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T-cell antigens of *Mycobacterium immunogenum*, an etiological agent of occupational hypersensitivity pneumonitis

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ABSTRACT

The T lymphocyte-mediated immune lung disease hypersensitivity pneumonitis (HP) in machinists is poorly understood for disease mechanisms and diagnosis due in part to lack of information on causative T-cell antigens of the etiological agent *Mycobacterium immunogenum* (MI). Therefore, overall objective of the current study was to identify T-cell reactive antigens of this recently recognized pathogen. In this direction, purified recombinant form of five of the seroreactive proteins (reported in our initial study), including three cell wall-associated (arbitrarily designated as antigens A through C) and two secretory (AgD & AgE), were examined for their potential to activate antigen-presenting cells (APCs) viz. alveolar macrophages and human monocyte-derived dendritic cells (DCs) and for T-cell reactivity. All five proteins strongly activated APCs by inducing inflammatory cytokines (TNF-α, IL-6 & IL-1α) and nitric oxide (NO), albeit to a varying extent (AgE ≥ AgD > AgB ≥ AgA ≥ AgC), implying their differential potential for activation of APCs. However, only two of the five proteins (AgA, AgD) showed significant T-cell response (T lymphocyte proliferation and IFN-γ secretion) when tested using sensitized T-cells from MI-induced HP mouse model. These antigens also activated the human naïve $CD4^+$ T cells in presence of autologous DCs as measured using ELISPOT for IFN-γ. Immuno-informatic analysis predicted that the identified T-cell antigens (AgA and AgD) bind more number of class I and class II HLA alleles as compared with the reference immuno-dominant antigens ESAT-6 and CFP-10 from the tuberculous mycobacterial species *M. tuberculosis*. Predicted human population coverage for the epitopes of AgA (90.87%) and AgD (88.09%) was also higher as compared to those for the reference antigens ESAT-6 (82.42%) and CFP-10 (80.21%). These two antigens were further predicted to be highly immunogenic for class I peptide MHC (pMHC) complex as compared to the reference antigens. Collectively, our results imply that AgA and AgD are T-cell antigens with a high HLA binding frequency as well as population coverage for HLA alleles. This first report on T-cell antigens and epitopes of *M. immunogenum* is significant as it is expected to open up avenues for understanding pathogenesis mechanisms and developing T-cell-based immunodiagnostic tools for this poorly investigated occupational lung disease.

1. Introduction

Hypersensitivity pneumonitis (HP) is an interstitial lung disease which has been associated with various occupational exposures and designated accordingly, such as farmer's lung, pigeon breeder's lung, machine operator's lung (MOL), among others (Grunes and Beasley, 2013). The MOL form of HP has been reported for more than a decade among machinists exposed to industrial metalworking fluid (MWF) in automotive plants and other machining operations (Rosenman, 2009). In the United States alone, an estimated 1.2 million workers are annually exposed to MWF. More than 200 cases of MWF-associated HP have been described in North America (Rosenman, 2009) and 27 clusters of the disease have been reported in the literature (Rosenman, 2015). Industrial in-use contaminated MWFs are known to be colonized by species of nontuberculous mycobacteria (NTM) of the *M. chelonae-M. abscessus* (MCA) complex, notably *Mycobacterium immunogenum* (MI). MI is a frequently cultured NTM species from in-use MWFs (Shelton et al., 1999; Wilson et al., 2001) and is a widely recognized etiological agent of occupational HP in machinists exposed to these fluids (Beckett et al., 2005; Falkinham, 2003). In this context, our laboratory has isolated multiple genotypes of MI from MWF sampled from diverse industrial MWF operations (Khan et al., 2005; Yadav et al., 2003).

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Despite the recognized etiological role of MI in MWF-linked HP, little is known on the causative antigens and immunodiagnosis of this form of HP. Mycobacterial pathogens are known to activate both humoral and cell-mediated immune responses in other human infections. HP being a T cell-mediated immune disorder, MI may be expected to cause innate activation of antigen-presenting cells viz. macrophages (Chandra et al., 2013; Gupta et al., 2009) and/or dendritic cells (Bellanger et al., 2013) and development of T cell-mediated immunity as critical steps in mycobacterial HP disease process. Identification of specific MI immunoreactive proteins which can elicit strong innate and cell-mediated responses could therefore unveil the critical causative antigens of this mycobacterial lung disease that in turn could serve as important tools for understanding its poorly understood pathogenesis and development of immunodiagnosis and possibly vaccine.

Our initial immunoproteomic study (Gupta et al., 2009) on MI led to the first identification of 33 seroreactive proteins, comprised of 4 secretory, 6 cell wall-associated, 11 membranous, and 12 cytosolic proteins. In a recent study, three of the cell wall-associated seroreactive proteins (arbitrarily designated as antigen A through antigen C) and two of the secretory proteins (AgD & AgE) were cloned and expressed in *E. coli* and their affinity-purified endotoxin-free recombinant forms were generated; the 5 recombinant seroreactive proteins were then evaluated for their potential in serodiagnosis of physician-diagnosed MWF-linked HP cases (Chandra et al., 2015). In the current study on identifying T-cell antigens, these recombinant proteins were examined for activation of the antigen-presenting cells (APCs) using murine alveolar macrophages and human monocyte-derived DCs and for their T-cell response potential. The T-cell response was assessed in terms of IFN-γ induction and T-cell proliferation in sensitized splenocytes isolated from an MI-induced chronic HP mouse model and by using a human DC-T cell coculture assay based on ELISPOT (IFN-γ) analysis. The MI recombinant antigen candidates producing a strong T-cell response (IFN-γ response) were subjected to a comparative immunoinformatic analysis against well-known potent T-cell antigens ESAT-6 and CFP-10 of the tuberculosis (TB)-causing species of mycobacterium, *M. tuberculosis*, in terms of epitope binding to HLA class 1 and HLA II alleles and human population coverage for these alleles.

2. Material and Methods

2.1. Strains and culture conditions

Mycobacterium immunogenum genotype 700506 (designated as MI) originally isolated from contaminated in-use HP-linked metal-working fluid (Wilson et al., 2001) was maintained by subculturing on Middlebrook 7H10 agar or in Middlebrook 7H9 broth (Difco Laboratories, Sparks, MD, USA). *E. coli* strains *DH5α* and Rosetta Blue (DE3) used for gene cloning and expression work were grown in Luria-Bertani broth medium with or without kanamycin (50 μg/ml) by shaking (225 rpm) at 37 °C.

2.2. Recombinant MI candidate antigens

Five recombinant immunoreactive proteins of MI encoding three cell-wall associated proteins (arbitrarily designated as AgA through AgC), namely DNA helicase (60.86 kDa), GTP binding translation elongation factor (71.16 kDa) and N-acetylmuramoylanyl-D-glutamate-2,6-diaminopimelate ligase (53.36 kDa) and two secretory proteins (AgD & AgE), namely elongation factor Tu (43.52 kDa) and trehalose phosphatase (90.81 kDa) were generated in our previous study (Chandra et al., 2015) by cloning and expression in *E. coli* Rosetta Blue DE3 cells. The soluble His-tagged recombinant proteins were purified using Ni-NTA column and depleted for the contaminating endotoxin as confirmed by LAL assay (Chandra et al., 2015).

2.3. Human peripheral blood mononuclear cells (PBMCs)

For the purpose of isolation of human immune cells (DCs and T cells) for this study, blood specimens from individual unidentified healthy human volunteer donors were obtained from the Hoxworth Blood Center of the University of Cincinnati, according to the IRB approved protocol. Peripheral blood mononuclear cells (PBMCs) were isolated based on density gradient centrifugation, using Ficoll-Paque (Sigma).

