

PERIPHERAL BLOOD CD8 T CELL RESPONSE
IN DIFFERENT PHASES OF MTB INFECTION

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Abstract

A reliable approach differentiating between active and latent TB infection (LTBI) is yet unavailable. Recent data suggest the involvement of CD8 T cells in protective response to mycobacterium tuberculosis (MTB). We compared the antigen-specific responses and subset composition of peripheral blood CD8 T cells in patients with active infection (ATB), recent household contacts (RC), highly-exposed health-care workers (HW), and BCG-vaccinated healthy controls (HC). Study groups included 20 ATB, 21 RH, 21 HW and 15 HC. Active TB was diagnosed according to microbiological and clinical criteria. RD1-specific CD4 and CD8 T cells were determined by intracellular cytokine staining (ICS). CD8 T subsets were defined as naïve (CD45RA+CD27+), memory (M, CD45RA-CD27+), effector (E, CD27-CD45RA-), and terminal effector (TE, CD27-CD45RA+), and analyzed by flow cytometry. The levels of RD-1-specific CD4 T cells were highest in ATB but not significantly different from LTBI groups. A significant RD-1-specific CD8 T cell IFN γ response (0.289%) differentiated RC from ATB, HE and HC subjects (0.053, 0.027 and 0.011, $p < 0.05$) and was associated with decreased M/TE ratio. Finally, an increased M/E CD8 T ratio distinguished HW from RC and ATB subjects (7.6 vs. 2.4 and 2.6, $p < 0.05$). The study of MTB-specific CD8 T-cell response in combination with CD8 T subset composition adds to the differentiation between active and latent TB.

Key words: CD8 T-cell response, RD-1-specific T cells, ICS, MTB infection

Introduction. Mycobacterium tuberculosis (MTB) is one of the most frequent causes of infectious morbidity worldwide [1]. A large majority of *M. tuberculosis*-infected individuals remains asymptomatic while unable to clear the bacterium, with a lifelong probability to develop active TB of about 5–10% [2]. This probability is highest within the first two years after infection, and in subjects with recently-established LTBI preventive therapy can be effective. Thus the distinction between the different forms and stages of MTB infection is fundamental for the effective control of TB transmission.

The correct diagnosis of LTBI remains a challenge. Until recently, tuberculin skin test (TST) has been the most frequently used screening method, leading to a high rate of false positive results in *M. bovis* BCG-vaccinated individuals, and false negative tests in immunosuppressed persons. The new interferon gamma release assays (IGRAs) employing RD-1 antigens specific for virulent mycobacteria have excellent specificity unaffected by BCG vaccination and increased sensitivity [3]. Yet IGRAs cannot discriminate between individuals with latent and active TBI either. Moreover, contradictory results have been obtained with recently-exposed subjects [4]. A new reliable approach for diagnosis of TB infection is based on the flow cytometry evaluation of T cell MTB-specific intracellular cytokine expression [5].

While CD4 Th1 cells are the well-known effectors of MTB-specific immune response, a lot of evidence from humans and from animal models indicate a critical role of CD8 T cells for MTB control as well [6–8]. However, data about the characteristics of CD8 T cell responses in individuals with LTBI and in patients with active tuberculosis are still scarce.

In this study, using intracellular cytokine staining (ICS) and flow cytometry, we compared the CD8 T cell responses elicited against the RD-1 antigens ESAT-6, CFP-10 and TB7.7, in parallel with CD8 T cell subset characteristics in patients with active TB, and two groups of subjects with high probability of LTBI: healthcare workers highly exposed to MTB, and recent contacts of ATB patients.

Materials and methods. Study groups. The following groups were recruited from February to December 2009 at the Regional Dispensary for Tuberculosis and Lung Diseases, Sofia: 20 MTB culture-positive confirmed pulmonary ATB patients, 21 RC of newly-diagnosed ATB patients (with no documented previous history of exposure to TB, no sign of ATB or other active diseases, negative chest X-ray and microbiological screening for MTB), 21 HW (with more than 100 admissions of TB patients per year), and 15 BCG-vaccinated HC with no known history of MTB infection. Informed consent was obtained from all patients and controls prior to the study.

