Material and Methods
3.1. Fungal cultures

*Candida albicans* ATCC 10231 was used throughout this study. Other fungal strains used for cross reactivity studies were *C. albicans* ATCC 60193, *C. albicans* ATCC 66027, *C. albicans* ATCC 14053, *Candida parapsilosis* ATCC 22019, *Cryptococcus neoformans* ATCC 66031, *Aspergillus fumigatus* and *Trichophyton mentagrophytes*. All these fungi were grown on Sabouraud Dextrose Agar (SDA) (Diffco USA) slants at 35°C for 24 to 72 h and stored at 4°C. Bacterial strains used for cross reactivity studies were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC BAA-427, *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 27736, *Bacillus cereus* MTCC 430. The bacterial cultures were grown on nutrient agar (Hi media) at 35°C for 24h and stored at 4°C. Other culture media and conditions used were Sabouraud dextrose broth (SDB) (1% peptone and 2% dextrose), Yeast nitrogen base (YNB) (minimal media supplemented with amino acids, Sigma) with 2% dextrose, YNB with 2% N-acetyl-D-glucosamine (NAG) (Sigma) and 2% Dextrose, RPMI 1640 with 10% Fetal bovine serum (FBS) (Hybridoma tested, Sigma), DMEM with 10% FBS and serum free media (Hybridoma tested, Sigma).

3.2. Culture conditions for *C. albicans*

The yeast phase of *C. albicans* was grown in Sabouraud dextrose Broth or YNB (pH 4.5) in an Erlenmeyer flask on a rotator shaker (200 rpm) at 28°C for 16h by inoculating approximately $10^6$ cells per ml. (0.05 OD at 600nm wavelength) from a 24h old SDA slant. Alternatively, for growing *C. albicans* in mycelial form, yeast cells from late log phase (24-36h culture) were inoculated in YNB (pH 6.5) supplemented with 10% FBS at a concentration of $10^6$ cells per ml. and incubated overnight (12-16h) at 37°C without shaking. For conversion of yeast cells to mycelial form under serum free conditions, FBS was replaced with mycelial inducers such as N-acetyl-D-glucosamine. Conversion of yeast to mycelial form was assessed by microscopic observation. Both yeast cells and mycelia were harvested by centrifugation at
3000 rpm for 5 min, pellets were washed three times with chilled distilled water and were either used for further experiments or stored at -80°C.

3.3. Protoplast preparation and their regeneration

For the preparation of protoplast CellLytic™ Y Plus kit (CYP1, Sigma) was used with minor modifications. Yeast cells or mycelia in mid-log phase (grown for 12-16h) were pelleted by centrifugation at 3000 rpm for 5 min in a pre-weighed centrifuge tube. The tube was re-weighed along with yeast cells or mycelia to estimate the wet weight of the fungal mass. The fungal pellet (yeast or mycelia) was re-suspended in two volumes of pre-treatment solution containing 10 mM HCl-Tris (pH 9.0), 5 mM EDTA, and 1% v/v 2-mercaptoethanol at 35°C with shaking at 80 rpm for 30 min. The cells were then centrifuged again at 3000 rpm for 5 min to remove the pre-treatment solution. The cell pellet was washed twice with 1 M sorbitol and re-suspended in reaction buffer provided with the kit. Reaction volume for protoplasting varied for each strain. For *C. albicans*, the reaction volume standardized was found to be 1500 µl for every 1g of cell pellet (yeast or mycelia) (1455 µl reaction buffer, 45 µl of 1 M dithiothreitol, and 25 µl of lyticase provided in the kit). The reaction tube was then incubated at 37°C with gentle shaking for 15-20 min. Protoplast formation was constantly monitored by measuring the optical density of the reaction sample at 800nm. Briefly, 10 µl of yeast protoplast sample from the reaction mixture was added in 990 µl triple distilled water (TDW) and OD was read at 800nm at regular intervals after addition of the enzymes. The OD values were compared with the value before the addition of the enzymes. Incubation was stopped when the OD values were 10% to 20% of the pre-lysed sample. Protoplasting (yeast and mycelia) was also monitored by regular observation under a microscope. During protoplast formation, yeast cells or mycelia slowly became translucent and thin. Protoplasts were then pelleted by centrifugation at 1500 rpm for 5 min and washed thrice with YNB containing 1 M sorbitol to eliminate any traces of lyticase.
Protoplast regeneration was induced in YNB containing 1 M sorbitol and protease inhibitor cocktail (Sigma). Yeast protoplasts were allowed to regenerate at 28°C with gentle shaking (50 rpm) and mycelial protoplasts at 37°C. Mycelial regeneration media also contained 2% N-acetyl D glucosamine. After 2h incubation, the cells were pelleted at 2000 rpm for 5 min, supernatant filtered through a 0.22 μm nitrocellulose syringe filter and concentrated in a speed vacuum to one third volume of the initial amount. Protein amount in the supernatant was estimated using 2D Quant protein estimation kit from GE healthcare, USA.

3.4. Isolation of cell wall and protein extraction

Cells of C. albicans stored at -80°C were thawed and the pellet was resuspended in chilled 20 mM tris-Cl (pH 7.4), supplemented with protease inhibitor cocktail (to a final concentration of PMSF 1 mM, EDTA 1 mM, AEBSF 1 mM, 1,10-phenanthroline 5 mM, pepstatin A 20 μM, E-64 10 μM), to a cell density of 10^8 cells/ml. To 10 ml of this cell suspension, an equal amount of acid washed glass beads (425-600 micron) were added and vortexed vigorously in the jacketed mixing chamber of bead beater (Hamilton Beach/Proctor-Silex, Inc.) that was cooled with glycerol at -20°C. The instrument was operated with cycles of mixing for 1 min followed by a pause of 2 min. After a total of 10-12 cycles, the lysate was observed under phase contrast microscope and the cell wall was pelleted by centrifugation. The cell wall pellet was extensively washed with chilled triple distilled water supplemented with PMSF and benzemidine hydrochloride to a final concentration of 1 mM each, and stored at -80°C. For cross reactivity studies, cell walls of C. parapsilosis, C. neoformans, A. fumigatus and T. mentagrophytes were similarly isolated and stored.

For protein extraction, cell walls were suspended in extraction buffer containing 40 mM β-ME, 50 mM EDTA, 50 mM tris-HCL (pH 8.5) and 2% (w/v) SDS and kept in boiling water for five minutes. Supernatant containing the cell wall proteins was collected by centrifugation at 10000 rpm for 5 min at room
temperature (RT). The protein content was estimated by 2D Quant protein estimation kit (GE healthcare, USA) as per the manufacturer's instructions.

3.5. Protein estimate on using 2D Quant kit

Protein estimation was done using 2D Quant kit (GE health care, USA) throughout this study. The kit utilizes only a small amount of protein sample and protein estimation can be done even in the presence of interfering substances (SDS, β-ME, urea, thiourea, sorbitol etc) that otherwise is not possible using traditional methods of protein estimation (Lowry method, Bradford method etc.). Estimation of the protein samples was carried out in 2 ml microfuge tubes. The required amount of BSA (2 mg/ml) provided in the kit was used as standard. For each test sample two different volumes in duplicate were taken in microfuge tubes. To each tube 500 μl of precipitant provided in the kit was added and vortexed briefly for 2-3 min at RT. To this 500 μl of co-precipitant was added and mixed briefly by vortexing as mentioned above and the tubes were centrifuged at 10000 rpm for 5 min at RT. The supernatant was removed carefully avoiding the loss of the pellet. The pellets were resuspended in 100 μl of copper solution using a micropipette. To each tube 400 μl of TDW was added and mixed. A colour reagent provided in the kit was prepared by mixing 100 parts of colour reagent-A with 1 part of colour reagent-B. To each tube, 1 ml of this colouring reagent was added and incubated at RT for 20 min. After incubation 200 μl of solution from each tube was dispensed into micro titre plate and the absorbance was read at 480 nm in a spectrophotometer with TDW serving as a blank. The protein quantity was estimated by Versamax® soft ware using the standard curve generated.