2.4. Generation of human dendritic cells

For isolation of DCs, the human PBMCs were suspended in 25 ml T cell media [RPMI 1640 containing 10% FBS, 10 mM HEPES, 1 mM L-glutamine, 0.5X essential amino acid mixture, 1X nonessential amino acid mixture, 1 mM sodium pyruvate, 0.1KU/100 μg/ml antibiotic (penicillin/streptomycin) and 2 mM 2-mercaptoethanol] containing 75 μl GM-CSF (300 units/ml) and were incubated in a 75-cm^2 tissue culture flask for adherence. One hour later, non-adherent cells (lymphocytes) were removed and frozen (10% DMSO in FBS) for further use; adherent cells were gently washed (3x) with PBS buffer. For generating DCs, adherent cells were cultured (4 days) in T cell culture medium (containing GM-CSF, 300 units/ml and IL-4, 200 units/ml). Cells were incubated (37 °C) in a humidified 5% CO_2 incubator for all experiments. No cytokines and fresh media were added during the 4 days of differentiation. GM-CSF- and IL4- treated monocytes differentiated into DCs which were washed (3x) in T cell media for further use as APCs in subsequent experiments.

2.5. Isolation of human naïve $CD4^+$ T cells

Naïve CD4-positive T cells were purified from the monocyte-depleted PBMCs by immuno-magnetic depletion using Dynabeads untouched Human $CD4^+$ T cell isolation kit (Invitrogen) according to the manufacturer's instructions. Isolated cells were counted and used for further experiments. Purified cells were further confirmed by staining with labeled antibodies (Biolegend) against CD3-FITC and CD4-APC and were analyzed by flow cytometry (BD Canto II) using FACSDiva software (BD Biosciences).

2.6. Antigen priming of the differentiated human DCs

The differentiated DCs were pulsed with individual MI recombinant antigens or the MI cell lysate (final concentration range of 5–50 μg/ml) in T cell media for 48 hours. The culture supernatant was harvested and analyzed for cytokines using Ready-set-go ELISA kits (eBioscience). The resulting antigen-primed DCs were washed for further use in T-cell response assays.

2.7. ELISPOT assay for human T-cell response analysis

Enzyme-linked immunospot (ELISPOT) assay for detection of T-cell response in terms of IFN-**γ** secretion was performed by co culturing antigen primed DCs (20,000 count) with $CD4^+$T cells (16,000 count). Briefly, a multiscreen ELISPOT plate (Millipore S2EM004M99) was coated with 2.5 μg/ml anti human IFN-γ antibody (Mabtech) and incubated at 4 °C overnight. The plate was then washed (2x) with sterile PBS and blocked with T cell media (150 μl) before incubation at 37 °C for 1.5 hours. The DC-T cell mixture was transferred to the individual wells and the plate incubated for two days at 37 °C in a humidified 5% CO2 incubator. The plate was then

washed twice, using PBS containing 0.5% tween 20 and tap water, followed by repeated washing (3x) with PBST. Biotinylated anti-human IFNγ monoclonal antibody (Mabtech) was added (100 μl of 0.4 μg/ml stock) to each well followed by incubation for 2 hours at room temperature. The plate wells were washed again as described above and treated with ExtrAvidin^R-alkaline phosphatase buffered aqueous solution (Sigma-Aldrich, Cat # E2636) (100 μl of 1:1000 diluted stock) for one hour at room temperature. The plate wells were washed and developed with 100 μl of BCIP/NBT substrate (Sigma) in dark for 2–10 minutes till the appearance of dark spots. The reaction was stopped by washing the plate with tap water. Spots were then imaged and analyzed using CTL ImmunoSpot® Analyzer.

2.8. Alveolar macrophages

Murine alveolar macrophage cell line MH-S (CRL-2019) originally acquired from the American Type Culture Collection (ATCC), was routinely maintained in RPMI 1640 cell culture medium containing 10% fetal bovine serum (Atlanta biologicals) and 1% streptomycin-penicillin-glutamate solution (Invitrogen) in a T–75 cm^2 Tissue culture flask. Cells were incubated at 37 °C in a humidified 5% CO_2 incubator and split after 48 h, for further experiments.

2.9. Macrophage activation by MI recombinant proteins

MH-S cells (1×10^6 cells/ml) in 24-well culture cluster plates were treated with increasing concentrations using both a lower dose range (1–5 μg/ml) and a higher dose range (5–20 μg/ml) of each candidate protein antigen for 24 hours. Supernatants were analyzed for pro-inflammatory mediators including different cytokines namely TNF-α, IL-6 & IL-1α using ELISA kits (eBioscience) and nitric oxide as nitrite using Griess reagent (Promega).

2.10. Preparation and purification of MI-sensitized splenocytes

An MI-induced chronic HP mouse model optimized in our laboratory (Unpublished data) was used as a source of MI-sensitized splenocyte T-cells. Briefly, six-weeks old male C57BL/6J mice were instilled by oropharyngeal aspiration method with 50 μl of MI whole cell lysate (equivalent to 10 μg protein), prepared in PBS from mycobacterial cells grown to 120 Klett reading, for three consecutive days each week for three weeks. Animals were sacked 4 hours after the last instillation. All practices involving animals were approved by the University of Cincinnati's Institutional Animal Care and Use Committee (IACUC). Spleens were harvested and their splenocytes were isolated (Eweda et al., 2010). Briefly, spleens were removed aseptically and single cell suspensions prepared by passing through sterile 70 micron nylon mesh and lysing the erythrocytes. Finally the cell population were placed in a complete RPMI 1640 medium (Hyclone) supplemented with 10% fetal bovine serum (FBS) (Atlanta Biologicals) and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) and allowed to adhere overnight. Non-adherent splenocytes were isolated and used for the following T-cell response assays.

2.11. Splenocyte T-cell proliferation assay

MI-sensitized splenocytes (0.2×10^6 cells/100 μl/well) were incubated with various concentrations of individual test MI proteins for 48 hours in a 96-well cell culture cluster plate and the assay reaction was developed using the CellTiter 96® AQueous One Solution Reagent (Promega). Stimulation Index was calculated by dividing the mean OD_{492nm} value of the treated wells with that of the vehicle-treated control wells.

2.12. Splenocyte IFN-γ secretion analysis

Supernatants from cultures of murine splenocytes (0.5×10^6 cells/250 μl) treated with various concentrations of the individual test MI proteins for 48 hours were analyzed for IFN- γ using a commercial ELISA kit (eBioscience).

2.13. Immunoinformatic analysis

HLA class I- and class II- binding peptides for MI proteins AgA and AgD and the known *Mycobacterium tuberculosis* antigens CFP-10 and ESAT-6 were predicted using ProPred-I and ProPred server as described elsewhere (Singh and Raghava, 2001) which predicts promiscuous binding regions for 47 and 51 different HLA class I and class II alleles, respectively. Population coverage analysis and T-cell epitope immunogenicity analysis of these predicted epitopes was done by an online population coverage calculator tool and a T-cell class I pMHC immunogenicity predictor available in Immune Epitope Database and analysis resource (IEDB) server (http://tools.immuneepitope.org). This tool can predict the population coverage for a given set of epitopes on the basis of HLA genotypic frequencies assuming non-linkage disequilibrium between HLA loci (Bui et al., 2006; Sakib et al., 2014). All populations available in the server were included in our analysis.

2.14. Statistical analysis

Data from three independent experiments were analyzed for means and standard errors. Statistical analysis was performed using one-way ANOVA and Post hoc Tukey test was used for multiple comparisons. The p-values ≤ 0.05 were accepted as statistically significant.

