Molecular mimicry between Mycobacterium leprae proteins (50S ribosomal protein L2 and Lysyl-tRNA synthetase) and myelin basic protein: a possible mechanism of nerve damage in leprosy

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Abstract

Autoantibodies against various components of host are known to occur in leprosy. Nerve damage is the primary cause of disability associated with leprosy. The aim of this study was to detect the level of autoantibodies and lympho-proliferative response against myelin basic protein (MBP) in leprosy patients (LPs) and their correlation with clinical phenotypes of LPs. Further, probable role of molecular mimicry in nerve damage of LPs was investigated. We observed significantly high level of anti-MBP antibodies in LPs across the spectrum and a positive significant correlation between the level of anti-MBP antibodies and the number of nerves involved in LPs. We report here that 4 B cell epitopes of myelin A1 and Mycobacterium leprae proteins, 50S ribosomal L2 and lysyl tRNA synthetase are cross-reactive. Further, M. leprae sonicated antigen hyperimmunization was responsible for induction of autoantibody response in mice which could be adoptively transferred to naive mice. For the first time our findings suggest the role of molecular mimicry in nerve damage in leprosy.

Keywords: Leprosy; Autoimmunity; Autoantibodies; Myelin basic protein; Molecular mimicry

1. Introduction

Leprosy is primarily a granulomatous disease of the peripheral nerves and skin. Nerve damage is the major cause of disability associated with leprosy. Mycobacterium leprae (M. leprae) is remarkably non-toxic organism and most of the tissue damage and neural damage in leprosy is known to be due to the host’s immune response to M. leprae antigens [1]. It has also been reported that this immune response by the host may lead to auto-antibody response against nerve antigens [2] and could be responsible for demyelination.

Elevated level of anti-myelin basic protein (MBP) autoantibodies has been reported in multiple sclerosis (MS), an autoimmune disease of CNS [3,4] and autism [5]. Presence of anti-MBP autoantibodies in MS patients showed site-specific proteolytic cleavage of the MBP molecule that might be responsible for pathological destruction of the myelin sheath [6]. Earlier, it was reported that MBP is one of the constituents in the circulating immune complexes (CIC) of lepromatous
leprosy (LL) patients and was suggested that MBP released after nerve damage caused by *M. leprae* was responsible for production of anti-MBP antibodies, which further may lead to demyelination and nerve damage [7]. Molecular mimicry may be the underlying mechanism for production of autoantibodies and might be responsible for pathological destruction in leprosy [8,9]. Molecular mimicry is defined as immunological cross-reactivity between infectious agent and host antigenic determinant/s [10]. In the recent past, Vardhini et al. (2004) [11] pointed out that molecular mimicry between mycobacterial proteins (ferredoxin-NADP-reductase and a conserved mycobacterial membrane protein) with myelin P0 (a protein that aids in compacting myelin through homotypical interactions) might be responsible for demyelination. Though the molecular mechanism leading to axonal degeneration and/or demyelination in leprosy have rarely been explored in detail, the present data indicate that molecular mimicry might result in immune mediated degeneration of myelin that contribute to nerve damage leading to deformities and permanent loss of nerve function in leprosy patients.

Hence, the purpose of our study was to find out presence of anti-MBP antibodies and lymphocyte proliferative response to MBP in leprosy patients and their correlation, if any, with the disease spectrum. We also aimed to investigate the probable role of molecular mimicry for such an immune response leading to nerve damage.

## 2. Research design

### 2.1. Proteins

MBP from bovine brain was procured from Sigma−Aldrich, USA. Non-irradiated *M. leprae* bacilli derived from Armadillo liver was procured from Colorado State University, Fort Collins, Colorado, U.S.A. (under WHO contract number NIH-NO1-AI-25469, Leprosy Research Support). *M. leprae* sonicated antigen (MLSA) was prepared by sonication of bacilli according to the published protocol [12]. The protein concentration of MLSA was assayed by Bradford's method (Sigma, USA) [13].

