Chapter Three

MP65 amyloid fibril based vaccine imparts protection against experimental murine disseminated candidiasis
3.1. Introduction

*Candida albicans* (*C. albicans*), an opportunistic fungal pathogen can inflict infections ranging from mild superficial lesions to life threatening systemic candidiasis in the immunocompromised or otherwise modified host (Pietrella et al. 2002. Systemic infection caused by *C. albicans* ranks among the four most frequent nosocomial infections and is most often a consequence of general immunosuppression, prolonged treatment with antibiotics, or breachment of anatomical barriers (e.g., surgery or central venous catheter) (Bär et al. 2012, Pfaller and Diekema 2010). It is associated with a mortality of more than 30% and a high morbidity in those who survive. The superficial *Candida* infections of skin, the oropharyngeal mucosa, and the vagina are however less severe and not themselves life-threatening yet they constrict the patient’s quality of life enormously (Puel et al. 2010, de Repentigny et al. 2004, Fidel et al. 2005). The increasing incidence of resistance to antifungal drugs and an inherent drug tolerance of some fungi cause major obstacles to an efficient treatment. This situation evokes an urgent need for the development of novel therapeutic approaches against fungal infections and has renewed the interest in the development of vaccination strategies against mycoses.

Generally, a crucial role in containing the pathogen establishment in the host is played by pathogen specific Abs. However, the protective immunity against intracellular pathogens is a bit complicated as such organisms adapt intracellular parasitism to avoid Ab onslaught. To suppress intracellular pathogens, host immune system relies on activation of IFN-γ producing cytolytic T lymphocytes (Chauhan et al. 2011a). The role of immunological responses against dimorphic fungi, such as *C. albicans*, has remained a contentious issue (Ahmad et al. 2012). Several research groups contend that *C. albicans* specific Abs may be protective against experimental disseminated candidiasis (Ding et al. 1988). On the contrary, various lines of evidences militate against protective function of pathogen specific Abs to play any role in constraining the infection (Kirby et al. 1984). In fact, the controversies can be attributed to the ability of the fungus to reversibly switch between unicellular yeast to filamentous forms in the infected host (Han and Cutler 1995). It is imperative that successful elimination of infection like *C. albicans* could be made possible by activation of
unmoral as well as cell mediated responses directed against both forms of *C. albicans* (Wagner et al. 1996).

A critical role in pathogenic potential as well as in elicitation of the host immune response against *C. albicans* has been ascribed to fungal cell wall components. In this context, proteins and glycoproteins, with the latter being mostly mannoproteins, appear to have a prominent function (Pietrella et al. 2008). Mannoproteins (MPs), natural glycol-conjugate usually containing between 80 and 90% mannose, are expressed mainly on the fungal surface and released into the external medium during growth. MPs are abundant in the cell wall of *C. albicans* and constitute about one-third of the dry mass (Pietrella et al. 2002). One of them, an immune-dominant 65-kDa-MP Ag (MP65), has been extensively characterized both biochemically and immunologically (Gomez et al. 2000, Gomez et al. 1996). MP65 of *C. albicans* has been shown elsewhere to possess strong immunogenic properties, including DTH licitation and induction of T-cell responses (Pietrella et al. 2002, Pietrella et al. 2008). It has been recognized to contain Th1 inducing dominant T-cell epitopes and is used to expand specific T cells, in particular CD4+ T-cell clones producing IFN-γ. Partial protection against systemic challenge by *C. albicans* in mice has also been attributed to an anti-MP65 response.

Amyloid fibrils, the cross β-sheet rich ordered nanostructures arising from misfolded proteins or peptides, are the entities involved in the progression of various neurodegenerative diseases including Alzheimer's and Parkinson's diseases (Maji et al. 2008). However, amyloids have also been found to perform normal biological function as demonstrated by fungal prions, which are involved in prion replication, and the amyloid protein Pmel17, which is involved in mammalian skin pigmentation. Peptide and protein hormones in secretory granules of the endocrine system have also been found to be stored in an amyloid-like cross β-sheet-rich conformation (Maji et al. 2009c). Thus, in contrast to the original association of amyloids with diseases, functional amyloids in the pituitary and other organs can contribute to normal cell and sue physiology. Moreover, some recent findings enumerate that artificially or in vitro synthesized amyloids can also perform beneficial biological activities *in vivo* (Maji et al. 2008, Gupta et al. 2010). It has been demonstrated that amyloids of
insulin or gonadotrophin releasing hormone can release peptides in a slow and sustained manner. It is speculated that amyloids release bioactive protein/peptide \textit{in vivo} since activating the downstream signalling cascade requires a perfect complementarity between the hormones and their receptors which is possible only when the amyloids release biologically active conformational form of peptides \textit{in vivo}. In an earlier study we found that the biophysical characteristics of the released proteins from the amyloids were similar to native protein. Also, amyloid immunization was found to induce development of Abs capable of recognizing fibril as well as native protein (unpublished data). These findings seem to favour the hypothesis that amyloids may release biologically active peptides/proteins retaining the native Ag epitopes and thus may act as depot for the sustained release of Ag. Our previous findings also indicate that amyloids can be avidly captured by macrophages. These observations persuaded us to evaluate the prophylactic potential of amyloids.

Considering the need of a protective strategy against \textit{Candida}, induction of T-cell mediated immune response by MP65 and the potential of amyloids to generate native Ag specific immune response, in the present study, we evaluated the prophylactic potential of amyloid based preparation of MP65 against experimental murine disseminated candidiasis.
Chapter 3: Materials and methods

### 3.2. Materials and methods

#### 3.2.1. Chemicals and reagents

All the reagents used were of the highest purity available. Thioflavin T, Congo Red and fetal calf serum were purchased from Sigma-Aldrich® (St Louis, MO). IgG2a (R35-95) isotype control was procured from eBiosciences (San Diego, CA). IgG1, IgG2a isotypes (550487) and cytokines viz. IL-4, IFN-γ, IL-12 cytokine estimation kits were procured from BD OptEIA (Franklin Lakes, NJ). RPMI 1640, antimycotic solution, and plasticwares were purchased from BD Biosciences (San Diego, CA). $[^{3}H]$-thymidine was procured from Amersham Pharmacia Biotech. The following Abs were procured from e-Biosciences: fluorochrome-labeled antimouse Abs; fluorescein isothiocyanate–conjugated CD4 (GK 1.5), CD8 (53.67), PE-conjugated CD44 (IM7), CD80 (B7-1), CD86 (GL1), PerCP conjugated CD62L (MEL-14) IgG2a (R35-95) isotype control. Nitrocellulose membrane filter of 0.8-mm-pore-size was purchased from Millipore Corp., Bedford, Mass. Dialysis bag of 10-kDa molecular mass cutoff was procured from Diaflow Ultrafilter YM10; Amicon Corp., Danvers, Mass.