3. Results

3.1. Activation of APCs by MI candidate antigens

All five candidate antigens (AgA through AgE) strongly stimulated murine alveolar macrophages (MH-S cells) at different test doses in terms of induction of proinflammatory cytokines (TNF-α, IL-1α and IL-6) and NO, albeit to a variable extent (Figs. 1 and 2). TNF-α was induced in a dose-dependent manner by all candidate antigens, at both higher (5–20 μg/ml) and lower (1–5 μg/ml) dose ranges, except AgE which showed dose-independent response at both dose ranges. Similarly IL-6 induction was dose-dependent at both dose ranges except AgE which was dose-independent at the higher (5–20 μg/ml) dose range. All candidate antigens significantly induced 1L-1α in the tested range in a dose-independent manner. All the antigens significantly induced NO in a dose-independent manner except AgD. LPS served as a positive control in the assay (Fig. 2- panels c & d). However, the LPS induction of the two cytokines tested (TNF and IL-6) showed a mutually contrasting trend, with TNF but not IL-6 showing a dose response, in the tested dose range (5–100 ng/ml). The IL-6 trend is consistent with a recent study (Vigano et al., 2015) that demonstrated IL-6 induction dose-response for only >100 ng/ml LPS concentrations.

In human dendritic cell stimulation assays (Fig. 3) using different antigen doses (5–50 μg/ml), all candidate antigens significantly induced TNF-α. The level peaked at lower concentrations (5 μg/ml for AgA, AgB, AgD, AgE and 10 μg/ml for AgC), following which it decreased significantly at higher concentrations. Treatment with MI whole cell lysate however showed the TNF-α peak at a higher concentration (50 μg/ml) and the induction was dose-independent. The LPS control showed a dose-dependent induction of TNF-α in DCs, with a significant induction at 10 ng/ml but none at 0.2 ng/ml.

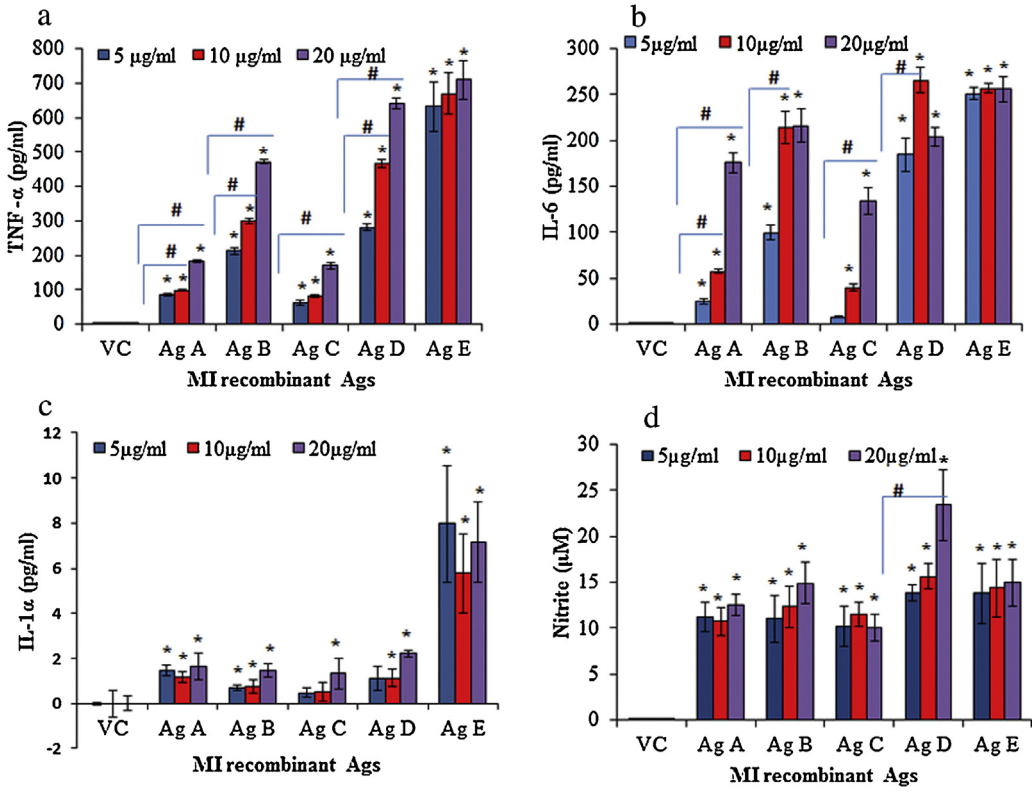

Fig. 1. Activation of cultured murine alveolar macrophages by the MI recombinant candidate antigens at a higher dose range (5 to 20 µg/ml). Panels a, b, and c: Cytokines (TNF-α, IL-6 & IL-1α, respectively). Panel d: Nitric oxide measured as nitrite content. Values are presented as means ± standard errors based on three independent experiments. Asterisk (*) and number sign (#) indicate statistically significant ($p \leq 0.05$) difference as compared to the vehicle control and between the doses, respectively.

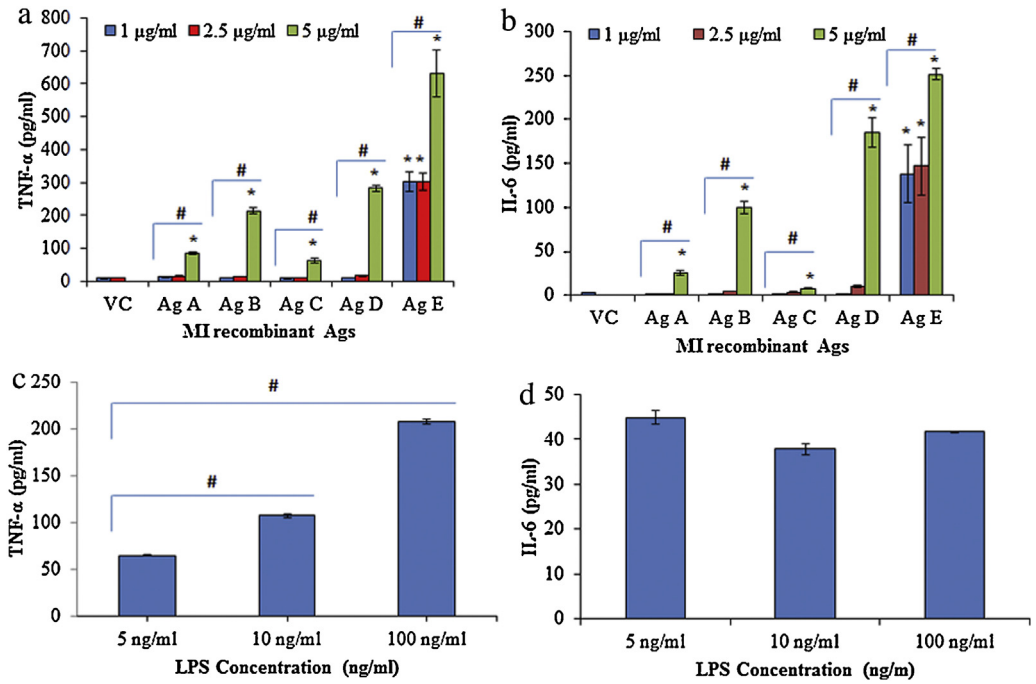

Fig. 2. Activation of cultured murine alveolar macrophages by the MI recombinant candidate antigens at a lower dose range (1 to 5 µg/ml). Panels a, b: Cytokines (TNF-α, IL-6) induced by MI antigens. Panels c and d: Cytokine induction (TNF-α, IL-6) by LPS. Values are presented as means ± standard errors based on three independent experiments. Asterisk (*) and number sign (#) indicate statistically significant ($p \leq 0.05$) difference as compared to the vehicle control and between the doses, respectively.

