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ORIGINAL ARTICLE

Evaluation of CD27 Expression on Mycobacterial Antigen-Specific CD4$^+$ T Cells as an Immunological Marker for Diagnosis of Active Pulmonary Tuberculosis

[1]Mona A. Gadallah[*], [1]Abo Elfetoh E. Elanany, [1]Marwa A. Abd Elwahab[1], [2]Basem I. El Shaffey, [1]Wageih S. El-Naghy, [2]Ayman S. El-dib, [2]Dalia El Sharawy, [1]Nadia M. Dahy, [3]Mahavir Singh, [3]LIONEX Scientific Research Group

[1]Medical Microbiology & Immunology Department, Faculty of Medicine, Tanta University
[2] Department of Chest diseases, Faculty of Medicine, Tanta University
[3] Lionex Diagnostics and Therapeutics, SalzdahlumerStraße 196, 38126 Braunschweig, Germany

Key words:
Tuberculosis, flow cytometry, CD4$^+$ T cells, CD27, ESAT-6 & CFP-10 antigens

Corresponding Author:
Mona Abd El-Aziz Gadallah, Demonstrator at Medical Microbiology & Immunology Department, Faculty of Medicine, Tanta University, Egypt.
Tel.02-01013326853
mona.gadallah@med.tanta.edu.eg

ABSTRACT

Background: *Until now, there is a need for novel tools helping the prompt diagnosis tuberculosis (TB).* ***Objective:*** *This study aims to explore the extent of CD27 expression on ESAT-6 & CFP-10 antigen-specific CD4+ T cells as a recent biomarker for rapid diagnosis of active pulmonary TB in Egypt.* ***Methodology:*** *Based on IFN-γ+ expression, ESAT-6 & CFP-10 antigen-specific CD4+ T cells were identified and the CD27 expression was analyzed for 60 M. tuberculosis-infected patients and 20 healthy controls.* ***Results:*** *The present data, in fact, displayed that patients with pulmonary TB had significantly lowered CD27 expression than healthy controls (P <0.001). The persistent active TB patients had much lesser percentages of CD27+ T cells than culture-positive recently infected TB patients (P =0.014) and healthy controls (P< 0.001). On evaluating the diagnostic performance of CD27 expression, it has been declared that at a cutoff value of 43.64%. CD27 had a sensitivity, specificity, positive predicted value (PPV), and negative predicted value (NPV) 86.7%, 100%, 100%, 71.4% respectively, for discrimination between patients with TB from healthy subjects.* ***Conclusion:*** *Thereby, proportion of CD27+ antigen-specific CD4 T cells could be used as an immunological marker for active TB.*

INTRODUCTION

Tuberculosis (TB) is one of the oldest human infections documented along history. Also, it stills a major public health priority. TB afflicts about 10.4 million people worldwide and account for about 1.8 million deaths according to WHO report in 2015. Also, in the same year, the incidence of tuberculosis (per 100, 000 people) in Egypt was 15 as measured according to the World Bank [1,2]. Moreover, the number of TB victims is escalating again due to the spread of multidrug-resistant TB (MDR-TB) [3].

The lack of gold standard diagnostic test or a rapid reference laboratory test due to the limitations of Current methods for TB diagnosis, including microbiological, radiological and clinical examinations makes TB a serious uncontrolled disease, especially if mycobacteria cannot be detected in the sputum of the cases[4]. More than fifty percent (58%) of patients infected with pulmonary TB in 2014 were confirmed to be positive by laboratory routine such as smear or culture, the remaining 42% of patients were diagnosed by means of clinical criteria only including history, symptoms, or by chest X-ray, highlighting that diagnostics availability in the resource-limited sites is needed [5].

Therefore, Blood-based host biomarkers exhibit attractive alternatives to tests that rely on identifying mycobacteria for diagnosing TB [6].

It has been declared that the cellular immune response, chiefly by T lymphocytes, plays a crucial role in limiting *Mtb* replication. Mycobacterium tuberculosis-specific CD4+ T-cells seem to work via cytotoxicity against the infected target cells. Their effect is virtually mediated by cell production of interferon gamma (IFNγ) in response to M. tuberculosis antigens[7,8].