### 2.2. Study subjects

#### 2.2.1. Patients and healthy controls

One hundred twenty four clinically diagnosed (based on cardinal signs) leprosy patients, attending the outpatient department of National JALMA Institute for Leprosy and Other Mycobacterial Diseases (NJIL & OMD), ICMR, Agra were recruited in the study. Leprosy patients were classified according to the classification laid by Ridley and Jopling scale [14] which included 20 borderline tuberculoid (BT), 21 borderline borderline (BB), 23 borderline lepromatous (BL), 29 lepromatous leprosy (LL), 21 type 1 reaction (T1R), 10 erythema nodosum leprosum (ENL). Blood samples from 43 healthy controls (HC), individuals with no signs and symptoms of leprosy and any other illness were taken as control. This study was approved by the Institutional Ethical Committee and all participants consented to the study.

#### 2.2.2. Experimental animals

Outbred female New Zealand white rabbits (weighing about 1 kg and 3–5 months old) and inbred strains of female BALB/c mice (6–8 weeks, weighing 20 ± 2 gms) were obtained from the Laboratory Animal Division of Central Drug Research Institute (CDRI), Lucknow. All animals were maintained under standard conditions in the Department of Animal Experimentation of NJIL & OMD, Agra.

All animal experiments included in this manuscript were approved by Institutional Animal Ethical Committee and we followed the guidelines laid down by Animal Research Ethics Board at our institute.

### 2.3. Production of polyclonal rabbit antibodies to MLSA and MBP

Rabbits were hyperimmunized with 250 µg of protein concentration of MLSA as well as 250 µg of host protein MBP to produce polyclonal antibodies against these proteins. Both the proteins were emulsified in Freund's Incomplete Adjuvant (IFA) (Sigma, USA) and 0.2 ml emulsion was injected intradermally (i.d.). The booster doses were given weekly up to 8th week. Control group of rabbits were administered with the emulsion of normal saline with IFA at the same time intervals. Blood samples (5 ml each time) were drawn from marginal ear vein before the booster doses with the antigen emulsion.

### 2.4. MLSA-hyperimmunized mice

Mice of the control (n = 10) and experimental (n = 15) group were hyperimmunized with saline and 25 µg of protein concentration of MLSA emulsion respectively as previously described [8].

#### 2.5. Protocol for transfer of auto-reaction by adoptive transfer

The adoptive transfer experiment was carried out as described in Singh et al., 2012 [8]. Briefly, adoptive transfer was done in three groups of mice by inoculating 100 µl (1X10⁷ cells/ml) of cell suspension. In the first control group of mice (n = 5), mixture of suspensions of cells of splenocytes and lymph nodes cells in isotonic saline obtained from naive mice were intravenously (i.v.) administered in tail vein. Second experimental group (n = 5) were similarly i.v. administered with immune cells (whole cells) acquired from MLSA hyperimmunized mice. Third group of mice (n = 5) were i.v. inoculated with nylon wool separated T cells [15] obtained from MLSA hyperimmunized mice.

### 2.6. Estimation of auto-antibodies against myelin basic protein by ELISA

#### 2.6.1. Human sera

ELISA was performed by using the protocol with some modifications as described earlier [8]. Briefly, each well of microtitre plates (Nunc, Denmark) was coated with 5 µg/ml of MBP (Sigma, USA) for 18 h at 4 ℃. Wells were blocked with 3% BSA (Sigma,
USA) in PBS for 1 h. Serum (1:100) was added in duplicate wells followed by incubation at 37 °C for 2 h. After 3 washings, 1:10,000 diluted anti-human IgG peroxidase conjugated (Sigma, USA) was added into each well. After 1 h incubation, plates were washed 3 times and 100 µl of substrate solution [orthophenylene diamine (OPD) tablet of 0.5 mg/ml and 0.2 µl/ml H2O2] was added and was kept at room temperature for 30 min in dark. Reaction was stopped by adding 50 µl/well of 3 M H2SO4 (stop solution) and absorbance was recorded at 492 nM using Spectramax-M2 Reader (Molecular Devices, Sunnyvale, CA, USA).

2.6.2. Experimental animal sera

We quantified the level of autoantibodies against MBP in MLSA hyper-immunized rabbits and mice sera/plasma using above mentioned ELISA protocol with minor differences as follows —

For rabbit serum, peroxidase conjugated anti-rabbit IgG was used as secondary antibody.

For mouse plasma, fifty fold diluted mouse plasma was used for reaction and peroxidase conjugated anti-mouse IgG was used as secondary antibody.

