage, and depends on shared differentiation pathways, the characteristics of antigen burden, and cytokine background [33]. Overall, Th17 responses fit well with the situation of stable LTBI, whereby the stability of granulomas is important, while the prevalence of Th1 over Th1/Th17 lymphocytes, is the basis of protection against replicating MTB. However, overproduction of IL-17, accompanied by increase of IL-10-producing Treg in LTBI was associated with a high risk of reactivation [47]. Similarly, too strong proinflammatory Th1 reponse, leading to too strong

Treg-mediated inhibition may be detrimental for efficient antigen-specific response.

In conclusion, we describe a clear-cut distinction between the effector/regulatory T subset balance in ATB, and LTBI, and propose the combination of Th17 Treg and Th1/Th17 subset as accessible markers differentiating between latency and disease.

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