#### 3.2.2. Purification of MP65

The MP65 was purified from components spontaneously released from *C. albicans* mycelial cultures, as described elsewhere (Gomez et al. 2000) and slightly modified in our laboratory. Briefly, *C. albicans* in the log phase (0.5 optical density at 560 nm) were incubated in RPMI media at 37°C for 24 h to obtain cells in hyphal form. After checking the cell morphology by microscopy, the supernatants were collected by rapid filtration through a 0.8-mm-pore-size nitrocellulose membrane filter. The soluble mannoproteins were concentrated and dialyzed in an ultrafiltration device equipped with a low-adsorbance membrane with a 10-kDa molecular mass cutoff. Thereafter, the mannoproteins from hyphal cells were further fractionated by gel filtration chromatography on Sephacryl S300 HR, as reported earlier (Gomez et al. 1996), to obtain a fraction enriched in the MP65 component. The purified Ag was substantially free from other mannoproteins or proteins, as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), silver staining. Total polysaccharide and protein compositions of MP65 were determined by the
phenol-sulfuric acid method (Dubois et al. 1956) and the BCA protein assay, respectively.

3.2.3. Fibril formation

MP65 was dissolved in an eppendorf in 1ml of PBS (pH 7.0) at a concentration of 1mg/ml and incubated at room temperature under continuous agitation at 180 rpm as described elsewhere (Gupta et al. 2010) with slight modification in our laboratory. Aliquots collected at various time points were pelleted down at 15,000 rpm for 15 mins. The pellet obtained was monitored for fibril formation by ThT fluorescence, CR binding and TEM analysis. Three independent experiments were performed for each sample.

3.2.4. Rayleigh Scattering measurements

Rayleigh scattering measurements were performed on a Hitachi F-4500 fluorescence spectrophotometer at room temperature using a cell with a 1 cm light path. The excitation and emission wavelengths were both set at 350 nm, and the slit length was 5 nm.

3.2.5. Turbidity measurements

For turbidity measurements, the incubated samples collected at various time points were monitored by UV absorbance at 350 nm using a Perkin Elmer UV/VIS spectrometer model lambda 25 in a 1 cm path length cuvette at room temperature.

3.2.6. ThT binding studies

MP65 aggregates (100 µg) obtained at various time points were incubated with 30µM ThT solution (30µl of 1mM ThT stock solution) at room temperature and fluorescence was measured on Hitachi F-4500 fluorescence spectrophotometer after 30 minutes. The bound ThT amyloid was excited at 450nm and spectra were recorded from 460nm to 560 nm. The excitation and emission slit widths were fixed at 5nm and 10nm respectively.

Interaction of various amyloid species obtained at various time points with ThT was also assessed by fluorescence microscopy. The aggregates obtained were incubated
with 20μM of ThT for 30 minutes at room temperature and then transferred onto a glass slide to be analysed by fluorescence microscope (Axio, HBU 50/AC; Zeiss, Gottingen, Germany).

3.2.7. CR binding studies

CR solution (20μM) prepared in PB (pH 7.4) (using a stock solution (1mM) of CR prepared in ethanol) was incubated with 100μg of aggregates for 30 minutes at room temperature. UV absorbance was measured in the spectral range 300-700nm using Perkin Elmer UV/VIS spectrometer model lambda 25. CR solution (20μM) in PB served as a control (CR-only spectrum), and absorbance of 100μg of native MP65 mixed with 20μM of CR solution was also measured.

3.2.8. CD measurements

JASCO spectropolarimeter (J-815) was used for circular dichroic measurements using quartz cell with 0.1cm pathlength. The temperature was controlled at room temperature using Peltier Thermostat with Multitech water circulator and the instrument was calibrated using D-10-camphorsulfonic acid. Scan speed of 100nm/min and response time of 2s were used for spectra collection. Scans were performed for each sample in the range of 200-250nm with final protein concentrations being 200μg/ml.

3.2.9. Transmission electron microscopy

For electron microscopy, 6 μl of protein sample (100μg) was spread on carbon coated copper grid which was further negatively stained with 2% (w/v) uranyl acetate. The grid was examined under JEOL transmission electron microscope operating at an accelerating voltage of 200 kV.

3.2.10. Preparation of liposomes

The MP65 bearing liposomes were prepared using egg PC essentially by following the published procedure as standardized in our lab (Unanue and Allen 1987, Chauhan et al. 2011a). Briefly, egg PC/cholesterol (2:1 molar ratio, total lipid 20 mg) or E. coli lipids (total lipid 20 mg) were reduced to thin dry film under N2
atmosphere. The film was hydrated, followed by sonication in a bath-type sonicator for 1 h at 4 °C under N₂ atmosphere. The liposomes thus formed were mixed at this stage with an equal volume of MP65 (30 mg/ml). The mixture was flash frozen and thawed (3 cycles), and then lyophilized. The free flowing, dried powder thus obtained was rehydrated with distilled water (120 µl) and finally re-constituted with PBS. The preparation was centrifuged at 14,000g and the pellet was further washed at least 3 times with PBS to remove the traces of the un-entrapped solute. The protein entrapped in the liposomes was estimated as described elsewhere (Romani et al. 1997). Briefly, the liposomes (given volume) were lysed with 10% Triton X-100 solution (the final concentration of Triton X-100 was maintained 1%). Protein concentration was determined using the BCA reagent and a calibration curve was prepared in the presence of triton X-100.

3.2.11. In vitro release kinetics

MP65 (1mg/ml of PBS, pH 7.0) samples incubated for fibrillation were withdrawn at various time points. The amyloids formed were isolated by pelleting at 15,000g for 15 min. The pellets obtained were washed thrice with PB (pH 7.4) and re-suspended in PB (pH 7.4). The kinetics of MP65 release into PB (pH 7.4) was monitored spectrophotometrically at 280nm (Gupta et al. 2010) by analysing the supernatant after centrifugation (Binger et al. 2008a), for approx. 15 days using Perkin Elmer UV/VIS spectrometer model lambda 25.

3.2.12. Animals

Inbred female BALB/c mice (6–8 weeks old, 20 ± 2 g) were obtained from the Institute's Animal House Facility. The animals were housed in commercially available polypropylene cages and maintained under controlled temperature conditions on a 12hr light-dark cycle and had free access to food and water ad libitum.

3.2.13. Ethics statement

All animal experiments were approved by the Institutional Animal Ethics Committee of the Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh, India. All animal experiments were performed according to the National Regulatory
3.2.14. Mode and schedule of immunization

Animals were immunized subcutaneously in the lower abdominal region aseptically with a dose of 25 μg of MP65 amyloid bodies (obtained from fibrillation reaction at various time points). A booster was given 3 weeks after the first immunization with 12.5 μg of the corresponding form of protein fibril.

3.2.15. Collection of sera

The blood was collected from mice by retro-orbital puncture after various time intervals in centrifuge tubes, then centrifuged at 1500 g for 10 minutes at 4°C. Finally, the supernatant was collected to be used for further experiments.