3.6. 2D gel electrophoresis

Proteins secreted by the regenerating yeast and mycelial protoplasts and cell wall proteins of both the forms were resolved according to their pI and molecular mass by performing 2D gel electrophoresis.
3.6.1. First-dimension isoelectric focusing (IEF)

Isoelectric focusing was carried out on Ettan IPGphor 3 apparatus (GE Healthcare) using pre-caste dry immobilised pH gradient (IPG) strips (3-10 pI, 13 Cm) from GE healthcare, USA.

A. Sample Preparation

Proteins secreted by regenerating protoplasts as well as cell wall proteins of both the forms were precipitated by 12% TCA and kept at 4°C for 4h. The precipitated protein was pelleted at 14000rpm for 15 min. Pellet was redissolved in lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 2% (v/v) pharmalyte (3-10), 40 mM DTT. Protein content was quantified by 2D Quant protein estimation kit (GE healthcare, USA) as per the manufacturer's instructions. The protein concentration was adjusted to 1 mg/ml with the same lysis buffer.

B. Rehydration of IPG strip

The immobilized dry IPG strip were rehydrated with the sample in rehydration buffer. During rehydration the proteins in the sample get adsorbed onto the strip randomly, ready to be focused according to their isoelectric point (pI). The rehydration solution volume per immobiline strip varied with the strip size. For 13 cm strip, 250 µl rehydration solution was required. 50 µl of lysis buffer containing sample protein was mixed with 210 µl of rehydration buffer containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 2% (v/v) Pharmalyte (3-10), 0.002% (w/v) bromophenol blue. DTT was added just before using this stock solution (7mg per 2.5 ml stock solution). The solution was spread in the rehydration tray and IPG strips were placed such that its gel portion is immersed in the rehydration solution. Upper protective polythene sheet was removed from the strip by simply stripping off just before rehydration. Mineral oil was poured such that it covered the entire strip to reduce evaporation and strips were left overnight for rehydration (18-20 h).
C. Isoelectric focusing

The rehydrated strips were placed in the strip holder manifold of the Ettan IPG3 unit such that the gel portion faces up and taking care the anodic and cathodic sides. Pre-cut electrode pads acting as wicks, wetted with TDW (150 μl per wick) were placed on both the ends of the strips. Electrode assembly was placed over the top of the pads such that the pins were positioned under the external lip of the manifold to seat the electrode in place. The strip holder was filled with 110 ml of mineral oil to cover the strips completely to prevent evaporation. Isoelectric focusing was carried out with conditions as mentioned by the manufacturer's manual with some minor modifications. An additional step of 150v for 2h was included to remove contaminating particles and salt, if any. IEF was performed for a total of 26300 Vh (volt-hours) with gradual increase in the voltage as mentioned below.

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3.6.2. SDS PAGE in second dimension

A. Equilibration of IPG strips

After isoelectric focusing was complete, the IPG strips were removed and processed for second dimension electrophoresis immediately or stored at -20°C wrapped in a plastic sheet or in a clean screw cap tubes, until further use.

For second dimension resolution of the proteins, the IPG strips were equilibrated with SDS equilibration buffer (6 M urea, 75 mM tris-Cl (pH 8.8), 29.3% (v/v) glycerol, 2% (w/v) SDS, 0.002% (w/v) bromophenol blue) to saturate the isoelectro-focused strips and bring them to an appropriate pH range compatible with the SDS buffer system and also to make the m/z values
equal for all proteins by appropriate binding of SDS molecules with the proteins. Equilibration was done twice (10 ml per strip per step), once with DTT (100 mg/10 ml) for reducing the thiol groups and then with iodoacetamide (250 mg/10 ml) for alkylation of the free thiol groups, for 15 min. each with constant shaking. The strips were placed such that the support film on one side of the strip touched the tube in which equilibration is done.

B. SDS-PAGE

After equilibration, the isoelectro-focused proteins in the IPG strips were further resolved in the second dimension according to their molecular weight. Single gel system containing only resolving gel (10%) buffered with Tris-CI (pH 8.8) was followed. The concentrations of individual components in the stock solutions used for the preparation of resolving gel are as follows

- 30.8% acrylamide (30% acrylamide and 0.8% bis-acrylamide)
- 1.5 M Tris pH 8.8
- 10% SDS
- 10% ammonium per sulfate (APS)

The gel constituents were mixed in appropriate amounts to prepare 10% resolving gel. APS and TEMED were added just before pouring of the solution in between the preassembled glass plates, after gently swirling the mixture avoiding gas bubbles. Water-saturated isobutanol was overlaid on the top of the resolving gel to minimize the exposure of the gel to oxygen (hinders the gel formation) and to get a smooth surfaced resolving gel top. Equilibrated IPG strip was first dipped in electrophoresis buffer and was placed on the top of the resolving gel by gently pushing the strip down to touch the resolving gel surface avoiding air bubbles between them. The immobiline strip was then sealed with agarose sealing solution (0.5% agarose with 0.002% (w/v) bromophenol blue in Laemmeli’s SDS electrophoresis buffer. After this, the glass cassette was clamped onto the gel unit and the electrophoresis buffer. Electrophoresis was performed at a constant current of 10 mA until the bromophenol blue (tracking dye) reached the bottom of the gel. At this point
the gel was removed and transferred either into a fixative solution (10% acetic acid, 30% methanol) for fixing the resolved proteins and silver staining or in the transfer buffer when electro-blotting had to be done.

3.7. Silver staining of gels

Silver staining was performed by the method of Shevchenko et al. (1996) (206). The proteins on the gels were fixed in a fixative (48% TDW, 40% methanol and 12% acetic acid) for 1h and subsequently washed thrice TDW. The gels were then sensitized by placing them in a solution of 0.02% sodium thiosulfate for 1 min and washed with twice (1 min each) with TDW. Subsequently the gels were transferred to a solution of 0.2% silver nitrate and gently agitated. After 10 min the silver nitrate solution was removed and the gels were washed with two changes of TDW, 1 min each. The developing solution (100 ml of 6% sodium carbonate and 500 μl formaldehyde) was added quickly and the reaction was stopped by addition of 10% acetic acid solution, as soon as the spots have appeared. Finally the gels were washed with at least two changes of 1% acetic acid and documented with the help of a document scanner.

3.8. Generation of hybridomas and production of monoclonal antibodies

3.8.1. Cell line used

Mouse myeloma cell line (Sp2/O) was obtained from National Institute of Immunology, New Delhi and maintained in RPMI containing 10% FBS at 37°C and 5% CO₂ atmosphere. It was also cryopreserved (10 %DMSO, 40 %RPMI 1640 and 50% hybridoma tested FBS) in liquid nitrogen in aliquots for future use as well.

3.8.2. Mice

Male BALB/c mice were used throughout this study. All the animals were maintained and cared in the animal house facility of the institute according to the norms laid down by the animal ethics committee. Prior clearance from the animal ethics Committee was obtained before performing any experiment on animals.
3.8.3. Immunization of mice

Ten BALB/c mice were allowed to acclimatize to the laboratory environment for 5-7 days. The mice were immunized subcutaneously with 100 μl of mycelial protoplast secreted protein antigen (100 μg/dose) preparation in Freund's complete adjuvant (1:1). This was followed by three consecutive booster doses at 15 days interval, subcutaneously with the same antigen in incomplete Freund's adjuvant and the mice were observed daily for secondary infection if any. After 30 days since the last booster 20-50 μl of blood was drawn from the tail vein of each animal to checking the optimum immunization by performing ELISA and Western blot as described later. Two to three mice that had the highest antibody titre were selected for fusion experiments. The mice selected for fusion were given a final booster dose of 100 μl (1mg/ml) of mycelial secreted protein (without any adjuvant) intraperitoneally, 5-6 days prior to fusion experiment.