Flow cytometry analysis of lymphocyte subsets. Multicolour immunophenotyping of whole blood was performed by a standard lysis/wash technique. Naïve (CD45RA+CD27+), memory (M, CD45RA-CD27+), effector (E,

CD27-CD45RA-) and terminally-differentiated effector (TE, CD27-CD45RA+) CD8 T cells were defined using CD45RA-FITC/CD27-PE/CD4-PerCP/CD8-APC mAb combination (BD Biosciences). At least 5000 CD8^{high}-gated cells were collected from each sample using a FACSCanto II flow cytometer and analyzed using BDFACS Diva 6.1.2. software (BD Bioscience, San Jose, CA).

Intracellular cytokine detection. The intracellular expression of IFN- γ was evaluated using flow cytometry. To this end, 250 μ l of whole blood were incubated either in a tube of Quantiferon TB gold in-tube assay with TB antigen (containing the ESAT-6, CFP-10 and TB7.7 peptide cocktail) or – with no antigen, at 37°C and humidified 5% CO₂ atmosphere. Two hours after the start of culture, 12.5 μ g/ml Brefeldin A (BD Biosciences San Jose, CA) were added for the next 14 h. Samples were processed using a BD FastImmune Intracellular Cytokine Detection kit, according to manufacturer's instructions and stained with IFN γ -FITC/CD69-PE/CD4-PerCP/CD8-APC. At least 50 000 lymphocytes were acquired from each sample and analyzed by FACS as described above.

Statistics. *T*-test for unpaired samples was used to evaluate differences between patients and control groups. *P* values less than 0.05 were considered statistically significant. Statistical analysis was performed using GraphPad 4.0 software.

Results. 1. RD-1 specific CD8 but not CD4 T cell response differentiates between active and latent MTB infection. According to the ICS results, 85% (17/20) of the ATB patients, 38% (8/21) of HW and 43% (9/21) of RC subjects displayed an MTB-specific CD4 T cell response. The level of RD-1-specific CD4 T cells was significantly increased in the patients and the two contact groups as compared to HC (average % 0.215, 0.069 and 0.166 for ATB, HE; and RC respectively, vs 0.012% for HC, $p < 0.01$ for all comparisons.) The established frequency of MTB-specific CD4 T cells did not discriminate either between ATB and RC, or between RC and HW groups ($p > 0.05$ for both comparisons) (Fig. 1a). At the same time, a significantly higher frequency of RD-1-specific CD8 T cells was observed in RC subjects (average % 0.245), which differentiated them from the other three groups (average % ATB 0.053, HW 0.028 and HC 0.011, $p < 0.01$ for all comparisons) (Fig. 1b).

2. Peripheral blood CD8 T cell subset composition differentiates between patients with active TB and subjects with high probability of LTBI. We further characterized the subset composition of the CD8 T cell pool in each studied group. An accumulation of M (CD45RA-CD27+) CD8 T cells, accompanied with an increased M/E (CD45RA-CD27-) cell ratio, was observed in HW, and distinguished them significantly from ATB, RC and HC subjects (7.7 vs. 2.6; 2.5, and 3.5, respectively, $p < 0.05$ for all comparisons) (Fig. 2a). At the same time, the RC group was characterized by accumulation of TE (CD45RA+CD27-) CD8 T cells and their M/TE ratio was correspondingly decreased, as compared to ATB, HW and HC subjects (1.1 vs 2.5; 4.5 and 3.2 respectively, $p < 0.05$ for all comparisons) (Fig. 2b).