3.2.16. Determination of MP65-specific total IgGs by ELISA

Ag-specific total IgGs against the native MP65 as well as MP65 aggregates were determined in the sera of mice immunized with MP65 aggregates as well as fibril following the protocol as described elsewhere (Ahmad et al. 2012). Briefly, 96-well microtiter plates were incubated overnight with 100μL (2 μg) of native MP65 as well as MP65 amyloids in carbonate–bicarbonate buffer (0.05 M, pH 9.6) at 4°C. After the usual washing and blocking steps, the plates were finally incubated with serially diluted sera at 37°C for 2 hours. After excessive washing of the plates, 100μL of (1:5000 dilution of stock) HRP conjugated goat anti-mouse Abs were added to each well and the plates were incubated at 37°C for 1 hour. The plates were washed again before adding 100μL of substrate solution (6 mg OPD) in 12 mL of substrate buffer with 5μL of 30% H₂O₂ and were finally incubated at 37°C for 40 minutes. The reaction was terminated by the addition of 50μL of 7% H₂SO₄. The absorbance was read at 490 nm with a microtiter plate reader (Bio-Rad Laboratories Inc, Hercules, CA).
3.2.17. Determination of Ab isotype in sera of immunized mice

Sera collected from mice immunized with MP65 aggregates as well as fibril were analyzed for Ab isotypes using the protocol described elsewhere (Ansari et al. 2012). Briefly, ninety-six well microtitre plates were incubated overnight with 2μg (100μl) Ag/well in carbonate bi-carbonate buffer (0.05 M, pH 9.6) at 4°C. After washing and blocking steps, the plates were incubated with serially diluted sera at 37°C for 2 h. After excessive washing of the plates, 100μl of (1:5000 dilution of stock) goat anti-mouse anti-IgG1, and IgG2a Abs were added in each well and incubated for 1 h at 37°C. After washing steps, 100μl of (1:5000 dilution of stock) HRP conjugated rabbit anti-goat Abs were added to each well and each plate was incubated at 37°C for 1 h. The plates were washed again followed by adding 100μl of substrate solution (6 mg OPD in 12 ml of substrate buffer with 5 μl of 30% H₂O₂) and were finally incubated at 37°C for 40 min. The reaction was stopped by the addition of 50μl of 1 M H₂SO₄. The absorbance was read at 490 nm with a microtitre ELISA plate reader (Bio-Rad Laboratories Inc, Hercules, CA).

3.2.18. Measurement of DTH response

The animals were immunized with MP65 amyloid bodies in their inguinal region and DTH response was assessed at various time points. The mice were footpad tested to determine their DTH reactions to native MP65 as well as various amyloidal forms. DTH reactions were elicited by the injection of MP65 fibril (50μg) as well as native MP65 in the right footpad of each mouse and PBS into the left footpad. The thickness of each footpad was measured just before and after 36 hrs post-injection of MP65 aggregates and PBS using a digital gauge caliper (Aerospace and Engineering Tools Ltd, Bolton, UK). DTH reactions were evaluated by the increase in footpad thickness as determined by the following formula (right footpad at testing time – right footpad at 0 hours): (left footpad at testing time – left footpad at 0 hours).

3.2.19. Determination of NO production

Mice were sacrificed one week post booster and peritoneal macrophages were isolated. The peritoneal macrophages were grown in culture plates and pulsed with MP65 (final conc. 10μg/well). After 24 hrs, 100μl of culture supernatant was
collected from each well, mixed with an equal volume of Griess reagent [1% sulfanilamide and 0.1% N-(1-naphthal) ethylenediamine dihydrochloride in 2.5% H$_3$PO$_4$], and further incubated for 10 min at 25 °C and absorbance determined at 550 nm in an ELISA reader (Bio-Rad Laboratories Inc, Hercules, CA).

3.2.20. T lymphocyte isolation from spleens of immunized mice

Mice immunized with native as well as fibrillar MP65 were sacrificed on day 5 post last booster. T lymphocytes were isolated from the spleens of sacrificed mice as described elsewhere (Ansari et al. 2011). Briefly, spleens isolated from animals belonging to various immunized groups were macerated and suspension was treated with ACK lysis buffer (0.15 mol/L ammonium chloride, 10 mmol/L potassium bicarbonate, and 88 mmol/L edetic acid) for lysis of the red blood cells. The cell suspension was centrifuged at 1500 g for 5 min and cell pellet was washed with HBSS solution 3 times and re-suspended in RPMI 1640 medium containing 10% fetal calf serum and 0.1% antimycotic cocktail.

3.2.21. Lymphocyte proliferation assay

Lymphocyte proliferation assay was performed as described elsewhere (Ansari et al. 2011). Briefly, lymphocytes isolated from the spleens of mice belonging to various immunized groups were incubated in round bottomed 96-well plates (2x10$^5$ cells/well) in 200mL of RPMI 1640 medium with 10% fetal calf serum. Splenic cells isolated from groups of mice immunized with native or appropriate MP65 amyloids were incubated with 20μg of corresponding matching forms of MP65. After 72 hrs, the cultures were pulsed with 0.5 mCi of $[^{3}H]$- thymidine. After 16 h, the plates were harvested onto glass-fiber filter mats by the use of Tomtec-Harvester-96 (Tomtec). The incorporated radioactivity was measured with liquid scintillation spectroscopy (Wallac-1450 Microbeta Trilux; Perkin Elmer).

3.2.22. Determination of IFN-γ, IL-4 and IL-12 by sandwich ELISA

Th1 as well as Th2 cytokines induced by lymphocytes upon their culture in the co-presence of MP65 amyloid bodies were estimated using appropriate and specific biotinylated Ab pairs according to the manufacturer's protocols. Briefly, polystyrene
microtiter plates were coated with 50 µl of the purified capture Abs in carbonate bicarbonate buffer of pH 9.5 at 4°C. After usual washing and blocking steps, 50 µl of the supernatant (isolated from cultured splenocytes after 48 h) was poured in each well for determining the level of cytokine induced. After stipulated incubation time, the plates were washed with PBST and incubated with biotinylated polyclonal goat anti-mouse cytokine detection Ab. Further, after washing the plates with PBST, 100 µl of streptavidin-HRP conjugate was added to each well and plates were incubated for 30 min at room temperature. Again plates were washed three times with PBST and finally colored complex was developed with tetra methyl benzidine. The absorbance was read at 450 nm with a microtiter plate reader (Bio-Rad). A known specific recombinant cytokine was used as standard for calculating level of given cytokine in the samples tested and concentration was expressed as pg/mL.

3.2.23. Staining of T cells for memory and macrophages for co-stimulatory markers

Splenocytes belonging to various immunized groups were harvested as described earlier for the proliferation assay. T cells (CD4⁺ and CD8⁺) were prepared for staining with labelled Abs as described elsewhere. Viable cells were counted using the Trypan blue dye exclusion method. The FACS express plus analysis demonstrated that isolated cells were CD4⁺ with 97% purity while the CD8⁺ population was found to be 98% pure. Further, 1 × 10⁶ CD4⁺ and CD8⁺ cells were washed twice in FACS buffer (PBS with 1% BSA and 0.1% sodium azide) in different tubes. We isolated the macrophages by adherence on petri-plates which were then washed three times with HBSS and incubated at 37°C. Moreover, we determined F4-80 expression on cells before assessing the expression level of costimulatory molecules in order to ascertain macrophage lineage of this population. Various isolated immune cells were incubated with Fc block (2.4G2) and FITC/PE tagged monoclonal Abs (CD44, CD62L, CD80, CD86, and isotype control) for 30 minutes at 4°C. After the appropriate washing steps, cells were fixed with 1.0% paraformaldehyde. The cytometry data were acquired using fluorescence activated cell sorter (GUAVA, Billerica, MA) and were analyzed with Express-Plus software (DME MAC Express Plus, National Government Services Common Electronic Data Interchange (CEDI), NHIC Corp, Hingham, MA). The total number of cells of a definite phenotype
(CD4^high\cdot CD62L^high/low, CD8^+\cdot CD4^high\cdot CD62L^high/low) was calculated by taking the percentage of a gated cell type, as determined by flow cytometry, and multiplying it by the total number of cells obtained per sample, which was further divided by 100.

