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ORIGINAL ARTICLE

Cellular immune response in MDR-TB patients to different protein expression of MDR and susceptible *Mycobacterium tuberculosis*: Rv0147, a novel MDR-TB biomarker

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Abstract
Tuberculosis (TB) is a crucial public health problem with prevalence of multidrug resistant (MDR) rising. An accurate TB biomarker is urgently needed to monitor the response to treatment in patients with MDR tuberculosis. To analyze interaction between selected MDR-TB purified protein and immune cells, dendritic cells from MDR-TB patients and healthy subjects were stimulated by 55KDa protein fractions (Rv0147). The purified proteins identified by proteomic techniques (two-dimensional gel electrophoresis, mass spectrometry) and peptide sequences are known to bind a MHC class I alleles which are extracted from the Immune Epitope Database and Analysis Resource database (www.iedb.org). T cells were isolated from PBMC by negative selection and cells were cultured in RPMI-1640 at 37 °C and 5% CO_2. Cell culture was assayed for cytokine IL-10 and INF-γ by ELISA. We found that INF-γ production was significantly (335 ± 35.5 pg/ml, $P < 0.05$) upregulated after protein candidate (Rv0147) stimulation by dendritic cells from MDR-TB patients, whereas IL-10 production was greatly reduced compared with production in healthy subjects (212 ± 9.94 pg/ml, $P < 0.05$). In fact, the purified protein, Rv0147, stimulated dendritic cells from MDR-TB patients, failed to produce IL-10 and directly stimulates INF-γ production by T cells. These results suggest that the purified protein, Rv0147, may stimulate Th1 type protective cytokine response in MDR-TB patients but not in normal subjects. The production of INF-γ but not IL-10 in the presence of purified protein, Rv0147, may be shifted to Th1 responses in MDR-TB patients and supports its potential as protein vaccine candidates against TB.

Keywords MDR-TB patients · TB proteins · Dendritic cells · Cytokines · IEDB

Introduction

Although the number of tuberculosis (TB) deaths decreased by around 22% from 2000 to 2015, TB persisted as one of the highest ten sources of death worldwide in 2015 [1]. According to the 2016 WHO Global TB Report, it was estimated that in 2015, there were 10.4 million TB cases globally [2]. Tuberculosis control requires a potent vaccine, but the only accessible vaccine against TB, Bacillus Calmette-Guérin (BCG), has shown flexible protective efficacy in different parts of the world [3]. Over the last two decades, considerable progress has been made in the field of vaccine development with many innovative preclinical candidates and more than a dozen vaccines in clinical trials. These vaccines are developed either as boosters of the current BCG vaccine or as novel prime vaccines to replace BCG [4]. Few selective *Mycobacterium tuberculosis* antigens have been tested for their ability to stimulate immune responses in order to use them as a vaccine. On the other hand, study of cytokine responses in multi-drug-resistant (MDR)-TB patients and healthy subjects plays an important role to understand better the mechanisms of protection and pathogenesis in TB as well as to identify antigens as subunit vaccine candidates. INF-γ is a critical cytokine involved in the control of *M. tuberculosis* infections and two cytokines; IL-10 and transforming growth

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factor (TGF)-β1 are associated with the increased production and activity of immunosuppressive molecules in active pulmonary TB [5]. Accordingly, a favorable vaccine should induce strong CD8 and Th1 memory responses and at the same time prevent the induction of immune tolerance mechanisms [6]. In this study, we have investigated the immune response of supernatants obtained from dendritic cell cultures from PBMC of MDR-TB patients and healthy individuals for the production of the cytokines INF-γ and IL-10, stimulated by purified protein Rv0147 of *Mycobacterium tuberculosis.*

Materials and methods

Study groups

Blood was collected and cultured from 13 diagnosed and confirmed MDR-TB patients admitted at the Masih Daneshvari Hospital, Tehran, Iran. Seventeen heparinized blood from healthy subjects was obtained from the Tuberculosis unit, Pasteur Institute of Iran, Tehran. All of these donor groups were negative for HIV infection. Consent from all individuals was obtained before recruiting into the study. The study was approved by the Ethical Research Committee at the Pasteur Institute of Iran, Tehran.