Pathogen-specific CD4 T cells maturation is associated with changes in the cell surface proteins expression pattern, such as CD27[9]. CD27 which is a 120-kDa transmembrane homodimeric molecule is expressed on the mainstream of T cells, B cells, and NK cells. It is a member of the tumor necrosis factor

receptor family and it has a pivotal role in T cell immunity [3, 10]. On the naïve T cells and early effector T lymphocytes, CD27 is constitutively expressed but at late stages of effector cell differentiation, it is down-regulated; thereby, late effector lymphocytes exhibit less to no expression of CD27. Based on these considerable changes that *Mtb*-specific CD4 T-cells undergo as they differentiate in response to virulent mycobacteria, this work aims to evaluate CD27 as an immunological biomarker in favor of diagnosis of active TB and through estimation of CD27 expression on mycobacterial antigen-specific CD4 T cells that reacted to *Mtb*-antigens by the production of IFN-γ in patients with pulmonary TB and healthy controls via multi-parameter flow-cytometry.

METHODOLOGY

Subjects

This study was carried out on 60 *Mycobacterium tuberculosis* patients and 20 healthy controls. *Mtb* patients were recruited from Tanta Chest Hospital, El-Mahalla Chest Hospital, during the period from May 2016 to June 2017. This study was approved by the Ethical Committee, Tanta Faculty of Medicine approval number (30799\03\17) Each individual participated in the present study was fully informed concerning the character of the disease and the diagnostic procedures. Patients group with active tuberculosis was subdivided into three subgroups:(20 recently infected active pulmonary TB patients (13 males and 7 females) based on positivity of sputum smear and/ or culture, 20 persistent active TB patients (16 males and 4 females): with persistent positive culture and lesions in chest X-ray after a minimum of 2 months of standard anti-TB chemotherapy regimen and 20 clinically and radiologically diagnosed TB cases (8 males and 12 females): proved by clinical & radiological assessment and all of them had ESR and positive Gene Xpert device results but they had negative smear & culture results.

Exclusion criteria: Individuals who were known to have any immunosuppressive condition, and HIV positive patients.

Microbiological study

All patients and controls included in the present study were subjected to full medical history and clinical examination. Also, they were subjected to ZN stain (Hexabiotch kit) for 3 successive times then they were cultured on LJ mediaat EL-Mahalla Chest Hospital laboratory.

Immunological study

Peripheral blood mononuclear cells (PBMCs) preparation:

Four ml venous blood samples were withdrawn by venipuncture from all studied subjects on evacuated tubes containing EDTA as an anticoagulant, and samples were processed within 4 hours of blood collection. Then, PBMCs were purified from whole blood by centrifugation using high-density HISTOPAQUE 1077 (SIGMA ALDRICH, United Kingdom). The cells were counted with the hemocytometer and their viabilities were determined with trypan blue. Then, the cells were re-suspended in RPMI 1640 (BioWhittaker, Switzerland) supplemented with 10% fetal bovine serum, penicillin 0.1 mg/ml, and streptomycin 0.3 mg/ml (Dingguo Biosciences, Beijing, China) to a concentration of 2×10^6 cells/ml.

Antigen Stimulation:

M. tuberculosis region of difference 1 (RD1) antigens including lyophilized ESAT-6 & CFP-10 (Lionex, Braunschweig, Germany**)** were used as stimulating antigens and reconstituted according to the manufacturer's instructions followed by incubation at 5% CO_2, 37°C incubator for 16–18 hrs**.** This stimulation was performed within 5 hours of blood collection. At the end of incubation, the PMNCs cells for each sample were washed twice with sterile phosphate buffer with 2% fetal bovine serum (PBS-FBS) (Bio Whittaker, Switzerland) with saving the pellet.