3.2.24. Dot blot assay

Native form or amyloidal forms of MP65 (10µg) were spotted onto PVDF strips (with dimension of approximately 10 by 4 mm), which were further allowed to dry at room temperature. The strips were rinsed briefly in PBST and were incubated overnight at 4°C in 5% non-fat dry milk in PBST to block the residual binding sites on the paper. The strips were rinsed three times in PBST. The strips coated with MP65 amyloids were incubated with Abs obtained from the animals immunized with corresponding aggregates. The native MP65 coated strips were then incubated with various anti-amyloidal MP65 Abs produced in mice. After stipulated incubation, the strips were washed thrice in PBST and further incubated for 1 hr at 37°C with horseradish peroxidase-conjugated goat anti-mouse Ab (1: 5000). The strips were washed with PBST three times and finally the immunoreactive dots were developed on X-ray film by ECL using ECL kit, BioRad. A clearly defined greyish to black spot at the site where the Ag was spotted was considered a positive result.

3.2.25. Western blot assay

For Western blot analysis, native form of MP65 (2µg/well) was resolved by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel and transferred onto PVDF membrane. After blocking in 5% non-fat dry milk prepared in PBST, the membrane was washed three times with PBST and incubated for 2 hours at 37°C with Abs raised in mice against amyloidal MP65 (fibrillar as well as non-fibrillar) aggregates. After incubation and stipulated washing steps, the membrane was incubated with horseradish peroxidase-conjugated goat-anti-mouse Ab (1:5000) for 1 hour at 37°C. Finally, post washing, the bands onto the membrane were developed on X-ray film by ECL using ECL kit, BioRad.

3.2.26. Macrophage interaction with amyloids

Macrophages (1×10^6) were isolated from the intraperitoneal cavity of thioglycolate-primed BALB/c mice following the protocol as described elsewhere (Chauhan et al.
The macrophages were incubated with 100μg of FITC-tagged amyloidal aggregates for 1-2 hours at 37°C in a humidified atmosphere of 5% CO₂. The cells were fixed on a slide for observation using a fluorescent microscope (Axio, HBU 50/AC; Zeiss, Gottingen, Germany).

3.2.27. Challenge of animals with *C. albicans* Infection

*C. albicans* (ATCC 18804, preserved in 10% glycerol at −20 °C) was cultured in 5% dextrose. The cells were harvested after 24 h and pelleted at 2000g for 20 min at 4°C. The cells were counted by hemocytometer and were diluted with normal saline in such a way that an aliquot of 200 μl contained $6.5 \times 10^5$ cells. On the day 25th (fourth day after the final booster) each animal was challenged with $6.5 \times 10^5$ cells (in 200 μl) of *C. albicans* intravenously (I.V. route). The establishment of infection was assessed on the basis of survival rate and CFU count in various vital organs of mice challenged with *C. albicans* infection.

3.2.28. Determination of fungal load in various vital organs

The establishment of infection in immunized mice was assessed by determining fungal load in different vital organs. The animals belonging to various immunized groups were sacrificed on day 6th post infection. Vital organs viz. liver, kidney, lung, spleen, were taken out aseptically. The organs were minced separately in normal saline (5 ml) and an aliquot (200 μl) of this suspension was plated on YPD agar plates after appropriate dilution. The plates were incubated for 48–72 h at 37 °C. The development of colonies in a given vital organ suspension belonging to a specific group was noted and the fungal load was calculated by multiplying with the respective dilution factors. The animals survived on day 60 post-infection were also screened for fungal load in various vital organs using the same method.

3.2.29. Statistical analysis. Data were analyzed and two groups were compared using Student’s t-test and one-way ANOVA (Holm–Sidak method) was used to compare all groups to each other. P values < 0.01 were considered significant. SigmaPlot (v 10 and 11; SigmaPlot software, San Jose, CA) software was used for data presentation.
3.3. Results

3.3.1. Characterization of purified MP65 for size and biochemical as well as immunological properties

Chemical determinations of three different batches of purified MP65 preparation showed an average polysaccharide (mannose)/protein ratio of 1:2. Figure 3.1A shows the electrophoretic pattern of MP65 purified from the mixture of proteins released in the culture supernatant of *C. albicans* as seen by silver staining. SDS-PAGE revealed a prominent protein band of 65kDa which is in concordance with earlier reports. The immunogenicity of the MP65 purified protein in terms of Ag-specific Ab production was assessed by Western blot analysis as elucidated in Figure 3.1B.

![SDS-PAGE and Western blot profile of purified MP65 from secretory fraction of C. albicans](image)

Figure 3.1. SDS-PAGE and Western blot profile of purified MP65 from secretory fraction of *C. albicans*. (A) SDS-PAGE profile of purified MP65 as revealed by silver stain. Lane i corresponds to molecular weight markers and lane ii shows the protein profile. (B) Western blot profile of the same protein probed with mouse antisera.

3.3.2. Monitoring aggregation of soluble MP65 by light scattering and turbidity measurements

To detect aggregation of soluble MP65 upon shaking we performed light scattering studies. A five-fold increase in light scattering as compared to native protein has been
reported to indicate aggregation (Santiago et al. 2010). MP-65 agitated till seven 
hours exhibited less than five-fold increase in fluorescence intensity (data not shown) 

hence, aggregation of MP-65 is ruled out till seven hours of incubation. The extent 
of light scattering in aggregates obtained after seven hours of incubation reveals an 
increase in fluorescence intensity, however, the light scattering was found to be 
maximised for 24hr-aggregate (Figure 3.2A). Aggregates obtained beyond 24 hours 
of incubation exhibited a negligible increase in fluorescence intensity as compared to 
that observed for 24hr-aggregate. Turbidity measurements also revealed a similar 
result. With increase in incubation (agitation time period), the turbidity of the MP65 
solution increased and reached saturation after 24 hours of shaking (Figure 3.2B).

3.3.3. Analyzing fibril formation by ThT fluorescence measurements

Fibril formation by MP65 was monitored by ThT fluorescence as ThT exhibits an 
increase in fluorescence upon binding to amyloidal aggregates (Hudson et al. 2009). 

As shown in Figure 3.3A, in concordance with the results obtained for light scattering 
and turbidity measurements an increase in ThT fluorescence with time was observed, 
which reached a maximum value for 24hr-aggregate indicating formation of fibril 
after 24 hrs of incubation. Fluorescence microscopy also revealed explicit binding of 
fibril (obtained after 24 hrs of shaking incubation) with ThT as compared to 12hr-
aggregate (Figure 3.3B).

3.3.4. Transmission Electron Microscopy

The MP65 aggregates obtained after various incubation periods were assessed for 
their morphology using TEM. As shown in Figure 3.4, aggregates obtained after 8 
and 12 hrs of incubation exhibited un-ordered oligomeric structures in contrast to 24hr 
and 30hr aggregates that exhibited ordered fully formed fibrillar morphology.
Figure 3.2. Continuous agitation induces aggregation of soluble MP65. (A) Rayleigh scattering measurements at 350 nm for aggregates formed at various time points. (B) Turbidity measurements for the same aggregates. Error bars exhibit standard errors. Three independent experiments were performed for each sample data are representative of at least two independent experiments with similar observations.
Figure 3.3. Fibril formation monitored by ThT fluorescence studies. (A) ThT emission spectra were obtained by excitation at 450nm and emission in the range of 460-560nm for M65 aggregates obtained at various time points. (B) ThT binding to various aggregates revealed by fluorescence microscopy. ThT (30μM) was incubated
with 100μg of aggregates for 30 minutes; 5 μl of the suspension was placed onto the glass slide and observed under fluorescence microscope. Three independent experiments were performed for each sample and data are representative of at least two independent experiments with similar observations.