Extraction of *Mycobacterium tuberculosis* antigen

Sensitive (MTB-1140PII) and MDR-TB (MTB-1503PII) strains were obtained from the Iranian *Mycobacterial* Collection, Pasteur Institute of Iran, Tehran. Two strains were cultured in middle brook 7H9 broth at 37 °C for 4 weeks. After centrifugation at 5000 rpm and washed with PBS, pH 7.4 (containing PMSF 1 mM, EDTA 20 mM, Triton X114 0.5%, glycerol 10%, sucrose 12.5 mM, DNAse 1 lg/ml, and DTT 10 mM), the MTB bacilli were subjected to sonication for 1 h at 50 Hz. Proteins were precipitated by ethanol. After centrifugation (13,000 rpm), pellets were dialyzed against saline [7]. The quantity of protein was measured using Bradford’s assay [8].

SDS-PAGE and two-dimensional gel electrophoresis

SDS-PAGE was carried out with the Bio-Rad system for vertical electrophoresis by the Laemmli method [9]. Isoelectric focusing was performed using the GE Healthcare system. The gel strips (4–7) were focused on an isoelectric focusing based on the following condition: 500 V for 1 h, 1000 V for 1 h, 6000 V for 3 h, for 15 KVh, running conditions consist of temperature 20 °C, current 50 μA per strip. Proteins were segregated in second dimension on 12% SDS-PAGE electrophoresis and visualized with Coomassie Brilliant Blue R-250 [10].

Mass spectrometry procedures

Mass spectrometry was performed in the Chemistry Department, York University, Britain (www.york.ac.uk/biology/technology-facility/proteomics). Protein spots of interest were separated from the gel, and digestion was carried out after reduction with S-carbamidomethylation with iodoacetamide. Spectral processing and peak list generation were performed by using Bruker Flex Analysis software (version 3.3) [11]. Result was filtered to accept only peptides with an expect score of 0.05 or lower by the Mascot program (Matrix Science Ltd., version 2.4). Protein sequences were recovered from Pasteur Institute-Paris, Tuberculist server (http://genlist.pasteur.fr/Tuberculist/). An alignment of sequences was done using the Blast 2 algorithm (http://blast.ncbi.nlm.gov/Blast.cgi.). The abundance of proteins was evaluated by calculating the PAI (protein abundance index) [12].

Purification procedures

Size-exclusion chromatography

In order to purify the 55KDa proteins (Rv0147) from the MDR-TB protein contents, size-exclusion chromatography was performed in Hiprep 26/60 chromatographic column (GE Healthcare). The column was prepacked with Sephacryl S-300 HR resins and it connected directly to Bio-Rad chromatography systems (Bio-Rad, Biologic DUO flow FPLC system). The phosphate buffer 0.05 M (pH 7) was used for the elution. The flow rate was less than 1 ml/min. Total volume, sample concentration, and voids volume were 318.396 ml, 4 mg. and 95.51 ml, respectively [13]. The purified proteins run on SDS polyacrylamide gel electrophoresis and protein bands in SDS-PAGE visualized with Coomassie Brilliant Blue.

Affinity chromatography

For the purpose of antibody purification against MDR-TB antigens, affinity chromatography was performed in affinity glass column according to the use of MDR-TB antigens covalently combined with cyanogen bromide-activated Sepharose 4B as described previously [14].

Western blotting procedures

Protein banding of purified protein (Rv0147), isolated from size-exclusion chromatography, was transferred to nitrocellulose membranes (Schleicher & Schuell Bioscience, Germany) in a Bio-Rad blot cell unit (Bio-Rad, USA). Transferred proteins were stained using ponceau solution (Sigma). The membrane was blocked by 3% bovine serum albumin (Sigma).

Washing is performed in Tris-buffered saline (TBS) with Tween-20 detergent to help remove nonspecifically bound material. The membrane is then incubated with primary antibody (MDR-TB Ab) for 2 h. The blot was washed again and secondary antibody, anti-rabbit-IgG conjugated to peroxidase (Sigma) is added. Finally, for detecting the antigen-antibody complex, 3, 3′-diaminobenzidine-tetrahydrochloride-dihydrate (DAB) was used as a chromogen and the reaction was stopped by water [15].