Phenotypic Staining and Cytometric Analysis:

At the flow cytometry unit at Centre of Excellence for Cancer Research, the cells were stained with labeled (FITC) conjugated monoclonal anti-human CD4 (MiltenyiBiotec1, Germany) , (PE) conjugated mono clonal anti-human CD27 (MiltenyiBiotec1, Germany) then permeabilizing solution (BD CellFIX, Shannon, Ireland) and incubated for 10 minutes. 'This step was done for permealization (APC) conjugated monoclonal anti-human IFN-γ (MiltenyiBiotec1, Germany) at room temperature in the dark for 30-45 minutes. Then the pellet was washed again by adding 2ml PBS and finally the cells were resuspended in 200 µl PBS and mixed thoroughly for acquisition by flow Cytometry. Flow cytometric analysis was performed using BD FACSCanto II (BD Biosciences, San Jose, CA) flow cytometer for acquisition; BD FACSCDiva and flowjo software were used for data analysis and interpretation. Gating protocol for CD27 positive IFN-γ positive CD4 positive cells is demonstrated in (Fig. 1, 2).

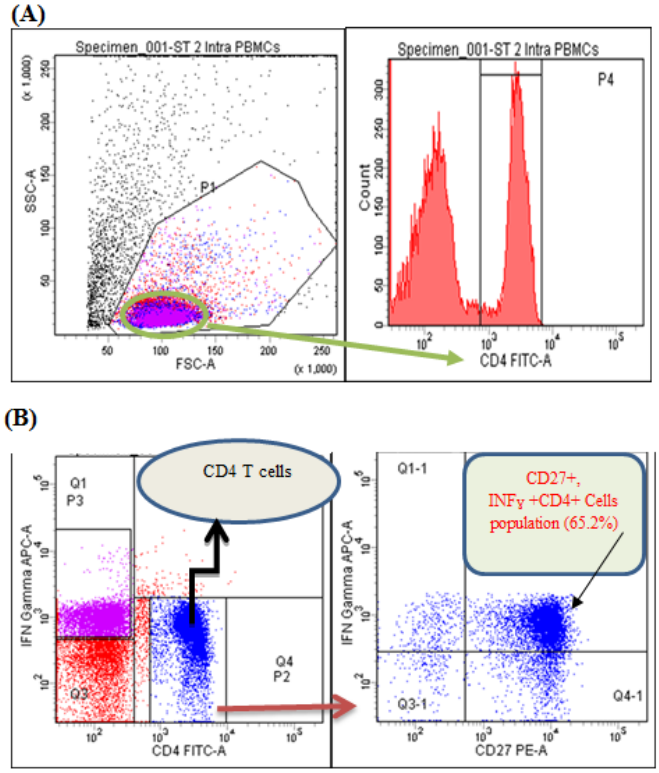

Fig. 1: A representative flow cytometric analysis (CD27, INF gamma, CD4) in the peripheral blood PMNCs in healthy control. (A) Scatterogram and histogram showing forward and side scattering of CD4 cells. (B) CD4 versus INFγ plot was used to identify MTB specific CD4 T cell response then gating of CD4 +INFγ+T cells to show expression of CD27.