Figure 3.4. Fibril maturation as revealed by TEM. Various aggregates (100μg) were coated onto the grid. (A) and (B) 8hr and 12hr aggregates respectively revealing unordered oligomeric structures. (C) and (D) represent 24hr and 30hr aggregates respectively exhibiting typical fibrillar morphology. Images shown are those selected after similar observation of at least two independent experiments performed for each sample.
Figure 3.5. MP65 fibril releases the precursor proteins in a sustained manner over extended time period. In vitro release kinetics of MP65 from various MP65 preparations as monitored spectrophotometrically at 280 nm over a period of 15 days. Data are representative of three independent experiments with similar observations.

3.3.5. MP65 fibrils release MP65 in a slow and sustained manner

To assess whether the MP65 fibril can act as a depot for the sustained release of MP65, release kinetics of MP65 from its various amyloid aggregates was examined. The release of MP65 from amyloid reservoir was also compared with its release from liposome. Liposome as well as 12hr-MP65 aggregate showed an initial burst release that maximised within 3-5 days. The MP65 aggregates obtained till 16 hrs of incubation were found to be unstable showing major release during the initial few days (data not shown). Aggregates obtained after 18hrs and 20 hrs of shaking incubation exhibited a slow release (data shown only for 18hr aggregate, Figure 3.5). MP65 fibril formed after 24 hrs of shaking exhibited a slow and sustained release of MP65 over a period of 15 days. The 18hr aggregate although showed a slow release but it was not as good as that exhibited by 24hr fibril. The fibril generated beyond 24hrs of incubation showed very slow release as can be observed for 30hr fibril (Figure 3.5). On the basis of release, amongst the non-fibrillar and fibrillar amyloids,
12hr-aggregate and 24hr-fibril were selected respectively for further characterization by CR binding, CD spectroscopy and studying immune response. Considering the morphologies of the aggregates, the '12hr aggregate' and '24hr fibril' are termed only as 'aggregate' and 'fibril' respectively in the succeeding sections of the manuscript.

3.3.6. Congo-Red Binding
CR upon binding to the β-sheet core in amyloids exhibits a red shift and therefore is a useful probe to detect amyloid formation (Klunk et al. 1999). CR upon binding to the MP65 fibril exhibited a marked red shift indicating presence of β-sheet in the fibrillar nanostructure. However, the non-fibrillar aggregate failed to exhibit a red shift although a little increase in absorbance was observed as compared to native MP65 (Figure 3.6A).

3.3.7. Far UV-CD spectra
To gain insight into the secondary structure of the formed MP65 fibril, we performed far-UV CD spectral studies. As shown in Figure 3.6B, under conditions that favor amyloid fibril formation (24 hr incubation with shaking), MP65 yields a characteristic far-UV CD spectrum that shows the generation of cross-β-sheet structure as assessed from the ellipticity observed in the region of 215-220 nm in contrast to native MP65 that revealed the typical α-helix conformation bearing minima at 208nm and 220nm. The MP65 aggregate exhibited β-turn like CD spectrum.
Figure 3.6. MP65 fibril harbors β-sheet rich core. (A) Steady state absorption spectra of CR bound to various M65 preparations. The aggregates were scanned in the 400-700nm range. (B) Secondary structure measurements native MP65, Aggr MP65 and MP65 fibril by Far UV-CD. The protein solutions (200μg/ml) were scanned in
the range of 200-250nm. Three independent experiments were performed for each sample and data are representative of at least two independent experiments with similar observations.

3.3.8. MP65 released from its fibril exhibits biophysical characteristics of native MP65

MP65 proteins released from amyloid aggregates were analysed for light scattering and ThT binding. The released MP65 showed light scattering and ThT binding similar to native form of MP65 (Figure 3.7).

3.3.9. Abs generated in response to MP65 fibril immunization recognise native Ag

ELISA, dot blot and western blot analyses were performed to assess the immune response evoked in model animals upon immunization with MP65 aggregate and fibril. For ELISA, 96-well plates were coated with native form of MP65 and allowed to react with Abs generated upon immunization with the amyloidal aggregate and fibril of MP65. As observed in Figure 3.8, the Abs generated upon immunization with MP65 aggregate as well as fibril were found to react explicitly with the native form of Ag indicating generation of anti-native MP65 Abs. ELISA results were corroborated by dot blot and Western blot assay which also showed reactivity of Abs (generated upon fibril immunization) with native form of MP65.

3.3.10. Uptake of amyloidal MP65 aggregates by macrophages

The FITC-labelled non-fibrillar as well as fibrillar aggregates of MP65 were analysed for uptake by macrophages. As shown in Figure 3.9, the MP65 aggregate as well as fibril were found to be successfully taken up by the macrophages as revealed from the fluorescence localized throughout the intracellular compartment of the macrophages.
Figure 3.7. **Biophysical characteristics of released MP65.** (A) Rayleigh Scattering studies (B) and ThT binding assay of the MP65 released from fibrillar MP65. At least two independent experiments were carried out for each sample and data obtained with similar results are presented.
Figure 3.8. MP65 fibril immunization induces native Ag specific Abs. (A) ELISA. Polystyrene plate (96-well) was coated with native MP65 Ag as specified in Materials and Methods section and reacted with sera obtained from the animals immunized with MP65 fibril as well as aggregate. (B) MP65 transferred onto the PVDF membrane was reacted with Abs produced in response to immunization with MP65 amyloid aggregates by Western blot assay and (C) similarly dot blot assay was performed by coating native MP65 onto PVDF membrane and incubating it with Abs generated against MP65 aggregates (both fibrillar as well as non-fibrillar). Three independent experiments were carried out for each sample and data are representative of two independent experiments with similar observations.
Figure 3.9. Uptake of MP65 amyloids by macrophages. Uptake of MP65 aggregate A) and MP65 fibril B) by macrophages isolated from thioglycolate primed Balb/c mice. Three independent experiments were performed for each sample and data are representative of atleast two independent experiments with similar observations.

3.3.11. Humoral response upon immunization with MP65 amyloid fibril

Our preliminary studies employing both ELISA as well as transblot assays (Western and dot blot assays) argue in favour of specificity of fibril induced Ab against native Ag MP65. To determine magnitude of the humoral response observed upon immunization with various MP65 formulations, we analysed the Ab titres. Mice were immunized subcutaneously with the same amount of Ag present in MP65 aggregate as well as fibril or encapsulated in the liposomes and evaluated for subsequent immune response. As shown in Figure 3.10, significantly higher levels of total IgGs were observed in the animals immunized with fibril MP65 as compared to MP65 aggregate, liposome entrapped MP65 and native MP65 at post booster and post-challenge time points (p<0.001). At post immunization time point, the liposome-entrapped MP65
was found to exhibit better response than all other formulations. The total IgGs produced in response to MP65 aggregate were found to be slightly higher than liposome-entrapped MP65 at all the time points (p<0.05). The anti-native MP65 Abs were found to persist in the animals immunized with fibril MP65 even after four weeks post challenge.

