Mycobacterial Hsp65 potentially cross-reacts with autoantibodies of diabetes sera and also induces (in vitro) cytokine responses relevant to diabetes mellitus†

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Diabetes mellitus is a multifactorial disease and its incidence is increasing worldwide. Among the two types of diabetes, type-2 accounts for about 90% of all diabetic cases, whereas type-1 or juvenile diabetes is less prevalent and presents with humoral immune responses against some of the autoantigens. We attempted to test whether the sera of type-1 diabetes patients cross-react with mycobacterial heat shock protein 65 (Hsp65) due to postulated epitope homologies between mycobacterial Hsp65 and an important autoantigen of type-1 diabetes, glutamic acid decarboxylase-65 (GAD65). In our study, we used either recombinant mycobacterial Hsp65 protein or synthetic peptides corresponding to some of the potential epitopes of mycobacterial Hsp65 that are shared with GAD65 or human Hsp60, and a control peptide sourced from mycobacterial Hsp65 which is not shared with GAD65, Hsp60 and other autoantigens of type-1 diabetes. The indirect ELISA results indicated that both type-1 diabetes and type-2 diabetes sera cross-react with conserved mycobacterial Hsp65 peptides and recombinant mycobacterial Hsp65 protein but do not do so with the control peptide. Our results suggest that cross-reactivity of mycobacterial Hsp65 with autoantibodies of diabetes sera could be due to the presence of significantly conserved peptides between mycobacterial Hsp65 and human Hsp60 rather than between mycobacterial Hsp65 and GAD65. The treatment of human peripheral blood mononuclear cells (PBMCs) with recombinant mycobacterial Hsp65 protein or the synthetic peptides resulted in a significant increase in the secretion of cytokines such as IL-1β, IL-8, IL-6, TNF-α and IL-10. Taken together, these findings point towards a dual role for mycobacterial Hsp65: in inducing autoimmunity and in inflammation, the two cardinal features of diabetes mellitus.

1. Introduction

Mycobacterium avium subsp. paratuberculosis (MAP) is an obligate pathogen linked to Johne’s disease in ruminants and is characterised by chronic inflammation of intestines. This zoonotic bacterium is also described as a probable causative agent of a similar inflammatory condition of the gut in humans known as Crohn’s disease. MAP exists in two forms, bacillary and sphaeroplast; the latter facilitates its survival during pasteurization and chlorination. Furthermore, infant nutrition studies point to the possibility that early exposure to cow’s milk might lead to increased risk of type-1 diabetes mellitus (T1DM). As the number of diabetes cases around the world shoot up, India is fast emerging as the ‘diabetes capital of the world’. The International Diabetes Federation in 2006 has projected around 40.9 million people to be diabetic and this figure is expected to rise to 69.9 million by 2025. TIDM/ juvenile diabetes is characterized by the humoral response directed against human autoantigens such as GAD65, insulin, insulinoma associated protein, Znt8 and Hsp60. Furthermore, it has been indicated that multiple factors such as genetic susceptibility, microbial infections, lifestyle related factors and environmental toxins act cumulatively/individually as triggers of T1DM or type-2 diabetes mellitus (T2DM). Recent studies have indicated that T1DM patient’s sera can cross-react with mycobacterial Hsp65, possibly because of epitope homology between the mycobacterial Hsp65 and human GAD65. Epitope homology between mycobacterial
Hsp65 and GAD65 (molecular mimicry) is considered as one of the mechanistic processes that lead to T1DM in genetically susceptible individuals. Hsp65 and GAD65 are highly conserved and constitutively expressed proteins in almost all prokaryotes and eukaryotes and function as molecular chaperones to prevent the aggregation and misfolding of partially folded protein intermediates. It has been indicated that under pathological conditions such as necrotic cell death, Hsps are released to the extracellular environment. Then they act as pro-inflammatory mediators and/or antigenic carriers and induce cross-presentation of antigens to sensitized Th and CTL cells. Autoantibodies and T-cells reacting to Hsps have been identified in patients with numerous autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus (SLE), T1DM and multiple sclerosis. The role of mycobacterial Hsp70 in the stimulation of proinflammatory and anti-inflammatory cytokine responses and its synthetic peptides to be involved in the stimulation of mechanisms behind the cross-reactivity of diabetes sera with mycobacterium Hsp65, GAD65 and human Hsp60, and to determine the innate immune response of mycobacterial Hsp65. We used the peptide stretches of MAP Hsp65 that are shared with human GAD65 and/or Hsp60 together with a non-conserved peptide (without any homology with human GAD65 or other human antigens of T1DM, such as human Hsp60). Our observations explain that epitope homology (molecular mimicry) between the mycobacterial Hsp65 and the host autoantigen, human Hsp60, might constitute an important mechanism behind the cross-reactivity of diabetes sera with mycobacterial Hsp65. Further, we found mycobacterial Hsp65 and its synthetic peptides to be involved in the stimulation of proinflammatory and anti-inflammatory cytokine responses relevant to diabetes mellitus.