2.7. Inhibition of binding of rabbit anti-MBP autoantibodies to MBP by leprosy sera

Polystyrene microtitre plates were coated with MBP as described in previous section. Fifty microliter of 1:500 diluted MBP hyper-immunized rabbit serum was added to each well and incubated for 1 h at 37 °C. Then 50 µl of three different leprosy patients sera were added at five different dilutions (1:5, 1:10, 1:20, 1:40 and 1:80) and incubated for 1 h at 37 °C followed by three washings. 100 µl of peroxidase conjugated anti-human IgG (1:10,000) (Sigma, USA) was added to each well. After incubation for 1 h at 37 °C plates were washed for 3 times, color was developed and absorbance was taken as described in previous section.

Fig. 1. (A) Levels of antibodies against MBP in leprosy patients across the spectrum and healthy controls. Dotted horizontal line represents the cut off O.D. value (mean OD value of HCs + 2SD). Smooth line with horizontal lines represents the mean OD value with SD of each group. Each dot represents the OD value at 492 nm obtained from each individual. (B—G) correlation between anti-MBP antibodies level and number of nerves involved in (B) TT/BT (C) BB (D) BL (E) LL (F) T1R (G) ENL (H) Inhibition of binding between anti-MBP rabbit sera and MBP antigen with leprosy patients' sera. (A: p value < 0.0001, One way ANOVA and post test Dunnett's multiple comparison test ***p value < 0.0001, **p value < 0.001, *p value < 0.05; B—G: Spearman's correlation test).
2.8. Effect of MBP on lymphocyte proliferation of leprosy patients and healthy individuals

Lymphocyte proliferation assay was done with some modifications as described earlier [8]. Cultures were set in triplicate with or without 20 μg/ml MBP in culture medium into 96 well flat bottom culture plates (Nunc, Denmark) and incubated for 5 days in a humidified CO2 incubator (Forma Scientific Inc, USA) at 37 °C with 5% CO2 in air. Phytohaemagglutinin (PHA) was included in this assay as positive control. After 5 days cells were pulsed with 1 μCi/well of [3H] thymidine and incubated for 18 h. The cells were then harvested on filter mats using Skatron cell harvester. The radioactivity incorporated into the DNA was determined by liquid scintillation counting (LKB Wallac, Rackbeta, Finland).

2.9. Characterization of cross-reactive proteins of MLSA and MBP

Denatured proteins of MLSA and MBP (30 μg/lane) were electrophorezed by SDS-PAGE [16] using 10% resolving gel (Bio-Rad Laboratories, USA) and blotted [17] to nitrocellulose membrane (NCM) (Bio-Rad Laboratories, USA). Two-dimensional PAGE, isoelectric focusing (IEF), was carried out employing the method of Gorg et al. (2000) [18]. IPG strips (Bio-Rad Laboratories, USA) of pH 4–7 for MLSA, pH 3–10 for MBP and length 7 cm were loaded with 100 μg of protein samples (MBP/MLSA). Proteins were separated in second dimension in vertical 10% SDS–PAGE gels followed by transfer to NCM.

Blotted NCM was washed with PBS and blocked with 3% BSA (Sigma, USA) for 1 h at RT with gentle shaking, then probed with 1:50 diluted MLSA-hyperimmunized rabbit sera while NCM of separated proteins of MLSA was probed with 1:50 diluted MBP-hyperimmunized rabbit sera. These antibody-treated NCMs were incubated overnight at 4 °C. NCMs were then washed for 3 times with PBS containing 0.05% Tween-20 and incubated with 1:10,000 diluted peroxidase conjugated anti-rabbit IgG (Sigma, USA) for 1 h. Afterwards, NCMs were washed for 5 times with PBS-T and antigen antibody reactivity were visualized by the color development with diaminobenzidine (Sigma, USA) solution. Image was captured by Chemidoc (Bio-Rad Laboratories, USA). Data of 1-D western blot was analyzed by using Quantity One Software (Bio-Rad, USA) while the data of 2-D blot was analyzed by PD Quest Software (Bio-Rad, USA).