![Graph showing antibody titre](image)

**Figure 3.10. MP65 fibril induces a strong humoral response.** For the determination of total anti-MP65 Ab concentration, mice immunized with various MP65 preparations were bled at different time points. Standard ELISA was used to analyze the sera obtained by coating the microtitre plates with native MP65 as described under Materials and Methods section. The data are mean ± standard error of three independent experiments.

3.3.12. MP65 fibril induces Th1 cytokines in the host

The Th1/Th2 polarization of fibril M65 formulation was assessed by evaluating Th1 (IFN-γ and IL-12) and Th2 (IL-4) in splenocyte culture supernatant belonging to various immunized groups. **Figure 3.11** depicts that fibril MP65 induces significantly higher Th1 cytokines at various time points when compared to native MP65, aggregate MP65 or liposome-encapsulated MP65 (p<0.001). On the contrary,
insignificant levels of Th2 cytokine, IL4 were observed for MP65 fibril as compared to native form of the Ag (Figure 3.12A). No significant expression of either Th1 or Th2 cytokines was detected in the control group (Saline) at post booster as well as post challenge time points when compared to immunized groups.

Ab isotype class switching was also determined by evaluating MP65 fibril specific IgG1 and IgG2a response in the sera of immunized animals. Animals immunized with MP65 fibril exhibited significantly higher levels of IgG2a/IgG1 ratios than native form of Ag at both post booster as well as post challenge time points (p<0.001) (Figure 3.12B). However, at post immunization time point IgG2a/IgG1 ratio for MP65 fibril was found to be nearly 1 which was lower than the ratio obtained for native MP65. MP65 aggregate was found to induce better IgG2a/IgG1 levels as compared to liposome-entrapped MP65 at post booster as well as post challenge time points (Figure 3.12B).

3.3.13. MP65 fibril stimulates T cell proliferation
Dose dependant T cell proliferation was observed in all the MP65 formulations although fibril MP65 induced a significantly higher dose dependent proliferation than all other formulations (p<0.001) (Figure 3.13). The lymphocytes isolated from the animals belonging to control group (administered saline) did not induce considerable proliferation even at higher dose of the Ag. At a dose of 24µg, lymphocytes were found to exhibit highest proliferation in their respective groups. Therefore, this dose was selected for studying lymphocyte proliferation at various time points. The lymphocytes isolated from the animals immunized with MP65 fibril exhibited higher T cell proliferation potential at all the time points, when activated with matching MP65 fibril formulation. There was statistically significant difference in T cell proliferation in MP65 fibril when compared with native MP65, its aggregate or liposome-entrapped MP65 at post booster as well as post challenge time points (p<0.001). The data shown in Figure 3.13 demonstrates that T cell proliferation was more prominent in the animals immunized with MP65 fibril even after 4 weeks post challenge with infection.
Figure 3.11. MP65 fibril induces Th1 polarized response in immunized animals. Th1/Th2 bias was ascertained by determining cytokine response in splenocyte culture supernatant belonging to various immunized groups at different time points prior and post challenge with *C. albicans*; (A) IFN-γ, (B) IL-12. To activate splenocytes belonging to group of animals immunized with native MP65, native form of 24μg of MP65 was used while splenocytes isolated from animals immunized with MP65 fibril
and aggregate were co-cultured with 24μg of MP65 fibril and MP65 aggregate respectively. Lymphocytes isolated from group immunized with liposome-entrapped MP65 were activated with liposome-entrapped MP65 (24 μg Ag). The data represent mean of three determinants ± S.D. and are representative of two different experiments with similar observation.

Figure 3.12. MP65 fibril induces lower production of Th2 cytokine and higher production of IgG2a in immunized animals. (A) Similar to the determination of IFN-γ and IL-12, IL-4 was estimated in the culture supernatants of splenocytes
belonging to various immunized groups. (B) To further confirm the Th1/Th2 polarization upon immunization with MP65 fibril, the sera of immunized animals were analysed for the presence of IgG2a and IgG1 isotypes by sandwich ELISA method as detailed in Materials and Methods section. Data represent mean three independent experiments ± S.D.

3.3.15. MP65 fibril immunization induces increased expression of co-stimulatory and memory markers as revealed by FACS analysis

The lymphocytes isolated from various immunized groups of animals were stained with conjugated Ab markers specific for cell surface molecules and subsequently analyzed by flow cytometry. Expression of co-stimulatory markers CD80 (B7-1) and CD86 (B7-2), was higher in the group of animals immunized with MP65 fibril when compared to native MP65, on 4th week post fungal challenge. Animals immunized with MP65 aggregate and liposome-entrapped MP65 exhibited more or less similar expression of co-stimulatory molecules on APCs (Figure 3.14). CD4+ as well as CD8+ T cells obtained from animals immunized with MP65 fibril exhibited both effector memory (CD44\textsuperscript{high}CD62L\textsuperscript{low}) and central memory phenotypes (CD44\textsuperscript{high}CD62L\textsuperscript{high}). MP65 aggregate and liposome entrapped MP65 immunized animals showed moderate levels of memory phenotypes which were significantly lower than MP65 fibril administered groups (p<0.01). Mice vaccinated with native MP65 exhibited only feeble expression of memory markers on CD4+ as well as CD8+ T cells (Figure 3.15).
Figure 3.13. T cell proliferation response in various immunized groups upon stimulation with MP65. To determine the effect of the amount of MP65 on the proliferation of lymphocytes, lymphocytes isolated from the spleens of immunized mice of various vaccinated groups including controls, were cultured in flat-bottomed 96-well plates. The cells were co-cultured in the presence of increasing doses (1–24
µg) of corresponding formulations of MP65 as well as controls. After 72 hours [3H]-thymidine was added and its incorporation into multiplying cells was measured after 16 hours of incubation, using liquid scintillation spectroscopy. The CPM values of stimulated culture were used to represent Ag-specific stimulation. (A) Dose-dependent proliferative response of lymphocytes at 1-week post-booster upon stimulation with various MP65 formulations. (B) Lymphocyte proliferation in terms of CPM values in various immunized groups at 1 week PB and 4 weeks PC time points. Data represent the mean of three determinants ± SD. The figures are representative of three independent experiments.

Figure 3.14. MP65 fibril induced increased expression of co-stimulatory molecules on Ag-presenting cells. The expression of costimulatory molecules, CD80 (B7-1) and CD86 (B7-2), on macrophages was determined by staining target cells with specific Abs and subsequent analysis by flow cytometry at 4 weeks post-challenge. The dot plots for CD80 (A) and CD86 (B) represent various immunized groups: (I) Saline as control (II) Native MP65 (III) Liposome-encapsulated MP65 (iv) MP65 aggregate (v) MP65 fibril. Data were analyzed with Student's t-test and are representative of three independent experiments.
Figure 3.15. Augmentation of CD8⁺/CD4⁺ T cell effector and central memory response upon immunization with MP65 fibril. The CD4⁺ and CD8⁺ T cells were harvested and their purity was determined as described in the ‘Materials and methods’ section. The CD4⁺CD44⁺CD62L⁻ (A) and CD8⁺CD44⁺CD62L⁻ (B) phenotypes were analyzed using flow cytometry at 4 weeks post-challenge, representing various immunized groups: (i) Saline as control (ii) Native MP65 (iii) Liposome-entrapped MP65 (iv) MP65 aggregate (v) MP65 fibril. Three independent experiments were performed and data are representative of at least two independent experiments with similar observation.