2. Materials and methods

2.1 Study population and clinical presentation

Our patient group comprised a total of 66 individuals of either sex (22 healthy, 22 T1DM and 22 T2DM). The diabetic individuals were classified and identified as T1DM or T2DM based on established clinical criteria and were negative for Mycobacterium tuberculosis (Mtb) infection by routine diagnosis. 5 ml of blood was collected from each subject and the sera obtained were stored at −80 °C for ELISA. Isolation of human peripheral blood mononuclear cells (PBMCs), approximately 10 ml of blood was taken from three healthy individuals. Written informed consent was obtained from all subjects and the study was approved by ethics committees of the participating hospital(s) and institutes.

2.2 T-coffee analysis, homology modeling and SAS analysis

The protein sequence of MAP Hsp65 (gi|438181|) was compared with the sequence of human autoproteins (against which the humoral response was observed in T1DM sera) namely GAD65 (gi|1352216|), human Hsp60 (gi|129379|), insulin (gi|386828|), insulinoma associated protein-2 (gi|145309322|) and Znt8 (gi|190358866|) using the online T-coffee multiple sequence alignment tool (EMBL-EBI). Five peptides were identified and selected for the study. The synthetic linear peptides were synthesized at Vimta Labs Limited, India. The HPLC analyses of peptides indicated a purity of 96–99%. The amino acid sequences of MAP Hsp65, GAD65 and human Hsp60 were submitted to I-TASSER (iterative threading assembly refinement algorithm), a 3D protein structure prediction tool, in order to predict the full length 3D structure of the protein. The rough models generated from I-TASSER were subjected to energy minimization with the help of the steepest descent technique using the GROMOS96 force field for the elimination of bad contacts in the protein. The energy minimized models were further evaluated by checking their stereo-chemical quality using PROCHECK server. Molecular visualization and superpose predications were carried out using PyMOL. To analyse the solvent accessibility surface area (SAS) of 14 peptides (representing MAP Hsp65, GAD65 and human Hsp60), we used the NetSurfP web based tool.

2.3 Purification of recombinant mycobacterial Hsp65

The protein sequences of Hsp65 from the Mtb strain H37Rv and MAP Hsp65 revealed 98% similarity (data not shown). The plasmid construct for the expression of Mtb Hsp65 was obtained from Shekhar Mande (CDFD, Hyderabad). Luria-Bertani broth was inoculated with overnight grown cultures of BL-21 cells expressing recombinant Mtb Hsp65 and placed in a shaking incubator at 37 °C until an OD$_{max}$ of 0.5 was reached. The culture was then induced with 1 mM IPTG and incubated at 37 °C for 4 h in a shaking incubator. The culture was then pelleted at 3200 × g for 10 min. The pellet was suspended in re-suspension buffer (50 mM Tris, 150 mM NaCl, 15 mM imidazole, 0.3% sarcosyl and 1 mM PMSF). After sonication, the cell lysate was centrifuged at 10000 × g for 30 min at 4 °C. The supernatant was collected and loaded on Ni$_2$-NTA beads in the column. After passing of the flowthrough, the column was washed with wash buffer (50 mM Tris, 500 mM NaCl, 30 mM imidazole). His-tagged protein was eluted using elution buffer (50 mM Tris, 500 mM NaCl, 100 mM imidazole). The purified protein was then dialysed in 1× PBS buffer. After concentrating the protein using Amicon ultracentrifugation filters (Millipore), the protein concentration was determined by Bradford’s method. The protein aliquots were then stored at −80 °C. The purified protein was treated with polymyxin B (Sigma) to neutralize the effect of any endotoxin contamination as described previously.