Fig. 2. (A) Levels of lymphoproliferation in the presence of MBP in leprosy patient across the spectrum. (Box and Whiskers graph). Each bar represents the minimum to maximum values with median as the horizontal line and SD as error bars. + Sign in each bar represents the mean value. Dotted line represents the S.I. = 2. (p = 0.0011, One way ANOVA test) and unpaired t test (two tailed) was used to compare HC with each group of leprosy **p < 0.001, *p < 0.05) (B) Levels of antibodies against MBP in MLSA hyperimmunized rabbit sera at different time intervals. Each dot with error bar represents the mean OD value with SD. (C) Comparison in levels of autoantibodies against MBP in plasma of MLSA hyperimmunized female BALB/c mice, pre-immunized mice and control mice. Each dot represents individual OD obtained from mouse plasma. Solid horizontal lines with error bars represent mean OD with SD. (D) Level of antibodies against MBP in adoptively transferred naïve female BALB/c mice. Each dot represents the individual OD. Horizontal line with error bars represents mean OD with SD. (B: p < 0.0001, Unpaired t test (two tailed) C, D: ***p < 0.0001, **p < 0.001 One way ANOVA and post test Bonferroni multiple comparison test).
2.10. Identification of cross-reactive proteins of MLSA and MBP by MALDI TOF analysis

In-gel digestion with trypsin [19] was done with slight modification. Protein spots of interest were picked up from coomassie-blue stained 2-D gels using spot picker (Model Investigator ProPic, Genomic Solutions Ltd., UK). In-gel automated robotic digestion with trypsin (Promega MS grade) of these picked proteins was done by using protein digester (Model Investigator ProPrep, Genomic Solutions Ltd., UK). Extracted peptides were desalted and concentrated using ZipTipC18 (Millipore, USA) according to manufacturer’s protocol and applied to an AnchorChip (Bruker Daltonik GmgH, Leipzig, Germany) with 2 μl of matrix (saturated solution of α-cyano-4-hydroxycinnamic acid (HCCA) formed in 50% ACN and 0.2% TFA). Mass spectra of digested peptides were acquired by Autoflex II TOF/TOF50 (Bruker Daltonik GmgH, Leipzig, Germany) in positive reflectron mode and in detection range of 500–3000 m/z. Mass spectra of digested peptides were analysed using Mascot Wizard program (Matrix Science, Ltd., London, United Kingdom; http://www.matrixscience.com).

Peptide mass fingerprint of cross-reactive protein of MBP with anti-MLSA rabbit sera was submitted to Mascot search engine and search parameters used for the identification were peptide mass tolerance ±30 ppm, peptide charge state 1+, and maximum missed cleavages 1. However, search parameters used for the identification of the cross-reactive protein of MLSA (≈ 30 kDa protein) were peptide mass tolerance ± 150 ppm, maximum missed cleavages 2. MS/MS ion search was done by submitting different peaks (mass − 1179.769) of the cross-reactive protein of

![Fig. 3. Reactivity of anti-MLSA rabbit sera with MBP (A-D) anti-MBP rabbit sera with MLSA (E-H). (A) Protein profile of MBP on SDS-PAGE gel stained with coomassie-blue, (B) Western blotting pattern of reactivity of anti-MLSA rabbit sera with MBP. Lane 1: Molecular weight marker, Lane 2: MBP (C) Protein profile of MBP on 2-D gel stained with coomassie-blue, (D) Western blotting pattern of reactivity of anti-MLSA rabbit sera with MBP (E) Protein profile of MLSA on SDS-PAGE gel stained with coomassie blue, (F) Western blotting pattern of reactivity of anti-MBP rabbit sera with MLSA. Lane 1: Molecular weight marker, Lane 2: MLSA (G) Protein profile of MLSA on 2-D gel stained with coomassie blue, (H) Western blotting pattern of reactivity of anti-MBP rabbit sera with MLSA.](image)

### Table 1

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<th>Nominal mass</th>
<th>pI</th>
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<td>MLSA cross-reacted with anti-MBP rabbit sera</td>
<td>50S ribosomal protein L2 - <em>M. leprae</em> Lysyl-tRNA synthetase (EC 6.1.1.6) (Lysine–tRNA ligase) (LysRS) – <em>M. leprae</em> 1179,769</td>
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MLSA (≈51 kDa protein) with anti-MBP rabbit sera to Mascot search engine and the search parameters used for the identification were peptide mass tolerance ± 100 ppm, fragment mass tolerance ± 0.5 Da, maximum missed cleavages 1.

2.11. Prediction of mimicking B cell epitopes from MALDI identified proteins of host and M. leprae

B cell epitopes of all MALDI identified cross-reactive proteins were predicted using BCPREDS server 1.0 (http://ailab.cs.tastate.edu/bcpreds/index.html). BCPREDS server used aap prediction method. Predicted B cell epitope length was of 20 amino acids and classifier specificity used was 75% [20].