3.3.16. MP65 fibril evokes delayed type hypersensitivity in immunized animals
DTH, is an in vivo manifestation of CMI and parallels the development of protective immunity in mice (Ahmad et al. 2012). As evident from Figure 3.16A, the animals immunized with MP65 fibril formulation revealed significantly higher DTH response as compared to other formulations at all the time points (p<0.01). Liposome-entrapped MP65 and aggregated MP65 exhibited similar DTH responses. The DTH response was minimal in animals receiving native MP65, while that in the groups receiving saline was negligible.

3.3.17. Nitric oxide production
Formation of NO is used as an index of the activated macrophage population (Ahmad et al. 2012). Generation of NO was evaluated in macrophages isolated from mice immunized with various MP65 formulations. MP65 fibril formulation induced
generation of significantly higher levels of NO at post immunization as well as post booster time points as compared to native MP65 and other formulations (p<0.01) (Figure 3.16B). The NO formation in the culture supernatants of macrophages from the groups receiving saline was negligible as compared to MP65 fibril formulation.

3.3.18. Protective efficacy of MP65 fibril formulation against candidiasis
Protective efficacy of various vaccine preparations was evaluated on the basis of their potential to suppress fungal load in kidney and spleen of the immunized mice. As shown in Figure 3.17, MP65 fibril immunization showed significant reduction in fungal burden in immunized animals when compared to native MP65. The liposome-entrapped MP65 and MP65 aggregate stand protective when compared with native MP65 at 4 week post challenge time point. On 8th week post challenge time point, the fungal load in MP65 fibril immunized animals was lower than that observed at two week post challenge time point for the same group. In comparison to saline control, MP65 fibril exhibited a significant reduction in CFU count (p<0.001) in spleen as well as kidney at both the post challenge time points. Moreover, the survival studies were performed for mice immunized with various formulations of Ags and subsequently challenged with 1×10⁶ live C. albicans cells. The unimmunized mice (administered saline) exhibited a mere 5% survival. Among the formulations used, MP65 fibril offered best protection against the fungal challenge with 90% survival. On the contrary native MP65 immunized animals showed only 40% survival. The animals immunized with MP65 aggregate and liposome-entrapped MP65 exhibited 65% and 55% survival respectively (Figure 3.18).
Figure 3.16. MP65 fibril immunization induces strong DTH response and enhances NO production. (A) To ascertain the induction ability of MP65 fibril to evoke cell-mediated immune response in the immunized mice, mean DTH response was evaluated by measuring footpad swelling in the groups immunized with saline, native MP65, liposome-entrapped MP65, MP65 aggregate and MP65 fibril after various time intervals. Data are representative of three independent experiments ± SD values. (B) NO concentration was determined in the supernatants of peritoneal macrophages isolated at two week PI and one week PB time points from various
immunized groups as described in Materials and Methods section. Data are mean of three independent experiments ±SD values.

Figure 3.17. MP65 fibril immunization successfully depleted fungal burden in vital organs. Fungal load, in the kidney (A) and spleen (B) of vaccinated mice, belonging to various groups of animals, was enumerated by plating tissue homogenates of kidney and spleen of vaccinated mice followed by counting the numbers of CFUs at 2 and 4 weeks post challenge following method as described in
methodology section of the text. The data were expressed as means of three determinants ± S.D. and are representative of 3 independent experiments.

Figure 3.18. MP65 fibril enhanced the survival of immunized animals. Survival of animals immunized with various preparations of MP65 and subsequently challenged with virulent strain of *C. albicans*. Each group consisted of 10 animals. The animals were challenged with $1 \times 10^6$ cells of *C. albicans* virulent strain through intra-venous route. The survival of challenged animals was monitored for 30 days. The values represent the mean of three independent experiments.
3.4. Discussion

The group of ordered nanostructures that are self-assembled from a wide range of polypeptides/proteins are referred to as amyloid fibrils. Amyloids have been associated with both numerous neurodegenerative diseases and benign functions ranging from skin pigmentation to storage of pituitary hormones (Maji et al. 2008, Maji et al. 2009c, Mankar et al. 2011). Amyloids have classically been viewed to be highly rigid and irreversible assemblies but growing evidences indicate that they may be dynamic and reversible and may release monomers or completely dissociate to monomers (Kardos et al. 2011, Meersman et al. 2006, Torrent et al. 2006, Hirota-Nakaoka et al. 2003, Legge et al. 2009, Binger et al. 2008b, Carulla et al. 2005).

Discovery of functional amyloids highlight their possible use in designing novel nanostructure materials. In fact, recent findings enumerate that in vitro synthesized amyloids can perform biological functions when delivered in vivo (Maji et al. 2008, Gupta et al. 2010). The biological functions performed by amyloids in vivo are attributed to the slow and sustained release of the bioactive peptides/proteins from amyloid depot. In vitro synthesized pre-amyloid and amyloid bodies formed of insulin and gonadotrophin releasing hormone respectively have been found to perform biological functions upon their release in systemic circulation. It has been proposed that they release bioactive conformation of the monomers in vivo which can activate downstream signalling cascade to produce desired biological outcome yet no direct evidence has been provided to state that amyloids release proteins bearing native conformation. Reckoning with these facts, in our earlier study, we investigated the Abs produced upon amyloid fibril immunization. We found that fibril immunization induces production of Abs specific for fibril as well as native protein (unpublished data). Moreover, the proteins released in vitro from amyloid depot were found to recognize native protein specific Abs (unpublished data). These findings supported the idea that amyloids may act as depots for the release of native Ag. The encouraging results persuaded us to evaluate vaccine potential of amyloids where they can act as Ag releasing depots.

In the present study, we evaluated the prophylactic potential of MP65 amyloid fibril in combating murine disseminated candidiasis. The present study was performed considering the fact that MP65 evokes potent T cell response required to contain the
infection by *C. albicans*, an intracellular pathogen. The data of the present study suggest that immunizing animals with amyloid fibril of MP65:

i) strongly upregulates Th1 type cytokines (IFN-γ and IL-12) in the host
ii) produces higher ratio of IgG2a type Abs to IgG1 in the sera of immunized animals
iii) induces Ag specific T lymphocytes
iv) helps in upregulation of costimulatory molecules (CD80/CD86) on the surface of APCs
v) induces CD4+ and CD8+ T cells with effector (CD44hiCD62Llo) as well as central memory (CD44hiCD62Lhi) phenotype
vi) evokes strong DTH response
vii) induces higher production of NO from macrophages
viii) reduces fungal burden in vital organs of infected mice
ix) increases survival of immunized animals

The higher efficacy of MP65 fibril can be owed to the slow and sustained release of MP65 from the amyloid depot. The amyloid depot not only facilitated sustained release of native MP65 for extended time duration but the particulate nature of amyloids also facilitated their specific uptake by both macrophages (Figure 3.9) and dendritic cells; potential APCs of the host. It seems that MP65 fibril harbouring macrophages as well as dendritic cells act as secondary depots that help in disseminated distribution of Ag to various lymphoid organs of the body. Moreover, the presence of mannann moiety in MPs which is considered critical for interaction with PRRs, in particular TLRs, may drive the cytokine response by APCs which plausibly govern subsequent T-cell activation (Pietrella et al. 2008). In fact, it has been demonstrated that interaction of the native mannosylated MP65 with human DCs leads to TNF-α and IL-6 release and to IL-12 gene expression (Pietrella et al. 2006) which lead to Th1 polarization. MP65 induce DC maturation by increasing the expression of costimulatory molecules, decreasing CD14 and Fcγ receptor (FcγR) molecule expression, and enabling DC to activate the T-cell response (Pietrella et al. 2006). Therefore, the depot effect provided by amyloid fibril of MP65 along with the adjuvant properties bestowed to MP65 due to the presence of mannose residues may
be responsible for the overall greater efficacy of MP65 amyloid fibril against candidiasis.