MHC I binding predictions

The MHC I binding predictions was made using the Immune Epitope Database and Analysis Resource (IEDB) analysis resource ANN tool [16, 17].

Cell preparations

Peripheral blood mononuclear cells (PBMCs) from the whole blood of MDR-TB patients and buffy coat of healthy individuals were isolated by density gradient separation method [18–20]. In brief, whole blood was mixed with equal volume of 6% (*w*/*v*) dextran (average molecular weight 200,000–300,000, MP.USA) and was incubated at 37 °C for 1 h to allow the sedimentation of red blood cells (RBCs). The pellet containing RBCs was discarded and the supernatant was centrifuged at 500×*g* for 15 min at 4 °C. Cell pellets were washed twice in phosphate buffered saline (PBS). For isolation of mononuclear cells, 3 ml NycoPrep 1.077 (Axis shield) was added to the centrifuge tube and carefully layer the washed cell pellets (4 ml) onto the NycoPrep solution. The tube was centrifuged at 600×*g* for 30 min, leaving the mononuclear cell layer undisturbed at the interface between the sample layer and the NycoPrep solution.

Cell culture

The viable and dead cells were determined by Trypan blue cell counting, and the cells (1×10^7 per well) were resuspended subsequently in RPMI 1640 (penicillin 100 U/ml, streptomycin 100 μg/ml, gentamycin 40 μg/ml, 10% fetal calf serum, GiBco) and cultured in 24-well plates at 37 °C and 5% CO_2. Dendritic cells were generated by culturing monocytes in tissue culture flasks with 800 U/ml GM-CSF (Sigma) and 1000 U/ml IL-4(Sigma) for 5 days and after day 5 of culture, with 20 ng/ml TNf-α (Sigma) and 50 ng/ml Poly I:C (Sigma) in RPMI 1640 with supplements as described above. T cells were purified from the PBMCs of MDR-TB patients and healthy contacts using Pan T cell isolation kit (Miltenyi Biotec) based on isolation of untouched T cells by depletion of nontarget cells or negative selection method. The both recovered DCs and T cells were determined by flow cytometry with anti CD11c and CD_3 antibody, respectively. These DCs were stimulated with purified 55KDa protein fractions (Rv0147) of MDR-TB antigens at 2 μg/ml or left unstimulated as a negative control for 48 and 72 h in the presence of autologous T cells.

Cytokine assay

To measure the concentration of secreted cytokines, the supernatants of stimulated cells are tested by commercial ELISA Kits for IL-10 and INF-γ according to the instructions of the manufacturer. The results of cytokine production in the study groups were statistically analyzed for significant differences ($P < 0.05$) by Mann-Whitney *U* test.

Results

Proteomic analysis

All the detected MDR and susceptible *Mycobacterium tuberculosis* protein extracts in the Coomassie Brilliant Blue-stained SDS-PAGE and two-dimensional gel electrophoresis were excised and subjected to in-gel digestion followed by MALDI-TOF/MS analysis (Fig. 1). Analysis of two independent experiments, using image master Melanie analysis software (version 6.0) revealed that a vast majority identified proteins were common to both strains. Most of the identified proteins were found in the range from less than 14.4 to more than 45.0 kDa. As shown in Fig. 1, several different MDR-TB protein spots were seen to be differentially expressed as compared to sensitive isolates. The identified proteins were classified according to the expressed proteins of MDR-TB isolates and upregulated or overexpressed proteins on the two-dimensional gel electrophoresis (2DE). Mass spectrometry analysis showed that majority of expressed/upregulated proteins belonged to the cellular metabolism and respiration category (Rv2215, Rv3161c, Rv3057c, Rv1392, Rv3028c, Rv0147 and Rv1876). The identified proteins were heat shock protein HSPX (Rv2031c), two information pathways proteins (Rv0009 and Rv1189), secreted Ag85A (Rv3804c), two hypothetical proteins (Rv3699 and Rv0443), and one secretion-associated protein (Rv3614c) belonging to cell wall and cell process pathways (Table 1).