2.4 Indirect ELISA using recombinant mycobacterial Hsp65 protein and MAP Hsp65 peptides

The wells of the ELISA plates (Axygen, India) were individually coated with the purified recombinant Mtb Hsp65 protein (Fig. S1, ESI †) and MAP Hsp65 peptides (conserved and non-conserved) of approximately 500 ng concentration in bicarbonate buffer. The plates were incubated at 4 °C overnight. The plates were then washed thrice with 1× PBS (1× PBS with 0.05% Tween 20). The uncoated sites of the wells were blocked with 1% bovine serum albumin. The plates were incubated with the sera obtained from the patient group for 60 min. The plates were washed and incubated with the HRP-conjugated antibodies. The enzyme activity was detected using 3,3',5,5'-tetramethylbenzidine (TMB) as a chromogenic substrate. The enzymatic reaction was stopped by 2 M H$_2$SO$_4$. The absorbance was measured at 450 nm using a microplate reader. The data were then subjected to statistical analysis using Graphpad prism software.
albumin (BSA, Sigma) in 1× PBS for 2 h by incubating the plates at 37 °C. Each plate was washed thrice with 1× PBST. The 1:50 diluted sera samples (healthy, T1DM, T2DM) were added, as appropriate, to each corresponding well and incubated for 1 h at 37 °C. After incubation, the plates were washed thrice with 1× PBST and 1:15 000 diluted anti-IgG human conjugated peroxidase antibody (Sigma) was added and incubated for 1 h at 37 °C. The plates were then washed thrice with 1× PBST and once with 1× PBS. The plates were developed with OPD (o-phenylenediamine, Sigma, USA) and H2O2 in citrate buffer. The reaction was stopped with 2 N H2SO4. The intensity of the colour so developed was read at 490 nm using an ELISA reader (Infinite M200, TECAN).

2.5 Cytokine analysis of culture supernatants of human PBMCs

Human PBMCs were isolated from heparinised blood of healthy individuals using the Ficoll-Hypaque (HiSepTM LSM, Himedia, India) method of Boyum.26 A total of about 0.5 million PBMCs per ml of complete RPMI1640 (Hyclone, USA) media supplemented with 10% foetal bovine serum (FBS, Hyclone) were treated with different concentrations (1 μg, 5 μg, 10 μg) of recombinant Mtb Hsp65 protein and 0.5 μg of LPS (E. coli, Sigma). The culture supernatants were collected at 24 and 48 h respectively and stored at −80 °C until the cytokine analysis was performed. Separate aliquots of about 0.5 million PBMCs per ml of complete RPMI1640 media supplemented with 10% FBS were treated individually with four conserved peptides (each separately) and a non-conserved peptide, all representing MAP Hsp65 (at different concentrations: 1 μg, 5 μg, 10 μg), and 0.1 μg of LPS, in separate wells. The culture supernatants were collected at 24 h and stored at −80 °C until cytokine analysis was carried out. The concentrations of cytokines IL-1β, IL-6, IL-8, IL-10 and TNF-α in culture supernatants were measured using the BD CBA Flex kit (BD Biosciences, USA) on a BD FACS Canto II flow cytometer by plotting the standard curve for each cytokine using FCAP array software (Soft Flow/BD Biosciences).

2.6 Statistical analysis

The ELISA data were analyzed by Mann–Whitney’s U test or the Wilcoxon rank sum test. We preferred Mann–Whitney’s U test when compared to Student’s t-test because of its robustness and also because the samples in our study were of the same size and also because the samples in our study were of the same size and also because the samples in our study were of the same size and also because the samples in our study were of the same size and also because the samples in our study were of the same size and also because the samples in our study were of the same size and also because the samples in our study were of the same size and also because the samples in our study were of the same size. The level of significance was determined by p value. The plotting of ELISA graphs and calculation of median values, values of U and level of significance were all carried out using online GraphPad Prism software. The U values of each test are presented in a table (Table S1, ESI†). The p value < 0.05 was considered significant, indicating that the median value of the two groups significantly differs from each other. Statistical analysis and plotting of graphs for the level of cytokine secretion by human PBMCs on treatment with Mtb Hsp63 protein in the culture supernatants was carried out by using GraphPad Prism software. The plotting of graphs for the level of cytokine secretion by human PBMCs on treatment with MAP Hsp65 peptides was performed by using Sigma plot. The mean and standard deviation were calculated for each experiment conducted in triplicate. Two tailed Student’s t-test was considered significant if p values were <0.05; the p value was calculated using online GraphPad Prism software.