3.8.4. Pre-fusion preparation

For all fusion experiments mouse myeloma cell line, Sp2/O was used. The cell line was essentially grown and maintained by regular culture on every fourth day in RPMI 1640, supplemented with 15 mM L-glutamine, 200 mM sodium bicarbonate, 5% FBS (Hybridoma tested, Sigma, USA), 50 U/ml ampicillin, 25 μg/ml streptomycin, 500 μg/ml gentamicin, and 8 μg/ml amphotericin B. As a rule, the sub culturing was performed by dislodging the cells from a confluent flask and seeding 500 μl of this cell suspension into 5 ml of fresh medium in tissue culture flask.

The preparation for fusion experiment started nearly 15 days before its commencement. Sufficient number of Sp2/O cells (3 x 10^9) was harvested from 36 h old cultures. Prior to expansion, Sp2/O cells were checked for their selectivity on HAT (final concentration in media, 0.1 mM hypoxanthine, 4 μM aminopterin, and 16 μM thymidine) media. This was done by sub-culturing an aliquot of cells in the RPMI media supplemented with HAT.
3.8.5. Isolation of splenocytes

On the day of fusion one of the immunized mice was sacrificed after under anaesthesia. Spleen was taken out under aseptic conditions in a sterile petri plate, washed with 5 ml of incomplete RPMI media (without FBS) and transferred to a fresh sterilized petri dish. To this 5 ml incomplete RPMI media was added and the spleen was macerated with a sterile plunger on a sterilized sieve to release the splenocytes. Small pieces of the spleen thus obtained were further disintegrated by pipetting them a few times with 5 ml disposable plastic pipette. The cell suspension was collected in a 50 ml centrifuge tube and centrifuged at 800 g for 5 min. The supernatant was removed and the cell pellet was resuspended in 10 ml of fresh medium and kept on a water bath at 37°C until for 5 min.

3.8.6. Fusion of splenocytes with Sp2/O myeloma cells

Fusion of the splenocytes from immunized mouse with Sp2/O myeloma cell line was carried out in a ratio of 1:2 using PEG/DMSO solution (50% polyethylene glycol and 10% DMSO) as a fusogen. On the day of fusion, the Sp2/O myeloma cells were dislodged and collected aseptically in 50 ml centrifuge tube. The cells were centrifuged at 800 g for 5 min and the supernatant was preserved. The Sp2/O cell pellet was washed twice with incomplete RPMI media, to remove FBS sticking to the cells. The pellet was resuspended in incomplete RPMI media and mixed with the above spleen cells. The mixture was centrifuged first at 800 g for 5 min and subsequently at 2000 g for 2-3 min. The supernatant was removed as completely by a pipette, without disturbing the pellet. The tube containing pellet was equilibrated for 3-4 min at 37°C on a water bath. To this pellet, 1 ml of PEG/DMSO already equilibrated to 37°C was added with gentle swirling of the suspension in such a manner that addition of 1 ml of the fusogen was spread over a period of 1 min. and the mixture was allowed to stand for one more minute. To this suspension, 5 ml of incomplete RPMI 1640 was slowly added with gentle swirling of the tube at 37°C for 3-5 min on water bath. The process was repeated again with 10 ml of same media as above and PEG/DMSO was diluted immediately by adding RPMI 1640 containing 10% FBS up to 50 ml
mark on the centrifuge tube. The contents of this tube were then transferred to a tissue culture flask containing 150 ml of RPMI 1640 supplemented with 10% FBS. To this flask 50 ml of Sp2/O cell culture supernatant was added and mixed gently.

Subsequently HAT supplement was added to this flask to attain the final concentration as described above and mixed by inverting the flask for a few times. To confirm proper fusion 10-20 µl of this cell suspension was placed on a glass slide and observed under microscope. The content of the flask were distributed in aliquots of 200 µl/well in 96 well tissue culture plates and incubated in an atmosphere of 5% CO₂ at 37°C in an incubator. Each well of these plates was observed carefully from day two to rule out contamination if any and check hybrid cell multiplication. Wells that appeared contaminated were marked and later sterilized by removing carefully all the contents of the well and adding 200 µl of sodium azide (0.1 mg/ml). Three days later few cells appeared alive and dividing. At this stage, each well was supplemented with 100 µl of 3x HT supplement to attain a final concentration of 0.1 mM of hypoxanthine and 16 µM of thymidine. The cells were allowed to grow for two more days to have confluent growth and subcultured in a fresh 96 well culture plate. The cells were grown further for 3 more days and then the screening for positive hybridomas was started.

3.8.7. Screening of positive hybridomas

Screening for antibody producing hybridoma clones was started after 7 days of incubation when the clones started looking like a bunch of grapes. First round of screening was done by ELISA method as follows.

Screening by ELISA

Enzyme linked immunosorbent assay (ELISA) was performed for the initial screening of the hybridomas. Briefly, each well of micro-titre plates (Greiner Bio One GmbH) was coated with 50 µl of mycelial protoplast secreted antigen adjusted to a concentration of 50 µg/ml in 0.6 M bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The coated wells were blocked by adding 150
μl of 1% (w/v) bovine-serum albumin in PBS for 90 minutes at 37°C. Blocking solution was removed and the wells were washed once with PBS-T20 (5 min). 50 μl of cell culture supernatant was aseptically poured directly into antigen coated and blocked ELISA plates using an 8 channel pipette such that the ELISA plate was an exact replica of culture plate. Care was taken not to spill contents while pipetting and the pipette tips were changed for every well to avoid cross contamination from other monoclonal antibodies. The plates were incubated for 90 min at 37°C, washed thrice with PBS T-20 (5 min each) and were incubated with peroxidase-conjugated anti-mouse IgG, IgA and IgM diluted 1:5,000 (each) in PBS T-20 for 90 minutes at 37°C. After washing, 100 μl of substrate containing 0.05% (w/v) O-phenylene diamine dihydrochloride (OPD) (Sigma) and 0.03% H₂O₂ (v/v) in phosphate citrate buffer 0.15 M (pH5.0) were added to each well and the plates were incubated in dark at room temperature for 30 minutes. The reaction was stopped with 10 μl of 7% H₂SO₄ and optical densities were read on a micro titre plate reader (Molecular Devices, USA) at 450nm.

3.8.8. Single cell cloning and selection of positive hybridomas

The wells containing positive hybridoma clones were identified and sub cloned by two-fold serial dilution to reach single cell per well in 96 well culture plates. After 4-6 days when the colonies from these single cells reached to a size of at least 200 cells per well, 100 μl of culture supernatant was removed to perform ELISA. The ELISA positive colonies were again subjected to a second round of serial dilution and selection by ELISA. The clones’ positive for antibodies by ELISA were selected and grown in multiple wells. After the wells have become confluent, the cells were collected and transferred to a 50 ml tissue culture flask in 7 ml of media to increase the volume of antibody producing hybridoma clones.

3.9. SDS PAGE of proteins secreted by regenerating protoplasts

The ELISA positive hybridoma clones consistently producing monoclonal antibodies with very high titre were grown in 24 well culture plates and their supernatant was used for Western blot studies. SDS PAGE was performed to
resolve the proteins secreted by the regenerating mycelial protoplast and the
resolved protein bands were transferred on to a nitrocellulose paper (NCP) as
follows.