Protein candidate selection

Antibody purification and western blotting The MDR-TB antibody was firstly chromatographically purified based on the use of MDR-TB patient's sera covalently coupled to CNBr-activated Sepharose beads in an affinity chromatography column. In order to determine the specific proteins or to explore any significance of selective antigen recognition in terms of immune response with *M .tuberculosis* protein or

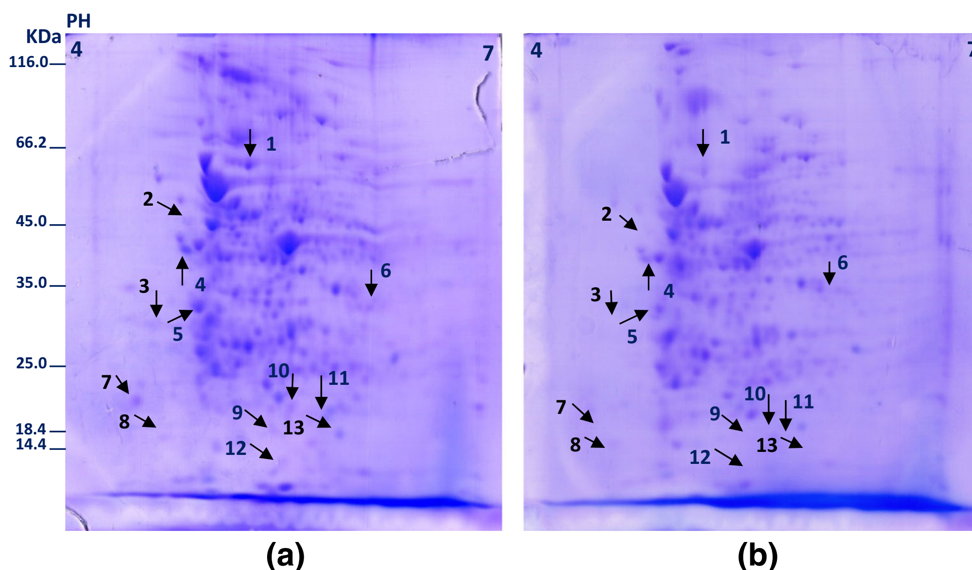

Fig. 1 Protein patterns of two-dimensional gel profiles of MDR and susceptible *M. tuberculosis* isolates. Arrows indicate a protein differentially expressed during growth in the MDR-TB isolates (**a**) compared with the susceptible TB (**b**)

vaccination status, MDR-TB antigens, which their protein profiles had been already obtained by electrophoresis procedures, were analyzed by western blotting technique. Results from the western blotting of protein bands versus MDR-TB antibodies are shown in Fig. 2. As shown, MDR-TB antibodies produced a stronger reaction with MDR-TB antigens and identified several prominent protein bands from less than 25 to 35 kDa in western blotting patterns (line 4), which was not apparent on the other areas of this profile. To identify non-immunogenic proteins in the white regions of the western blot film (Fig. 2, line 4), protein bands on the polyacrylamide gel electrophoresis (Fig. 2, line 3) were excised and remitted to *mass spectrometry laboratory* in the Department of Biology at the University of York (UK). Mass spectrometry analysis shows a conserved protein (Rv0147) belongs to the aldehyde dehydrogenases family. Hence, no visible bands (white regions or negative bands) on the 55 KDa areas can be due to absence of reaction between antigen and antibody. Therefore, it was one of the most important criteria for selection of Rv0147 as a protein candidate.