3. Results and discussion

3.1 Molecular structure prediction and selection of immunogenic peptides representing MAP Hsp65

In T1DM patients, a greater level of antibodies (humoral response) was observed against human autoantigen GAD65. T-coffee analysis of MAP Hsp65 and human GAD65 revealed 45% identity and the analysis led to the identification of four conserved peptides from MAP Hsp65 (amino acid stretches that are highlighted in red in Fig. S2, ESI†): peptide1 (ELEDPYEKIGAI8ELVEKVVAKK), peptide2 (DQUAAIA8SGDQS), peptide3 (LAKL8GV8IKAGA8TEVELK8HRK), peptide4 (AVEE8IV8AGG8V8ALLH8PA8LD).

To unravel the role of epitope homology between MAP Hsp65 and GAD65, it was significant to identify a non-conserved peptide of MAP Hsp65 that doesn’t cross-react with GAD65 and with any other autoantigens of T1DM. The T-coffee analysis was performed for MAP Hsp65 as compared to other autoantigens of T1DM such as human Hsp60, insulin, insulinoma associated protein-2 and Znt-8. The T-coffee analysis of MAP Hsp65 and human Hsp60 indicated 97% identity; MAP Hsp65 and insulin indicated 62% identity; MAP Hsp65 and insulinoma associated protein indicated 52% identity; and MAP Hsp65 and Znt-8 indicated 51% identity. As MAP Hsp65 and human Hsp60 have greater % identity, a non-conserved peptide of MAP Hsp65, from T-coffee analysis of human Hsp60 and MAP Hsp65, the peptide5 (V8GL8LES8ADI), was identified (Fig. S3, ESI†). The peptide5 was further checked for overlapping amino acid stretches in the protein sequence of GAD65, Znt-8, insulin and insulinoma associated protein and it was confirmed that peptide5 was indeed non-conserved with respect to the autoantigens of T1DM (Table S2, ESI†). The PDB structure search analysis of MAP Hsp65, GAD65 and human Hsp60 revealed that these sequences have corresponding templates with more than 92% sequence identity. Even though the sequences had enough identities to build a homology model, some of the important protein segments in MAP Hsp65, GAD65 and human Hsp60 were missing. For this reason, we reconstructed 3D models of MAP Hsp65, GAD65 and human Hsp60 by using I-TASSER web server. A total of five structures each were predicted for each of the above proteins. Only best structures were selected (Fig. S4, ESI†) based on the maximum C-score, TM-score and RMSD value (Table 1) and these values were found to be in correct topology with respect to reference values.27 After energy minimization of the 3D models of MAP Hsp65, GAD65 and human Hsp60, the stereochemical quality of the structures was validated by submitting the PDB files to PROCHECK server. The results of PROCHECK analysis indicated that a relatively low percentage of residues have phi/psi angles in the disallowed regions suggesting the acceptability of Ramachandran plots for the proteins.28 The percentage of residues in the allowed/core region were found to be 98.9, 99.2 and 98.8%
and those in the disallowed region were found to be 1.1, 0.8 and 1.2% for MAP Hsp65, GAD65 and human Hsp60, respectively (Fig. S5 and Table S4, ESI†).