3.2. *T -cell reactivity of the MI candidate antigens*

Three of the five recombinant MI candidate antigens (AgA, AgB, and AgD) were found to be T-cell reactive when analyzed in terms of their potential to induce IFN-γ release and/or T cell-proliferation in splenocyte rechallenge assays. Of these, AgA and AgD induced a strong response in terms of both endpoints (IFN-γ release and splenocyte T cell-proliferation) whereas AgB showed only IFN-γ

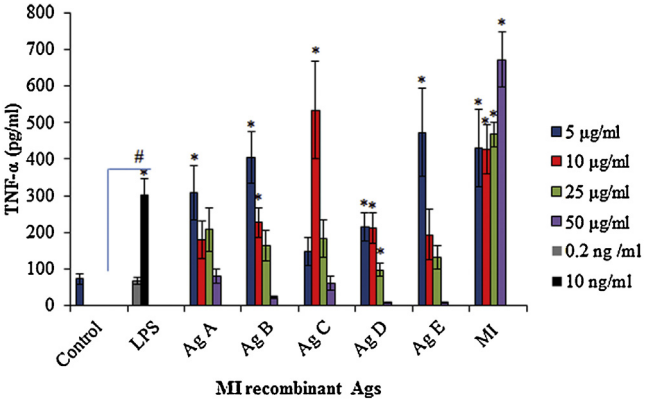

Fig. 3. Activation of human monocyte-derived dendritic cells by the MI recombinant candidate antigens and the whole cell lysate. The differentiated dendritic cells were treated with the candidate MI antigen (5- 50 µg/ml) or with MI lysate (5- 50 µg/ml) for 48 hours. The cells were also treated with LPS (0.2 ng/ml or 10 ng/ml) for use as positive controls. Supernatants were harvested and analyzed for TNF-α using ELISA as described in the Methods section. Values are presented as means ± standard errors based on three independent experiments; each experiment was performed using a different blood donor and testing each antigen dose in triplicate. Asterisk (*) and number sign (#) indicate statistically significant ($p \leq 0.05$) difference as compared to the vehicle control and between the doses, respectively.

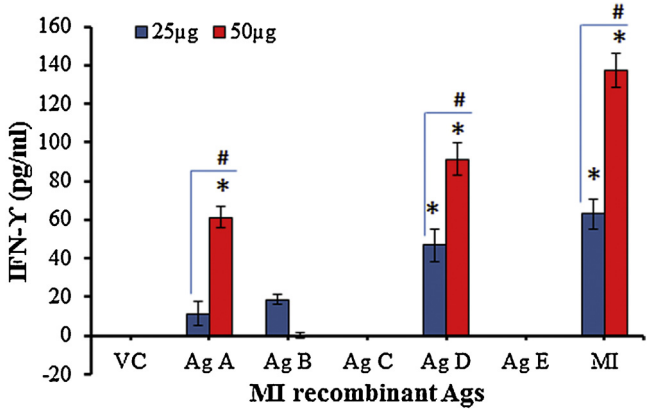

Fig. 5. Sensitized splenocyte T-cell response in terms of IFN-γ induction on exposure to the individual MI recombinant candidate antigens (AgA through AgE) and the MI whole cell lysate (MI). Values are presented as means ± standard errors based on three independent treatments of the pooled splenocytes (isolated and pooled from 9 MI-sensitized male C57BL/6 J mice) with a given antigen dose. Asterisk (*) and number sign (#) indicate statistically significant ($p \leq 0.05$) difference as compared to the vehicle control and between the doses, respectively.

release (that too with only one of the test doses), which was not statistically significant (Figs. 4 and 5). While induction of IFN-γ release was clearly dose-dependent, T-cell proliferation did not show a clear dose-dependence.

The T-cell response for the candidate antigens was further assessed using human naïve $CD4^+$ T cells and primed autologous DCs in an ELISPOT assay in terms of IFN-γ secretion. The results indicated that AgD was able to stimulate naïve $CD4^+$T cells significantly at the 5–10 µg/ml dose range whereas AgA and AgB stimulated significantly at higher dose ranges (10–25 µg/ml and10–50 µg/ml, respectively). The stimulation by AgC was not significant at any of the concentrations and AgE stimulated significantly at 5 µg/ml only. The MI whole cell lysate strongly induced the T-cell response in a dose-dependent manner. As a comparison control, the LPS did not induce significant T-cell response at 0.2 ng/ml concentration unlike the higher concentration (10 ng/ml) that significantly induced the response, as compared to the vehicle control (Fig. 6).

3.3. T-cell epitope predictions

Two of the recombinant MI proteins, AgA and AgD, that showed strong T-cell reactivity (IFN- γ response) in MI-sensitized cells were considered T-cell antigens and thus were subjected to *in silico* prediction of T cell-epitopes in comparison to the well-recognized immunodominant antigens ESAT-6 and CFP-10 of *Mycobacterium tuberculosis* (Doherty et al., 2002; Hill et al., 2005; Trajkovic et al., 2002). Based on ProPred-I analysis for HLA class I alleles, AgA and AgD were predicted to bind 47 (100%) and 43 (91%) of 47 class I alleles whereas ESAT-6 and CFP-10 were predicted to bind 38 (80.85%) and 33 (70.21%) of 47 class I alleles (Table 1), respectively. Also the number of epitope sequences binding to the HLA I alleles were higher in AgA (36) and AgD (30) than the ESAT-6 (14) and CFP-10 (7) antigens. Based on ProPred analysis for HLA class II alleles, AgA and AgD could bind to 51 of 51 class II HLA alleles analyzed (100%) whereas ESAT-6 could bind to 43 alleles (84.31%) and CFP-10 to 45 alleles (88.23%) (Table 1). ProPred predicted about 37 and 33 epitope sequences (HLA II binders) for AgA and AgD, respectively and only 8 epitope sequences (HLAII binders) each for ESAT-6 and CFP-10.

Eight epitope regions of AgA (aa145–153, aa203–211, aa219–227, aa239–247, aa362–370, aa421–429, aa462-470, aa536–544) and six epitope regions of AgD (aa35–43, aa115–123, aa126–134, aa141–149, aa170–178, aa368–376) were predicted to bind with at least eight or more HLA I alleles (See Table 1 and Table 2 in Ref (Chandra and Yadav, 2016)). In AgD, nanomer aa170–178 (APVVRVSAL) was found to be a promiscuous HLA

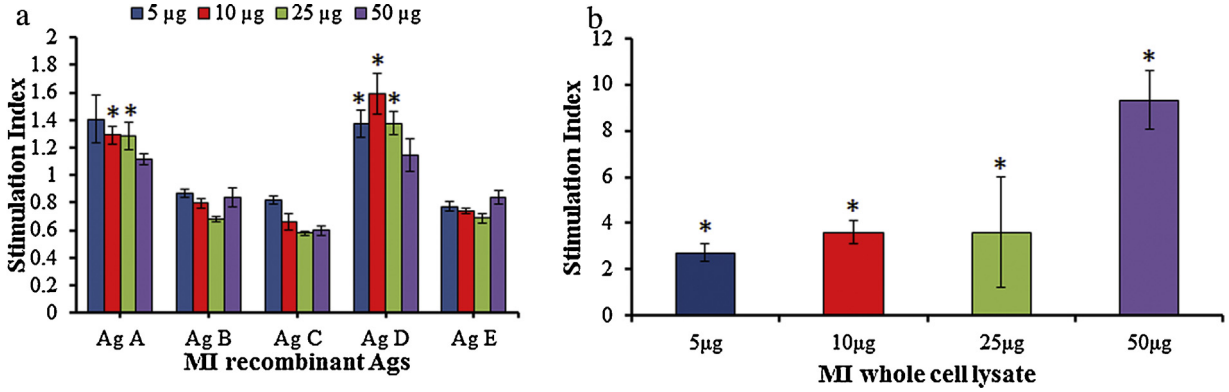

Fig. 4. Splenocyte T-cell proliferation response to the MI recombinant candidate antigens (a) and the whole cell lysate (b). T-cell proliferation is presented as Stimulation index calculated as described under Materials and Methods section. Values are presented as means ± standard errors based on three independent treatments of the pooled splenocytes (isolated and pooled from 9 MI-sensitized male C57BL/6 J mice) with a given antigen dose. Asterisk (*) indicates statistically significant ($p \leq 0.05$) difference as compared to the vehicle control.