MHC class I peptide binding predictions The identified proteins were measured by the Immune Epitope Database for MHC class I binding predictions (IEDB, www.iedb.org). This site provides a collection of tools for the prediction and analysis of immune epitopes from antigens of interest, or other

Table 1 Identification of MDR *Mycobacterium tuberculosis* proteins by MALDI-TOF mass spectrometry

Spot no	Accession no	Protein identified	Isolated from	Description	pI	Mr (KDa)	MASCOT score
1	Rv2215	Dihydrolipoamide acetyltransferase	2DE[a]	Metabolism, respiration	4.90	57.11	181
2	Rv3161c	Possible dioxygenase	2DE	Metabolism, respiration	5.00	45.50	213
3	Rv3057c	Short chain dehydrogenase reductase	2DE, SDS PAGE[b]	Metabolism, respiration	5.27	30.67	344
4	Rv1392	S-adenosylmethionine synthetase	2DE	Metabolism, respiration	4.95	43.13	238
5	Rv3804c	Secreted Ag85B	2DE, SDS PAGE	Lipid metabolism	6.82	34.72	120
6	Rv3028C	Electron transfer flavoprotein subunit alpha	2DE	Metabolism, respiration	4.71	31.67	414
7	Rv3614c	ESX-1 secretion-associated protein	2DE, SDS PAGE	Cell wall, cell processes	3.93	19.87	306
8	Rv2031c	Heat shock protein HSPX	2DE	Virulence, detoxification	4.75	16.22	76
9	Rv1876	Bacterioferritin	2DE	Metabolism, respiration	4.51	18.44	220
10	Rv1189	RNA polymerase SigI	2DE	Metabolism, respiration	5.39	24.72	76
11	Rv3699	Conserved protein	2DE	Conserved hypothetical	4.69	25.04	287
12	Rv0443	Conserved protein	2DE, SDS PAGE	Conserved hypothetical	4.60	13.27	173
13	Rv0009	Peptidyl prolyl cis trans	SDS PAGE	Information pathways	6.20	19.23	198
14	Rv0147	Aldehyde dehydrogenase	SDS PAGE,	Metabolism, respiration	9.21	55.11	32

[a] Two-dimensional electrophoresis gels

[b] SDS-PAGE electrophoresis gels

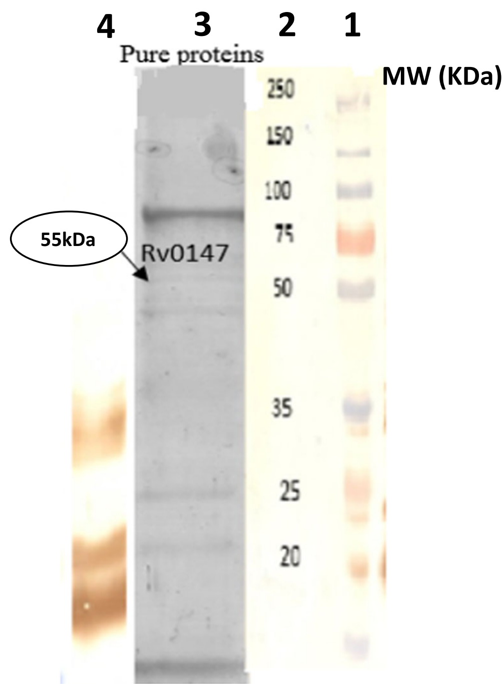

Fig. 2 Protein banding patterns of 55 KDa fractions (Rv0147) on the SDS polyacrylamide gel (10%) electrophoresis isolated from gel filtration chromatography followed by western blotting versus MDR-TB antibody. The band indicated by arrow (Rv0147) was excised and analyzed by mass spectrometry. Lanes 1 and 2: proteins ladder. Lane 3: protein fraction 55 KDa on the SDS gels. Lane 4: western blotting patterns

relevant applications. Table 1 shows the MHC-I binding predictive tool results of Rv0147 protein sequence. As shown, feedback was summarized into some categories. The anticipated result is given in units of IC_{50}nM. Accordingly, a lower number specified higher affinity. Fundamentally, peptides with IC_{50} values < 50 nM are regarded as high affinity, < 500 nM intermediate, and < 5000 nM low affinity. Moreover, in the IC_{50} values for each peptide, a percentile grad is created by comparing the peptide's IC_{50} value against those of a set of random peptides from SWISSPROT server. A small numbered percentile grad indicates high affinity. We made binding predictions for all identified peptides; however, theRv0147predicted output apparently leads to a better representation of binding specificity (Table 2). Thus, to perform dendritic cell (DC) reaction influenced by purified protein candidate, we choose the 55 KDa fractions (Rv0147) to stimulate patient and healthy subject DCs in the presence of autologous T cells.