Localization of peptides on the 3D structures of MAP Hsp65 and GAD65 is indicated in Fig. 1. Further, localization of peptides after superimposition of 3D structures of MAP Hsp65 and human Hsp60 is presented in Fig. 2. The SAS was calculated using the NetSurfP web tool for individual residues of the 14 test peptides representing MAP Hsp65, GAD65 and human Hsp60. NetSurfP Z-scores enabled identification of the most reliable/unreliable predictions for both buried and exposed amino acids (Table S3, ESI†). As shown in Table 2, MAP Hsp65 peptide1 showed 42.1% of exposed residues and its overlapping peptide in GAD65 showed more than 89% of exposed residues in its structure. Conserved peptides 2, 3 and 4 of MAP Hsp65–GAD65 revealed approximately equal ratios of buried and exposed residues. The non-conserved peptide of MAP Hsp65–human Hsp60 showed 70% and 80% of exposed residues respectively. Studies have indicated humoral immune response to human Hsp60 in both T1DM and T2DM individuals. Human Hsp60 is a mitochondrial stress protein that is induced during mitochondrial impairment. Though Hsp60 is an intracellular protein, its elevated levels have been found in the systemic circulation of T2DM patients. The exact mechanism of secretion of Hsp60 into the biological fluids of T2DM individuals is not yet known.29

### 3.2 Cross-reactivity of diabetes sera with mycobacterial Hsp65 protein and MAP Hsp65 peptides

The indirect ELISA results indicated that recombinant *Mtb* Hsp65 protein and conserved MAP Hsp65 peptides can cross-react with both T1DM and T2DM sera when compared to sera from healthy controls. T1DM is an autoimmune disease with antibodies produced against autoantigens such as GAD65, Zn-t-8, insulin, insulinoma associated protein and human Hsp60. Although T2DM is not an autoimmune disease, antibody response was previously reported against human Hsp60 in T2DM as well.29 Given that MAP Hsp65 is 97% identical to human Hsp60, we could observe T2DM sera reacting with *Mtb* Hsp65 protein and conserved peptides of the same.

Further, assay with the non-conserved peptide of MAP Hsp65 (peptide5) indicated no significant median difference between the healthy control, T1DM and T2DM sera (Fig. 3). It appears that the mycobacterial Hsp65 cross-reacts with autoantibodies in T1DM and T2DM sera due to overlapping peptides that mimic the epitopes of the host proteins, GAD65 and/or human Hsp60.

In a related study,30 the peptide3 sequence (LAKLAGGVAVIKAGAATEVELKERKHRI) was observed to bind to I-Ag7 (mouse MHC) with high affinity and induce enhanced proliferation of T-cells. However, the peptide4 sequence (AVEEGIVAGGGVALLHAIPALD) was indicated to bind to I-Ag7 with less affinity and induce lower proliferation of T-cells.30 Our results corroborate with this report and suggest that the above two peptides might be acting as T-cell and B-cell epitopes. The peptide5 (VGLSLESADI), although having surface probability, could not cross-react with T1DM or T2DM sera due to the lack of conserved/overlapping motifs in comparison to autoantigens of T1DM and T2DM.

### Table 1 Evaluation data of 3D protein models as gleaned from I-TASSER

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Protein</th>
<th>C-score</th>
<th>TM-score</th>
<th>RMSD</th>
<th>No. of decoys</th>
<th>No. of clusters</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>MAP Hsp65</td>
<td>0.99</td>
<td>0.85 ± 0.08</td>
<td>5.3 ± 3.4 Å</td>
<td>2261</td>
<td>0.3925</td>
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<tr>
<td>2</td>
<td>GAD65</td>
<td>1.16</td>
<td>0.57 ± 0.15</td>
<td>10.4 ± 4.6 Å</td>
<td>1047</td>
<td>0.0706</td>
</tr>
<tr>
<td>3</td>
<td>Human Hsp60</td>
<td>0.85</td>
<td>0.83 ± 0.08</td>
<td>5.8 ± 3.6 Å</td>
<td>2237</td>
<td>0.3436</td>
</tr>
</tbody>
</table>

*Confidence score (C-score) for estimating the quality of predicted models typically in the range of [−5, 2]. Template Modeling score (TM-score) measures the structural similarity between two structures; TM-score > 0.5 indicates a model of correct topology and a TM-score < 0.17 conveys random similarity. Heavy atoms root-mean-square deviation (RMSD) with respect to the experimental structure. Number of structure decoys (low temperature replicas) at a unit of space in the SPICKER cluster.*