**SDS PAGE**

The proteins secreted by the mycelial protoplasts of *C. albicans* were mixed
with one part of SDS sample loading buffer (125 mM tris, 2% SDS, 5% β-ME,
10% glycerol, and traces of bromophenol blue) and kept in boiling water for 5
min and centrifuged at 12,000 rpm for 5 min to remove debris. The
supernatant was collected and the protein value estimated as described
above. SDS PAGE was performed using Laemmili's discontinuous two gel
system was used to resolve the proteins. The lower gel or the resolving gel is
of higher percentage having acrylamide concentration of 12% (Acrylamide
12%, SDS 10%, Tris-Cl 1.5M, pH 8.8) and the upper gel (4%) of only 1-2 cm
in height (Acrylamide 4%, SDS 10%, Tris-Cl 0.5M, pH 6.8). The stock
solutions used for SDS PAGE were as follows:

- 30% acrylamide (29.3% acrylamide and 0.7% bis-acrylamide)
- 1.5 M Tris pH 8.8
- 0.5 M Tris pH 6.8
- 10% SDS
- 10% ammonium per sulfate (APS)

Resolving gel constituents were mixed in appropriate amount to prepare 12%
resolving gel. APS and TEMED were added just before pouring of the solution
in between the pre assembled glass plates, after degassing the mixture.
Water-saturated isobutanol was overlaid on the top of the resolving gel as
mentioned before. After polymerization of resolving gel the mixture of stacking
gel was poured on top of it and a comb placed to form the wells. A pre run of
the gel was carried out (10 mA, 30 min), the samples were loaded (40 μg/well
of protein value) and electrophoresis was carried out at a constant current of
10 mA till the dye front reached the bottom of the gel and subsequently, the
gel was placed in transfer buffer for equilibration for 15 min.
3.10. Electro-transfer of proteins and Western blotting

Electro-transfer of proteins to nitrocellulose paper (NCP) was performed as described by Towbin et al. (191) The above gel was placed in contact with NCP (sandwiched between blotting paper) in electro-transfer unit (GENEI, Bangalore) filled with blotting buffer and transblotted for 2-3h at 100 mA. After the desired time, the NCP was removed, rinsed once in TDW and allowed to air dry. To confirm the protein transfer, the NCP was stained with ponceau S dye (Sigma) for 5 min and washed to a desired background with TDW. The NCP was then carefully cut into strips of individual lanes and numbered before further processing. These individual strips were blocked using 2% fat free milk solution in tris buffered saline (TBS, pH 7.4) for 1h at 37°C. The strips were washed once with TBS containing 0.05% tween 20 to remove excess blocking reagent and the cell culture supernatant from single cell origin ELISA positive clones was added and incubated for 1h at 37°C. The blots were then washed thrice with TBS-T20, 5 min. each, probed with 1:10,000 dilution of secondary antibody (peroxidase-conjugated anti-mouse IgG, IgA, and IgM polyclonal antibody raised in rabbit), washed again, developed with DAB, and documented.

3.11. Production of monoclonal antibodies under serum-free conditions

The hybridoma clones which gave positive result in Western blotting were maintained in RPMI 1640 media supplemented with FBS. For production of monoclonal antibodies for few sensitive experiments, the hybridoma cells were first acclimatized to grow in serum free medium (serum-free low protein hybridoma medium’ Sigma, USA, supplemented with all the antibiotics described above) by gradually diluting the culture medium with increasing concentration of serum free medium. Essentially, this was done by slightly tapping the flask and all the supernatant was removed that contained many dead cells, taking care not to dislodge any dividing and growing cells. The supernatant was centrifuged and to every 3 ml of this was added 4 ml of serum-free medium, mixed and kept at 37°C in a CO₂ incubator. After 2 days when media appeared orange to yellow, the cells were dislodged and cultured.
in fresh serum free medium. The cells in this flask were allowed to grow for 3-4 days and then subcultured in 10 flasks in serum free medium (without any antibiotics) and incubated in a CO₂ incubator for 4 days. After 4 days the supernatant was collected and the flasks were replenished with fresh serum free medium. This supernatant containing MAbs was centrifuged to get rid of cell debris and preserved at -20°C.

The supernatant for each MAb was precipitated with 50% ammonium sulphate (313 g/L) in flasks at 4°C avoiding any accumulation of crystals. These flasks were left at 4°C for 16h and the precipitate was collected by centrifugation (15,000 g for 30 min) and reconstituted in minimal amount of PBS. The reconstituted antibody solution was dialysed using Mini dialysis kit (Amersham, USA) at 4°C, against PBS with 5 changes within duration of 14-16h to completely remove the salt. After dialysis the solution containing monoclonal antibodies was centrifuged at 12000 rpm and supernatant was collected in a sterile labelled microfuge tube. The protein content (antibody concentration) in the solution was estimated by 2D Quant kit and stored at -20°C until used further.

3.12. Cryopreservation of positive clones

Positive hybridoma clones were cryopreserved for long term storage as follows. The clones were passaged a day before cryopreservation, harvested and the cell count & viability was assessed using hemacytometer and trypan blue. The cells were then pelleted by centrifugation at 1000rpm for 5 min. at room temperature and resuspended in chilled Cryopreservation mixture containing 10% DMSO, 40% RPMI 1640 and 50% hybridoma tested FBS to a concentration of 1x10⁶ cells/ml. The cell suspension in aliquots of 1 ml was distributed in labelled cryovials and immediately kept in a cryopreservation box that contained Iso-propanol (at 4°C). The box was kept at -80°C overnight and transferred to liquid nitrogen container.
3.13. Iso-typing of the monoclonal antibodies (MAb 1A1 and MAb 1C1)

Two methods viz., capture ELISA method and readymade isostrip method, were followed to identify the isotype of the monoclonal antibodies (MAb 1A1 and MAb 1C1) produced by the hybridoma clones in the present study.

Capture ELISA method was performed using mouse monoclonal antibody Iso-typing kit from Sigma (ISO-2). Iso-type specific antibodies were diluted 1:1000 in PBS and 100μl of each diluted antibody was pipetted into each well of the ELISA plate. Replicates were used to minimize the error. The plate was incubated at 37°C for 1h. The coating solution was then removed and the plate was washed thrice (6 min each) with washing buffer (PBS with 0.05% T-20). 100 μl of the test MAbs in PBS were put in each well and incubated at 37°C for 1h. Plate was again washed thrice (6 min each) using washing buffer. Peroxidase conjugated goat anti-mouse IgG (Fab specific) antibody was diluted 1:600 in washing buffer. 100 μl of this diluted antibody was pipetted into each well and incubated at 37°C for 30 min. After 30 min the solution in the wells was discarded and washed thrice with washing buffer as above. Subsequently, 100μl of substrate solution (5-aminosalicylic acid at 1 mg/ml concentration in 0.02M phosphate buffer, pH 6.8 and 0.1ml of hydrogen peroxidase solution per 10 ml of substrate buffer) was added to each well and allowed to develop the colour in positive wells. The reaction was stopped by adding 50 μl of 3 N NaOH to each well.

The results were also confirmed by using Isostrip™ mouse monoclonal antibody isotyping kit from Santa Cruz Biotechnology, Inc. Briefly, 150 μl of the culture supernatant containing the monoclonal antibody was added to the development tube containing the latex beads bearing anti-mouse kappa and anti-mouse lambda antibodies, which reacted regardless of the isotype of the mouse monoclonal antibody and vortexed gently to suspend the beads completely in the supernatant. The suspension was allowed to stand for 30 seconds and an isostrip was placed into the development tube such that the black end which contained the wick was at the bottom. The isotyping strip provided in the kit contained immobilized bands of goat anti-mouse antibodies.
corresponding to each of the common mouse antibody heavy chain isotypes (IgG1, IgG2a, IgG2b, IgG3, IgM and IgA) and light chain isotypes (κ and λ). Both sides of the strip also contained a positive control band, which indicated that the antibody-coated beads have travelled up the strip. The liquid moved along with the beads due to capillary action. Within 5-10 minutes, blue bands appeared, indicating the isotype of the heavy chain as well as the light chain of the MAb. The reaction was stopped by removing the strip from the development tube once the positive bands appeared. The black portion of the strip was cut to avoid further development of the bands which might otherwise lead to false positive results and the strips were documented by scanning over a conventional scanner.