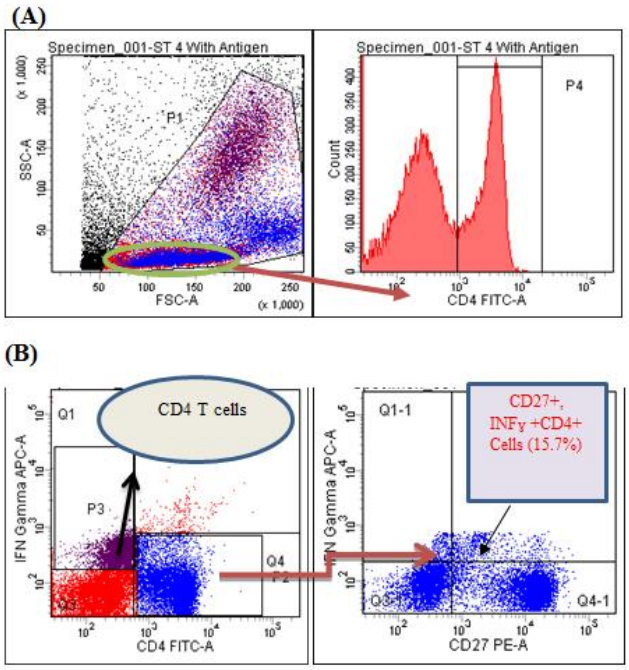

Fig. 2: A representative flow cytometric analysis (CD27, INF gamma, CD4) in the peripheral blood PMNCs in TB patient. (A) Scatterogram and histogram showing forward and side scattering of CD4 cells (B CD4 versus INFγ plot was used to identify MTB specific CD4 T cell response then gating of CD4 +INFγ+T cells to show expression of CD27.

Statistical Analysis:

In the present study, statistical analyses of data were carried out using SPSS version 23. Shapiro –Wilks test was used to test normal distribution of variables. Numerical data were expressed as mean ± standard deviation. Qualitative data were summarized as percentages. Correlations between different parameters were done using spearman's correlation coefficient. The Receiver Operating Characteristic (ROC) was constructed to obtain the most sensitive and specific cutoff value for $CD27^+$ antigen-specific CD4 T cells. The diagnostic sensitivity, specificity, positive predictive (PPV) and negative predictive (NPV) values were calculated. The probability (P) values of ≤0.05 were considered statistically significant indicated.

RESULTS

From May 2016 to June 2017, 80 subjects were enrolled in the following four groups: healthy controls (n = 20), recently infected active pulmonary TB patients (n = 20), persistent active TB patients (n = 20), and clinically and radiologically diagnosed TB cases (n = 20). Patient characteristics are summarized in table 1. Age was evenly distributed in the four groups, The mean age ± SD in years was 36.4± 12.93, 52.17 ± 15.15, 52.75±12.15, and 39.2±20.5 for the healthy controls, recently infected active pulmonary TB patients, persistent active TB patients, and clinically and radiologically diagnosed TB cases groups, respectively (P = NS). Whereas the first three groups were composed mostly of men (60%, 65%, and 80%), the last one had a higher percentage of females (60%), with statistically significant difference between the four groups *(P = 0.07).* Table 1 also shows the mean patients' body weight at diagnosis. The mean ± SD body weight among the 60 patients was 62.93±13.59 kg compared to 72.2±8.167 kg in healthy controls. Male patients weighed about 0.68 kg more than female patients. Patients with persistent TB had a lower weight than recently infected patients, and patients with smear-positive TB weighed less than patients with smear-negative pulmonary TB. Also, approximately sixty percent of controls and fifty-five percent of the patients were of urban. CD 27+ T lymphocytes (CD4 +ve, INF γ +ve & CD27 +ve %) were significantly different among the four groups *($P < 0{\cdot}001$)*. The healthy controls group was significantly higher than all active TB patients groups ($P < 0{\cdot}01$; pair-wise comparison), suggesting an association between disease and low CD 27+ T lymphocyte percentage (Fig. 3). Patients with persistent TB had the lowest percentage of CD 27+ antigen-specific CD4 T lymphocyte compared to patients with recently infected TB and those with clinically and radiologically diagnosed TB *($P=0.014$, $P=0.021$; respectively)*. This difference was lost when patients with clinically and radiologically diagnosed TB were compared to those with recently infected TB *($P =0.559$).*

Table 1: Demographic data and biochemical parameters of the patients and controls:

Variable / Groups	Healthy Controls (N=20)	New TB patients (N=20)	Persistent TB patients (N=20)	Clinical TB patients (N=20)	Total TB patients (N=60)
Age(Yrs.)	36.4 ±12.93	52.17±15.15	52.75±12.15	39.2±20.5	48± 16.59
Gender Male/Female Percentage of Male	12/8 (60%)	13/7 (65%)	16/4 (80%)	8/12 (40%)	37/23 (61.7%)
Body weight (Kg)	72.2 ± 8.167	65.83±11.29	51.75±12.74^{a*}	68.4±13.79	62.93±13.59
Residence Urban (%)	12/20 (60%)	9/20 (45%)	15/20 (75%)	9/20 (45%)	27/60(45%)
CD 27+ antigen specific CD4 T cells (%)	62.43±12.94	26.07±12.05^{a***}	6.002±4.33^{a***,b*}	31.18±15.9^{a**,c*}	22.42±15.44^{a***}
CD 27-ve antigen specific CD4 T cells (%)	4.1 ± 2.077	21.18 ± 9.3^{a**}	48.47 ± 16.46^{a***,b**}	13.77±6.84$^{ac:*}$	25.99±17.6^{a***}
INF γ response after RD1antigen stimulation	22.66 ± 5.7	53.3±11.84^{a**}	46.82 ±8.95^{a**}	63.5 ± 16.66^{a***,c*}	54.97±13.88^{a***}

- Data are expressed as Mean ± standard deviation
- TB: Tuberculosis; SD: standard deviation; N: Number of cases; CD: Cluster of Differentiation; INF γ: Interferon Gamma; RD1: Region of difference 1
- ^{a}: significant difference from Healthy Controls, ^{b}: significant difference from New TB patients, ^{c}: significant difference from Persistent TB (Treatment failure patients).
- *: P ≤0.05, **: P ≤0.01, ***: P ≤ 0.001.

Statistically significant differences were observed among the four groups in the analysis of the percentage of late differentiated memory/effector (CD27–) *(P < 0·001)* subsets within the CD4+ T-lymphocyte population. Patients with persistent TB had the highest percentage of (CD27–) CD4+ T cells. This was in contrast to the healthy subjects who had the lowest percentage. Pair-wise comparisons between these two groups were statistically significant for the percentage of late differentiated memory/effector (CD27–) subsets of CD4+ T-lymphocyte population (Fig.3). Also, a statistically significant difference was observed when patients with recently infected TB and those with clinically and radiologically diagnosed TB compared to healthy controls (P= 0.006 & P= 0.031; respectively). Additionally, patients with clinically and radiologically diagnosed TB showed a statistically significant difference in the percentage of CD 27-ve antigen-specific CD4 T cells compared to persistent TB patients (P =0.018). While no differences were noted in the percentage of late differentiated memory/effector (CD27–) when patient with clinically and radiologically diagnosed TB compared with those with recently infected TB.