Induction of cytokines by the 55 KDa (Rv0147) antigens in MDR-TB patients and healthy contacts Production of IL-10 and INF-γ by dendritic cell response to the 55 KDa Ag was determined by ELISA. The concentrations of each cytokine were expressed as mean concentration (pg/ml) ± SEM. Figure 3 shows the data on the production of cytokines, IL-10 and INF-γ, after treatment by protein candidates (Rv0147). The patients showed significant increases in mean production of INF-γ in response to the MDR-TB antigen from 5 μl (500 pg/ml) to 20 μl (2 μg/ml) for the 55KDa antigen (Rv0147) after 48 and 72 h of treatment. In contrast, the mean production of IL-10 in response to 55KDa Ag was not detectable in cell supernatants of the MDR-TB patients and normal subjects. After incubation time (48 and 72 h) of treatment, the cytokine responses and production of INF-γ to the 55KDa Ag (Rv0147) were increased in all MDR-TB patients. The purified protein, Rv0147, stimulated dendritic cells from MDR-TB patients, produced significantly higher levels of INF-γ (335 ± 35.5 pg/ml) than did those from normal subjects (212 ± 9.94 pg/ml) ($P < 0.05$).

Discussion

The slow decline in TB incidence globally and the persistent threat of MDR-TB both highlight the critical need for new effective TB vaccines [21]. Although there is significant evidence to support a function for cell-mediated immunity in the protective immune response against TB, little is known about Th1 regulatory cytokine production in MDR-TB [5]. There has been a tremendous effort in the last few years to represent specific *M. tuberculosis* antigens to design a novel TB vaccine or develop a rapid TB diagnostic test. This study provides a sizeable picture of the protein analysis of MDR-TB and susceptible isolates by 2DE followed by MALDI/TOF mass spectrometry to find out proteins with consistent increase in intensities in resistant TB isolates which may serve as valuable antigens for novel vaccination strategies. We have also investigated the interaction of selected MDR-TB purified protein, Rv0147, with human peripheral blood monocyte-derived dendritic cells and analyzed the cytokine expression from MDR-TB protein-infected cells focusing on their ability to stimulate INF-γ and IL-10 production on T cells.

In this current investigation, patients with MDR tuberculosis have demonstrated a stronger cellular immune response to the purified protein, Rv0147, of MDR *Mycobacterium tuberculosis* than healthy subjects do. This result was studied by examining the Th1 (gamma interferon, INF-γ) and Th2 (interleukin 10, IL-10) type cytokines produced in response to the purified protein, Rv0147, by peripheral blood mononuclear cells (PBMC) from patients with MDR tuberculosis. Interleukin 10 was not produced by purified protein Rv0147 stimulated cells from both MDR-TB patients and normal subject groups. In fact, the purified protein, Rv0147, stimulated PBMC from MDR-TB patients, failed to produce IL-10 by ELISA method, whereas these cells produced the INF-γ cytokines. A number of studies have been performed to determine cytokine profiles in the TB patients' PBMC stimulated with *Mycobacterium tuberculosis* antigens. In a study that describes the cytokine profiles in TB patients with pulmonary

Table 2 Details of T cell epitope prediction of MDR tuberculosis protein isolates identified by IEDB analysis resource

Rv no.	Allele	#	Start	End	Length	Peptide	Ic_{50}	Percentile
Rv0147	HLA-A*02:06	5	53	60	8	YVLADATV	4.00	0.2
	HLA-A*01:01	4	14	21	8	MTELVYRY	13.00	0.2
	HLA-A*15:01	8	15	22	8	MMVNHLAF	15.00	0.1
	HLA-A*02:06	8	22	29	8	FQVSTAKL	18.00	0.8
	HLA-A*02:06	3	43	50	8	YLTLGPAV	22.00	1
	HLA-A*02:06	3	52	59	8	AIAAGNAV	22.00	1
	HLA-A*02:06	3	11	18	8	LLLEVPQL	26.00	1.1
	HLA-A*02:06	1	15	22	8	TMMTTESV	27.00	1.2
	HLA-B*02:06	4	12	19	8	HLMTELVY	36.00	0.3
	HLA-A*02:06	2	44	51	8	YLADIATT	41.00	1.6
	HLA-A*02:06	5	4	11	8	AAPHLTPV	47.00	1.8
	HLA-B*02:06	3	5	12	8	WMRRRYLL	61.00	0.1
	HLA-A*02:06	6	33	40	8	RLSGYTAA	72.00	2.3
	HLA-B*07:06	9	3	10	8	RPDLSSFI	82.00	0.3
	HLA-A*02:01	3	4	18	8	LLLEVPQL	89.00	0.3
	HLA-B*15:06	9	7	14	8	SSFIYPPY	93.00	1.1