2.12. Three-dimensional modeling of identified proteins

Three dimensional (3-D) structure of mimicking proteins of M. leprae and MBP was formed by submitting the sequence to CPH models 3.2 server (www.cbs.dtu.dk/services/CPHmodels/) [21]. Modelled structure was visualized and analysed by VMD viewer (www.ks.uiuc.edu/Research/vmd/) [22].

2.13. Statistical analysis

Data were analyzed using GraphPad prism software version 5.0 (GraphPad, La Jolla, CA). ELISA data was presented as mean ± 2SD. P value <0.05 was considered to be statistically significant. Individual test used for the analysis of data of each experiment is mentioned in respective figure or table legend.

3. Results

3.1. Levels of antibodies against MBP in sera of leprosy patients

Highest mean OD value for anti-MBP antibodies was observed in the sera of patients with T1R (0.355 ± 0.27) and was followed by ENL (0.344 ± 0.26), LL (0.309 ± 0.23), BL (0.309 ± 0.28), BB (0.224 ± 0.21) and TT/BT (0.208 ± 0.15). Mean OD value of T1R is significantly higher than TT/BT (p < 0.05). Anti-MBP antibody level was significantly higher in lepromatous pole of leprosy and in reactions than HC (Fig. 1A).

Highest sero-positivity for anti-MBP antibodies was also found in T1R (61.9%) followed by ENL (50%), LL (41.3%), BB (23.8%), BT (25%) and HC (0%). Percentage positivity of T1R was significantly higher than BT group (p = 0.02).

3.2. Correlation of anti-MBP antibodies level and number of nerves involved in different groups of leprosy patients

Spearman’s correlation test showed anti-MBP antibodies level to be significantly correlated with number of nerves involved for TT/BT (R² = 0.748, p < 0.0001), BB (R² = 0.643, p = 0.001), BL (R² = 0.540, p = 0.02), LL (R² = 0.617, p < 0.0001), T1R (R² = 0.631, p < 0.0001) and ENL (R² = 0.594, p = 0.02) group of leprosy (Fig. 1B–G).

3.3. Binding of hyper-immunized rabbit anti-MBP antibodies with MBP was inhibited by leprosy patients’ sera in dose dependent manner

Binding of anti-MBP antibodies of hyper-immunized rabbit was maximum inhibited at 1:5 dilutions of leprosy patients’ sera and inhibition gradually decreased with the increasing dilutions of leprosy sera (Fig. 1H). Due to the unavailability of patients’ sera all Western blot experiments were conducted using hyper-immunized rabbit sera.

3.4. Lymphoproliferation of leprosy patients in the presence of MBP

The highest mean value of S.I. was obtained in N (4.43 ± 3.1) group of leprosy patients which was followed by T1R (3.72 ± 2.9), BL/LL (2.51 ± 1.3), TT/BT (2.39 ± 1.5), ENL (2.2 ± 1.5). The mean values of S.I. in the presence of MBP were found to be significantly higher in N (p = 0.007), TT/BT (p = 0.03), BL/LL (p = 0.01), T1R (p = 0.02) types of leprosy patients’ group in comparison to that of healthy controls (Fig. 2A).

3.5. MLSA hyperimmunization induce production of anti-MBP antibodies in rabbit

It was observed that hyperimmunization of rabbits with MLSA evoked induction of significantly higher levels of auto-antibodies against MBP (p < 0.0001) in comparison to control rabbits. The highest levels of antibodies against MBP were observed at 35th day of immunization with MLSA (Fig. 2B).

3.6. Levels of antibodies against MBP in MLSA-hyperimmunized mice

It was noted that MLSA hyperimmunization till 7th week induced significantly higher levels of anti-MBP antibodies (0.071 ± 0.018) (p < 0.0001) in hyperimmunized mice compared to those of normal saline inoculated control group (0.016 ± 0.014) and pre-immunized mice (0.012 ± 0.009) (Fig. 2C). However, no difference was observed in between pre-immunized and control mice.