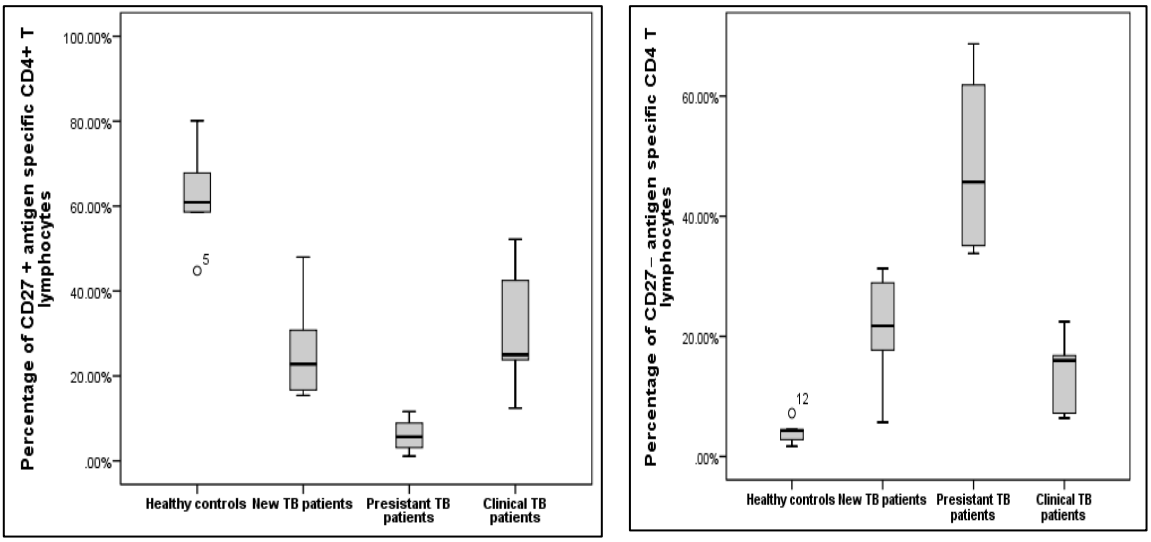

Fig. 3: Peripheral blood percentage of CD4+ T-lymphocyte subpopulations. CD27– late differentiated memory/effector cells and CD27+ memory/effector cells. The box stands for median and interquartile, and the whiskers represent minimum to maximum of indicated index.

CD4 T lymphocyte activation was assessed by the expression of INFγ after RD1 antigen stimulation. In comparison with the healthy controls group, the percentage of INFγ +ve CD4 T cells was high in the other groups particularly in the group of patients with clinically and radiologically diagnosed, and recently infected active TB (P≤0.001) suggesting an overall increase in cellular activation in response to ongoing MTB disease (Fig. 4).

A statistically significant difference was detected in the percentage of INFγ +ve CD4 T cells among the four groups (P < 0·001), with higher values observed in the clinical pulmonary tuberculosis group (22.66 ± 5.7, 53.3±11.84, 46.82 ±8.95, and 63.5 ± 16.66 for healthy controls, recently infected TB patients, persistent TB patients, and clinically and radiologically diagnosed TB patients groups, respectively).

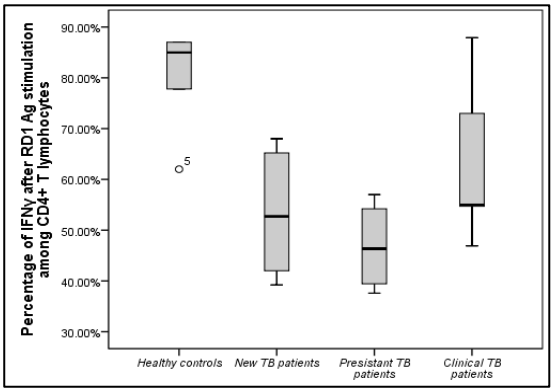

Fig. 4: Percentage of peripheral blood CD4+ T lymphocytes expressing activation markers INF γ after RD1antigen stimulation. The box stands for median and interquartile, and the whiskers represent minimum to maximum of indicated index.

Relationships between CD27 + antigen specific CD 4 T cells and other parameters:

Results of laboratory tests indicated that CD27 + T cells decreased in parallel with body weight (r =0.466, P = 0.038). However, percentage of CD27 + T cells was not significantly correlated with Age (r = -0.258, P=0.272), INFγ response after RD1 Ag stimulation (r = 0.275, P = 0.0.24), or residence (r = -0.142, P = 0.552) (table 2).

Table 2: Correlation between percentages of CD27 + antigen-specific CD 4 T cells and other studied parameters

Parameters	Concentration of CD27 + antigen-specific CD 4 T cells (%)	
	r	P-value
Age (Yrs)	-0.258	0.272
CD27 + antigen-specific CD 4 T cells (%)	1.000	-
INFγ response after RD1 Ag stimulation (%)	-0.275	0.24
Body weight (Kg)	0.466*	0.038
Occupation (%)	-0.387	0.092
Residence (%)	-0.142	0.552

Evaluation of expression of CD27 on CD4 antigen-specific CD4 T cells as a marker of tuberculosis:

Using ROC curve to differentiate between TB patients over the non-TB healthy controls group), showed that at a cutoff value of 43.64% CD27 has a sensitivity, specificity, PPV, and NPV 86.7%, 100%, 100%, 71.4% respectively, for differentiation patients with TB from healthy subjecs (table 3, Fig. 5).

Table 3: ROC curve shows sensitivity, specificity, PPV, and NPV of CD27 as a marker of tuberculosis.