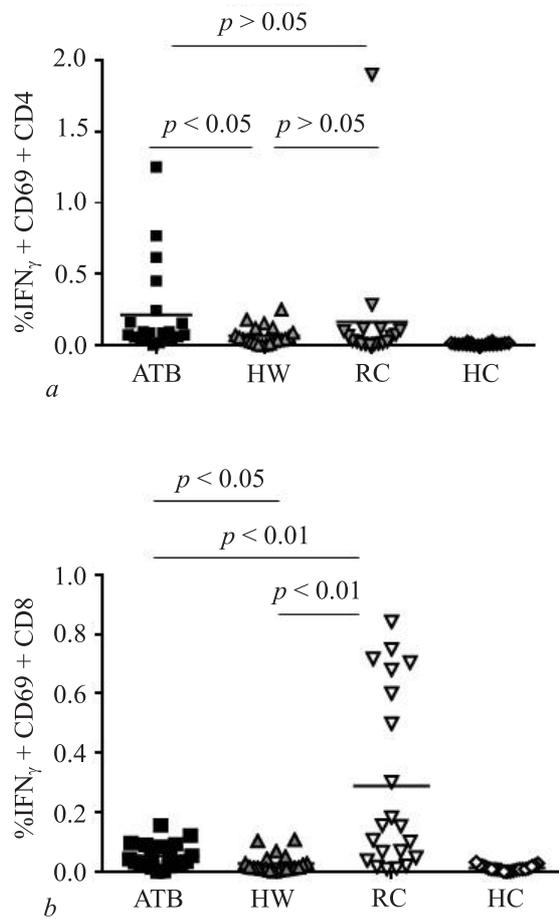


Fig. 1. RD-1-specific CD8 but not CD4 T cell response differentiated between RC and ATB, HW, HC groups. *a*) Comparison between the levels of RD-1-specific CD4 T cells (%IFN γ +CD69+CD4 T cells) in ATB, HW, RC and HC groups; *b*) Comparison between the levels of RD-1-specific CD8 T cells (%IFN γ +CD69+CD8 T cells) in the above groups (Man-Witney non-parametric test, $p < 0.05$ is considered significant)

Discussion. The worldwide revival of tuberculosis and the appearance of drug-resistant MTB strains present a global healthcare challenge [1]. Accurate testing and preventive treatment of individuals with LTBI at high risk of progression is an important part of the infection control [9, 10]. In the last years, flow cytometry evaluation of cytokine secretion profiles and cell surface phenotypes of peripheral blood T cells have been employed in the attempt to differentiate active and latent TB infection [5, 11, 12].

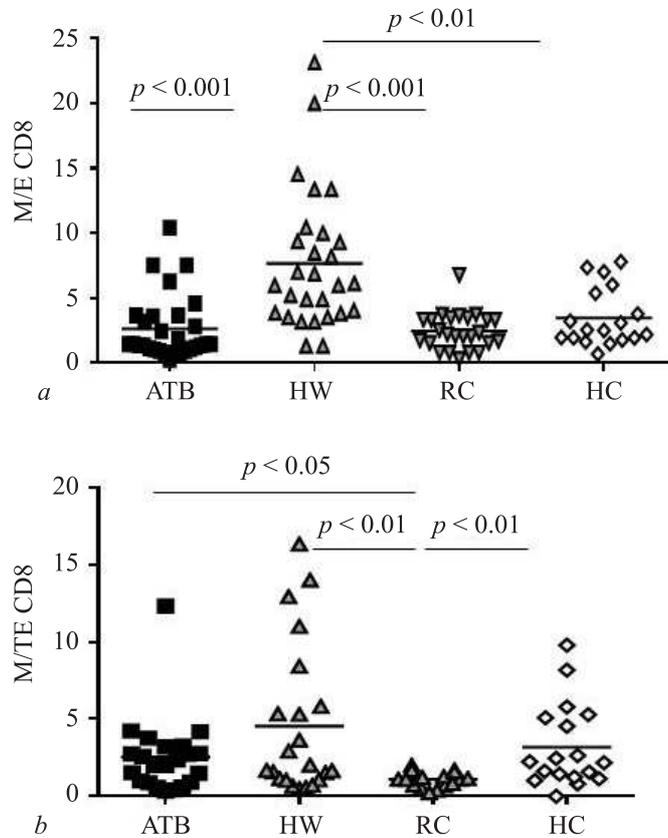


Fig. 2. HW contacts are characterized by an increased M/E ratio, while a low M/TE ratio is the hallmark of the RC group. *a*) The M/E (CD45RA-CD27+/-/CD45RA+CD27-) CD8 T cells ratio; *b*) the M/TE CD8 T cell ratio in ATB, HW, RC and HC groups. (Man-Witney non-parametric test, $p < 0.05$ is considered significant)