3.7. Adoptive transfer of whole immune cells and nylon wool separated T cells induce autoimmune response in naïve female BALB/c mice

After 21 days of adoptive transfer significantly higher level of anti-MBP antibodies were observed in the plasma of T cells transferred mice (0.063 ± 0.008) (p < 0.0001) or mixture of splenocytes and lymph nodes cells (0.053 ± 0.005) (p < 0.001) in comparison to those of pre-immunized mice (0.034 ± 0.009) and control group mice (0.032 ± 0.007) (Fig. 2D).

3.8. Cross-reactive proteins of host MBP and MLSA

The specific cross-reactive proteins between host MBP and MLSA was observed by 1-D western blot and 2-D western blot. It was noted that anti-MLSA rabbit sera cross-reacted at ≈20 kDa protein of MBP (Fig. 3A–B) with one isofrom of
pl ≈ 10.0 (Fig. 3C–D) by 1-D and 2-D western blots. On the other hand, anti-MBP rabbit sera cross-reacted at ≈ 51 kDa, 30 kDa and 22 kDa protein of MLSA (Fig. 3E–F) and at ≈ 51 kDa, pl 5.5 and 30 kDa, pl 6.0 (Fig. 3G–H).

3.9. Identification of cross-reactive protein/s of host MBP and MLSA

As shown in Table 1 cross-reactive host protein MBP that reacted with anti-MLSA rabbit sera was identified as MBP (myelin A1 protein) 20 kDa microtubule-stabilizing protein of Bos taurus (Bovine) (Fig. 1S A). The cross-reactive proteins of MLSA those reacted with anti-MBP rabbit sera, one was identified as ≈ 30 kDa protein 50S ribosomal protein L2 of M. leprae, (Fig. 1S B) and the second one was identified with the help of MALDI-TOF/TOF-MS/MS ion search by submitting different peaks (mass - 1179.769) of the cross-reactive protein of MLSA with anti-MBP rabbit sera to Mascot search engine as Lysyl-tRNA synthetase (Lysine–tRNA ligase) of M. leprae (Fig. 1S C and Table 1).

**Mascot Search Result.**

Match to: **MBP_BOVIN** Score: 104 Expect: 2e-006.

Myelin basic protein (MBP) (Myelin A1 protein) (20 kDa microtubule-stabilizing protein) - B. taurus (Bovine)

Nominal mass (M₀): 18312; Calculated pI value: 11.28.

Number of mass values searched: 30.

Number of mass values matched: 8.

Sequence Coverage: 46%

Matched peptides shown in Bold Red.

**Match to:** **RL2_MYCLE** Score: 55 Expect: 0.021.

50S ribosomal protein L2 - M. leprae.

Nominal mass (M₀): 30593; Calculated pl value: 11.41.

Number of mass values searched: 67.

Number of mass values matched: 8.

Sequence Coverage: 23%

Matched peptides shown in Bold Red.

3.10. Prediction of B cell epitopes of cross-reactive proteins of MLSA and MBP

As shown in Table 1S, three B cell epitopes of MBP were predicted with the help of BCPRED Server 1.0. Further, 8 B cell epitopes of 50S ribosomal protein L2 (Table 2S) and 12 B cell epitopes of Lysyl-tRNA synthetase (Lysine–tRNA ligase) of M. leprae were predicted (Table 3S).

3.11. Phylogenetic tree of predicted B cell epitopes of cross-reactive proteins of MBP and M. leprae

Phylogenetic tree of predicted B cell epitope of MBP and 50S ribosomal protein L2 of M. leprae was made with the help of Clustal W server to find out similarity between them (Fig. 2S).

As shown in phylogenetic tree 2 B cell epitopes of MBP and 50S ribosomal protein L2 of M. leprae resemble closely to each other. These epitopes are VVHFFKNIVTPRTPPPSQGK (MBP 85-104) and VSPWGKPEGRTRKPNKSSNK (50S ribosomal protein L2 of M. leprae 247-266) and GAPKRGSGKDGHHAARTTHY (MBP 48-67) and EQANINWGKAGRMWKGKR (50S ribosomal protein L2 of M. leprae 200-219) resemble very closely to each other (Fig. 2SA).