![Fig. 1](image-url) Identification of conserved peptides of MAP Hsp65 and GAD65. The conserved peptides are represented in different colours: peptides of MAP Hsp65 are shown in yellow and peptides entailing GAD65 are shown in cyan.
A previous study indicated association of mycobacterial Hsp65 with rheumatoid arthritis. It was shown that the peptide sequence of *M. bovis* Hsp65 spanning amino acids 180–188 acts as a major T-cell epitope for adjuvant arthritis; however, this T-cell epitope was unable to cross-react with sera of rheumatoid arthritis patients suggesting that this peptide is not recognized as a B-cell epitope. Further, Hsps are conserved proteins and Hsps homologous to mycobacterial Hsp65 might as well occur in many bacteria. The observed cross-reactivity of T1DM and T2DM sera with *Mtb* Hsp65 protein and MAP Hsp65 conserved peptides could be mostly due to the presence of autoantibodies rather than antibodies produced against any other bacterial Hsps. This is because no such cross-reactivity was observed in healthy controls.

### Table 2 NetSurfP prediction results for % solvent accessibility of the residues of MAP Hsp65, GAD65 and human Hsp60 peptides

<table>
<thead>
<tr>
<th>Protein</th>
<th>Domain</th>
<th>Peptide number</th>
<th>Peptide sequence</th>
<th>Buried residues (%)</th>
<th>Exposed residues (%)</th>
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<td>MAP Hsp65</td>
<td>Conserved domains</td>
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<td>ELEDPYEKGIAELVKEVAKK</td>
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<td>42.1</td>
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<tr>
<td></td>
<td></td>
<td>2</td>
<td>DQIAATAAAGDGQ5</td>
<td>46.6</td>
<td>53.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3</td>
<td>LAKLAVVAKAGAATEGFQKHERHI</td>
<td>71.5</td>
<td>28.5</td>
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<tr>
<td></td>
<td></td>
<td>4</td>
<td>AVEEGIVGGGVALHAIPLD</td>
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<td>27.3</td>
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<td></td>
<td>Non-conserved domain</td>
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<td>VGLSLESADI</td>
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<td>70</td>
</tr>
<tr>
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<td>LMWRAGTGTGFEAHVDKCLEAEYLNI</td>
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<td></td>
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<td>MVFDGKPQHTCWFVYIPSRL</td>
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<tr>
<td>Human Hsp60</td>
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<td></td>
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<td>20</td>
<td>80</td>
</tr>
</tbody>
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### 3.3 Pro- and anti-inflammatory cytokine profiles of human PBMCs

The cytokine analysis of culture supernatants of human PBMCs upon treatment with recombinant *Mtb* Hsp65 protein indicated...
increased secretion of cytokines IL-1β, IL-8, IL-6, IL-10 and TNF-α in a dose and time dependent manner, and in a dose dependent manner, when treated with MAP Hsp65 peptides (Fig. 4 and 5).

The non-conserved peptide (peptide5), although not cross-reacting with T1DM or T2DM sera samples as compared to the conserved peptides, could significantly induce cytokine responses. This suggests that this non-conserved peptide could be a potential epitope involved in innate immune response even if it is not conserved with respect to human Hsp60 or to any other autoantigen of T1DM. IL-1β, IL-6, IL-8, and TNF-α are pro-inflammatory cytokines and IL-10 is an anti-inflammatory cytokine. It has been suggested by another study that the pro- and anti-inflammatory cytokines and their signalling pathways play a key role in protection during mycobacterial pathogenesis and their balance likely determines the clinical outcome.32