Cross reactivity of the monoclonal antibody was assessed against the cell wall proteins of both mycelial and yeast forms of C. albicans and also against the proteins secreted by the regenerating yeast protoplasts. Cell wall proteins from both the morphologies were extracted as mentioned before, run on SDS-PAGE, transblotted on NCP and Western blotting was performed using the test MAb (MAb 1A1).

3.15. Cross reactivity of MAb 1A1 under native condition of secretory proteins

Native gel electrophoresis of the proteins secreted by the regenerating protoplasts was carried out under native conditions for performing Western blot. A two gel system consisting of resolving gel (12%) and stacking gel (4%) as mentioned before was used to resolve the proteins. All the constituents of the resolving gel as well as stacking gel were same except that SDS was replaced with same amount of TOW. Also, the sample loading dye did not contain any SDS and reducing agent (β-ME). After polymerization of the gel, the samples were mixed with sample loading dye in 1:1 ratio and loaded directly (40µg/well) in the wells without boiling. Electrophoresis was carried out at constant current (10mA) using borate buffer (0.1M, pH6.5) for 3h. After the electrophoresis was complete the gel was equilibrated in the transfer buffer.
and transblot was performed as mentioned earlier. Western blotting of the transblotted proteins under native conditions was carried out using MAb 1A1 as mentioned before and the results were documented.

3.16. Cross reactivity of MAb 1A1 with other fungi and different strains of C. albicans

Cell wall from different isolates of C. albicans and other species of fungi was isolated and stored at -80°C. The proteins from the cell wall of the fungi were extracted by boiling in extraction buffer as mentioned above and quantified using 2D Quant kit. SDS PAGE and electro-transfer was performed as described earlier. After Ponceau S staining and documentation of band profile, the NCP was immuno-probed against MAb 1A1 as described before.

3.17. Cross reactivity of MAb 1A1 with bacteria

Cross reactivity of MAb 1A1 was also tested against different bacteria (Escherichia coli, Pseudomonas aeruginosa, Staphylococcus aureus, Klebsiella pneumoniae, Bacillus cereus). The bacterial cultures were grown on nutrient agar (Hi media) slants at 35°C for 24h and harvested by adding 5 ml normal saline containing 0.05% tween 20 (NS-T20). The bacterial cells were pelleted down by centrifuging at 6000 rpm for 5 min at RT, washed once with NS-T20, resuspended in 500 µl of SDS sample loading buffer and kept in boiling water for 10 min. The contents were then centrifuged at 10000 rpm for 10 min at 4°C, the supernatant transferred to fresh labelled tubes and protein quantity was estimated using 2D Quant kit. Equal amount of protein was loaded in each well keeping C. albicans cell wall protein as positive control. SDS PAGE and electro-transfer was performed as described before and Western blotting was done using MAb 1A1.

3.18. Assessment of in vivo diagnostic potential of the monoclonal antibody

3.18.1. Biotinylation of the purified monoclonal antibody

Monoclonal antibody 1A1 purified by ammonium sulphate precipitation of serum free supernatants were biotinylated using biotinylation kit from GENEI
as per the instructions of the manufacturer. Briefly, 0.1 ml of conjugation buffer provided in the kit was added to 1 ml of monoclonal antibody solution (1mg/ml, dialyzed) in PBS. The contents were then transferred to a vial containing 0.5 mg BAH-Sulfo-NHS (Biotinamidohexanonate-N-hydroxy-sulfosuccinimide ester and incubated for 2h at 4°C with mild stirring. The biotinylated monoclonal antibodies were purified by affinity purification using streptavidin column provided in the kit. After the reaction was complete the reaction mixture was applied to the streptavidin column allowing the biotinylated monoclonal antibodies to bind to the column. The flow through was collected in a fresh tube and stored at -20°C. The column was washed with 10 bed volumes of PBS and eluted with 8M guanidine-HCl, pH 2.7. Monoclonal antibody (biotinylated) presence was checked using Bradford assay. The biotinylated monoclonal antibodies (1A1) were used in ELISA for checking the presence of antigens recognized by it in the serum of the infected mice.

3.18.2. Mouse model of C. albicans infection

*In vivo* diagnostic potential of the monoclonal antibody 1A1 was assessed in a mouse model of *C. albicans*. Six BALB/c mice were challenged intravenously with 200 μl (2 x 10⁵ cells) of yeast suspension of *C. albicans*. Uninfected BALB/c mice served as negative control. After every three days blood was drawn through the tail vein to check the presence of antigens recognized by the monoclonal antibody which might have been secreted during the course of infection from both infected as well as control mice. Serum (50 μl) of the blood from infected as well as uninfected mice was mixed with 150 μl of PBS and stored at -20°C until used further.

**Diagnosis using ELISA**

ELISA plates were coated with 100 μl of the stored serum (in PBS) of infected as well as control mice and kept overnight at 4°C. Wells coated with 100 μl (100 μg) of mycelial secreted protein antigen served as positive control. The coated plates were washed and blocked as mentioned earlier with 150 μl BSA (1%). The biotinylated monoclonal antibody 1A1 (1:1000) was used to probe the presence of *Candida* antigen in the serum of the infected mice. The plates
were then incubated with 100 µl of biotinylated monoclonal antibody for 1h at 35°C. Later the plates were washed thrice with PBS-T20 (5 min each) and incubated with 100 µl of horse radish peroxidise (HRP) enzyme conjugated streptavidin polymer (sigma) diluted 1:200 times at 35°C for 45 min. The plates were again washed thrice with PBS-T20 (5 min each) and developed with OPD as mentioned earlier.

3.19. Detection of antigens recognized by MAb 1A1 in Candida positive patient sera using ELISA

MAb 1A1 was checked for its ability to detect antigens of C. albicans in serum of the persons who have been confirmed for candidiasis in our laboratory by culture method as well as Candida specific antibody detection method. Eighteen such patient sera and nine sera from healthy donors were screened for presence of the antigens by ELISA method essentially as described before with minor changes. Briefly, patient sera (18 samples) were diluted in coating buffer (1:100) and 100 µl of diluted serum was added to each well of microtitre plates in duplicates and incubated overnight at 4°C. Mycelial cell wall proteins (100 µg) served as positive control. The plate was washed with PBS-T20 once for 5 min and the coated wells were blocked by adding 150 µl of 1% (w/v) bovine-serum albumin in PBS for 90 minutes at 37°C. Blocking solution was removed and the wells were washed once with PBS-T20 (5 min). Purified MAb 1A1 in PBS (100 µl, 1 mg/ml) was added to each well and incubated for 90 minutes at 37°C. Plate was washed thrice with PBS-T20 (5 min. each) and incubated with 100 µl of peroxidase-conjugated anti-mouse IgG, diluted 1:5,000 in PBS-T20 for 90 min at 37°C. Tetramethyl benzidine (TMB) substrate solution (RnD systems, USA) was prepared by mixing solution A and solution B in equal volumes just before use and 100 µl was added to each well and incubated in dark at RT for 30 min. The reaction was stopped with 10 µl of 7% H₂SO₄ and optical densities were read on a micro titre plate reader (Molecular Devices, USA) at 450 nm.
3.20. Inhibition of attachment of *C. albicans* with composite material

The cell adhesion assay was performed in 96 well ELISA plates. Exponentially growing culture of *C. albicans* was harvested by centrifugation and after washing the cells thrice with chilled DPBS (Dulbecco's phosphate buffered saline), the cells were suspended in the same buffer at a density of $2 \times 10^3$ cells/ml. To 100 µl of this culture suspension 100 µl of antibody (MAb 1A1) solution (100 µg/ml final concentration) was mixed and added to the ELISA plate. For control, DPBS was used in place of monoclonal antibodies. The plate was then incubated at 28°C for 1h without shaking after that the contents of the plate were discarded, and washed twice with DPBS. The number of cells sticking to the plate was counted under an inverted phase-contrast microscope (Olympus, Japan). The experiment was performed in triplicate and the data represented mean of all three experiments.