Firstly, we synthesized MP65 fibril by agitating native MP65 in neutral buffer. Fibrillar aggregates were obtained only after 24hrs of shaking as revealed by ThT fluorescence that got saturated beyond 24hrs (Figure 3.3). Moreover, TEM also exhibited fibrillar morphology for 24 hrs aggregate or aggregates generated at higher incubation periods but not obtained prior to 24 hrs (Figure 3.4). CR binding and CD spectroscopy demonstrated corroborating results demonstrating presence of β-cores, a feature of fibrillar amyloids (Figure 3.6). We analysed the release of MP65 from its aggregates as well as fibril (Figure 3.5). Aggregates formed till 16hrs of incubation exhibited a burst release (data shown only for 12 hr aggregate). A slow and sustained release was obtained for aggregates obtained at 18hr, 20 hr, 24 hr and 26 hr (data shown only for 24hr fibril). Fibrils obtained beyond 26hrs of shaking incubation released MP65 only feebly (data not shown). Therefore for further in vivo studies, amongst the ‘burst release’ showing aggregates we selected 12 hr aggregate and amongst those exhibiting ‘sustained release’, we chose 24 hr fibril so as to perform a comparative study between non-fibrillar and fibrillar amyloid. The ‘12hr aggregate’ is called only ‘aggregate’ and ‘24hr fibril’ is termed as only ‘fibril’.

The released MP65 from its fibril was found to exhibit biophysical characteristics of native MP65 as observed from ThT fluorescence of released MP65 which was similar to native state of protein (Figure 3.7) and CD spectroscopy (data not shown). In the next phase of study, mice were immunized with MP65 aggregate as well fibril. The Abs generated upon the immunization with MP65 amyloids were found to recognize native MP65 (Figure 3.8) in addition to the aggregate and fibril conformation (data not shown). As shown in Figure 3.10, animals immunized with MP65 fibril exhibited significantly higher native Ag specific Ab titres as compared to the groups vaccinated with native MP65, liposome-entrapped MP65 and aggregated MP65 (p<0.001). Moreover, MP65 fibril exhibited consistently higher Ab titres even at four week post challenge time point. This might have happened due to slow and sustained release of native MP65 from MP65 fibril which however did not happen for liposome-entrapped MP65 and aggregate MP65 because they exhibited a burst release of the native form
of Ag. The MP65 fibril induced primarily Th1 cytokines, IgG2a type Abs and feeble generation of Th2 cytokines (Figures 3.11 and 3.12). Native MP65, liposome-entrapped MP65 and aggregate form of MP65 evoked significantly lower levels of Th1 cytokines as compared to MP65 fibril (p<0.001). Moreover, lymphocytes isolated from animals immunized with MP65 fibril exhibited highest dose dependent proliferation amongst all the preparations tested (Figure 3.13A). Lymphocytes obtained from group vaccinated with MP65 fibril exhibited significantly higher proliferation as compared to native MP65 immunized group at post booster as well as post challenge time points (p<0.001). On the other hand, MP65 aggregate showed only a slight increase in T cell proliferation as compared to native MP65 (Figure 3.13B).

Once the presented Ags on APCs are recognised by TCRs (present on CD4 T cell as well as CD8T cell), they are simultaneously activated to induce co-stimulatory molecules, which provide co-stimulatory signals, and produce specific cytokines according to characteristics of Ag (Khan et al. 2012). The level of co-stimulatory molecules induced defines the immune activation ability of the Ag. Therefore it is important to analyse the co-stimulatory markers on the APCs. The maximum upregulation of co-stimulatory markers CD80 and CD86 was observed in animals vaccinated with MP65 fibril (Figure 3.14). In contrast, the animals immunized with MP65 aggregate, liposome-entrapped MP65 and native MP65 showed feeble expression of CD80 as well as CD86. Moreover, induction of memory response is a hallmark of any vaccine formulation (Gowthaman et al. 2011). MP65 fibril was found to induce central memory phenotype (CD44^{high}CD62L^{high}) as well as effector memory phenotype (CD44^{high}CD62L^{low}) on CD4 as well as CD8 T cells. Interestingly, effector memory was significantly higher in animals immunized with MP65 fibril as compared to other groups (p<0.01) (Figure 3.15). In general, central memory persists after rapid clearance of acute infections, and is more effective in controlling secondary infections involving intracellular pathogens (Wherry et al. 2003). On the other hand, the effector memory has been reported to be induced by chronic infections (Appay et al. 2002, Champagne et al. 2001). This indicates continued low-level presentation of Ag to both the CD4^+ and CD8^+ T cells by APCs.
at later time points and thus predicts an Ag-depot effect offered by fibrillar amyloids, which in turn results in producing a balanced central and effector memory in the host.

DTH is mediated by CMI and functions in the development of protective immunity (Ahmad et al. 2012). The mice immunized with MP65 fibril exhibited a significantly higher DTH response as compared to MP65 aggregate and other groups (p<0.01). Also, formation of NO which is used as an index of the activated macrophage population was observed to be highest in the group immunized with MP65 fibril (Figure 3.16). Lastly, protective efficacy of MP65 fibril was assessed by determining the fungal burden in various vital organs and performing survival studies. Fungal burden in kidney as well as spleen of mice immunized with MP65 fibril was found to be significantly lower even after four weeks of challenge as compared to animals immunized with MP65 aggregate as well as other groups (p<0.001) (Figure 3.17). Moreover, survival studies showed remarkable survival rates in animals immunized with MP65 fibril in comparison with MP65 aggregate, native MP65 and liposome entrapped MP65 vaccinated animals (Figure 3.18).

Conclusively, amyloid fibril based vaccine provides a platform to deliver Ag as well as trigger both arms of immunological response including memory. The heightened amyloid fibril based immune responses could be ascribed to the following scenarios. First, amyloid fibril mediated prolonged Ag release could offer prime-boost effect on memory CD8+ T cell generation. It has been shown that Ag persistence during infection correlates with a stronger T cell response (Ochsenbein et al. 1999). The slow release of protein from amyloid fibril may more closely approximate the presence of soluble Ag during an infection. Second, amyloid fibril due to slow release of Ag may provide better adjuvant effects by eliciting proper cytokine milieu that favor higher number or quality of memory CD8+ T cell formation. Lastly, fibril generates memory CD8+ T cells that elicit immediate effector functions in response to pathogen entry and rapid secondary expansion to rapidly clear the infection. The responses observed for amyloid fibril did not happen for amyloid aggregate as well as for liposome-entrapped Ag potentially due to instability issues of these two preparations leading to burst release of the Ag. In nut shell, the results of this work highlight that the pace of Ag release from the amyloid depot has its effect on overall
immune response, long-term CD4\(^+\) as well as CD8\(^+\) T cell memory responses and efficacy in pathogen recall.