Test	Best cut -off value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	AUC (95%CI)
Healthy control vs. total TB patients						
CD27 + antigen- specific CD 4 T cells	43.635%	86.7	100	100	71.4	0.973 (0.908-1.039)

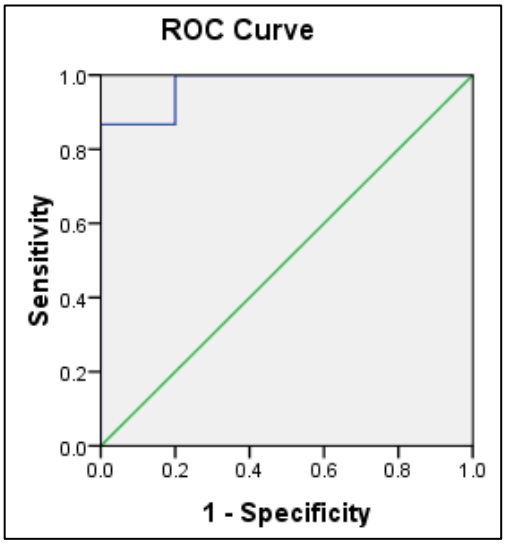

Fig. 5: ***Receiver operating characteristic (ROC) curve of*** CD27 + antigen specific CD 4 T cells indicates that at a cutoff value of 43.64% CD27 has a sensitivity, specificity, PPV, and NPV 86.7%, 100%, 100%, 71.4% respectively.

DISCUSSION

TB remains a principal public health burden[11]. In clinical practice, rapid diagnosis of pulmonary TB persists as a challenge for clinicians [12].

Current advances in TB research have drawn the attention to focus on new diagnostics thereby, Expression of CD27 have been evaluated for this purpose. It is expressed on the naïve T-cell surface and down-regulated when these cells proceed from a non-antigen experienced state to a terminal memory one [13]. Thus, Lack of CD27 expression on T lymphocytes labels highly differentiated functionally mature T cells [4].

M. tuberculosis is an intracellular pathogen. Consequently, Th1 phenotype is an eminent player against it that means that IFN-γ is the signature cytokine. Thus, intracellular cytokine production is utilized as the standard method of recognizing these cells [14]. Likewise, here the percentages of IFN-γ+ cells were used as a marker for stimulation.

In that context, most flow cytometric researches on TB depend on ex-vivo short-term T-cell stimulation by

M. tuberculosis- precise antigens; ESAT-6 and CFP-10. In humans, T-cell responses to either ESAT-6 alone [15, 16] or those to ESAT-6 and CFP-10 [15, 17] were sensitive and specific for recognition of active pulmonary or extrapulmonary TB**.**

As regard CD27 expression, it has been revealed that there is drop in the percentage of $CD27^+$ antigen-specific CD4 T cells in TB patients. These observations may reflect a higher cell differentiation and tissue homing after primary antigenic insult by *M. tuberculosis*, respectively [18]. Lymphocyte homing has been explained during tuberculosis in lymph nodes [19], lungs [20] and pleural spaces [21]. Furthermore, Lyadova *et al* also stated that these (CD27–) CD4 T cells can migrate from peripheral sites to the lung and can also be derived in situ from $CD27^+$ T-cells as they are infrequently found in lymph nodes [22].

In our work patients recently infected with TB showed a low percentage of $CD27^+$ antigen-specific CD4 T cells and a high percentage of (CD27–) antigen-specific CD4 T cells compared to healthy controls. Furthermore, persistent TB patients showed more statistically significant decrease in the percentage of $CD27^+$ cells and a statistically significant increase in the percentage of late effector (CD27–)cells compared to both healthy controls and new TB patients.

Besides, patients with clinical TB who had clinical and radiological data supporting TB diagnosis but their smear and culture were negative showed a statistical significant decrease in the percentage of CD 27^+ cells and a statistical significant increase in the percentage of late differentiated (CD27 cells compared to healthy controls.