3.21. Inhibition of germination of *Candida* cells by MAb 1A1

Exponentially growing cells of *C. albicans* were washed in DPBS and the cell density was adjusted to $2 \times 10^3$ cell/ml in DPBS. 100 µl of this cell suspension was poured in 96 well tissue culture plates and 100 µl of test monoclonal antibody (MAb 1A1) solution (1 mg/ml) was added to it and incubated at 37°C for 4h. The plate was observed under phase contrast microscope. The experiment was performed in triplicate and the percentage of germination was determined by the following formula:

$$\frac{\text{(Number of cell with germ tube)}}{\text{Total number of cells in the given field)} \times 100$$

3.22. Effect of MAb 1A1 on colony forming units (CFU)

The effect of monoclonal antibody (MAb 1A1) on CFU was evaluated by incubating $10^3$ cells of *C. albicans* with 100 µl of test antibody solution (0.5 mg/ml) in DPBS at 28°C for 16h and plating them onto SDA plates. The reduction in CFU was calculated by comparing it with control that contained an equal amount of DPBS without any antibody.
3.23. MTT assay

Candidacidal activity of MAb 1A1 was studied by MTT reduction assay. Briefly, 100 µl of cell suspension (~1x10^4 cells/ml) was incubated with 100 µl of antibody (MAb 1A1, 50 µg) and incubated for 16h at 35°C. After incubation, cells were centrifuged at 1x1000 g for 5 min and to the pellet 100 µl of MTT (0.5 mg/ml in RPMI) was added and incubated for 1h. This was centrifuged (3000 rpm for 5 min) and 100 µl of DMSO was added to the pellet and the absorbance of the dark formazan product was read at 550 nm. All controls and concentrations were kept same as in the above experiment. The optical density obtained for amphotericin B (AMB) was considered as 100% inhibition and that of DPBS as 0%. The formula used to calculate percent inhibition was

\[100 - \left(\frac{X - \text{OD}_{\text{AMB}}}{\text{OD}_{\text{DPBS}} - \text{OD}_{\text{AMB}}}\right) \times 100\],

where \(X\) is the OD of test antibody.

3.24. Phagocytic assay

Phagocytic assay was performed using mouse macrophage cell line J774. The cell line was maintained in DMEM with 10% FBS. The cell line was subcultured two days before performing the phagocytic assay. Sub culturing was done by gently dislodging the cells using a cell scrapper (Greiner bio-one, Austria). Small amount of cells were mixed with trypan blue solution in a ratio of 1:1 and cell counting was done on a Hemacytometer. 4x10^6 cells were seeded in a 250 ml tissue culture flask in DMEM with 10% FBS and incubated at 37°C in atmosphere of 5% CO₂. Actively growing yeast cells and germ tubes were used for phagocytic assay.

3.24.1. Treatment of yeast and germ tubes with MAb 1A1

Actively growing yeast and germ tubes were used for performing phagocytic assay. On day two, yeast cells were inoculated in YNB and incubated at 28°C for 12h. The cells were harvested by centrifugation at 3000 rpm for 5 min, washed twice with sterilized PBS by centrifugation and suspended in PBS containing purified 1A1 MAb (1mg/ml) at 1 x 10^6 cells/ml and incubated overnight (18h at 37°C) with mild shaking (50 rpm). Germ tube induction was carried out in mycelial induction media under serum free conditions as mentioned earlier. After 1h, germ tubes were harvested by centrifugation at
1200 rpm for 5 min, washed twice with PBS by centrifugation and resuspended in PBS containing purified 1A1 MAb (1mg/ml) at $1 \times 10^6$ cells/ml and incubated overnight as above with mild shaking (50 rpm). The yeast cells and germ tubes suspended in PBS without MAb 1A1 served as control.

3.24.2. Phagocytosis and Geimsa staining

On day three, macrophage cells were dislodged by scraping using a cell scraper and seeded into chambered slides (Nalgene) at $2 \times 10^6$ cells per ml (2ml per slide) and allowed to adhere and come to resting stage for 6h. Control and MAb 1A1 treated yeast and germ tubes were harvested and centrifuged at 3000 rpm and 1200 rpm respectively and washed twice with incomplete DMEM (without FBS) and resuspended at a concentration of $4 \times 10^6$ cells/ml in the same. The cells were pipetted out gently to separate cell aggregates. Media was removed from the chambered slides and the cells were washed with incomplete DMEM to remove traces of FBS. The yeast and germ tube cell suspensions were added to macrophages in a ratio of 1:4 and kept at 37°C in an atmosphere of 5% CO$_2$ for 2h. After 2h incomplete DMEM was replaced by DMEM with 10% FBS containing antimycotic and antibiotic solution and phagocytosis was allowed for one more hour. After 1h, the media was aspirated from the chambered slides and the cells were washed thrice with incomplete DMEM to remove non-phagocytosed yeasts/germ tubes adhering to the surface of the slide and the macrophages and it was washed subsequently twice with DPBS. The cells were then fixed with 100% methanol (1ml/slide) for 30 seconds at RT. Geimsa stain (diluted 1:10 with PBS) was added (1ml/slide) to each slide and allowed to stand for 30 min at 37°C. The excess stain was removed and slides were washed thrice with PBS (1min each). The upper covers of the chamber slides were removed to expose the inner side of the slide containing the cells to enable microscopic observation and documentation. The slides were observed under a phase contrast microscope (Olympus, Japan) and photographed using a high resolution camera (Nikon, Japan)
3.25. Epitope localization on the surface of *C. albicans*

Localization of the epitope recognized by MAb 1A1 on intact *C. albicans* cells (yeast cells, germ tubes and mycelia) was performed by indirect immunofluorescence. The exponentially growing yeast cells and germ tubes of *C. albicans* were collected by centrifugation, washed twice with PBS and suspended in the same at a cell density of 1x10^5 cells/ml. The cell suspension was placed on a cover slip (10 μl/cover slip) coated with poly-L-lysine (Sigma) and fixed with 4% paraformaldehyde. The cover slips were blocked by 1% BSA in PBS in a moist chamber at 37°C for 1 h and washed twice (2 min each) with PBS with mild shaking. To each blocked cover slip 15 μl of MAb 1A1 (adjusted to 50 μg/ml in PBS) was overlaid and incubated in a moist chamber for 1 h. Then the cover slips were washed with four changes of PBS as mentioned above and 15 μl of FITC-conjugated anti-mouse antibody (FITC – conjugated antimouse IgG, IgM, or IgA) at a dilution of 1:100 in PBS was overlaid on it and incubated for another hour. The cover slips were washed finally with four changes of PBS and mounted in mounting medium containing antifade by inverting them over a clean glass slide. The margins of the cover slips were sealed with nail enamel and the slides were observed under a phase contrast fluorescence microscope and documented.