involvement and BCG-vaccinated healthy subjects, Al-Attiyah et al. [3] showed that INF-γ was secreted in response to Ag85B, ESAT6, and MPT64 in TB patients and ESAT6 in healthy donors. Except for GroEL and DnaK, single antigens did not induce TNF-α and IL-10 secretion from PBMC in either donor group. Mensah et al. [22] recently described cytokine response to selected *Mycobacterium tuberculosis* antigens in TB patients from Ghana, before and at 2 weeks of anti-TB treatment. Cellular immune response in four MTB antigens, ESAT-6/CFP-10 fusion protein, and three DosR-encoded proteins (Rv1733c, Rv2029c, and Rv2628) were analyzed in Ghanaian TB patients. All antigens produced higher levels of IFN-γ, followed by granzyme B, TNF-α, and IL-17, and low levels of IL-10 and sIL-2R-α in PBMC prior to treatment and after 2 weeks of anti-TB therapy. These results are approximately similar to our findings. In contrast, Pinheiro et al. [23] have demonstrated that cells of MDR-TB patients stimulated with early secretory antigenic target (ESAT-6), but not purified protein derivative (PPD), showed a lower frequency of CD4+/INF-γ T cells and enhanced CD4+ CD25+ IL-10 T cell population. In addition, increased IL-10 secretion was observed in cultured MDR-TB cells following ESAT-6 stimulation, but not in untreated nonresistant TB or NTM patients. Another study showed that INF-γ production by the CD4+ cells stimulated by total lipid antigens from the MDR-TB patients was decreased significantly compared with the PPD positive individuals, whereas IL-4 production in the patients was elevated [24]. The discrepancy in findings between the present and an earlier study can be explained by the difference in selective *Mycobacterium tuberculosis* antigen. In these studies, early secretory antigenic target (ESAT-6), some single antigen, and total lipid antigens were used as antigens to study the profiles of INF-γ and IL-10 secreted by PBMC of TB patients and healthy contacts, whereas we have