Table 1
Immuno-informatic analysis of the identified T-cell antigens AgA and AgD of *M. immunogenum* against the reference TB antigens ESAT-6 and CFP-10.

Antigens	HLAI binders	HLAI alleles	HLAII binders	HLAII alleles	Population Coverage class I alleles	Population Coverage class II alleles	Total population Coverage (class 1+ class II alleles)
Ag A	36	47	37	51	65.90%	76.94%	90.87%
Ag D	30	43	33	51	56.68%	76.94%	88.09%
ESAT6	14	38	8	43	52.37%	67.23%	82.42%
CFP10	7	33	8	45	52.37%	62.60%	80.21%

Table 2
Predicted human population coverage for the MI antigen AgA.

Population/Area	Class I			Class II			Class I and II		
	Coverage [a]	Average hit [b]	PC90 [c]	Coverage [a]	Average hit [b]	PC90 [c]	Coverage [a]	Average hit [b]	PC90 [c]
East Asia	86.46%	4.76	0.74	80.29%	3.98	1.01	97.33%	8.74	4.13
Northeast Asia	73.07%	2.59	0.37	60.75%	2.58	0.51	89.43%	5.17	0.95
South Asia	65.27%	2.62	0.29	88.94%	4.18	1.81	96.16%	6.8	2.88
Southeast Asia	69.51%	2.55	0.33	60.81%	2.55	0.51	88.05%	5.1	0.84
Southwest Asia	70.36%	3.29	0.34	67.87%	3.13	0.62	90.47%	6.42	1.15
Europe	84.72%	4.75	0.65	95.18%	5.37	2.73	99.26%	10.13	5.24
East Africa	59.90%	2.78	0.25	86.01%	4.49	1.43	94.39%	7.27	3.45
West Africa	70.59%	3.81	0.34	91.18%	4.9	2.43	97.40%	8.71	4.1
Central Africa	62.31%	2.98	0.27	83.47%	4.17	1.21	93.77%	7.15	3.03
North Africa	71.91%	3.65	0.36	93.86%	5.2	2.44	98.28%	8.85	4.28
South Africa	55.86%	2.09	0.23	50.30%	1.98	0.6	78.06%	4.06	0.46
West indies	80.54%	4.42	0.51	81.50%	3.97	1.08	96.40%	8.39	3.72
North America	83.41%	4.53	0.6	95.46%	5.57	3.01	99.25%	10.09	5.2
Central America	4.35%	0.17	0.42	61.84%	2.72	0.52	63.50%	2.9	0.55
South America	58.66%	2.17	0.24	70.55%	3.39	0.68	87.82%	5.57	0.82
Oceania	57.48%	1.6	0.24	63.01%	2.72	0.54	84.27%	4.32	0.64
Average	65.90%	3.0475	0.38625	76.94%	3.80625	1.320625	90.87%	6.854375	2.59
(Standard deviation)	19.15%	1.25681343	0.15785542	14.70%	1.12246678	0.88420562	9.44%	2.19732555	1.7156668

[a] Projected population coverage.
[b] Average number of epitope hits/HLA combinations recognized by the population.
[c] Minimum number of epitope hits/HLA combinations recognized by 90% of the population.

Table 3
Predicted human population coverage for the MI antigen AgD.

Population/Area	Class I			Class II			Class I and II		
	Coverage[a]	Average hit[b]	PC90[c]	Coverage[a]	Average hit[b]	PC90[c]	Coverage[a]	Average hit[b]	PC90[c]
East Asia	79.85%	4.32	0.99	80.29%	5.32	1.52	96.03%	9.65	4.29
Northeast Asia	45.51%	1.88	0.37	60.75%	3.54	0.76	78.61%	5.41	0.94
South Asia	48.98%	2.09	0.39	88.94%	6.2	2.71	94.36%	8.28	3.76
Southeast Asia	48.20%	1.9	0.39	60.81%	3.43	0.77	79.70%	5.33	0.99
Southwest Asia	62.00%	2.98	0.53	67.87%	4.45	0.93	87.79%	7.43	1.64
Europe	80.59%	4.5	1.03	95.18%	7.52	3.89	99.06%	12.02	6.36
East Africa	58.02%	2.59	0.48	86.01%	6.52	2.14	94.13%	9.1	4.46
West Africa	68.99%	3.44	0.65	91.18%	6.87	3.7	97.26%	10.31	4.97
Central Africa	61.10%	2.83	0.51	83.47%	6.04	1.81	93.57%	8.86	4.1
North Africa	68.98%	3.54	0.64	93.86%	7.41	4.75	98.10%	10.95	5.48
South Africa	47.83%	2.11	0.38	50.30%	2.77	1.01	74.07%	4.88	0.77
West Indies	77.70%	4.18	0.9	81.50%	5.79	1.62	95.88%	9.97	4.39
North America	78.29%	4.2	0.92	95.46%	7.53	3.82	99.01%	11.73	6.13
Central America	4.35%	0.15	0.21	61.84%	4.01	0.79	63.50%	4.16	0.55
South America	45.58%	1.96	0.37	70.55%	4.83	1.02	83.97%	6.79	1.25
Oceania	30.92%	1.21	0.29	63.01%	3.6	0.81	74.45%	4.81	0.78
Average	56.68%	2.7425	0.565625	76.94%	5.364375	2.003125	88.09%	8.105	3.17875
(Standard deviation)	20.38%	1.23664331	0.26237299	14.70%	1.59857006	1.34876595	11.01%	2.61851103	2.1169408

[a] Projected population coverage.
[b] Average number of epitope hits/HLA combinations recognized by the population.
[c] Minimum number of epitope hits/HLA combinations recognized by 90% of the population.

binder as it was predicted to bind with 17 HLA I alleles. On the other hand, 11 epitope regions of AgA (aa107-115, aa119-127, aa269-277, aa-280-288, aa294-302, aa307-315, aa457-465, aa465-473, aa458-466, aa476-484, aa510-518) and 10 epitope regions of AgD (aa131-139, aa171-179, aa219-227, aa325-333, aa326-334, aa329-337, aa340-348, aa363-371, aa 369- 377, aa375-383) were predicted to bind with at least eight or more HLA II alleles (See Table 3 and Table 4 in Ref (Chandra and Yadav 2016)). In AgA, promiscuous binder nanomers aa280-288 (YRHLELFDS) and aa510-518 (VVSRDTLDA) were found to bind 21 and 18 HLA II alleles while in AgD the promiscuous epitopes were nanomers aa171-179 (VVRVSALKA), aa329-337 (YRPQFYFRT), aa326-334 (FNNYRPQF), and aa375-383 (LRFAIREGG) which were found to bind 34, 25, 18, and 24 different HLA II alleles, respectively.