Curiously, these described results were in accordance with Jiang *et al.*[10] and Nikitina *et al* who found that in patients with pulmonary TB, CD27 expression was significantly reduced compared with controls. The reduced expression was even more significant in patients with persistent active TB**.** Also**,** Nikitina *et al* demonstrated that the percentages of CD27 -ve IFN-γ +ve cells were significantly higher in TB patients compared to *Mtb* -unexposed individuals [4]**.**

In addition, the results of this study have followed the same trend of studies published in 2007 and 2008 which compared T-cell responses in patients with active pulmonary TB and controls. A striking difference was noted in term of CD27 expression on tuberculin-specific CD4 T-cells. The down-regulation of CD27 might occur following infection with TB [13, 23, 24].. The down-regulation of CD27 may be emerged from the existence of mycobacterial antigens in the host during active infection which eventually, might promote the deficit of CD27 expression as the antigen-primed T cell populations differentiate into effector cells. Thereby, raised incidences of $CD27^-$ T cells in pulmonary TB patients might presumably reflect the fact of the presence of high ratio of effector T cells and indicate the presence of antigens in vivo, which in turn can explain the observations that patients with persistent active TB or treatment failure group had the highest frequencies of $CD27^-$ antigen-specific T cells [10, 23]. In this context, following exposure, this response is boosted, and extra cells lose CD27. Therefore, the frequencies of $CD27^+$ T cells might have a significant diagnostic implication as an immunological marker for persistent active infection.

The state of CD4 lymphocytes cellular activation was assessed by the detection of antigen-specific INF γ production. In humans, antigen-specific IFNγ-producing $CD4^+$ T cells have been identified, which have a potential cytotoxic effect. The results presented here declared that there was a statistically significant increase in the percentage of $CD4^+$ IFN γ^+ T-cells induced by RD1 antigen stimulation in total TB patients compared to healthy control. This is in line with Nikitina *et al* [4] and Lichtner *et al* studies [25] which reported that the percentages of IFN-γ+ cells were higher in TB patients as compared to *Mtb* unexposed individuals**.**

This increase of INF γ response in TB patients can be explained as effector cells that have recently encountered antigen in vivo can promptly release IFN-γ when re-exposed to the antigen [26].

Moreover, the present results showed no significant correlation between CD27 expression and INF γ response after RD1 Ag stimulation. This matched well with the studies that showed no significant correlation between reactivity to tuberculin and expression of CD27 on T cells [4, 13].

Evaluation of the diagnostic performance of CD27 expression on antigen-specific CD4 T cells as a marker of tuberculosis on the receiver operating curve (ROC) declared that at a cutoff value of 43.64%, CD27 has a sensitivity, specificity, PPV, and NPV of 86.7%, 100%, 100%, and 71.4% respectively for differentiation of patients with TB from healthy controls. These results correspond well to the results by Streitz *et al* who first described an increase in the frequency of $CD27^-$ *Mtb* specific CD4 T Cells and delineated that evaluation of these cells can be used to discriminate TB patients from healthy controls. The cut off value discriminating TB patients from healthy control (43.64%) was slightly lesser than that stated by Streitz *et al* (49%). This may be due to technical variations or the difference in patients' selection [13].

CONCLUSION

The previous findings support that CD27 expression may have a substantial diagnostic implication for the detection of tuberculosis. However, a larger patient population is required to validate these outcomes as this study was a cross-sectional analyzing a relatively small number of subjects within each group. Moreover, the mechanisms of decreased CD27 expression in patients

with tuberculosis need to be more explored. Also, the progressive versus non-progressive/latent *Mtb* infection needs to be assessed, as well as the biomarkers ability to distinguish between TB and other inflammatory diseases. Significant effort is still required until the application of such novel diagnostic strategies on a large scale could be attained. However, this novel diagnostic host biomarker for early identification of progressive TB disease might herald a new era in TB diagnosis and early treatment.

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Compliance with ethical standards

Conflict of Interests:

The authors declare that they have no conflicts of interest.

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