3.26. Monoclonal antibody production by ascites growth

*In vivo* production of MAb 1A1 in bulk was carried out in BALB/c mice by developing ascites as follows. Five Male BALB/c mice were given Freund’s incomplete adjuvant (0.5 ml each) intraperitonealy to induce ascites formation. Hybridoma implanting was done after 7 days. The day before implanting hybridoma, cells were split 1:1 in fresh medium (RPMI 1640 with 10% FBS). The following day the cells were tapped gently and pooled and 200μl of this cell suspension was mixed with 200μl of Trypan blue solution and total numbers of viable cells were estimated using hemacytometer. The pooled hybridoma cells were centrifuged at 1000rpm for 5 min. at room temperature and the pellet was resuspended in sterilized PBS to a final concentration of 2x10^6 cells/ml. The primed mice were inoculated intraperitoneally with 0.5 ml
of the hybridoma cell suspension. The mice were observed daily for the enlargement of the peritoneum indicating the production of MAb. The formed ascites were tapped after approximately 10 days post inoculation using an 18-gauge needle followed by two more tapings on alternate days. The ascites was pooled and centrifuged at 1200 rpm for 5 min. at RT to remove the cells. The supernatant was further clarified to remove cell debris by filtration through 0.22 μm filter and stored at -20°C until further use.

3.27. Purification of monoclonal antibody

Monoclonal antibody 1A1 was purified from either culture supernatant containing FBS or from ascites. Both the sources contained high amounts of other contaminating proteins therefore their purification was carried out using pre-packed HiTrap Protein G HP column (GE healthcare) as per the manufacturer's instructions as follows. All the buffers were prepared in TDW and filtered through 0.22 μm filter before used further. A peristaltic pump set up was used to regulate the flow of the liquid through the column. Before proceeding, pump tubing was washed once with binding buffer (20 mM sodium phosphate, pH 7.0) and was filled with the same buffer. The protein G column was connected to the peristaltic pump at the inlet (top end) avoiding air bubbles. The snap-off end of the column outlet was removed and 10 bed volumes of binding buffer was passed through the column by the peristaltic pump at 1 ml per min to remove the storage buffer from the column and to wash it. Sample containing MAb 1A1 (culture supernatant or ascites) was diluted six times with binding buffer and was loaded onto the column at a rate of 1 ml per min. The flow through was collected in a fresh tube and the column was washed with 10 bed volumes of binding buffer to remove non-specifically bound protein molecules. This was assessed by mixing the washings with Bradford reagent in 1:1 ratio which gave no colour with the reagent when the column was washed thoroughly. Elution of the bound monoclonal antibody 1A1 was done by passing 10 column volumes of elution buffer (0.1 M glycine-HCl, pH 2.7). Fractions (1 ml) of eluent were collected in microfuge tubes containing 200 μl of neutralizing buffer (1 M PBS, pH 7.6) and the purity of the monoclonal antibody in the eluents were checked by SDS PAGE. Fractions
containing the monoclonal antibody were pooled and stored at -20°C after estimating the antibody concentration.

3.28. Monoclonal antibody 1A1 column preparation

The purified monoclonal antibody (MAb 1A1) was used for the preparation of the affinity column for the purification of the antigens recognized by it.

3.28.1. CNBr activation of sepharose 4B column material

Sepharose 4B (1.5 g) was weighed and allowed to swell in triple distilled water for 4h. The swollen beads were washed twice with 1 mM HCl and then two times with triple distilled water in a sintered glass funnel. The beads were washed again with 1 M sodium carbonate (pH 11.0), resuspended in 10 ml of 1 M sodium carbonate and transferred into a beaker. Cyanogen Bromide solution (1 g dissolved in 1 ml acetonitrile) was added constantly drop wise to the column material while maintaining the pH between 10.5 and 11.0 with addition of 2 N NaOH with mild stirring. The entire operation was carried out in a chemical fume hood. Under any circumstances the pH was not allowed to drop below 10.5. Stirring was continued for 10 min more and later the beads were washed with 100 mM Ferrous sulfate to inactivate the CNBr followed by washing with several times with triple distilled water and then by two washes with PBS (pH 7.4). The activated beads are then used for the coupling of the purified monoclonal antibody.

3.28.2. Coupling of monoclonal antibody

About 25 mg purified monoclonal antibody (1A1) solution was added directly to 5 ml of CNBr activated beads and incubated overnight at room temperature (RT) on a rocker. The beads are then centrifuged at 300 rpm and supernatant removed into a fresh tube. Binding of the MAb 1A1 to beads was confirmed by Bradford test (by comparing the antibody solution before and after coupling). The beads were washed with 0.5 M sodium phosphate (pH 7.5) followed by washing with 0.5 M sodium phosphate containing 1 M NaCl. Unblocked active groups on the beads were blocked by incubating these beads with 100 mM ethanolamine (pH7.5) at RT for 4h with gentle mixing. The beads were
washed finally with PBS and stored in PBS containing 0.01% merthiolate at 4°C till used further.

3.29. Antigen purification by monoclonal antibody affinity column

Monoclonal antibody 1A1 coupled beads were packed in glass column (Sigma) and washed with 10 bed volumes of antigen binding buffer (20 mM sodium phosphate, pH 7.0) using a peristaltic pump. Secreted proteins from regenerating mycelial protoplasts were diluted six times with binding buffer and applied to the column. The flow through collected in a fresh tube was again applied to the column for rebinding of the unbound antigen molecules. This was followed by washing with 10 bed volumes of binding buffer. Elution of the antibody bound antigen was done by passing 10 bed volumes of elution buffer (0.1 M glycine-HCl, pH 2.7). Fractions (1ml) of eluent were collected in microfuge tubes containing 200 μl of neutralizing buffer (1 M Tris-HCl, pH 9.0) and the purity of the eluted antigen was checked by SDS PAGE and 2D gel electrophoresis as mentioned above. Fractions containing the purified antigen were pooled and stored at -20°C after estimating the protein concentration. To check if the purified protein was the same protein that was recognized by the MAb 1A1, Western blotting of the purified protein was performed as mentioned before.

3.30. 2-D gel electrophoresis of the purified protein for Western blotting and N-terminal sequencing

The purified protein was subjected to 2D gel electrophoresis to confirm that it is the same protein recognized by the monoclonal antibody by Western blotting and to blot the protein on to PVDF membrane for N-terminal sequencing. About 20 μg protein was applied onto an IPG strip for performing Western blotting and 50 μg onto another strip for performing N-terminal sequencing. The strips were rehydrated overnight and Isoelectric focusing, second dimensional separation of the proteins was done as mentioned earlier. After the second dimension electrophoresis was complete, one gel was blotted onto nitrocellulose membrane and Western blotting was performed as
mentioned earlier, using monoclonal antibody 1A1. The other gel was blotted onto a PVDF membrane for N-terminal sequencing as follows.

3.31. Sample preparation for N-terminal protein sequencing

PVDF membrane was immersed in 100% methanol for 1 min and washed with two changes of triple distilled water (15-20 sec each) and dipped in the transfer buffer at least for 15 min for equilibration of the membrane. The 2D gel (as above) was also allowed to equilibrate with the transfer buffer for same period of time. The transfer buffer used for preparing blots for N-terminal protein sequencing contained 10 mM CAPS (3-[cyclohexylamino]-1-propane sulfonic acid, 10% methanol and 0.037% SDS adjusted to a pH of 11.0. The equilibrated gel and the PVDF membrane were sandwiched between Whatman filter paper and assembled in the blotting cassette. Transfer blot was performed at 100 mA for 4h at RT and subsequently the membrane was rinsed with three changes of triple distilled water and stained with amido blue black (0.1% amido blue black in 1% acetic acid) for few minutes. The stained PVDF was destained with several changes of 50% methanol and rinsed with 20 changes of triple distilled water (10 sec each) and air dried. The protein spots were cut carefully avoiding any contact or cross contamination, placed in a clean microfuge tube, labelled and stored at 4°C. N-terminal sequencing of the blotted protein was done commercially at Institute of Microbial Technology, Chandigarh.