While, one B cell epitope VVHFFKNIVTPRPPPSSQGK of MBP (MBP 85-104) and NTLSAPTFVKDFPVETTPL (Lysyl-tRNA synthetase of M. leprae 384-403) resemble closely to each other (Fig. 2SB).
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3.12. Similarity between the predicted B cell epitopes of MBP and 50S ribosomal protein L2 of M. leprae or Lysyl-tRNA synthetase (Lysine-tRNA ligase) of M. leprae

As shown in Fig. 4A two B cell epitopes of MBP98-104 with 50S ribosomal protein L2226-237 and MBP127-131 and MBP55-60 with 50S ribosomal protein L2 of M. leprae41-46 are mimicking epitopes. While, 2 B cell epitopes of MBP85-98 with Lysyl tRNA synthetase of M. leprae388-401 and MBP99-104 with Lysyl tRNA synthetase of M. leprae472-477 (Fig. 4B) are mimicking with each other.

3.13. Three dimensional structure of 50S ribosomal protein L2 of M. leprae

Three-dimensional structure was highlighted with mimicking epitopes of MBP and 50S ribosomal protein L2 of M. leprae. It was observed that 2 mimicking B cell epitopes (MBP94-104 with 50S ribosomal protein L2226-237 and MBP55-60, MBP127-131 with 50S ribosomal protein L241-46) are present on the surface of the 3-D structure of 50S ribosomal protein L2 of M. leprae (Fig. 5A).

Above mentioned 2 mimicking B cell epitopes are present on the surface of 3-D structure of MBP (Fig. 5B).

4. Discussion

In this study, we demonstrated the clinical correlation between level of antibodies against MBP and number of nerve involved in leprosy patients across the spectrum (Fig. 1B–G). Furthermore, we found presence of significantly high level of anti-MBP antibodies (Fig. 1A) in leprosy spectrum as well as significant high lympho-proliferation to MBP in all groups of leprosy patients except ENL (Fig. 2A). Sero-positivity for anti-MBP antibodies was found to be highest in leprosy patients’ sera of T1R (61.9%) followed by ENL (50%), LL (41.38%), BL (30.43%), BB (23.81%) and TT/BT (25%). These results indicate that antibodies are more associated with chronic phase of the disease. We also demonstrated that binding of hyperimmunized rabbit anti-MBP antibodies with MBP was inhibited by leprosy patients’ sera in dose dependent manner (Fig. 1H). Inhibition assay highlight that both anti-MBP antibodies raised in rabbit and antibodies present in leprosy patients' sera compete with same B cell epitopes. Neural damage is one of the characteristic features of leprosy disease. Earlier, it was reported that M. leprae has a strong demyelinating effect on infected Schwann cells (Sc) neuron culture [23]. Further, it was also showed by Antunes et al. (2006) [24] that M. leprae has direct destructive effect on the integrity of nerve fibers in leprosy patients. However, our findings suggest that anti-MBP antibodies are associated with extent of nerve involvement in leprosy patients. Recently, Mycobacterium avium subspecies paratuberculosis peptides have been shown to be recognized by anti-MBP antibodies in MS patients [25]. In MS, Ponomarenko et al. (2006) [6] provided a mechanistic explanation of anti-MBP antibodies in pathological destruction of myelin sheath. Presence of anti-MBP antibodies in leprosy patients may also be involved in the pathological destruction of myelin sheath. It was shown by Corsico et al. (1994) [7] that in LL patient circulating immune complexes contain MBP as an antigen. Authors suggested that presence of MBP could be correlated with pathogenesis of leprosy since liberation of MBP after nerve damage may induce anti-MBP autoantibodies production to myelin breakdown, which could react with peripheral nerve MBP also. For the first time our study provides evidence that anti-MBP antibodies are associated with the extent of nerve damage in leprosy patients. Although, M. leprae specific immunosuppression is reported in LL patients but presence of auto-reactive cells might be responsible for positive proliferation to MBP as observed in this study. A key question remains to be answered that whether these auto-reactive cells are induced by mycobacterial components? The experiments carried out by hyperimmunization of rabbits and mice with MLSPA evoked a high level of anti-MBP antibodies in their sera/plasma (Fig. 2B–D) and suggest that M. leprae components are able to induce an imbalance in the homeostatic mechanism of the host which in turn induces autoantibody response.