A large body of evidence suggests the important contribution of CD8 T cells in the MTB-specific immune response [6-8, 13]. The latter display specific effector functions performed upon recognition of mycobacterial antigens – production of IFN- γ , lysis of infected host cells, and direct killing of MT bacteria [14].

The established frequency of MTB-specific CD4 T cell responses in the studied groups was relevant to literature data and reflected a correlation between this response in MTB-infected patients and the antigen burden. ATB patients presented a positivity in the typical range of 70–85%, established with IFN γ -based assays [11]; recent contacts are reported to have about 50% probability of MTB infection (43% established in our RC group), and the positivity of 38% in HW does not differ from the OSHA annual occupational TB risk for 50 bed-wards

with more than 100 admissions for TB per year, equivalent to 34.5% [15]. Indeed, the response to the RD1 antigens was shown to be lower in MTB-infected subjects without active disease than in ATB patients, and decreased under effective chemotherapy treatment together with the bacterial load [16, 17].

Interestingly, it was not the CD4 but the CD8 MTB-specific response that differentiated between active MTB and different LTBI stages. An MTB-specific CD8 T response was detected in only 30% (6/20) of ATB and 24% (5/21) of HE subjects, while it was present in 81% (17/21) of RC subjects, and with a significantly higher IFN γ expression.

The high frequency of RD-1-specific CD8 T cells in RC, in line with a significantly decreased M/TE ratio, most probably reflects recent contacts with MTB antigens, leading to an effective activation and differentiation of MTB-specific CD8 T effectors, and redistribution of subsets at the peripheral blood level, therefore confirming a protective role for the CD8 rather than for the CD4 response in the settings of acute MTB infection. One possible explanation for the reduced antigen-specific CD8 T cell frequency in ATB patients would be the sequestering of large numbers of CD8 T cells at the locus of infection in active phase TB [8], and/or CD8 T cell apoptosis due to the continuous in vivo stimulation and the impossibility to contain mycobacterial spread. This is in agreement with the findings in a cattle model of MTB infection in which the CD8 response was present at the onset of the infection [13]. Further, a recent study indicates that the frequency of CD8 T cells against ESAT-6 and other 5 MTB-specific antigens is higher in subjects with LTBI than with active TB [18]. It should also be noted that in the present study we did not observe a high CD8 T cell response in HW. These subjects might have developed regulatory mechanisms to avoid the persistent high T cell activation due to the almost constant exposure to MTB. In this context, pathologically-increased inhibitory regulatory T-cells have also been observed in TB patients [19].

It should be underlined that no ICS of CD4 or CD8 T cells was established in the BCG control group, confirming the specificity and applicability of this methodology for testing a BCG-vaccinated population as the Bulgarian one.

The data from MTB-specific ICS assays were completed by the subset analysis of the CD8 T cell compartment in the studied groups. The significantly increased M/E CD8 T cell ratio that distinguished HW from RC, ATB and HC subjects was due to a simultaneous accumulation of CD8 M, and decrease of the effector cell population. In fact, CD27+ CD8 T cells are apoptosis-resistant memory cells retaining their replicative potential. Their accumulation reflects the beneficial homeostatic function of Treg cells in case of strong/long-lasting stimulation [20], and may be advantageous at the level of the CD8 pool during chronic viral infections.

Conclusion. The study of MTB-specific CD8 T cell response adds to the differentiation between active and latent TB. A prominent early MTB-specific CD8

T cell response prevents from the development of active infection. In the settings of chronic stimulation, the generation of CD8 memory pool is advantageous over the extreme differentiation of effector cells.

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