In terms of population coverage of putative class I and class II HLA-specific epitopes, the class I epitopes of AgA (65.90%) and AgD

Table 4
Predicted human population coverage for the TB antigen ESAT-6.

Population/Area	Class I			Class II			Class I and II		
	Coverage[a]	Average hit[b]	PC90[c]	Coverage[a]	Average hit[b]	PC90[c]	Coverage[a]	Average hit[b]	PC90[c]
East Asia	73.35%	3.72	0.75	65.92%	1.39	0.29	90.92%	5.17	1.11
Northeast Asia	42.57%	1.76	0.17	45.28%	0.78	0.18	68.58%	2.56	0.32
South Asia	43.53%	1.74	0.35	75.96%	1.25	0.42	86.43%	3	0.74
Southeast Asia	45.25%	1.75	0.37	42.08%	0.76	0.17	68.29%	2.59	0.32
Southwest Asia	59.03%	2.84	0.24	60.43%	1.03	0.25	83.79%	3.88	0.62
Europe	77.15%	4.2	0.44	86.21%	1.74	0.72	96.85%	5.96	1.73
East Africa	54.82%	2.45	0.44	80.90%	1.45	0.52	91.37%	3.91	1.08
West Africa	65.90%	2.96	0.29	85.54%	1.85	0.69	95.07%	4.8	1.51
Central Africa	52.08%	2.22	0.42	77.01%	1.26	0.43	88.98%	3.48	0.91
North Africa	64.98%	3.15	0.29	84.99%	1.53	0.67	94.74%	4.7	1.31
South Africa	37.09%	1.49	0.32	50.30%	0.55	0.2	68.73%	2.05	0.32
West Indies	72.00%	3.73	0.36	73.86%	1.36	0.38	92.68%	5.09	1.26
North America	74.38%	3.9	0.39	85.47%	1.8	0.69	96.28%	5.73	1.62
Central America	4.35%	0.15	0.21	57.19%	0.76	0.23	59.05%	0.91	0.24
South America	42.37%	1.8	0.17	64.25%	0.95	0.28	79.40%	2.77	0.49
Oceania	29.09%	1.19	0.28	40.21%	0.93	0.17	57.60%	2.13	0.24
Average	52.37%	2.440625	0.343125	67.23%	1.211875	0.393125	82.42%	3.670625	0.86375
(Standard deviation)	19.53%	1.12729451	0.13950956	16.40%	0.40508795	0.20509246	13.62%	1.47197585	0.52186684

[a] Projected population coverage.
[b] Average number of epitope hits/HLA combinations recognized by the population.
[c] Minimum number of epitope hits/HLA combinations recognized by 90% of the population.

Table 5
Predicted human population coverage for the TB antigen CFP-10.

Population/Area	Class I			Class II			Class I and II		
	Coverage[a]	Average hit[b]	PC90[c]	Coverage[a]	Average hit[b]	PC90[c]	Coverage[a]	Average hit[b]	PC90[c]
East Asia	73.35%	2.39	0.38	62.50%	1.26	0.27	90.01%	3.65	1
Northeast Asia	42.57%	0.99	0.17	39.68%	0.88	0.17	65.36%	1.87	0.29
South Asia	43.53%	1.03	0.18	57.45%	1.5	0.24	75.97%	2.52	0.42
Southeast Asia	45.25%	0.86	0.18	35.60%	0.76	0.16	64.74%	1.63	0.28
Southwest Asia	59.03%	1.6	0.24	54.95%	1.36	0.22	81.54%	2.97	0.54
Europe	77.15%	2.37	0.44	78.75%	2.26	0.47	95.14%	4.63	1.74
East Africa	54.82%	1.52	0.22	82.19%	2.47	0.56	91.95%	3.99	1.41
West Africa	65.90%	1.91	0.29	85.96%	2.71	0.71	95.21%	4.62	2
Central Africa	52.08%	1.52	0.21	75.70%	2.2	0.41	88.36%	3.72	0.86
North Africa	64.98%	1.76	0.29	78.73%	2.35	0.47	92.55%	4.11	1.38
South Africa	37.09%	1.02	0.16	50.30%	1.36	0.4	68.73%	2.38	0.32
West Indies	72.00%	2.24	0.36	67.98%	1.8	0.31	91.03%	4.04	1.11
North America	74.38%	2.14	0.39	80.40%	2.35	0.51	94.98%	4.49	1.73
Central America	4.35%	0.13	0.1	51.33%	0.95	0.21	53.45%	1.08	0.21
South America	42.37%	0.9	0.17	60.77%	1.35	0.25	77.39%	2.26	0.44
Oceania	29.09%	0.53	0.14	39.35%	0.74	0.16	57.00%	1.27	0.23
Average	52.37%	1.431875	0.245	62.60%	1.64375	0.345	80.21%	3.076875	0.8725
(Standard deviation)	19.53%	0.68201876	0.10178409	16.59%	0.66374568	0.16431677	14.40%	1.22849349	0.61795901

[a] Projected population coverage.
[b] Average number of epitope hits/HLA combinations recognized by the population.
[c] Minimum number of epitope hits/HLA combinations recognized by 90% of the population.

(56.68%) (Tables 2 and 3) were predicted to have larger coverage than the reference antigens from *M. tuberculosis*, ESAT-6 (52.37%) and CFP-10 (52.37%) (Tables 4 and 5). Class II HLA-specific epitopes from AgA and AgD also showed larger population coverage (76.94%-each) than ESAT-6 (67.23%) and CFP-10 (62.60%) (Tables 2–5). The combined projected population coverage for all predicted epitopes (for both class I and class II HLA) were also calculated. Epitopes from AgA and AgD showed the largest population coverage, 90.87% and 88.09% for both class I and class II HLA alleles combined; the corresponding values for the reference antigens were ESAT-6 (82.42%) and CFP-10 (80.21%) (Tables 2–5).

Further analysis of the immunogenicity of class I epitopes for eliciting a T-cell immune response as predicted by the IEDB immunogenicity prediction tool revealed that AgA and AgD are highly immunogenic as compared to the reference antigens ESAT-6 and CFP-10. Prediction results for AgA showed that 20 of 34 HLA class I binding epitopes are immunogenic (Fig. 7 panel a) and for AgD 21 of the 29 HLA I binding epitopes are immunogenic (Fig. 7 panel b). In comparison, both the reference mycobacterial antigens ESAT-6 and CFP-10 showed only 6 HLA I binding epitopes each to be immunogenic out of 14 and 7 epitopes, respectively (Fig. 7-panels c & d, respectively).