In our previous study,33 we analyzed the cytokines secreted by human PBMCs upon treatment with whole cell lysates of MAP; the results indicated increased secretion of IL-1β, TNF-α, IL-8, IL-6 and IL-10. In this study, we sought to analyze the profiles of cytokines secreted upon treatment with Mtb Hsp65 protein and peptides from MAP Hsp65. When we consider the two studies together, we observe an increased level of secretion of IL-6, IL-8 and IL-10 by human PBMCs on treatment with
Mtb Hsp65 protein when compared to treatment with MAP lysate. Further, the level of cytokines secreted by human PBMCs on treatment with MAP Hsp65 peptides was low when compared to treatment with recombinant Mtb Hsp65; this may be because the peptides are less immunogenic when compared to whole protein. Moreover, studies from another group identified that monocytes isolated directly from the blood of T1DM individuals spontaneously secrete pro-inflammatory cytokines such as IL-1β and IL-6 thereby inducing the expansion of Th17 cells that are mostly involved in the development of autoimmune diseases.34 Furthermore, studies have also indicated increased levels of IL-8 in both T1DM and T2DM individuals.35 The in vivo studies using non-obese diabetic (NOD) mice have shown that pancreatic IL-10 induces autoimmune diabetes via an ICAM dependent pathway.36 Another study reported induction of insulin resistance by TNF-α and IL-6.37 Taken together, our cytokine profiling of peptides/epitopes points to the understanding that mycobacterial Hsp65 has antigenic peptides that could bind/interact with immune cells and induce secretion of cytokines. Given this, the MAP infection appears to be an important, putative aetiological factor.
factor in the pathogenesis of diabetes mellitus wherein the MAP antigens such as Hsp65 can accelerate autoimmune destruction or cytotoxicity of pancreatic islet cells and thereby hasten the clinical progression of T1DM. In T2DM, however, the exact role of MAP antigens will be discerned only based on future mechanistic evidence.

4. Epitope homology from a system’s perspective

Recent studies have suggested the role of mycobacterial pathogens in diabetes mellitus, wherein epitope homologies of pancreatic antigens and mycobacterial proteins are proposed as underlying triggers of type-1 diabetes that operate through elusive mechanisms. Advancing this hypothesis further on, we demonstrate that the obviously conserved regions of some of the human autoproteins (Hsp60/GAD65) and mycobacterial Hsp65 could constitute critical hotspots of cross-reactivity of mycobacterial antigens with diabetes sera. Taking the example of above proteins, we espoused a detailed scenario entailing all such components that participate in the initiation and progression of autoimmune signalling leading to diabetes mellitus (Fig. 6). This scenario might as well hold true for all other possible cross-reacting microbial proteins described earlier and their human homologues (such as MAP3865c cross-reacting with ZnT8 antigen). Hsps are conserved intracellular proteins that are released to the extracellular space during mycobacterial infections. Further, human Hsp60 is also

Fig. 5 Cytokine analysis of culture supernatants of human PBMCs upon treatment with MAP Hsp65 peptides. The culture supernatants of human PBMCs treated with different concentrations of MAP Hsp65 peptides and LPS were assayed using the multiplex cytokine bead array kit. The assay indicated increased secretion of IL-1β, IL-6, IL-8, IL-10 and TNF-α in a dose dependent manner at 24 h. Student’s t test analysis of the cytokine concentrations observed in three independent experiments revealed p < 0.05; this indicated a significant increase in the secretion of cytokines upon treatment with different concentrations of individual peptides of MAP Hsp65 and LPS (positive control) in comparison to untreated cells (added 1× PBS; the solvent used for solubilizing the peptides).
released due to unknown mechanisms in both diseased and healthy individuals. When these cryptic proteins are accumulated, they could be cross-presented to the immune cells resulting in autoimmunity. Our study based on patient sera samples partly explains the above espousal. More concrete proof of molecular mimicry at the base of diabetes could emerge from future studies by developing monoclonal antibodies against the cross-reacting peptides and harnessing suitable animal models. Until then, molecular cross-reactivity as deduced from our work could be considered as a plausible mechanism of (autoimmune) diabetes in genetically susceptible hosts. Having this said, future efforts are indeed required because autoimmune diseases are complex in nature and the molecular basis of diabetes mellitus as a multifactorial disease with several interacting factors cannot be explained from the analysis of any single component. Therefore, autoimmunity as a putative mechanism of diabetes from the perspective of mycobacterial triggers has to be studied through a holistic approach that combines the systems level understanding of mycobacterial infections and diabetes while also integrating their systems epidemiology. Given this, we strongly believe that our observations in identifying the putative mechanism of molecular mimicry (epitope homology) of Hsp65 (which is conserved in most organisms) and human Hsp60, based on computational, clinical and immunological data,
constitute some of the baseline components of the ‘system’ comprising molecular pathways that lead to autoimmunity. Out of these components and pathways, a few would potentially serve as interventional targets to achieve reduction of circulating autoantibodies thereby mitigating the impact of diabetes in a given patient.

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Notes and references

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