3.32. Protein-A-monoclonal antibody covalent coupled column preparation

In another experiment monoclonal antibody 1A1 was coupled covalently to Protein A column under high salt conditions to purify the high molecular wt protein that could not be purified by CNBr coupled MAb 1A1 column used above. Ascites (4 ml) of mice containing the monoclonal antibody 1A1 was diluted with 50 mM sodium borate buffer (pH 9.0) to a final volume of 10 ml and NaCl was added such that the concentration of the salt in the final solution was 3 M. Protein A beads bind approximately 2-5 mg of antibody (Isotype IgG1) under high salt concentration hence binding was performed under
excess antibody concentration. Sepharose (5 ml) coupled protein A beads (GENEI) were added to this solution and incubated at RT for 1h with gentle rocking. Antibody coupled beads were then washed with 10 column volumes of binding buffer (50 mM sodium borate with 3 M NaCl, pH 9.0) by centrifugation (300 rpm) and aspiration. The beads were resuspended in 10 volumes of 200 mM sodium borate (pH 9.0) containing 3 M NaCl. 10 µl beads were taken out in a microfuge tube and kept separately for assessment of covalent coupling. Dimethylpimelimidate (covalent coupling reagent) was added to this suspension of antibody coupled protein A to a final concentration of 20 mM and incubated at RT for 30 min with gentle rocking. Again 10 µl beads were taken out in a microfuge tube and kept separately for assessment of covalent coupling. The covalent coupling reaction was stopped by washing the beads with 0.2 M ethanolamine (pH 8.0) twice and incubating them in the same solution for 2h at RT on a rocker with mild rocking. Finally the beads were washed thrice with PBS (pH 7.4) and stored in PBS containing 0.01% merthiolate. Coupling was checked by boiling the beads collected before and after covalent coupling, with SDS sample loading buffer (1µl with 9 µl of buffer) and SDS PAGE.

3.33. Antigen purification using Protein A coupled monoclonal antibody under high salt concentration.

Purification of the second antigen recognized by the monoclonal antibody 1A1 was done under high salt concentration. Mycelial cell wall protein was used for this purpose. Mycelial cell wall was prepared and its proteins were extracted as mentioned earlier using extraction buffer containing 2% SDS. SDS was precipitated out of the cell wall protein sample by using SDSOut™ precipitation kit (Pierce) according to the manufacturer's instructions. Briefly, one volume of precipitation solution supplied in the kit was mixed with twenty volumes of cell wall proteins and incubated on ice for 20 min. The precipitated SDS was removed by centrifugation at 10000g for 10 min. The supernatant was collected in a fresh tube and the pellet was discarded. The supernatant was further clarified in a spin column provided in the kit by centrifugation at
10000g for 1 min. The process was repeated until minimum frothing of the protein solution was observed.

Protein A coupled MAb 1A1 beads were packed in a glass column (Sigma) and washed with 10 bed volumes of antigen binding buffer (20mM sodium phosphate, pH 7.0) using a peristaltic pump. The SDS free cell wall protein was diluted six times with binding buffer and applied onto the column (1ml/min). The flow through was collected in a fresh tube and was again applied onto the column for re-binding of the unbound antigen molecules. The final flow through was collected in a fresh tube. This was followed by washing with 10 bed volumes of binding buffer. Elution of the antibody bound antigen was carried out by passing 10 bed volumes of elution buffer (0.1 M glycine-HCl, pH 2.7). One ml of eluent fraction was collected in microfuge tubes containing 200 µl of neutralizing buffer (1 M Tris-HCl, pH 9.0). Purity of the eluted antigen was checked by SDS PAGE. Fractions containing the purified antigen were pooled and stored at -20°C after estimating the protein concentration. The purified protein was separated by SDS PAGE and transblotted to perform Western blotting for the confirmation of the purified protein.

3.34. Cytokine response of mouse splenocytes against the purified antigens

Cytokine response against the two purified antigens (Ag1- high molecular weight antigen; Ag2- low molecular weight antigen) by the mouse splenocytes was checked using BD™ Cytometric Bead Array (CBA) for mouse Th1/Th2 cytokine kit (BD biosciences, USA).

3.34.1. Isolation of mouse splenocytes

Two BALB/c mice were sacrificed after anaesthetic treatment with ether. A longitudinal incision was made along the midline (inguinal region) on the ventral side in the abdominal region towards the pectoral girdle. Spleen was excised into a sterile petri plate and spleen cells were isolated as mentioned before by macerating the spleen in chilled incomplete DMEM containing heparin (10 U/ml). After complete isolation of the splenocytes from the spleen,
the cells were passed through 70μm nylon cell sieve (BD biosciences, USA) to remove clumps of cells and tissue parts. The splenocytes thus obtained were centrifuged at 1200 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in ice cold erythrocyte lysis buffer (150 mM NH₄Cl, 10 mM EDTA, pH 7.4) and incubated on ice for 10 min with occasional mixing. The cell suspension was then centrifuged at 1200 rpm for 5 min at 4°C, the supernatant was discarded and the pellet was resuspended in DMEM with 10% FBS. This cell suspension was devoid of erythrocytes. The cell number in the suspension was estimated using Hemacytometer and 1x10⁶ cells were distributed in each well of 48 well tissue culture plate with 400μl medium. The cells were incubated overnight (12h) at 37°C in an atmosphere of 5% CO₂.

3.34.2. Antigen stimulation and flow cytometric analysis

The splenocytes were stimulated with the purified antigen individually at different concentrations (5 μg, 10 μg and 15 μg) and kept at 37°C in an atmosphere of 5% CO₂ for 48h. Macrophages stimulated with the Toll like receptor ligand (PAM₃CSK₄) served as a positive control. For negative control no stimulation was given. Supernatant from each well was collected into sterilized individual microfuge tubes and centrifuged at 1200 rpm for 5 min at 4°C to remove cell debris. The resulting supernatant was collected into fresh sterilized microfuge tubes and stored at -20°C until further use.

Flow cytometric analysis: CBA kit for mouse Th1/Th2 cytokine estimation was used for the analysis of the cytokines released into the medium after stimulation by the antigens. The kit contains capture beads for 5 cytokines (IL-2, IL-4, IL-5, IFN-γ and TNF-α) in individual vials and have to be mixed before use. The capture beads for each cytokine were vortexed thoroughly to suspend the beads and 10 μl/test of each cytokine beads were mixed in a fresh sterilized flow cytometry tube. Cytokine standards provided in the kit were dissolved in the assay buffer provided in the kit by gently pipetting with a micropipette. Different concentrations of the standards were prepared by following two-fold serial dilution in 15 ml centrifuge tubes. Required number of flow cytometry tubes for standards as well as the test samples was pre-
labelled and 50 μl of the mixed cytokine capture bead suspension was added to each tube. To these tubes, 50 μl of the serially diluted standards or 50 μl of the test sample was added and mixed briefly. Mouse Th1/Th2 PE detection reagent was added to each tube (50 μl/tube) protecting from light. The contents in the tubes were mixed gently and incubated in the dark for 2h at RT. After the incubation, 1 ml of wash buffer provided in the kit was added to each tube and centrifuged at 200g for 5 min and supernatant was carefully aspirated from each tube avoiding loss of beads. To these beads 300 μl of wash buffer was added and the samples were analyzed on a flow cytometer (BD FACS CALIBER, USA) after setting the machine for compensation with the cytometric setup beads provided in the kit.