4. Discussion

HP-associated morbidity and mortality depends on the form and severity of the disease, and may reach extreme fibrotic stage requiring lung transplantation or may result in fatal outcome (Grunes and Beasley, 2013). HP pathogenesis in general is characterized by inflammation of the lung parenchyma which progresses from acute to chronic phases involving both humoral (antibody) and cell-mediated (T-cell) immune responses (Selman et al., 2012). Acute phase of HP is thought to be mainly initiated by tissue deposition of the antigen-antibody complexes formed in response to antigen exposure (Selman et al., 2012), whereas subacute and chronic phases of HP are mainly provoked by the T lymphocytes. The T

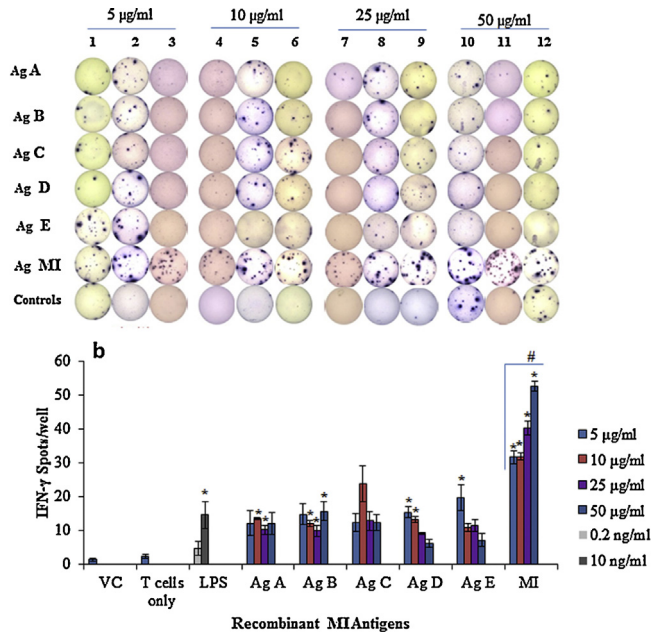

Fig. 6. ELISPOT-based IFN-γ response in human dentritic cell – T cell co-culture assay. ELISPOT analysis involved incubation of antigen-pulsed DCs with naïve purified $CD4^+$ T cells for 48 hours in a multiscreen ELISPOT plate precoated with anti IFN- γ antibodies. The spots developed using BCIP/NBT substrate (Sigma) were imaged and analyzed by CTL ImmunoSpot® Analyzer. Panel a: Representation of immunospots for different antigens and controls. For AgA – AgE and MI, lanes 1-12 denote antigen concentration range (5–50 µg/ml), with each concentration represented by three wells (each well representing an independent experiment performed using an independent blood donor; only one of the triplicate wells is shown here for each experiment); for example, lanes 1-3 represent triplicate experiments (each represented here by only one of the three treatment wells) for the first dose (5 µg/ml), and so on. For controls row, the lanes represent vehicle control or no antigen (lanes 1-3), T cells only (lanes 4-6), LPS at 0.2 ng/ml (lanes 7-9), and LPS at 10 ng/ml (lanes 10-12). Panel b: Quantitative presentation of immunopots as bar graph. Values are presented as means ± standard errors of 9 observations for a given antigen dose generated in the three independent experiments using triplicate treatments as explained above. Asterisk (*) and number sign (#) indicate statistically significant ($p \leq 0.05$) difference as compared to the vehicle control and between the doses, respectively.

cell-mediated process for HP development involves activation of antigen-presenting phagocytes by the offending antigens leading to surface display of the processed antigenic peptides that in turn interact with and cause differentiation of the T-cells into a variety of effector subsets critical in the progression or suppression of HP (Selman et al., 2012). HP being a Th1-driven disease, IFN-γ response (which is typical of Th1 polarized response) is a potent indicator of HP-relevant T-cell response. Knowledge of antigens and their epitopes eliciting Th1-cell response is therefore critical for understanding HP pathogenesis and designing T cell-based diagnostics and possibly a vaccine for mycobacterial HP.

For an immune-reactive protein to qualify as a T-cell antigen, it is expected to have the potential to interact with the antigen-presenting cells as well as have T-cell reactive epitopes. Hence identification of T-cell antigens for the HP etiological agent *M. immunogenum* in this study involved screening of the candidate proteins for their potential for activation of APCs (using macrophages and dendritic cells) and T-cell immune-reactivity. Specifically, we evaluated the five test recombinant MI proteins, available from our preceding studies, (Chandra et al., 2015; Gupta et al., 2009) using murine alveolar macrophages (MH-S cell line) and human monocytes-differentiated dendritic cells as APCs and splenocyte T-cells (derived from an MI-induced chronic HP mouse model) and autologous CD4+ naïve T cells (isolated from the healthy human blood donors).

Our results demonstrated that all five candidate MI proteins are potent activators of macrophages in a dose-dependent manner (except AgE), albeit to a variable extent. Furthermore these antigens were able to stimulate human dendritic cells for inflammatory cytokines such as TNF-α. However, this stimulation was greater in the lower concentration range (5–10 µg/ml) of the antigen as compared to the higher concentrations (25–50 µg/ml). A similar decrease in cytokine (TNF-α) induction after reaching the peak was also reported-recently (Chen et al., 2015) for a recombinant protein study in human macrophage cell line THP1; the response showed a dose-dependent increase in the initial concentration range (0.4–4 µg/ml) followed by a decrease in the higher concentration range (5–6 µg/ml). The observed stimulation of the APCs in our study was due to antigen protein activity, and not due to endotoxin contamination. This claim is supported by the facts that our samples were depleted free of endotoxin, with none or negligible remaining amount (0.0–0.0023 EU/µg protein) detectable by the LAL assay. Based on this highest detected residual value (0.0023 EU/µg protein), the maximum LPS amount that could get delivered in the assay well was never more than 0.1 ng/ml (corresponding to the well of highest antigen dose viz. 50 µg/ml tested). In this context, a positive control treatment with LPS at 0.2 ng/ml (which is equivalent to 2 EU/ml) neither stimulated APCs significantly for cytokines nor stimulated T-cell response in our assays in this study. According to the published literature, the reported lowest LPS concentration range that can stimulate macrophages is about 1–2 ng/ml in terms of TNF-α induction (Chen et al., 2004). Likewise, Biswas et al. (Biswas et al., 1995) showed that LPS can stimulate proliferation of murine splenocytes in a concentration range of 2–125 ng/ml (Biswas et al., 1995).

Observed induction of proinflammatory cytokines in alveolar macrophages by the MI candidate antigens is consistent with expression of increased proinflammatory and decreased anti-inflammatory cytokines in HP (Chandra et al., 2013; Gudmundsson et al., 1998; Thorne et al., 2006; Tillie-Leblond et al., 2011). The induced inflammatory cytokines are known key immunoregulatory molecules. For instance, TNF-α is important for controlling mycobacterial primary infection, as it helps in recruiting other inflammatory cytokines such as IL-1 and different chemotactic cytokines (Blanchard et al., 1987; Blanchard et al., 1989). TNF-α in particular, as also IL-1 and IL-6, is involved in the activation of both innate immunity (macrophage activation) and cell-mediated immunity (T-cell reactivity). Certain inflammatory cytokines are known to induce NO production (Blanchard et al., 1987). For instance, IL-1 family cytokines including IL-1β have been shown to act as co-inducers of NO (Bogdan, 2015). IFN-γ released during Th1-response to antigens is also known to stimulate release of NO in macrophages (Bogdan, 2015). Recent advances in emerging functions of NO have also noted its role in T- and B- cell differentiation.