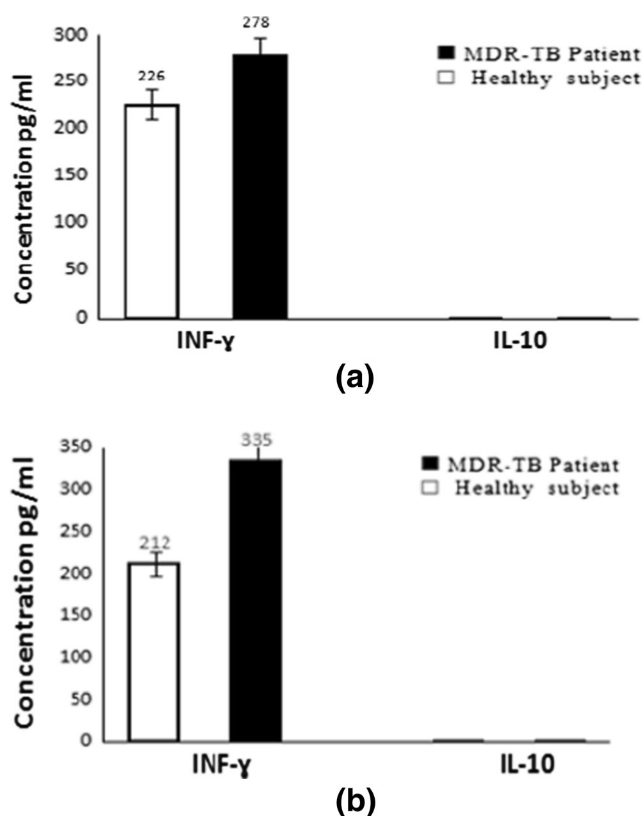

Fig. 3 Analysis of the immune response to the 55KDa Ag (Rv0147) in individuals with active MDR-TB and healthy contacts. Mean of cytokine production in MDR-TB patients and normal subjects with protein candidate based on incubation time; DCs were generated from PBMC at day 6 of culture stimulated with 55KDa Ag (Rv0147) for **a** 48 h and **b** 72 h. Results are expressed as Mean ± SE in picogram per milliliter [pg/ml]

studied the cytokine profiles in response to purified membrane/cell wall MDR-TB antigen (Rv0147) which was already confirmed by western blotting of its protein bands versus MDR-TB antibodies and binding predictions to the highly selective MHC molecules. Furthermore, secreted molecules, membrane, and whole protein extracts of *Mycobacterium tuberculosis* represent promising candidate in *Mycobacterium tuberculosis* vaccine development. Thus, we studied this protein (Rv0147) as antigens presented on MHC molecules. Because forceful vaccine should response to induce strong CD8 and Th1 immune responses and at the same time evade the induction of immune tolerance mechanism. In this way, the immudominant membrane proteins Rv0147, derived from the MDR-TB strain appeared robust INF-γ production, could maintain INF-γ production as Th1 type immune response and may represent candidates for vaccine development, diagnostic, or therapeutic strategies against TB. Our results are in agreement with those of Bai et al. [25], who also observed that Th1 cytokines and TGF beta are increased while Th2 cytokines are decreased in well-formed pulmonary granulomas of TB patients compared to controls.

As observed in Fig. 3, according to incubation time, there were differences in the magnitude of cytokine increase; the supernatants from all patients showed upregulation of INF-γ after 48 and 72 h of treatment, with no influence on the suppressive effect of IL-10, which suggest that this is not the only mechanism of regulation. On the other hand, Rv0147 probably is a potentially rich source of T cell antigens and shows potential for exploitation in vaccine development efforts; several issues need to be considered. Firstly, although INF-γ cytokine is clearly important for the control of TB, it is not necessarily the best predictor of protection against subsequent challenge [26]. The coetaneous production of other cytokines, such as IL-2, has been shown to be a significant element of a protective immune response [27]. Moreover, the phenotypic form of the responding cell population is also important [28], for instance, the presence of antigen specific central memory T cells correlates with protection [29]. Secondly, the immunogenic Rv0147 epitopes should be identified during active infection; thirdly, it may be possibly formulated with the suitable adjuvant in multiple animal models, and finally, because the number of MDR-TB patients is limited in this study, these data should be confirmed in larger cohort study and more investigation is needed regarding the potential of Rv0147 as a protein candidate for further designing of vaccine or preventive strategies against TB.

In conclusion, the development of a new generation of vaccine against tuberculosis has to elicit a strong activation of DC to stimulate the maximal Ag presentation, the production of INF-γ and IL-10 cytokines, and consequently a protective T cell response [30]. These alternative vaccines should induce the secretion of protection-associated type 1 cytokines, but not of disease-associated type 2 cytokines by PBMC [31]. Here, we showed that the 55KDa antigens (Rv0147) as MDR-TB components induce DC maturation leading to a Th1 shift in T cell measurement. Besides, secretion of INF-γ and TNF-α by PBMC in the presence of various preparations of the complex antigens supports the suggestion that protective antigens can be found in the cell wall, cell membrane, and cytosol of the mycobacterial cell [32], and hence protective antigens of *M. tuberculosis* should be searched for in various compartments of the mycobacterial cell. Hence, these findings may have a possible future decision for TB vaccine strategies.

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Compliance with ethical standards Consent from all individuals was obtained before recruiting into the study. The study was approved by the Ethical Research Committee at the Pasteur Institute of Iran, Tehran.

Conflict of interest The authors declare that they have no conflict of interest.

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