T1R in leprosy is known to occur even after the completion of the therapy [26] and the bacterial loads in these patients are significantly lower in comparison to LL patients. Therefore, the precipitating factor for the occurrence of T1R is still remains an enigma. Earlier, it has been hypothesized that cross-reactive proteins of M. leprae and host might be responsible for initiation of reaction [9,27]. Antigenic similarity between M. leprae and human nerve and skin components has been suggested as the possible mechanism for the development of auto-reaction in leprosy patients [8,9,11]. For the first time, we identified using anti-MBP antibodies and anti-M. leprae antibodies in 1-D as well as 2-D western blot (Fig. 3A–H) that the cross-reactive proteins are Lysyl-tRNA synthetase (Lysine − tRNA ligase) and 50S ribosomal protein L2 of M. leprae and MBP (myelin A1 protein) of host by MALDI-TOF/TOF (Fig. 1S and Table 1). The basic A1 protein is a major structural protein of the myelin and reported to be responsible for induction of EAE. Peptide fragments derived from the A1 protein are themselves immunogenic as demonstrated by their ability for induction of a delayed hypersensitive response [28]. We also find cross-reactive myelin basic protein as myelin A1 protein.

Fig. 4. (A) Multiple sequence alignment of predicted B cell epitopes of MBP and 50S ribosomal protein L2 of M. leprae. Figure showing full sequence of 50S ribosomal protein L2 of M. leprae and predicted B cell epitopes of MBP. (B) Multiple sequence alignment of predicted B cell epitopes of MBP and Lysyl-tRNA synthetase (Lysine-tRNA ligase) of M. leprae. Figure showing full sequence of Lysine-tRNA ligase of M. leprae and predicted B cell epitopes of MBP. A, B: Mimicking B cell epitopes are highlighted in yellow color in the sequence. Blue colored fonts are B cell epitopes of M. leprae and red colored fonts are similar sequence of B cell epitopes of MBP with B cell epitopes of M. leprae.
and it might be involved in the auto-reaction in leprosy patients. Further, using bioinformatics tools 2 mimicking B cell epitopes were identified between MBP and 50S ribosomal protein L2 of *M. leprae* while 1 mimicking B cell epitope was identified between MBP and Lysyl-tRNA synthetase (Fig. 4). Some of the mimicking B cell epitopes of MBP (MBP<sub>98-104</sub>; MBP<sub>85-98</sub>; MBP<sub>99-104</sub>) were found to be in the immunodominant region of MBP which is MBP<sub>85-101</sub>. MBP<sub>85-101</sub> is found to be encephalitogenic peptide [29,30,6]. Hence, these mimicking B cell epitopes might be responsible for demyelination of nerves in leprosy patients. This suggested that the high prevalence of anti-MBP in leprosy spectrum and western blotting reaction between both of these proteins (i.e. MBP and MLSA) might be because of the presence of common B cell epitopes. Vardhini et al. (2004) [11] have also pointed out the molecular mimicry enacted by mycobacterial proteins (ferredoxin-NADP-reductase and a conserved mycobacterial membrane protein) that could mimic myelin P0. Our findings are based on the wet lab experiments as well as bioinformatics tools while these authors suggested molecular mimicry on the basis of bioinformatics tools only. From our findings we suggest that molecular mimicry is responsible for high level of anti-MBP antibodies in leprosy spectrum and induction of anti-MBP antibodies in hyperimmunized rabbit and mice. Further, adoptive transfer experiment in present study also provides evidence for transferring autoreaction in naïve mice by T as well as B cells (Fig. 2D). We also have noted T cell response to MBP in leprosy patients (Fig. 2A). Recently, we have reported that CD<sub>4</sub><sup>+</sup>CD<sub>25</sub><sup>+</sup>FoxP3<sup>+</sup>Treg cells level goes down in the hyperimmunized (with MLSA) mice [8] that in turn give strong support to our hypothesis that *M. leprae* antigens are capable in inducing homeostatic imbalance in the immune system of the host and is a major factor for the development of the auto-reaction. Molecular mimicry shown in the present study suggests that mimicking molecules of host and *M. leprae* might be responsible for high level of autoantibody and CMI reaction in leprosy patients.
Thus, our study suggests the existence of molecular mimicry between MBP of host and *M. leprae* proteins (50S ribosomal protein L2 and Lysyl-tRNA synthetase). Correlation of anti-MBP antibodies with number of nerves involved and high CMI reaction to MBP showed that molecular mimicry is the most possible factor for the pathogenesis of nerve damage in leprosy.

**Conflict of interest statement**

The authors declare no conflict of interest.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.micinf.2014.12.015.

**References**