In terms of reactivity with adaptive immune cells, we recently showed B-cell reactivity of the five recombinant test proteins (AgA through AgE) against human HP patients' sera albeit with various specificities (Chandra et al., 2015). Interestingly, only AgA and AgD produced the strongest T-cell response in terms of both IFN-γ induction and cell proliferation in pre-sensitized murine splenocytes and were also able to stimulate naïve human T cells in a DC-T cell ELISPOT assay. The observed IFN-γ response is of particular importance as HP disease in general is primarily caused by the Th1-polarized response (Bellanger et al., 2013; Schuyler et al., 1999) although other T-helper responses such as Th17 have also been implicated (Simonian et al., 2009). Interestingly, specific T-cell antigens eliciting these responses have not been known for any of the HP types, including mycobacterial HP. The observed antibody- and cell- mediated responses of these two antigens are particularly important in the context of mycobacterial HP since natural immunity/protection against intracellular pathogens/parasites such as

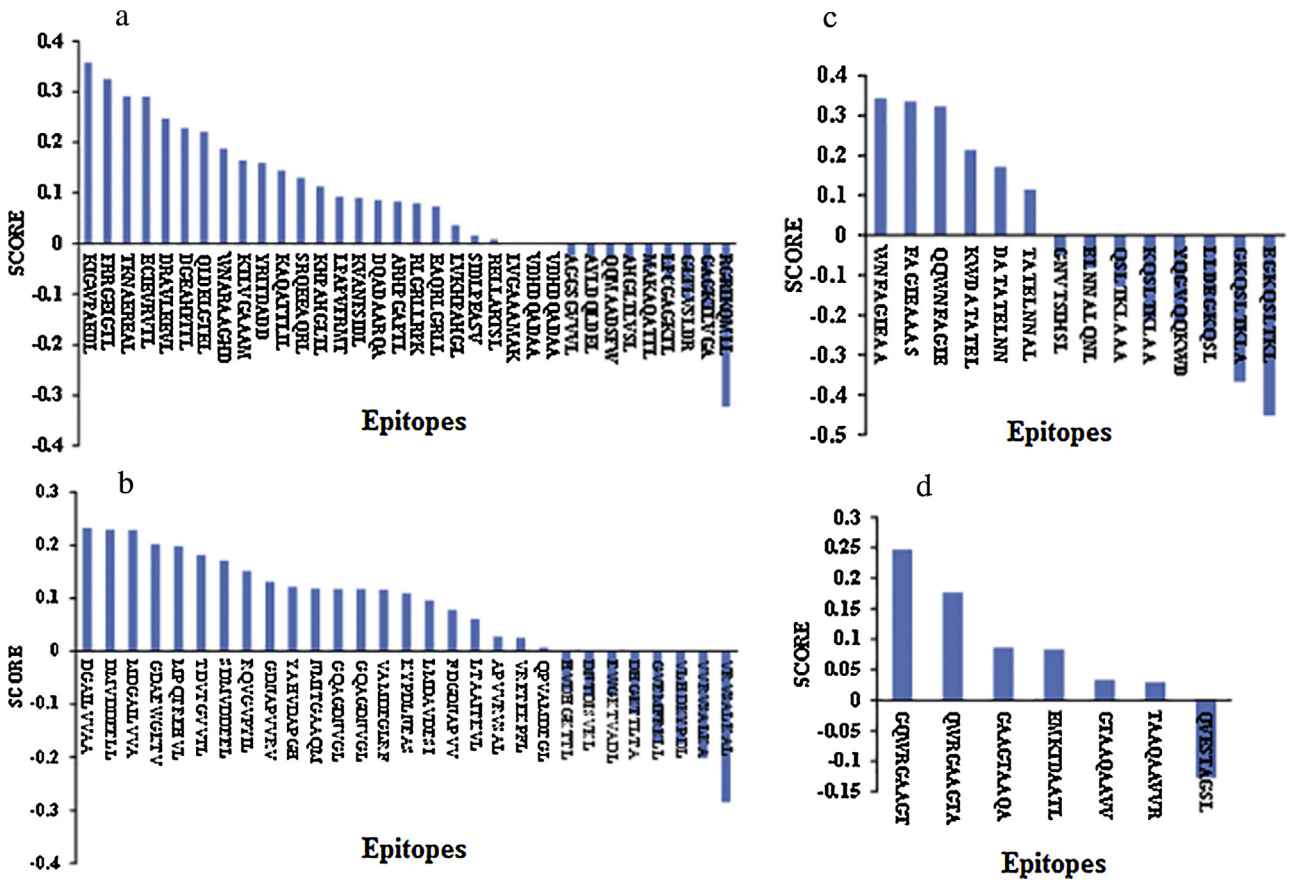

Fig. 7. *In silico* immunogenicity analysis of the predicted epitopes. Immunogenicity analysis was performed using a T-cell class I pMHC immunogenicity predictor tool available in Immune Epitope Database and analysis resource (IEDB) server (http://tools.immuneepitope.org). Panels a and b: Immunogenicity analysis of Ag A and AgD of *M. immunogenum*; Panels c and d: Immunogenicity analysis of the reference antigens ESAT-6 and CFP-10 of *M. tuberculosis*.

species of *Mycobacterium,* is usually mediated by both humoral and cellular immunity (Finco et al., 2011). Identification of such bivalent function antigens stimulating both arms of the immune system has been particularly desirable in vaccine discovery process (Finco et al., 2011). Thus, the two antigens with bivalent function identified in this study could be potentially useful as diagnostic tools or for further screening as vaccine candidates, which are high priority areas in mycobacterial HP research.

HP is a T cell-mediated immune disorder that is thought to depend on an intricate balance (ratio) between CD4 and CD8 cells (Caillaud et al., 2012). This balance may be governed by the relative abundance of HLA class-specific epitopes in the causative antigen. Therefore, the two identified MI T-cell antigens (AgA and AgD) were further analyzed for epitopes that can bind to HLA-I and HLA-II alleles, using the epitope prediction platforms namely ProPred and ProPred-I. ProPred analysis has been used for prediction of epitopes from the established antigens ESAT-6, CFP-10, and MPT-70 of the TB agent *M. tuberculosis* (Mustafa and Shaban, 2006). ProPred-I has been used for prediction of HLA-I binders from OmpC protein of Salmonella (Diaz-Quinonez et al., 2004). ProPred analysis predicted that AgA and Ag D can bind all (100%) of the class II HLA alleles analyzed as compared to the reference antigens ESAT-6 and CFP-10 which were predicted to bind fewer alleles (84.31% and 88.23% alleles, respectively). This analysis suggested that these candidate MI antigens strongly induce CD4 lymphocytes. ProPred-I analysis predicted AgA and AgD to bind 100% and 91% of the 47 class I alleles as compared to the reference antigens ESAT-6 and CFP-10 that were shown to bind to a much less extent (80.85% and 70.21%, respectively). These results indicate that AgA is capable of inducing more CD8 response than AgD. Collectively these *in silico* predictions suggest that AgA and AgD might be useful in eliciting both CD4- and CD8- specific immune responses. The combined projected human population coverage (for both class I and class II HLA) for all predicted epitopes were also higher than the reference antigens. AgA and AgD were predicted to have larger population coverage (90.87% and 88.09%, respectively) as compared to the reference antigens ESAT-6 (82.43%) and CFP-10 (80.21%). Further analysis using IEDB immunogenicity prediction tool showed that AgA and AgD are highly immunogenic as compared to the reference antigens ESAT-6 and CFP-10. Hence, MI antigens AgA and AgD can be useful for inducing both CD4- and CD8-specific T-cell immune responses in a larger human population than the widely studied reference TB antigens ESAT-6 or CFP-10.

In conclusion, while all five MI seroreactive proteins activated APCs, only two (AgA and AgD) proved inducers of T-cell response consistent with the HP-associated cellular immune response. Immuno-informatic analysis of these identified T-cell antigens, AgA and AgD, showed them to be immunogenic and highly specific for CD4 and CD8 type T cells. They were predicted to bind HLA-1 and HLA-II type alleles more efficiently and had higher total human population coverage than the widely studied reference TB antigens ESAT-6 and CFP-10. This first report on identification of T-cell antigens of *M. immunogenum* (and their epitopes) opens new avenues for the development of clinical diagnostic tools and therapeutic and/or vaccine targets for machinists HP, a poorly studied and difficult-to-diagnose occupational lung disease.

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